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The role of titin and nebulin in myofibril assembly in cultured embryonic chick muscle cells

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The role of titin and nebulin in myofibril assembly in cultured embryonic chick muscle cells

by

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GENERAL INTRODUCTION

Myofibrils are the contractile units of striated muscle (Huxley, 1957). The more than twelve different proteins that constitute a myofibril can be grouped, on the basis of their location in the myofibril, into thin filament and Z-line proteins, thick filament and M-line proteins, intermediate filament proteins, and elastic filament proteins.

Thin filaments (1 μm long, 6-8 nm in diameter) (Huxley, 1957) are assembled into the I band, the light band in the phase or electron microscope. The I band is bisected by a dark Z line. N_1 and N_2 lines are also structural components of the I band (Franzini-Armstrong, 1970; Locker and Leet, 1976; Wang, 1981), but are not always visible by electron microscopy (Przybylski and Blumberg, 1966; Fischman, 1970).

Thick filaments (1.5 μm long, 14-16 nm in diameter) are located in the A band, which appears dark in the phase or electron microscope. The lighter H zone is found in the middle of the A band. A dark M line bisects the light H zone (Knappeis and Carlsen, 1968).

The thin filaments consist primarily of actin, tropomyosin and troponin. In vivo, G-actin monomers (M_r=42,000) aggregate to form double-stranded, helical F-actin filaments (Huxley, 1957), which form the backbone of the thin filament. One end of the thin filament is associated with the Z line (Huxley, 1963). Beta-actinin (M_r=70,000) binds to the free end of the actin filaments, where it may function to regulate thin filament length or to prevent interaction between adjacent thin filaments in the myofibril (Maruyama et al., 1977).

Tropomyosin and the troponin complex are thin filament regulatory proteins which confer calcium sensitivity to the actin-myosin interaction in striated muscle (Ebashi and Endo, 1968). The tropomyosin molecule (M_r=68,000) is a dimer composed of either two alpha chains (M_r=33,308), two beta chains (M_r=33,072) or one of each arranged in a coiled-coil (Mak et al., 1980). A head to tail assembly of tropomyosin molecules lies in each of the two grooves of the actin filament (Ohtsuki et al., 1967). Troponin consists of three subunits, troponin-C (TN-C, M_r=17,900), troponin-T (TN-T, M_r=30,500), and troponin-I (TN-I, M_r=20,900). Skeletal muscle TN-C has four Ca^{2+} binding sites *in vivo*, while cardiac TN-C has three (Ebashi et al., 1971; Potter and Gergely, 1975). TN-C binds to TN-T and TN-I, but not to actin or tropomyosin (Weber and Murray, 1973). TN-I alone can inhibit actin-myosin interaction, but this inhibition is enhanced in the presence of tropomyosin (Cachia et al., 1983). TN-I binds to TN-C in a Ca^{2+} sensitive manner.

The major component of the Z line is alpha-actinin, a dimer of subunit M_r=100,000 (Suzuki et al., 1976). *In vitro*, alpha-actinin cross-links actin to form parallel arrays (Podlubnaya et al., 1975). Thus, *in vivo*, alpha-actinin may serve to bind and cross-link thin filaments from adjacent sarcomeres (Goll et al., 1972). Many detailed models of Z-line structure appear in the literature (Knappeis and Carlsen, 1962; Landon, 1970; Franzini-Armstrong, 1973) the most recent of which is the model proposed by Yamaguchi et al. (1985).

Yamaguchi's model is based on a pair of Z filaments, termed a Z

unit, which are linked near their centers at a 90° angle and form bridges between neighboring antipolar thin filaments. A square lattice of four Z-filament pairs defines the geometrical position of the I-square unit.

Other proteins reported to be at the Z line include filamin (Gomer and Lazarides, 1981), vinculin (Pardo et al., 1983), zuegmatin (Maher et al., 1985), Z protein (Ohashi et al., 1982), and synemin (Granger and Lazarides, 1980). Intermediate filaments, composed of the protein desmin in adult skeletal muscle, are also found at the Z line, where they may function to connect adjacent myofibrils (Gard and Lazarides, 1980).

The majority of the thick filament is composed of myosin. Two heavy chains (M_r=230,000), two essential light chains (M_r=16,000-20,000), and two regulatory (DTNB) light chains (M_r=16,000-20,000) make up the myosin molecule (Harrington and Rodgers, 1984). About 50% of the heavy chains form a coiled-coil rod, which binds to other myosin rods allowing myosin to assemble into thick filaments. The remainder of the heavy chains plus the light chains form two globular head regions per molecule. These regions, called subfragment-1 (S-1) regions, project from the thick filament to form cross-bridges capable of binding to actin (Huxley, 1963). Each head also contains a divalent cation binding site and a site for ATP binding and hydrolysis (Squire, 1975).

M-band proteins are found in the center of the A band, and serve to align thick filaments within the A band. The M band is composed of M bridges, M filaments and secondary M bridges (Carlsson and Thornell,

1987). M bridges connect adjacent thick filaments to form the characteristic hexagonal array. M bridges may be composed of the proteins myomesin (M_r=185,000) (Grove et al., 1985), M-protein (M_r=165,000) (Trinick and Lowey, 1977), and creatine-kinase (M_r=40,000) (Turner et al., 1973). M filaments run parallel to the thick filament and link the M bridges. The Y-shaped secondary M bridges connect adjacent M filaments. A pair of high molecular weight proteins called skelemins may also be present in the M line in striated muscle (Price, 1987).

C-protein (M_r=140,000) is located with myosin in the thick filament (Offer et al., 1973). In vitro, C-protein binds to myosin, (Moos et al., 1975; Starr and Offer, 1978), alters actin-activated ATPase activity of myosin (Offer et al., 1973), and binds to F-actin (Moos et al., 1978; Yamamoto and Moos, 1983). The in vivo function of C-protein is not known. It may serve a structural function (Moos et al., 1978), regulate thick filament length (Moos et al., 1975), or modify cross-bridge movement (Starr and Offer, 1978).

Morphological evidence for the existence of a third set of elastic, longitudinally running filaments in addition to the thick and thin filaments has existed since the 1960s. A set of very thin filaments were seen spanning the gap between separated A and I bands in overly stretched muscle (Huxley and Peachey, 1961; Sjostrand, 1962), and in myosin and actin extracted myofibrils (Guba et al., 1968; dos Remedios and Gilmour, 1978). Locker and co-workers also reported the existence of "gap filaments" in super-stretched bovine sternomandibularis muscle

(Locker and Leet, 1975). They observed that these filaments were highly extensible, had diameters of 2 to 6 nm, and were very susceptible to proteolytic degradation.

Maruyama attempted to identify the protein component of the third set of filaments in the mid-1970s. Maruyama subjected skeletal muscle myofibrils to many extraction solutions, including acid, alkali, SDS, phenol, KI, and urea. He called the insoluble residue remaining after such treatments connectin (Maruyama et al., 1976). At about the same time, Wang was studying the high molecular weight protein composition of skeletal muscle by SDS-PAGE of total SDS extracts of whole muscle. Wang discovered two high molecular weight myofibrillar proteins, which he named titin and nebulin (Wang et al., 1979; Wang and Williamson, 1980). Maruyama compared his connectin prepared from KI extracted myofibrils with Wang's titin purified by SDS-gel filtration chromatography in terms of electrophoretic mobility on PAGE, amino acid composition, and localization in myofibrils, and declared the two equivalent (Maruyama et al., 1981). It now appears that Maruyama's original connectin preparation was actually a mixture of titin, nebulin, several other myofibrillar proteins, and connective tissue (Wang, 1985). Both titin and connectin are names used to identify this protein in current literature.

Titin is the third most abundant myofibrillar protein, making up 9-10% of total myofibrillar protein in skeletal and cardiac muscle (Wang et al., 1979). Titin has been found in many vertebrate and invertebrate striated muscles (Gassner et al., 1985; Hu et al., 1986). Titin

migrates as a doublet on low porosity SDS polyacrylamide gels. The upper band of the doublet is known as T_1 (alpha-connectin) and the lower band as T_2 (beta-connectin). There are two different estimates for the molecular weight of titin; 1.4 or 2.8 x 10^6 for T_1, and 1.2 or 2.1 x 10^6 for T_2 (Maruyama et al., 1984a; Wang, 1984a), using cross-linked myosin heavy chains as artificial molecular weight markers. The differences in reported molecular weight are due to the fact that Maruyama believes the maleimide derivative cross-linker created 2x dimers while Wang assumes dimers of myosin heavy chain were produced by the cross-linker. T_1 and T_2 are chemically and immunologically related, and it appears that T_2 is a proteolytic breakdown product of T_1. In fresh muscle, the doublet is present in a T_1:T_2 ratio of 3-5:1 (Wang et al., 1979; Maruyama et al., 1981). However, T_2 concentration increases and T_1 decreases upon storage, either at or near room temperature (Maruyama et al., 1981), or at 0° C (Seki and Watanabe, 1984). Because titin does not appear to be composed of small lysine-derived crosslinked polypeptides (Robins and Rucklidge, 1980; Gruen et al., 1982; Maruyama, 1986), it may be the largest protein synthesized as a single chain.

The large size of titin and its unique solubility properties have resulted in many technical difficulties in purification and characterization. Intact titin can be purified only in the presence of strong denaturants such as SDS (Wang et al., 1979; Maruyama et al., 1981), urea, or guanidine-HCl (Gruen et al., 1982), or from KI residues of myofibrils (Maruyama et al., 1981). Recently, T_2 (beta-connectin) has been purified in the native state (Kimura and Maruyama, 1983;

Maruyama et al., 1984a; Trinick et al., 1984; Wang et al., 1984b). T_1 (alpha-connectin) has still not been purified in the native form.

In freshly prepared, intact isolated adult myofibrils, both polyclonal and monoclonal antibodies to titin label predominantly the A-I junction (Wang, 1985; Wang and Greaser, 1985; Gassner, 1986; Hill and Weber, 1986; Maruyama 1986). In addition, M line, Z line, A band, and I band staining has been observed (Wang et al., 1979; Maruyama et al., 1984b). Selective removal of thick or thin filaments results in antibody staining that indicates translocation of titin to the Z line or M line (Wang and Greaser, 1985). Titin antibodies also label the 6-8 nm filaments spanning the gap between A and I bands in overstretched myofibrils (LaSalle et al., 1983; Maruyama et al., 1984b; Gassner, 1986). These filaments appear to be morphologically indistinguishable from "end filaments" (Trinick, 1981) and "gap filaments" (Locker and Leet, 1976).

Nebulin has been purified only in the denatured form by SDS gel filtration chromatography (Wang, 1982). To date, the physicochemical properties of nebulin have not been well characterized. The molecular weight of nebulin is 5-6 x 10^5 daltons by SDS-PAGE, it accounts for 3-5% of total myofibrillar protein, and is chemically and immunologically distinct from titin (Wang and Williamson, 1980; Wang, 1981). In intact isolated myofibrils polyclonal nebulin antibodies label a pair of bands within the I band that coincide with the N_2 line (Wang, 1985). N_1 and N_2 lines are transverse structures located in the I band on both sides of the Z line. N_1 lines are fixed in position, 0.1-0.2 micrometers from

the Z line (Wang and Williamson, 1980). N_2 lines are found farther from the Z line, but their exact distance depends on the state of contraction of the myofibril. When sarcomeres are lengthened, the N_2 line moves away from both the M line and Z line, but maintains the same proportional distance. This stretch-dependent movement suggests that N_2 line associated proteins are not strongly bound to either thick or thin filaments (Wang, 1985). The N_2 line is also located in the I band in the region where the thin filaments change from the tetragonal array found at the Z line to the hexagonal array found in the overlap of the A and I bands (Franzini-Armstrong, 1970). In a more recent study on mechanically skinned skeletal muscle fibers, a monospecific nebulin antiserum labeled 6-7 pairs of bands within the I and A bands when examined in the electron microscope (Wang and Wright, 1987).

Results of these immunochemical experiments can best be explained by the supposition of titin and nebulin as a set of fragile, elastic longitudinal filaments that run through a sarcomere. Mechanical damage or proteolytic degradation can break the filaments, which then snap back, or translocate, to more stable anchoring points such as M lines, Z lines or N_2 lines. Several third filament models have been proposed. Locker first proposed that one gap filament formed the core of two thick filaments (Locker and Leet, 1976). The elastic, or gap, filament would emerge from the A-I junction end of one thick filament, pass through the I band and the Z line, then end as the core of a second thick filament in the adjacent sarcomere. This model now seems unlikely in light of current structural evidence. In Maruyama's model, determined by the use

of polyclonal antibodies, connectin filaments link myosin filaments to Z lines starting 0.15 micrometers away from the A-band center (Maruyama, 1986). Nebulin filaments are not considered in Maruyama's model. Wang proposes a model which does include nebulin filaments. Titin filaments are anchored at the M line and run parallel to myosin thick filaments, possibly wrapped around them. Titin filaments run through the A-I junction and the I band to the N_2 line. Nebulin filaments then travel through the rest of the I band to the Z line. It is not known if the M-line and Z-line attachments are direct or mediated through other proteins (Wang, 1985). Wang and Wright (1987) have more recently suggested that nebulin constitutes a distinct set of filaments in parallel, but not in series, with titin filaments. They propose a four filament sarcomere model consisting of thick filaments and thick filament (A segment)-linked titin filaments, and thin filaments plus thin filament (I segment)-linked nebulin filaments.

Based on many structural studies, (Allen et al., 1979) the differentiation sequence of skeletal muscle cells has been described. The mesodermal germ layer gives rise to presumptive myoblasts, which differentiate to form myotubes. Presumptive myoblasts are mononucleated, actively dividing and incapable of fusion and contain no myofibrils. Myoblasts are also mononucleated, but are capable of both fusion and contractile protein synthesis. The committed step in myogenesis is the fusion of myoblasts to form myotubes. Synthesis of contractile protein increases significantly after fusion, as does myofibril assembly (Devlin and Emerson, 1978; Allen et al., 1979).

Fusion, however, is not a prerequisite for myofibrillar protein accumulation in skeletal muscle (Allen et al., 1979). Cardiac muscle is also a form of striated muscle, and also arises from the mesoderm. In contrast to skeletal muscle, cardiac muscle cells do not fuse but are mechanically linked by intercalated discs. Also, unlike skeletal myotubes, cardiac myocytes containing myofibrils still undergo mitosis, although some reports say transient myofibril disorganization occurs (Hay and Low, 1972; Kaneko et al., 1984).

A significant portion of current research focuses on the characterization of myofibrillar protein isoforms and their sequence of appearance and disappearance during the stages of myofibril maturation. Since the research presented here concentrated on the organization of titin, myosin, actin, and nebulin in the striated muscle myofibril, only these four proteins will be discussed in terms of protein isoforms.

The myosin molecule is coded for by many genes which are tissue and developmental-stage specific. The transition of myosin heavy chain (MHC) from an embryonic to a neonatal to an adult form has been described for chicken pectoralis (Bandman et al., 1982; Lowey et al., 1983; Winkelmann et al., 1983), rat (Whalen et al., 1981; Mahdavi et al., 1985), and mouse (Weydert et al., 1987) muscle development.

By using a cDNA probe and monoclonal antibodies to adult rat slow MHC, Narusawa et al. (1987) showed that the same beta-MHC gene expressed in adult soleus and fetal ventricle tissue is also expressed in all fetal rat limb muscles, regardless of whether they are future slow or future fast muscles. Gauthier et al. (1982) used immunocytochemical

methods to show that all fibers of developing mammalian (rat) or avian (chicken) muscle react with antibodies specific for slow and fast myosin. Terminal differentiation to a slow or fast twitch type depends on the muscle itself. Gauthier suggested that all fibers of developing fast muscles have an embryonic MHC distinct from adult fast or slow MHC that can associate with a fast, slow, or embryonic light chain.

Whether myosin isoform transitions occur in vitro has been the focus of many investigations. Whalen et al. (1981) found that only the embryonic form of MHC was expressed in rat L6 cells in culture. Adult MHC was never detected as a major component in rat primary muscle cell cultures. Pectoral muscle cell cultures from chick embryos expressed only embryonic MHC even after thirty days in culture (Bandman et al., 1982). Cultured pectoralis muscle cells also failed to undergo immunochemical transitions in the MHC as detected by monoclonal antibodies in vitro (Bader et al., 1982). Zadeh et al. (1986) labeled chick embryonic heart cell cultures with monoclonal antibodies specific for atrial, ventricular, and conductive system MHC. Epitopes specific for the MHC appeared to be altered in the culture system, because atrial and ventricular staining was lost with increased time in culture and the conductive system epitope was never present.

In contrast, Schafer et al. (1987) found that while fetal chicken myoblasts and mouse cell line myoblasts initially synthesize embryonic fast MHC, some myotubes synthesize a neonatal fast MHC after several days in culture. Transition from fetal to perinatal/adult myosin, as defined by monoclonal antibody labeling, occurred within one week in

cultures of mouse C_2C_{12} cells (Silberstein et al., 1986). This transition occurred in the absence of nerves or connective tissue components. Weydert et al. (1987) also examined myosin transitions in mouse cell lines cultured in the absence of nerves. Embryonic, perinatal, and adult MHC mRNA were found to accumulate. The levels of perinatal and adult MHC mRNA were influenced by culture conditions.

Stockdale and co-workers showed that three types of myotubes were formed in cultures of 5 or 6-day chick embryo pectoral muscle cells: one that contained only a fast MHC, one that contained only a slow MHC, and one that contained both fast and slow MHC (Miller et al., 1985). In cultures derived from day 8 or older embryos, only the fast class of MHC was synthesized. The class of MHC synthesized appeared to depend on time in culture (Schafer et al., 1987). Fetal quail myoblasts in short-term cultures formed both fast and fast/slow myotubes. In long-term cultures (> 20 days), quail, duck, and human fetal myoblasts formed a large percentage of fast/slow myotubes, whereas less than 1% of myotubes in fetal chicken myoblasts were of the fast/slow class. Stockdale suggests that a single cell gives rise *in vitro* to two types of myoblasts which are committed to forming two types of myotubes.

Six different actin isoforms are found in vertebrates; all are products of separate genes. Nonmuscle beta- and gamma-actins are present in greater concentration than muscle specific alpha-actin in immature cardiac and skeletal muscle cells (McKenna et al., 1985), both in primary culture and clonal muscle cell lines (Garrels and Gibson, 1976; Pardo et al., 1983). Sarcomeric alpha-actin was first detected in

stage 8 hearts of chick embryos (Wiens and Spooner, 1983). Myofibrillar organization and initiation of heart beat occurs in stage 10 chick embryos. Urea/SDS electrophoresis of SDS-extracted embryonic chick skeletal muscle proteins showed that only brain type actin is present in young embryos (less than 10 days in ovo). As development progressed, muscle type actin became relatively more abundant. In 20-day chick embryos, muscle actin was the predominant species (Storti et al., 1976).

Pardo et al. (1983) used immunofluorescence to show that gamma-actin associates with muscle mitochondria, but not with myofibrils in adult skeletal muscle cells. Antibody to molluscan actin was found to cross react with cytoplasmic vertebrate actins but not myofibrillar actin (Lubit and Schwartz, 1980). By immunofluorescence, this antibody localized along the sarcolemma and along membranes surrounding individual adult skeletal myofibrils.

In addition to beta- and gamma-actins, both alpha-skeletal and alpha-cardiac actins are found in differentiating striated muscle. Bains et al. (1984) used isotype-specific cDNA probes to follow the expression of alpha-skeletal and alpha-cardiac actins in differentiating mouse skeletal muscle cells. They found that within 24 hours of differentiation, alpha-cardiac actin mRNA was six times higher than alpha-skeletal actin mRNA. Alpha-cardiac actin mRNA, however, is not a major gene product in mature skeletal muscle (Bains et al., 1984).

Maruyama and co-workers have found differences in electrophoretic mobility among embryonic, neonatal, and adult chicken breast muscle connectin (Yoshidomi et al., 1985). Embryonic alpha-connectin is

present from day 7 of incubation to day 3 posthatch and neonatal alpha-connectin from hatch to day 7 posthatch. Adult alpha-connectin was found from day 5 posthatch. Embryonic beta-connectin was found until day 3 posthatch, and adult beta-connectin was present from day 7 of incubation. Also, they found that nebulin was not easily detectable until day 7 of incubation.

Myofibrils are assembled de novo from the newly-synthesized muscle specific isoforms of contractile proteins. Several investigators have utilized the electron microscope in an attempt to describe this process of skeletal myofibril assembly. Initially, myosin and actin filaments are synthesized in ribosome-rich areas of the cell (Allen and Pepe, 1965; Kelly, 1968). It is not apparent whether myosin-containing thick and actin-containing thin filaments are synthesized simultaneously, or whether thin filament formation precedes thick filaments (Allen et al., 1979). Myosin monomers can self-assemble into thick filaments of 1.5 micrometer length before incorporation into A bands (Allen and Pepe, 1965). It is not known if thin filaments also polymerize to correct I band length before incorporation. Many free myofilaments found just under the cell membrane are oriented parallel to the longitudinal axis of the skeletal muscle cell. Thus, the majority of myofibril assembly appears to occur in close association with the sarcolemma (Fischman, 1970).

The earliest phase of skeletal myofibril assembly may involve the alignment of polarized thick and thin filaments into primitive A and I bands (Kelly, 1968; Holtzer et al., 1972). Filament interdigitation

into a hexagonal array might be due to myosin cross-bridges linking the two sets (Fischman, 1967; Taniguchi and Ishikawa, 1982). Other investigators suggest that electron-dense plaques seen on immature myofibrils may be Z line precursors (Kelly, 1968; Warren, 1973) and act as important initial sites of myofilament aggregation (Peng et al., 1981). While in transmission electron microscopy, Z bands are visible in early stages of myofibril development and appear as dense plaques on thin filament bundles, a scanning electron microscope study by Isobe and Shimada (1983) reports that Z bands are not recognizable until the myofibrils have become well developed. Non-striated myofibrils are found before striated myofibrils are seen (Allen and Pepe, 1965). Holtzer (1961) also suggests that myoblasts contain fine, nonstriated longitudinal myofibrils. Mature myofibrils with definitive A bands, I bands, and Z and M lines move from a position near the cell membrane into the cytoplasm. Diameter growth occurs by association of additional thick and thin filaments to the initially formed hexagonal nuclei. Fully assembled sarcomeres can also add to the ends, or growth tips, of preexisting myofibrils (Dlugosz et al., 1984).

Several investigators have implicated a Z line precursor (Z substance) as having an important role in cardiac myofibrillogenesis. It has been proposed that Z-line-thin-filament bundles provide a scaffold for thick filament incorporation (Saetersdal et al., 1976; Myklebust et al., 1978). Results of immunoelectron localization studies of alpha-actinin and actin in embryonic hamster heart cells suggests that myofibrillogenesis is initiated at the plasma membrane, and Z

plaques are precursors of Z lines and may serve as organizing centers for myofibrils (Hill and Lemanski, 1985). Half-sarcomeres may interdigitate to form myofibrils (Markwald, 1973; Ronnau, 1977), or Z substance itself may differentiate into myofilaments (Manasek, 1968; Legato, 1972). In contrast, Hiruma and Hirakow (1985) have described three phases of chick cardiac myofibril organization. Phase I involves the assembly of thick and thin filaments into irregular bundles. In Phase II, filaments are attached to an electron-dense precursor Z substance. In Phase III, the myofibril consists of a series of regularly striated bands. As in skeletal muscle, new sarcomeres can add to the ends of preexisting cardiac myofibrils (Dlugosz et al., 1984). Despite the amount of information gathered by electron microscope studies, ultrastructural observations alone have not been able to provide enough evidence to determine the sequence or mechanism of assembly of either skeletal or cardiac myofibrillar proteins.

Several structures have been implicated in playing a role in directing the process of myofibril assembly, such as microtubules, stress fibers, or stress fiber-like actin cables. Holtzer and co-workers have cultured myogenic cells in the presence of several drugs, some of which affected microtubule stability, in their studies of myofibrillogenesis (Antin et al., 1981; Croop et al., 1982; Toyama et al., 1982). Treatment with 12-tetradecanoylphorbol-13-acetate (TPA) causes degradation of myofibrils in immature myotubes. If TPA is removed from the myogenic cell culture medium, striated myofibrils are rapidly assembled, and are indistinguishable from normal, mature

myofibrils. Taxol stabilizes microtubules in vitro. This drug blocks fusion of postmitotic myoblasts. It also induces the formation of interdigitating thick filaments and microtubules instead of thick and thin filaments. In the presence of taxol, myosin filaments still maintain a length of approximately 1.5 micrometers and form laterally aligned, bipolar, solitary A bands. Holtzer suggests that A bands self-assemble from myosin and M band protein monomers (Antin et al., 1981). Postmitotic myoblasts and myotubes exposed to Colcemid form rounded myosacs. Colcemid induces disruption of thick and thin filament alignment, patchy Z band formation, and intermediate filament cable formation. Colcemid does not block contractile protein synthesis nor does it inhibit assembly of hexagonal arrays of thick and thin filaments. Cytochalasin B and trypsin also induce patchy Z bands, which are insertion sites for thin filaments.

When myogenic cells cultured in TPA are then exposed to Taxol, the A bands consist of aligned thick filaments and microtubules, while I bands consist only of microtubules. Myogenic cells cultured in TPA and then in Colcemid form unaligned myofibrils. Patchy Z bands with attached polarized thin filaments are numerous, and may or may not be associated with poorly aligned thick filaments. Patchy Z band formation suggests that Z lines may function as actin-organizing centers for individual sarcomeres.

Stress fibers were first observed in nonmuscle cells in culture, and were found to consist of bundles of 7.5 nm actin microfilaments (Peng et al., 1981). Stress fibers have also been seen in muscle cells,

both in vivo and in vitro. There is evidence for a relationship between developing myofibrils and stress fibers in both skeletal and cardiac muscle, although the precise nature of this relationship is a source of controversy. Peng et al. (1981) have studied myofibril assembly in cultured myotome cells and have found that nonstriated bundles of microfilaments, similar to nonmuscle stress fibers, appear before nascent myofibrils. Striated and nonstriated regions can be detected on a single nascent myofibril. They also observed the formation of sarcomeres on preexisting microfilament bundles, suggesting that microfilaments are myofibril precursors. Sanger and co-workers also believe that microfilaments are nascent myofibrils (Sanger et al., 1986). By microinjection of rhodamine labeled alpha-actinin into living cells, they have found that alpha-actinin periodicity along fibrils increases with development time of cells in culture, unlike alpha-actinin periodicity in stress-fibers. Secondly, skeletal muscle myosin antibody labels the same fibers which contain myofibril-like alpha-actinin periodicities. All fibers that contain alpha-actinin also contain skeletal muscle myosin, whether alpha-actinin was present in adult Z lines or the closely spaced minisarcomeres. The skeletal myosin antibody did not label stress fibers in fibroblasts.

Holtzer and co-workers (Dlugosz et al., 1984; Antin et al., 1986) demonstrated that in both ethyl methanesulfonate (EMS) recovering chick skeletal myosheets and in normal cultured chick cardiac myocytes, assembly of thick and thin filaments occurred in close association with a single preexisting stress-fiber-like-structure (SFLS). EMS kills

replicating myoblasts, but postmitotic myoblasts fuse to form flat, multinucleated myosheets that contain extensive SFLS. EMS blocks the synthesis of myofibrillar, but not cytoplasmic myosin isoforms. When removed from EMS, nascent myofibrils assemble in recovering myosheets. As the myofibril matures, it is no longer closely associated with SFLS. In regions of muscle cells which contain many mature myofibrils, no SFLS are visible. When chick myoblasts were cultured in EMS and allowed to recover in normal medium, alpha-actinin first stained SFLS with irregular periodicity, then stained fibers with a periodicity equal to that of mature, striated myofibrils (Antin et al., 1986). In chick cardiac cells in culture, alpha-actinin first appears to bind to SFLS in a punctate pattern, then to Z lines of well formed myofibrils (Dlugosz et al., 1984). Holtzer suggests that SFLS act as transitory assembly sites for myofibrils.

With their immunofluorescence techniques, Holtzer and co-workers could not determine whether muscle-specific isoforms of myofibrillar proteins such as myosin and actin formed heteropolymers with the nonmuscle isoforms of the same proteins previously incorporated into SFLS, or whether muscle isoforms formed homopolymers near SFLS. They found that anti-chicken brain myosin (CBM) labeling often colocalized along structures that labeled continuously with muscle specific anti-light meromyosin (MS-LMM). In areas where anti-MS-LMM labeled myofibrils, anti-CBM showed a diffuse cytoplasmic fluorescence. This observation agrees with that of Fallon and Nachmias (1980). In rat skeletal muscle cell culture, cytoplasmic specific myosin antibody

localized in SFLS, on the cytoplasmic side of the membrane, and diffusely throughout the cell. Skeletal muscle specific myosin antibody was found in myofibrils. They also observed that some stress fibers appeared to be continuous with structures containing skeletal myosin. While they could not rule out a zone of copolymerization of nonmuscle and muscle myosin in this region, their results suggested that the myosin isozymes are mainly compartmentalized in the cell.

McKenna et al. (1985) found that nonmuscle and muscle cells did not discriminate between microinjected muscle and nonmuscle actin isoforms. In fibroblasts, both actins were primarily associated with stress fibers. In cardiac myocytes, both actins incorporated into sarcomeres. Lin and Lin (1986) provide biochemical and immunofluorescence evidence that both nonmuscle and muscle forms of actin and tropomyosin can assemble into heteropolymers. They propose that a preexisting microfilament containing nonmuscle tropomyosin (or no tropomyosin at all) may serve as a precursor for the assembly of muscle-specific actin and tropomyosin containing thin filaments.

During the course of this dissertation research, Hill et al. (1986) published a paper in which they analyzed the temporal assembly of titin and myosin heavy chain (MHC) into myofibrils in cultured embryonic chick skeletal muscles using double-label immunofluorescence. They found that titin and MHC synthesis is initiated simultaneously in postmitotic mononucleated myoblasts. The appearance and localization of titin is linked to the appearance of MHC. Both titin and MHC localize first to nonstriated, then striated myofibrils. This temporal and spatial

relationship between titin and MHC is also observed in myofibril assembly in Colcemid-treated cells.

In summary, it is still not known whether myosin-containing thick filaments or actin-containing thin filaments polymerize first in the developing muscle cell. Regardless, the two filament types first interdigitate to form the characteristic hexagonal array found at the A-I junction in close association with the sarcolemma. Initial assembly events may be directed by microtubules, stress-fibers or stress-fiber-like actin cables. Much information has been gathered pertaining to the appearance and disappearance of myofibrillar protein isoforms with muscle development, but little is known about the temporal sequence of assembly of these muscle-specific isoforms to form the adult, striated myofibril.

The main body of this dissertation consists of two full length manuscripts which will be submitted for publication. The first paper examines the appearance and localization of titin, muscle-specific myosin, and actin in normal and EMS-treated embryonic chick skeletal muscle cells. Emphasis was placed on determining the temporal sequence of assembly of these three proteins into an adult striated muscle myofibril. The second paper compares the temporal appearance and organization of nebulin, titin, actin, and myosin in the same culture system. Double-label immunofluorescence techniques were emphasized in both the first and second papers. The possible role of titin and nebulin in striated muscle myofibrillogenesis is discussed.

SECTION I. EXAMINATION OF THE ORGANIZATION OF TITIN DURING MYOFIBRILLOGENESIS BY IMMUNOFLUORESCENCE LOCALIZATION OF TITIN, ACTIN, AND MYOSIN IN EMBRYONIC CHICK SKELETAL MUSCLE CELL CULTURES

EXAMINATION OF THE ORGANIZATION OF TITIN DURING MYOFIBRILLOGENESIS BY IMMUNOFLUORESCENCE LOCALIZATION OF TITIN, ACTIN, AND MYOSIN IN EMBRYONIC CHICK SKELETAL MUSCLE CELL CULTURES

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FOOTNOTES

[1]Abbreviations used in this paper: SFLS, stress-fiber-like-structures; EMS, ethyl methanesulfonate.

ABSTRACT

To examine the time course of organization of titin during myofibrillogenesis in skeletal muscle, a series of double-label immunofluorescence localization studies was done to localize simultaneously titin and myosin or titin and actin in embryonic chick myogenic cell cultures. Titin was localized by indirect immunofluorescence using polyclonal rabbit antibodies prepared against purified adult chicken breast muscle titin and rhodamine-conjugated goat anti-rabbit second antibody. Actin was localized using either NBD-phallacidin or indirect immunofluorescence with monoclonal anti-actin antibodies and fluorescein-conjugated goat anti-mouse second antibody. Myosin was localized by indirect immunofluorescence with a monoclonal antibody specific for muscle myosin isoforms. During myogenesis, titin organization progressed from a diffuse pattern in mononucleated myoblasts to a filamentous organization along actin-containing stress-fiber-like-structures (SFLS) in myoblasts and early myotubes. Titin organization in later myotubes progressed first to a punctate pattern and finally to a myofibril-like banding pattern in more mature myotubes. Organization of titin into a myofibril-like arrangement occurred before the organization of actin into discreet I bands. Titin organization appeared to occur simultaneously with the organization of myosin into A bands. These results demonstrate that titin and myosin are organized concurrently into myofibrils along SFLS before the final organization of actin. The relative time course of titin, myosin, and actin assembly into myofibrils was confirmed by labeling of cultures recovering from

treatment with ethyl methanesulfonate (EMS). EMS treatment results in formation of large myosheets that do not synthesize or assemble myofibril proteins; after removal of EMS the myosheets rapidly assemble myofibrils. These results suggest that SFLS serve as the site of assembly of myofibrils in cultured skeletal muscle cells, and suggest further that titin may play a role in the organization of the A band.

INTRODUCTION

Titin is a high molecular weight protein (M_r = 1.4 x 10^6 daltons), first purified by Wang et al. (1979). Titin migrates as a doublet on low percentage SDS-polyacrylamide gels. The lower band of the doublet (T_2) appears to be a proteolytic breakdown product of the upper band (T_1) (Wang, 1985). Evidence suggests that titin is identical to the high molecular weight protein connectin (Maruyama et al., 1981).

In freshly prepared, intact, isolated adult myofibrils, both polyclonal and monoclonal (Maruyama et al., 1981; Wang, 1985; Wang and Greaser, 1985; Hill and Weber, 1986) titin antibodies predominantly label the A-I junction. In addition, M line, Z line, A band, and I band staining has been observed (Maruyama et al., 1984a; Wang et al., 1979). Selective removal of thick and thin filaments in myofibrils results in antibody staining that indicates translocation of titin to the Z line or M line (Wang and Greaser, 1985). Thus, fluorescent antibody labeling suggests that titin is not a component of either thin or thick filaments.

Evidence from several investigators currently suggests that titin is a component of a third set of longitudinal, elastic filaments in addition to the thick and thin filaments in the striated muscle myofibril (Maruyama et al., 1984b; Trinick et al., 1984; Wang et al., 1984). Although morphological evidence for a third set of filaments has existed since the 1960s, their existence remained controversial (for reviews, see Locker, 1984; Wang, 1985; Maruyama, 1986). Locker and Leet (1975) provided additional evidence for the existence of these filaments

with the identification of "gap filaments" in overly stretched bovine muscle. Subsequent to the purification of titin, several laboratories have demonstrated labeling of these filaments with anti-titin antibodies, suggesting that titin is a component of the gap filaments (LaSalle et al., 1983; Maruyama et al., 1984b; Wang, 1985; Gassner, 1986). Titin also appears to comprise the "end filaments" that can be seen at the ends of isolated thick filaments (Trinick, 1981; Gassner, 1986). Current evidence suggests that titin filaments extend into both the A band and I bands, and may connect thick filaments to Z lines. While Maruyama says titin- containing filaments run from thick filaments to Z lines (Maruyama, 1986), Wang suggests that titin filaments run to the N_2 line in the I band (Wang, 1985). Nebulin-containing filaments run from the N_2 line to the Z line.

Possible functions of titin in mature sarcomeres include roles as elastic components (Maruyama et al., 1977; Wang, 1985), in maintaining resting tension (Horowits et al., 1986), and in maintaining positional stability of thick filaments (Horowits and Podolsky, 1987). Less attention has been paid to the role of titin in skeletal muscle myofibril assembly. Hill and Weber (1986) suggested that titin may serve as a scaffold for the subsequent assembly of myofibrillar proteins. Hill et al. (1986) subsequently reported that the appearance of titin is correlated with the appearance of myosin heavy chain, but not with the appearance of desmin. The temporal sequence of assembly of titin with respect to other myofibrillar proteins, however, has not been established.

We have utilized double-label immunofluorescence techniques to follow the appearance and localization of titin, myosin, and actin in cultured embryonic chick skeletal muscle cells, in an effort to clarify the temporal sequence of titin, myosin, and actin organization in striated muscle myofibrillogenesis. In addition, we followed the organization of these three proteins in ethyl methanesulfonate (EMS) treated cultures. EMS has been shown to kill replicating cells in culture (Dlugosz et al., 1984; Holtzer et al., 1985; Antin et al., 1986). Surviving postmitotic myoblasts fuse to form very large, multinucleated myosheets. These myosheets are ideal for immunofluorescence studies because of the thinness of the sheets. The myosheets do not synthesize many of the muscle-specific proteins, but upon removal of EMS, the recovering myosheets assemble striated myofibrils over a period of a few days. By slowing down the normal time course of myofibril assembly, the temporal sequence of myofibrillar protein incorporation can be more carefully documented.

In this paper, we present evidence that titin and myosin assembly are linked in early skeletal muscle myofibrillogenesis, but titin and actin assembly is not. Some of these results have been presented previously in abstract form (Kurpakus and Huiatt, 1986).

MATERIALS AND METHODS

Antibody production and characterization

Denatured titin was prepared by gel filtration of SDS-extracts of adult chicken breast myofibrils on Biogel A50 (Bio Rad Laboratories, Richmond, CA) as described by Wang (1982). Myofibrils were prepared according to the method of Dayton et al. (1976). Polyclonal antibodies to titin were produced in New Zealand white rabbits by the method of Richardson et al. (1981). Serum was stored at -80° C and used for characterization and immunofluorescence studies.

Titin antibody was characterized by Western blot analysis. Proteins in 96 h skeletal muscle cell cultures, and adult isolated chicken skeletal muscle myofibrils (see below) were solubilized in sample buffer consisting of 0.2M Tris-HCl, pH 8.0, 10mM EDTA, 10% SDS, 0.1mM PMSF, 80mM DTT, 2% deoxycholate. 20 µl of tracking dye solution (50mM MES, pH 6.5, 5% SDS, 45% sucrose, 2mM EDTA, bromphenol blue) and 10 µl of 2-mercaptoethanol was added to 100 ul of the culture and myofibril homogenates and proteins were separated by SDS-PAGE on low percentage acrylamide gels (5% acrylamide, 100:1 acrylamide:bis acrylamide) according to the method of Laemmli (1970). Samples were run in triplicate sets which were cut into sections after completion of the electrophoretic separation. One gel section was stained with Coomassie blue dye, and the remaining sections were used for blots. Proteins were electrophoretically transferred to Zeta-Probe membrane (Bio-Rad) by a modification of the method of Towbin et al. (1979) using a Trans-Blot cell (Bio-Rad) with a 25mM Tris, 192mM glycine, pH 7.4 blotting buffer

without methanol. Transfer was done for 16-18 h at 50V and room temperature. One of the blots was stained for total protein with Bio-Rad Biotin-Avidin Protein Detection Kit, whereas the other blot was reacted with anti-titin antibody after first blocking the membrane with BLOTTO (5% nonfat dry milk in phosphate buffered saline, PBS, 0.01M sodium phosphate, 0.15 M NaCl, pH 7.2) (Johnson et al., 1984). Blots were then incubated with a 1:50 dilution of titin polyclonal antibody in BLOTTO at room temperature for at least 1 h, rinsed with 3 changes of BLOTTO for 1 h, and then incubated with a 1:1000 dilution of horseradish peroxidase conjugated goat-anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) at room temperature for at least 1 h. Blots were rinsed with 3 changes of PBS for 30 min, then reacted with 4-chloro-1-naphthol and H_2O_2 for antibody detection. Gels and blots were photographed with Kodak Technical Pan Film, and developed in D-19 or Technidol-LC for negatives of gels and blots, respectively..

Hybridoma cells from clone MF20 (Bader et al., 1982) were a generous gift of Dr. Donald Fischman (Cornell Univ. Medical College, New York). Myosin monoclonal antibody was partially purified from culture supernatants by ammonium sulfate precipitation according to Dennis et al. (1984). Actin monoclonal antibody culture supernatant from clone JLA20 (Lin, 1981) was purchased from the Developmental Studies Hybridoma Bank (Johns Hopkins University School of Medicine, Baltimore, MD).

Muscle cell culture

Skeletal muscle cells were isolated from mixed leg and thigh muscles of 12-day chick embryos by mechanical dissociation according to Ridpath et al. (1984). After filtration, the single cell suspension was preplated at 37° C for 40 min to differentially remove fibroblasts. Cultures were plated at 1.2 x 10^7 cells per 60 mm collagen coated Falcon tissue culture dish. Cells were maintained by daily feeding in Dulbecco's MEM, 10% horse serum, 1% antibiotics (GIBCO, Grand Island, NY), and 2.5% chick embryo extract. Cultures were incubated at 37° C in an atmosphere of 5% CO_2 and 95% air. Cells were processed for double-label immunofluorescence after 24, 48, 72, and 96 h in culture.

Some cultures were maintained in normal medium containing EMS (methanesulfonic acid, ethyl ester; Sigma) as described by Antin et al. (1986). Briefly, after 24 h in normal medium, cells were fed with 2 ml normal medium containing 7.5mM EMS. Cells were fed daily with EMS medium for a total of 3 d. After 3 d in EMS medium, cultures were returned to normal medium and allowed to recover for an additional 1 to 6 d before labeling studies were done.

Double-label immunofluorescence

Cells were double-labeled with antibodies to titin and myosin or titin and actin. NBD-phallacidin (Molecular Probes, Junction City, OR) was also used as a probe for actin. Cultures were fixed on the plastic culture dishes in (a) room temperature 2% paraformaldehyde for 20 min followed by 50% ethanol for 15 min (Fig. 8a-c; Fig. 9a-i), or (b) room

temperature 2% paraformaldehyde for 20 min followed by -20°C acetone for 10 min (Fig. 1d-i), or (c) -20°C methanol containing 50mM EGTA for 6 min. For labeling with phallacidin, cultures were grown on sterile glass coverslips, fixed by method (b), incubated with NBD-phallacidin (100 ul at 1:40), washed and labeled with anti-titin antibodies as for double-antibody labeling. For double antibody labeling, dishes were washed in PBS and stained with a mixture of 100 µl of 1:50 polyclonal anti-titin IgG and either 100 µl of 1:20 monoclonal anti-myosin IgG or 200 µl undiluted monoclonal anti-actin IgM. After incubation at 37°C for 30 min in a humid chamber, cells were washed in several changes of PBS, pH 8.6 for 15 min. Cells were then incubated with a mixture of 100 µl of 1:200 rhodamine-labeled goat-anti-rabbit IgG (ICN Immuno-Biologicals, Lisle, IL) and 75 µl undiluted FITC-labeled goat-anti-mouse IgG (titin-myosin pair), or 100 µl 1:200 FITC-labeled goat-anti-mouse IgG, mu chain specific (Cappel) (titin-actin pair). After washing in PBS, coverslips were mounted in a 9:1 solution of glycerol:PBS containing 1% n-propyl gallate (Giloh and Sedat, 1982).

Cells were examined in a Zeiss Photomicroscope III equipped with epifluorescence optics, using a 63x oil immersion objective. Images were recorded on Kodak Tri-X Pan film developed in D-76.

Myofibrils for immunofluorescence studies were prepared from adult chicken thigh muscle by a modification of Knight and Trinick (1982). Strips of tissue were stretched to 1.2x rest length, tied to wooden sticks, and soaked in Buffer A (100mM NaCl, 2mM KCl, 2mM $MgCl_2$, 3mM EGTA, 6mM potassium phosphate, 0.1% glucose, 2mM NaN_3, 5mM PMSF, and

10mg/ml each of pepstatin, E-64, alpha$_2$ macroglobulin, and trypsin inhibitor) at 4° C for 1 h. Strips were incubated in Buffer A containing 0.5% Triton X-100 for 1 h, in Buffer A without Triton for 1 h, then overnight in fresh Buffer A. Subsequent incubations were in Buffer C (100mM KCl, 10mM potassium phosphate, 5mM EDTA, 1mM DTT, 2mM NaN_3, 5mM PMSF, and 10mg/ml each of pepstatin, E-64, alpha$_2$-macroglobulin, and trypsin inhibitor) at 4° C for 1 h. Strips were placed in fresh Buffer C, minced with scalpel blades, homogenized in a Lourdes Multi-Mixer at setting 50 for 30 s, then centrifuged at 2000 x g for 5 min. The pellet was resuspended in Buffer C. This step was repeated two more times. The final pellet was resuspended in Buffer C, and glycerol was added to 50%. Myofibrils were stored at -20° C. For immunofluorescence, myofibrils were allowed to settle on to glass microscope slides, fixed in -20° C methanol for 6 min, briefly washed in PBS, then incubated with a mixture of actin and titin or myosin and titin antibodies in a humid chamber at 37° C for 30 min. After a PBS wash, myofibrils were incubated with a mixture of rhodamine-labeled goat-anti-rabbit and FITC-labeled goat-anti-mouse second antibody as described for cell culture labeling studies.

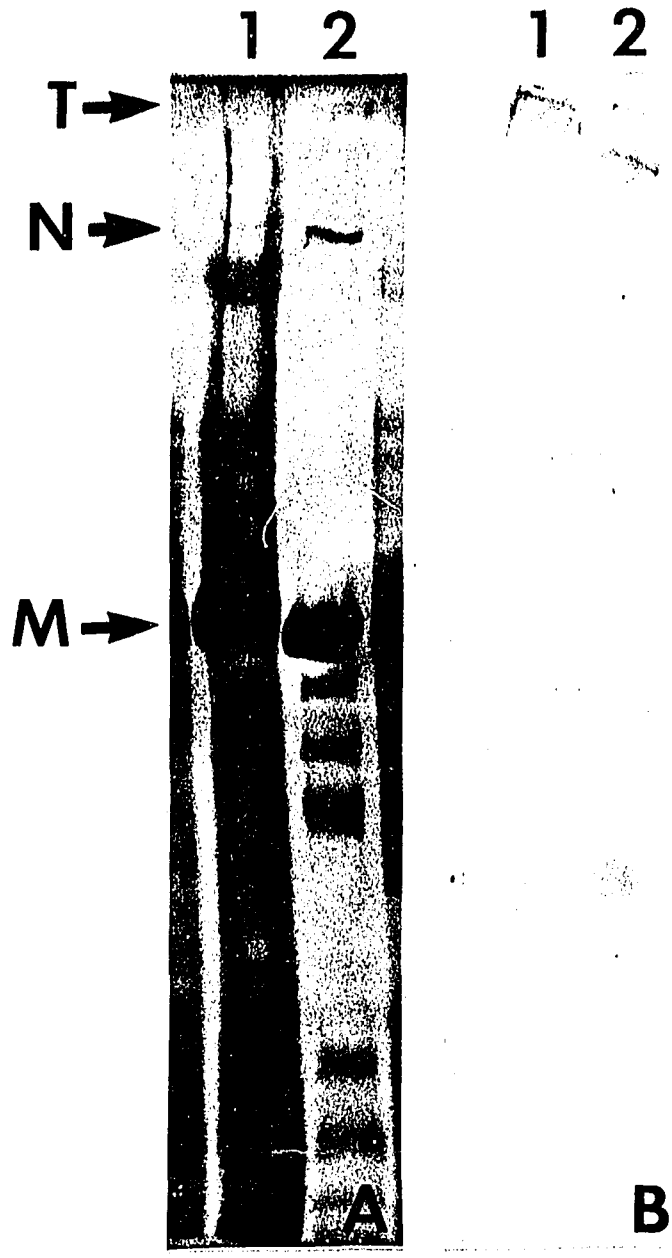
RESULTS

Characterization of antibodies

The antibodies used in this study were characterized by Western blotting techniques and by double-labeling of isolated adult skeletal muscle myofibrils. Zeta-Probe membrane was used as the blotting matrix, and methanol was removed from the buffer in order to improve the transfer efficiency of titin and nebulin. Immunoblotting analysis of the polyclonal titin antibodies (Fig. 1) shows that the antibody recognizes only titin in both embryonic cell culture and adult myofibril homogenates.

The myosin monoclonal antibody used, MF20, is an IgG_{2b} prepared against adult chicken pectoralis myosin heavy chain. It binds to a site in the LMM portion of myosin (Shimizu et al., 1985). Bader et al. (1982) have shown this antibody to be myosin heavy chain-specific in embryonic, neonatal and adult chicken pectoralis, as well as in muscle cell culture derived from embryonic chicken pectoralis muscle by immunoautoradiography. This antibody does not react with the cytoplasmic form of myosin. The actin monoclonal antibody, JLA20, is an IgM prepared against a cytoskeletal protein preparation from chicken gizzard. This antibody reacts with all actin isoforms, and has been shown to label the I bands of mammalian myofibrils by indirect immunofluorescence (Lin et al., 1982). The specificity of the myosin and actin monoclonal antibodies was also checked by immunoblotting as described above for polyclonal anti-titin (results not shown).

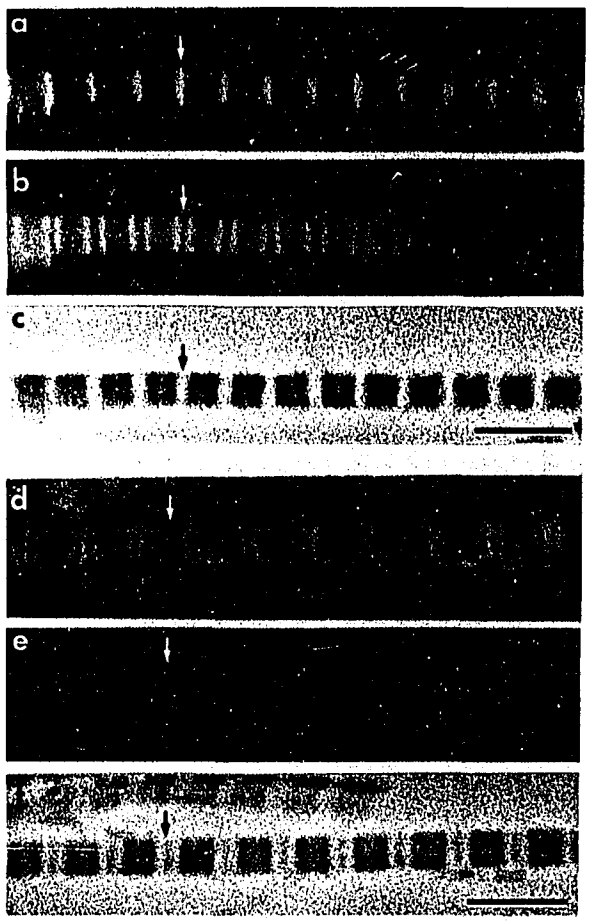
Fig. 1. Immunoblot characterization of polyclonal titin antibodies Duplicate samples of a 96-h embryonic chick myogenic cell culture homogenate (lane 1) and adult chicken thigh muscle myofibrils (lane 2) were separated by SDS-PAGE on a 5% acrylamide gel and transferred electrophorectically to Zeta-probe membrane. Blot A was stained for total protein using a biotin-avidin-HRP staining procedure; blot B was labeled with anti-titin antibodies and HRP-goat-anti-rabbit IgG. The titin antibodies reacted only with the titin in both samples. Positions of titin (T), nebulin (N) and myosin (M) are indicated.


1 2
T
N
M
A
1 2
B

Myofibrils prepared from adult chicken breast muscle were fixed with -20°C methanol prior to double-label immunofluorescence (Fig. 2). Myofibrils were fixed by this method because the majority of the cell cultures in this study were also methanol fixed, and we wished to correlate antibody labeling patterns in cell cultures and in adult myofibrils under the same conditions of fixation. Monoclonal anti-actin antibody labeled the non-thick-filament-overlap region of the I band of intact myofibrils (Fig. 2a). A non-fluorescent line corresponding to the Z line is not seen in I bands labeled with anti-actin antibody. This type of fluorescence labeling pattern was also shown by Lin et al. (1982). The lack of a well-defined Z line may be a consequence of the large size of the IgM actin antibody. Monoclonal anti-myosin antibody labeled the entire A band region (Fig. 2d). The bright band of fluorescence found in the middle of A bands in intact myofibrils is characteristic of the labeling pattern obtained with this antibody (Shimizu et al., 1985). Anti-titin antibody fluorescence is found at the A-I junction (Fig. 2b, 2e), with staining extending into the A bands and I bands themselves. The distance between titin doublet bands on either side of the Z line depends on the state of contraction of the myofibril (compare Fig. 2c and 2f). The labeling patterns of all three antibodies were identical on unfixed, methanol fixed, or paraformaldehyde fixed myofibrils (results not shown).

Fig. 2. Double-immunofluorescence labeling of adult chicken skeletal muscle myofibrils

(a-c) Paired immunofluorescence images of a myofibril double-labeled with monoclonal anti-actin (a) and polyclonal anti-titin (b) antibodies, and phase-contrast image of the same myofibril (c), shows anti-actin staining in the I-band and anti-titin staining primarily at the A-I junction. (d-f) Paired immunofluorescence images of a myofibril double-labeled with monoclonal anti-muscle myosin (d) and polyclonal anti-titin (e), and phase contrast image of the same myofibril again demonstrates that the anti-titin antibodies label at the A-I junction. Arrows indicate the position of Z-lines. Bar, 5μm.


a
b
c
d
e

Actin and titin localization in normal cultures

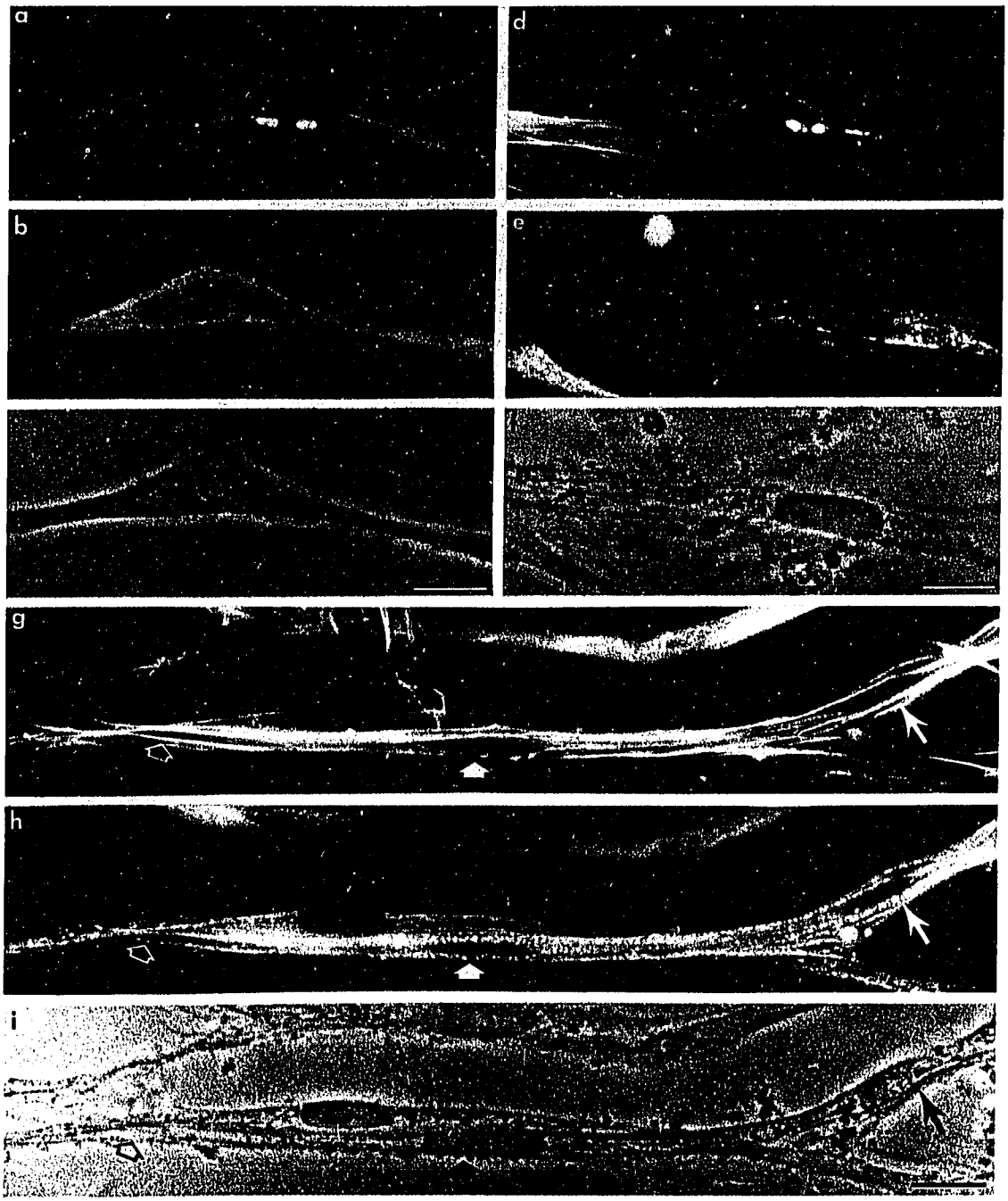
To establish the sequence of assembly of titin filaments into the myofibril with respect to assembly of actin filaments into I bands, myogenic cells derived from 12-d chick embryos were cultured for 1-4 d, fixed, and labeled with anti-titin and anti-actin (or NBD-phallacidin). By using this primary culture system (Ridpath et al., 1984), the majority of fusion occurs between 2- and 3-d in culture. Thus, 2-d cultures are primarily myoblasts and early myotubes, 3-d cultures are a mixture of myoblasts and myotubes, and 4-d cultures contain more mature myotubes. Typical results of titin and actin labeling on 2-d cultures are shown in Fig. 3. Some myoblasts (presumably postmitotic myoblasts) and all myotubes in these cultures are labeled with the anti-titin, initially in a diffuse pattern throughout the cell (myotube in Fig. 3a-c; myoblast in lower left of Fig. 3d, e). In immature myotubes, titin staining (Fig. 3b, e, h) is most intense along actin bundles located either near the sarcolemma or the nucleus (Fig. 3a, d, g). These actin bundles or cables normally run parallel to the long axis of the cell and appear morphologically identical to the stress-fiber-like-structures (SFLS) described previously in developing myotubes by Holtzer and co-workers (Dlugosz et al., 1984; Holtzer et al., 1985; Antin et al., 1986). Thus, the term SFLS will be used subsequently to refer to these actin-containing fibers. The relative amount of diffuse titin labeling appears to be inversely related to the amount of titin labeling along SFLS, with more titin located along SFLS in more elongated myotubes (compare Fig. 3b with Fig. 3e and h). Thus, the first step in

organization of titin appears to be the localization in a continuous pattern along SFLS. A cell that appears to be a fibroblast in Fig. 3d, e is labeled with anti-actin in a typical filamentous pattern, and is negative for titin labeling. Also, several round structures associated with the cell membrane exhibit bright fluorescence with anti-actin (Fig. 3a, d) or NBD-phallacidin (Fig. 3g), but not with anti-titin. These actin-containing structures appear to be "macules" (Denning and Fulton, 1985; Denning et al., 1988), structures that contain primarily cytoplasmic isoforms of actin that are discarded by muscle during early stages of myofibrillogenesis.

At least three different patterns of titin localization along SFLS can be seen in different myotubes and even within the same myotube in Fig. 3. The first is a continuous labeling of the entire actin-containing fiber. The second is a series of dots or broad bands in a punctate pattern (Fig. 3e, h), and the third is a banded pattern similar to the pattern seen with labeling of adult muscle myofibrils (Fig. 3h), i.e., as a series of double bands. A single SFLS that exhibits continuous actin labeling in Fig. 3g shows titin localization in both a punctate pattern (open arrow, Fig. 3h) and a double-banded pattern (closed arrow, Fig. 3h). Nascent myofibrils with distinct I bands as seen by actin labeling and titin bands are found within this same myotube. Actin organization into I bands is always accompanied by organization of titin in an adult-like banding pattern, but titin can be organized into punctate or banded patterns along SFLS or nascent myofibrils that exhibit a continuous pattern of actin labeling.

Fig. 3. Double-immunofluorescence localization of actin and titin in early myotubes in normal embryonic chick myogenic cell culture

Immunofluorescence images showing localization of actin (a, d, g), with either monoclonal anti-actin (a) or NBD-phallacidin (d, g), and titin (b, e, h), with polyclonal anti-titin. Phase-contrast images are shown in (c, f, i). (a-c) Very early myotube in a 1-d culture. Titin staining (b) is distributed diffusely throughout the cytoplasm and is most intense along actin-containing filamentous structures located near the cell nuclei (a). (d-f) Cells in a 2-d culture, showing titin (e) in a punctate pattern along actin cables near the cell periphery (d). In the lower left, a cell exhibits a diffuse titin staining pattern (e) but shows a fibrous distribution of actin (d). Actin-containing structures ("macules") are visible in both (a) and (d), but are not labeled with anti-titin (b, d). (g-i) Myotubes in a 2-d culture showing several stages of myofibrillogenesis. Wide, open arrows indicate an actin cable (g) labeled in a punctate pattern with anti-titin (h). Wide, closed arrows point to an area in which titin is organized in a distinct double-banded pattern (h), but actin remains in a fiber-like pattern (g). Narrow arrows indicate a nascent myofibril with both discreet actin (g) and titin (h) banding. Bar, 10 μm.

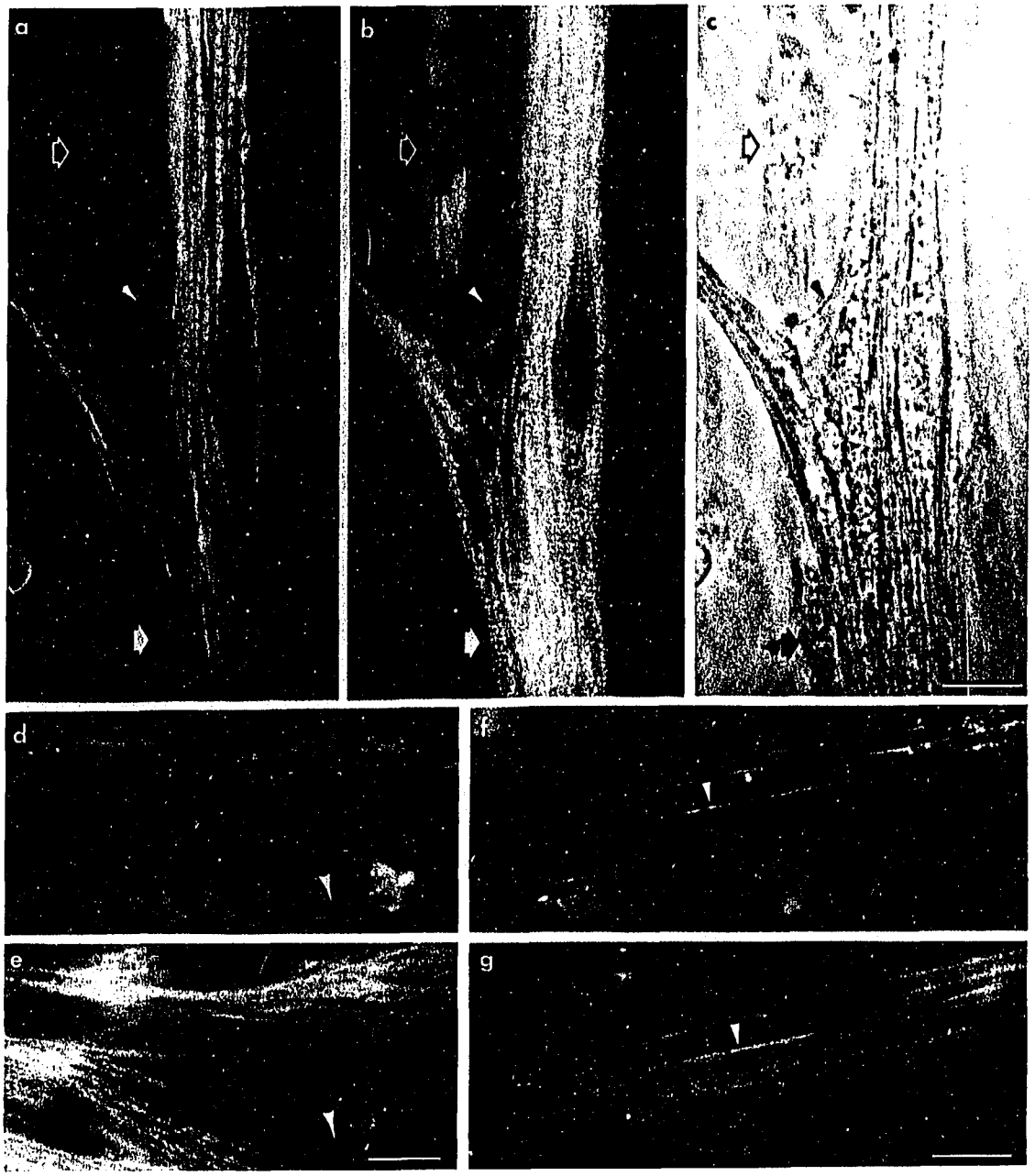

a
d
b
e
g
h
i

Localization of actin and titin in more mature myotubes in 3- and 4-d cultures more clearly shows the progression of titin organization from a continuous pattern to a punctate pattern and finally to a banded pattern along continuous actin bundles that appear to be SFLS. The myotubes in Fig. 4d, e and f, g show many SFLS. Some of these SFLS are labeled in a continuous pattern with both anti-actin and anti-titin, indicating an equivalent state of organization for both proteins and suggesting that these fibers represent an early stage in myofibril assembly. Other fibers show titin periodicity along the actin bundles, indicating a more advanced stage of organization. Fig. 4a-c shows a mononucleated myoblast that appears to be in the process of fusing to the adjacent large myotube. In the myoblast, titin is localized in either a diffuse pattern or along actin-containing SFLS, while the myotube contains predominantly nascent myofibrils. Thus, this image provides additional evidence for the progression of titin organization along SFLS.

Actin and titin localization in EMS-treated cultures

Myogenic cells were cultured in the presence of the alkylating mutagenic agent EMS for 3-d, then allowed to recover in regular medium. The relatively protracted period of myofibril assembly in EMS-treated cells as compared to normally cultured myogenic cells (Dlugosz et al., 1984; Antin et al., 1986) allows for a more careful examination of the temporal sequence of actin and titin assembly. Results of double-label immunofluorescence experiments are shown for cultures allowed to recover

Fig. 4. Double-immunofluorescence localization of actin and titin in 3- and 4-d normal embryonic chick myogenic cell cultures (a-c) Immunofluorescence images of a 3-d culture double-labeled with anti-actin (a) and anti-titin (b) antibodies and the corresponding phase-contrast image (c). Open, wide arrows indicate a mononucleated myoblast that has not yet fused with the nearby myotube; this cell is labeled in a largely diffuse pattern with anti-titin (b) and a more fibrous pattern with anti-actin (a). Many nascent myofibrils are visible in the myotube itself; wide, closed arrows point to a portion of the cell where both actin (a) and titin (b) are arranged in a myofibril-like pattern. The small arrowhead points to an actin fiber (a) that shows a punctate, "beads-on-a-string" arrangement of titin (b). Other myotubes in a 4-d culture (d, e) and a 3-d culture (f, g) labeled with anti-actin (d, f) and anti-titin (e, g) show a number of stress-fiber-like structures that are labeled with both antibodies. Large arrowheads (d, e) and small arrowheads (f, g) point to fibers in which titin organization appears to be more advanced than actin organization. Bar, 10 μm.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for 2 d (Fig. 5a-d), 3 d (Fig. 5e-g; Fig. 6a, b and e, f) or for 4 d (Fig. 6c, d). In initial stages of recovery both titin and actin are localized in a continuous pattern along variable diameter SFLS (Fig. 5a, b; Fig. 6a, b). In Fig. 5c, d however, while anti-titin antibodies now label in a punctate pattern, actin still appears to be at the same level of organization as the SFLS in Fig. 5a, b.

Many stages of myofibril assembly are found in a single myosheet at all times of recovery in normal medium. With increasing recovery time, the total number of SFLS in an individual myosheet decreases, and the number of striated myofibrils increases, in agreement with the observations of Holtzer and co-workers (Dlugosz et al., 1984; Antin et al., 1986). Another characteristic of recovering myosheets is the increased state of myofibrillar organization from the outside of the cell to the inside, illustrated in Fig. 5e-g. In this myosheet anti-titin antibody labeling patterns include nonstriated SFLS, a series of evenly spaced dots (single arrow), and a series of evenly spaced bands (double arrows). Actin is found predominately in a cable pattern (single arrow), although the double arrows point to an area on an actin cable where the staining is more punctate.

The progression of titin organization is marked by the appearance of a punctate or "beads on a string" labeling pattern in fibers whose diameters are similar to those of the continuously labeled SFLS (single arrowhead in Fig. 5f, double arrowheads in Fig. 6d). In relatively larger diameter nascent myofibrils, titin exists first as a series of evenly spaced fluorescent bands (double arrowheads in Fig. 5f; open

Fig. 5. Double-immunofluorescence localization of actin and titin in EMS-treated embryonic chick myogenic cell cultures Cultures were allowed to recover in normal medium for 2 (a, b and c, d) or 3 d (e-g), and labeled with anti-actin and anti-titin. Micrographs show localization of actin (a, c, e) and titin (b, d, f); (g) is phase-contrast image corresponding to (e, f). In myosheets allowed to recover for 2 d, titin is localized primarily along actin-containing fibers. The fiber indicated by the open arrows in (a, b) shows apparently equivalent levels of organization of both proteins, while the fiber indicated by the closed arrows in (c, d) shows titin in a periodic, punctate pattern (d) along an actin cable (c). In the myosheet allowed to recover for 3 d (e-g), the fiber indicated by the single arrowhead shows titin (f) in a periodic pattern along an actin cable (e), although interruptions in the actin staining suggest that actin organization has begun. In the nascent myofibril indicated by the double arrowheads, titin (f) is localized in a series of evenly spaced bands, rather than an adult-like pattern of double bands; initial organization of actin into I-bands (e) is also apparent although myofibrillar organization is not evident in the phase contrast image (g). Bar, 10 μm.

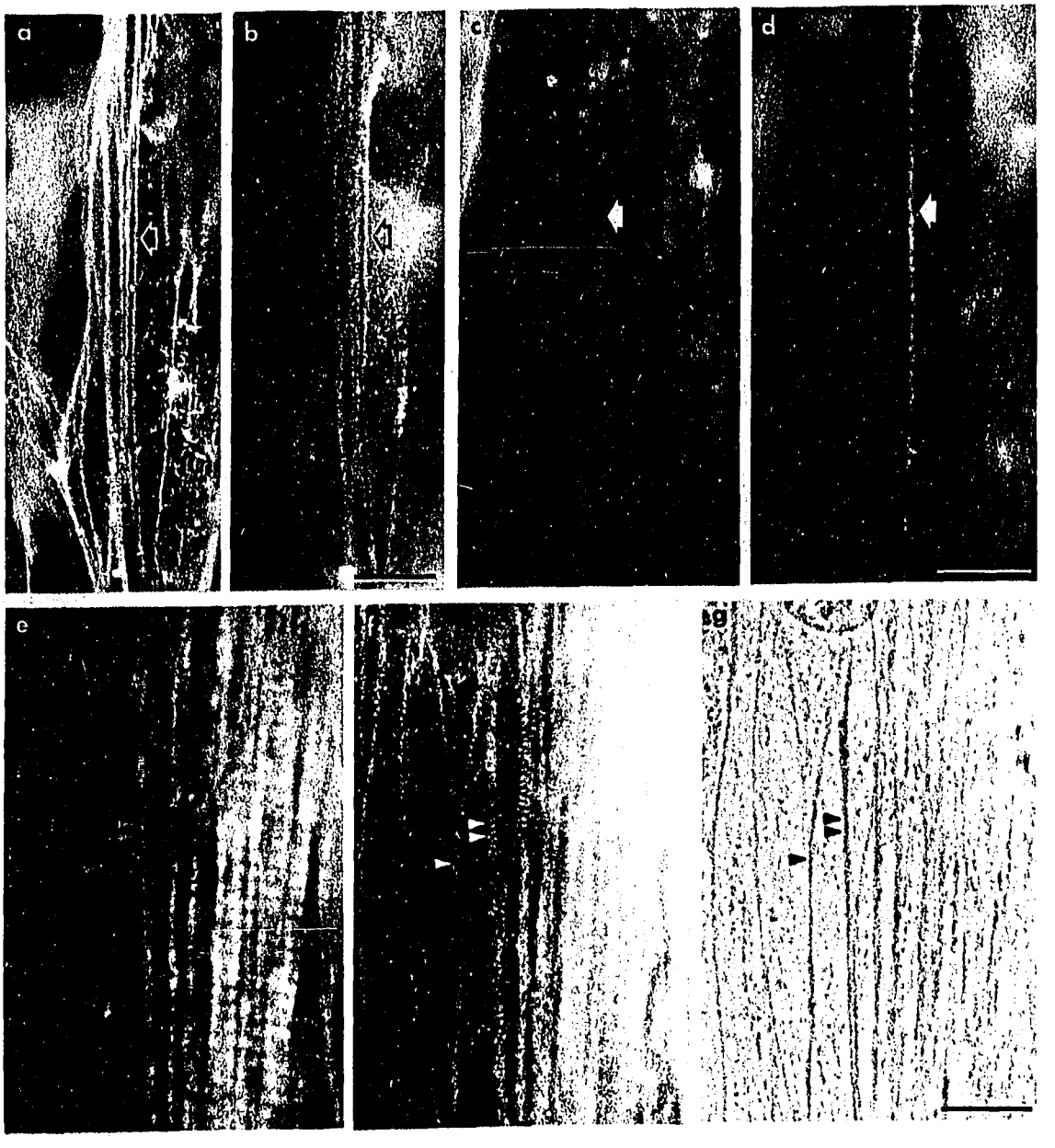
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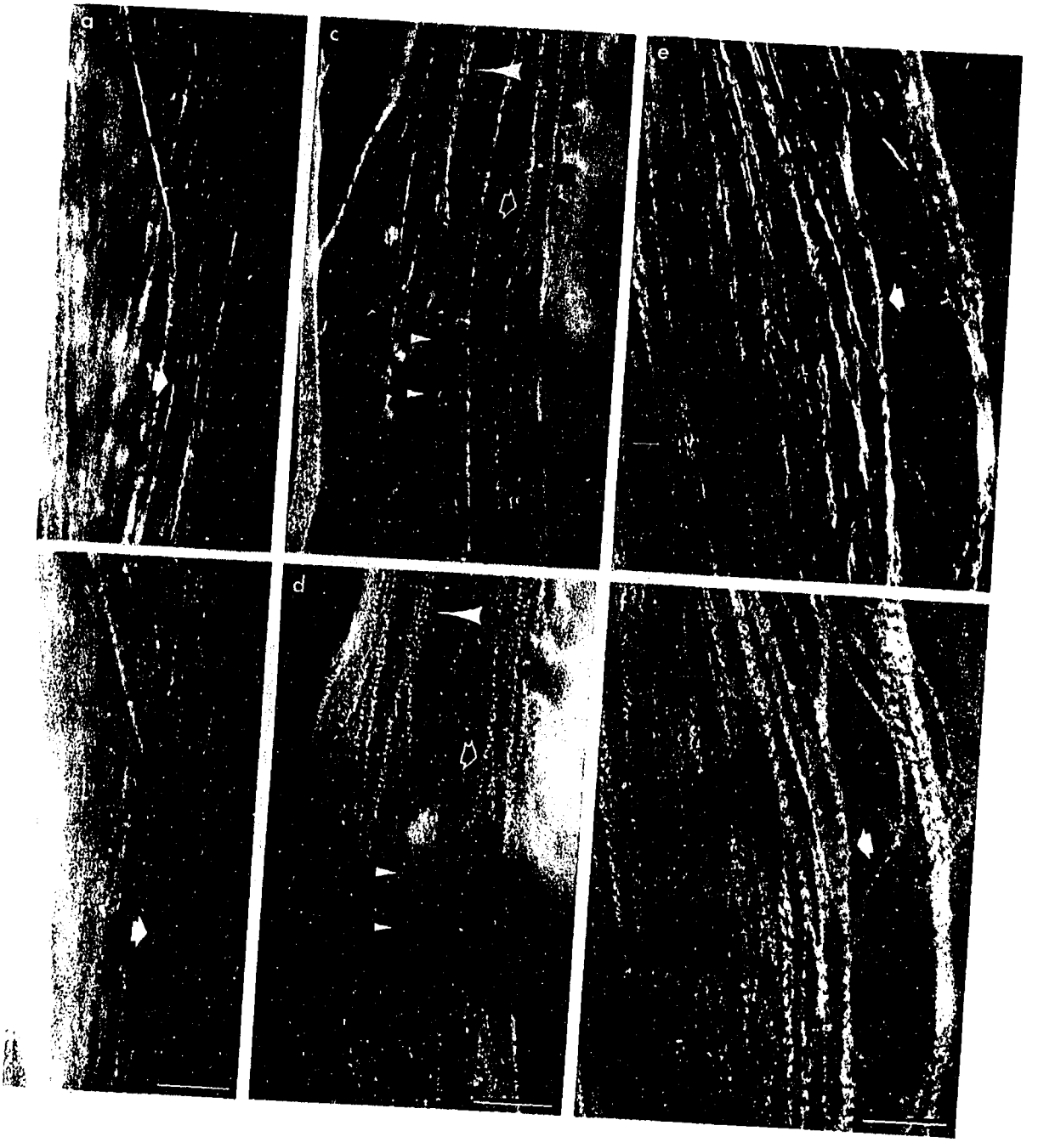
Fig. 6. Double-immunofluorescence localization of actin and titin in EMS-treated embryonic chick myogenic cell cultures Paired immunofluorescence micrographs showing localization of actin (a, c, e), with anti-actin, and titin (b, d, f), using anti-titin, in cultures treated with EMS and allowed to recover in normal medium for 3 d (a and b, e and f) or 4 d (c and d). Wide arrow in (a, b) indicates the filamentous localization of titin (b) along an actin cable (a), typical of early stages in myofibril assembly. The myosheet in (c, d) shows several stages in myofibrillogenesis: small arrowheads show titin in a periodic arrangement (d) along an actin cable (c); open arrows show titin in evenly spaced bands (d) along an actin fiber that is begining to exhibit I-bands (c); and large, closed arrowheads show a more mature myofibril that exhibits an unusual extra band of titin fluorescence apparently at the level of the Z-line (d). The myosheet in (e, f) also shows several stages of assembly; wide closed arrows indicate an adult-like pattern of titin fluorescence (f) along a nascent myfibril that is just beginning to exhibit arrangement of actin into I-bands. Bar, 10 μm.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arrow in Fig. 6d), then in an adult-like double-banded arrangement (large closed arrowhead in Fig. 6d; closed arrow in Fig. 6f). While cases of titin organization into either a punctate pattern (Fig. 5c, d) or into a discreet banded pattern (Fig. 6e, f) along actin SFLS can be found, the opposite situation of actin banding along a titin SFLS is not seen. Where actin shows labeling in distinct I bands, titin is localized at the A-I junction.

In recovering myosheets, we have observed that many of the SFLS appear to branch. In some cases two fibers are in close apposition (Fig. 5g) while in others several fibers seem to combine to form a larger diameter fiber. The nascent myofibril indicated by the large closed arrowhead in Fig. 6d is interesting for two reasons. First, it appears to arise from the coalescence of several narrow diameter fibers. Second, an extra band of titin fluorescence can be seen at the level of the Z line. This extra band can be seen more easily in Fig. 7. This band is found relatively frequently in mature myofibrils in myosheets recovering from EMS treatment, but is seen very rarely in normal myotubes.

Myosin and titin localization in normal cultures

In a second series of experiments, myogenic cell cultures were double-labeled with anti-titin and anti-muscle-myosin antibodies to follow the temporal organization of titin filaments with respect to the organization of myosin-containing thick filaments into A bands. Fluorescence images of 1-d cultures (Fig. 8) illustrate myosin and titin

Fig. 7. Double-immunofluorescence localization of actin and titin in an EMS-treated culture allowed to recover for 4 d in normal medium

Fluorescence images of anti-actin (a) and anti-titin (b) localization and corresponding phase-contrast image. Fibers indicated at arrows show presence of titin banding (b) in the absence of discreet actin banding (a). The anti-titin localization (b) also demonstrates the presence of an "extra" band of titin labeling, both in the nascent myofibrils indicated by arrows and at the Z-line in the more mature myofibril located at the bottom of the micrograph. Bar, 10 μm.

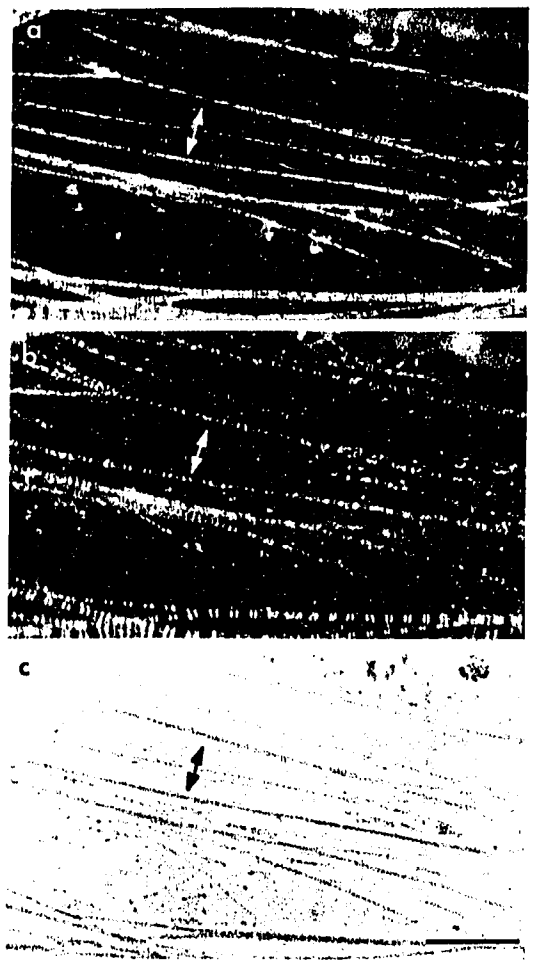
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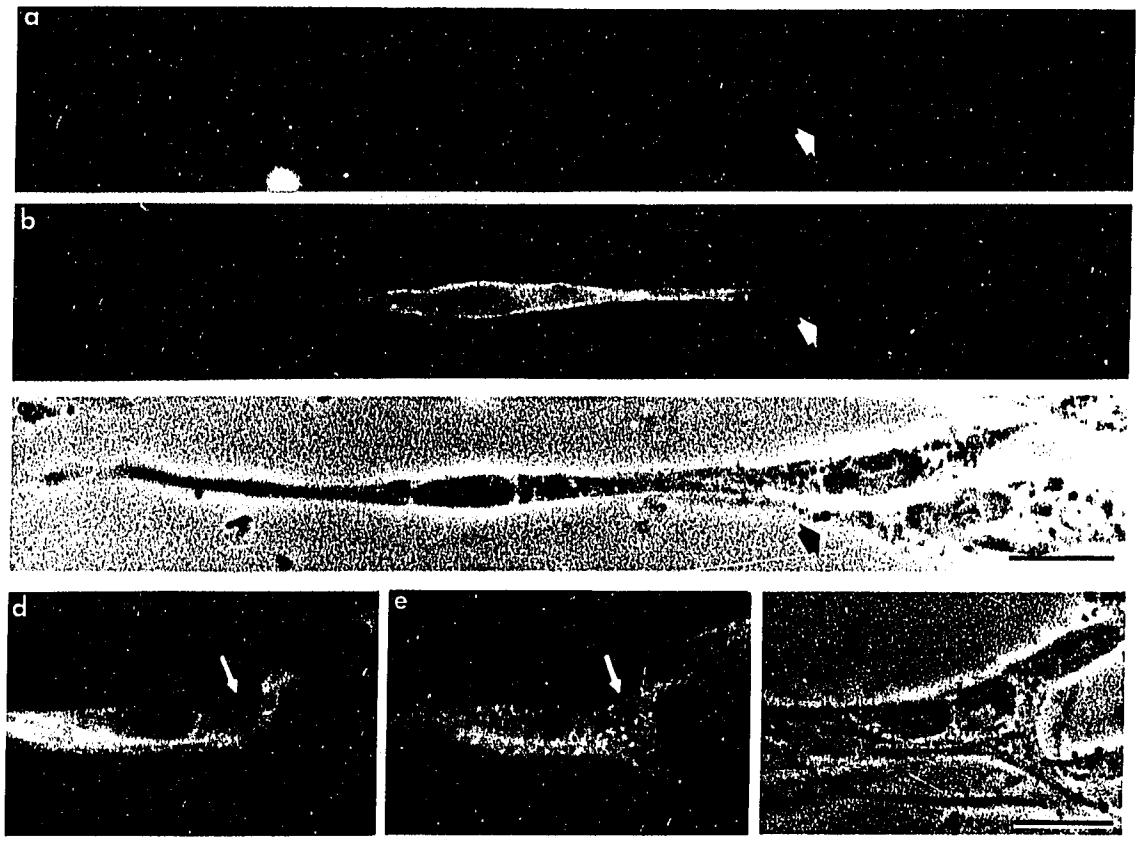
Fig. 8. Double-immunofluorescence localization of myosin and titin in 1-d normal embryonic chick myogenic cell cultures Paired fluorescence images of anti-myosin (a, d) and anti-titin (b, e) staining and corresponding phase-contrast images (c, f). In (a-c) three myoblasts are fusing to form a myotube. The elongated cell in the center shows diffuse labeling with both anti-myosin (a) and anti-titin (b), with a brighter line of fluorescence along the edge of the cell nearest the top of the picture; cells indicated by arrows are only faintly labeled with anti-titin and are not labeled with anti-muscle myosin. The early myotube in (d-f) shows a punctate pattern of titin labeling (e) that corresponds to a more filamentous pattern of myosin fluorescence. Bar, 10 μm.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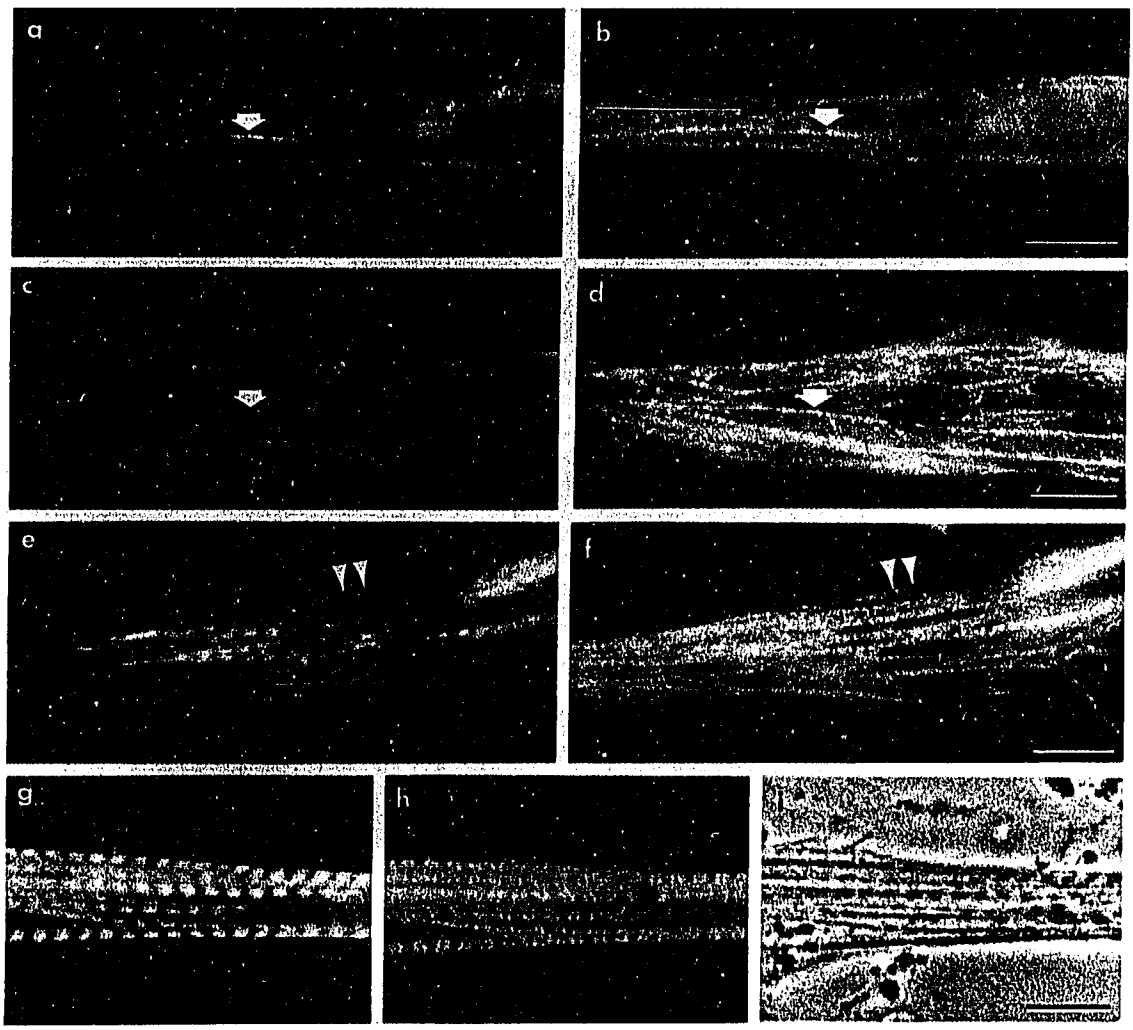
localization in a diffuse pattern in mononucleated myoblasts (Fig. 8a-c). The intensity of staining often appears brighter along the edges of mononucleated cells than within the cytoplasm. The three myoblasts in Fig. 8a-c have begun to align and fuse. While the myoblast in the center of this figure is both myosin and titin positive, the adjacent two myoblasts show much less labeling with both myosin and titin. Fig. 8d-f shows an immature myotube formed from the fusion of three myoblasts. In a small proportion of cells such as this one, anti-titin antibody labels in a punctate pattern along a continuous distribution of myosin.

The micrographs of normal cultures of 3-d (Fig. 9a-f) and 4-d (Fig. 9g-i) myotubes demonstrate the temporal sequence of myosin organization with respect to titin. In myotubes typically found in older, normal cultures, anti-titin labels in a nonstriated (closed arrows in Fig. 9a-b), or a punctate, "beaded" pattern (Fig. 9c-d), but myosin is arranged in bands. While titin periodicity is evident along the nascent myofibril shown in Fig. 9f (double arrowheads), indicating a more advanced state of organization relative to the organizational states shown in Fig. 9a, b and Fig. 9c, d, anti-myosin antibodies label the same myofibril in nearly adult-like bands. Adult-like banded patterns of both myosin and titin are found in mature myotubes containing striated myofibrils identifiable by phase contrast microscpy (Fig. 9g-i). When both titin and myosin are in mature, banded patterns, titin labels the A-I junction.

Fig. 9. Double-immunofluorescence localization of myosin and titin in 3-d and 4-d normal embryonic chick myogenic cell cultures

(a-f) Paired immunofluorescence images of myotubes in 3-d cultures showing localization of muscle myosin (a, c, e) and titin (b, d, f). Closed arrows in (a-d) indicate nascent myofibrils in which muscle myosin (a, c) is localized in a banded pattern while titin is arranged in a filamentous pattern (b) or a beaded pattern (d). Double arrows in (e, f) indicate that myosin (e) has advanced to a nearly adult-like organizational pattern, but titin (f) has remained in a beaded array. (g-i) Fluorescence images of anti-myosin (g) and anti-titin (h) labeling and corresponding phase-contrast image (i) of a myotube in a 4-d culture. In this cell, both proteins exhibit adult-like patterns in more mature myofibrils. Bar, 10 μm.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Myosin and titin localization in EMS-treated cultures

As for EMS-treated cultures double-labeled with anti-actin and anti-titin antibodies, myogenic cells were cultured in the presence of EMS for 3-d and allowed to recover in normal medium. Localization of myosin and titin is shown in cultures after 2-d (Fig. 10a, b) and 3-d (Fig. 10c, d and e, f) of recovery. As in normal cultures, the initial organizational state of myosin and titin is a nonstriated, filamentous distribution (Fig. 10a, b and c, d). Patches of brighter myosin and titin fluorescence (Fig. 10a, b) visible along SFLS may indicate areas of initial organization of these two proteins. These myosin and titin containing patches appear to coincide. In myosheets in early stages of recovery, SFLS are abundant and run parallel to the long axis of the cell, as illustrated in Fig. 10a-f. The particular fiber indicated by the arrow in Fig. 10e, f shows myosin organization into distinct A bands, but titin is still distributed in a nonstriated pattern. The portion of the myosheet in Fig. 10g, h demonstrates a staining pattern typical of all three of the antibodies used. Bright, diffuse staining around groups of nuclei is seen at all stages of myosheet recovery from EMS treatment, but is especially prevalent at early stages of recovery.

Myosheets at late stages of recovery; 5-d (Fig. 11d-f) and 6-d (Fig. 11a-c) are illustrated. Myosin is localized to adult-like A bands in the two nascent myofibrils indicated by small arrowheads, and in the myofibril indicated by the large arrowhead in Fig. 11a-c. At the large arrowhead, A bands show the bright fluorescence at the M line seen with anti-myosin antibody staining of adult isolated myofibrils

Fig. 10. Double-immunofluorescence localization of myosin and titin in EMS treated embryonic chick myogenic cell cultures at early stages of recovery

Paired fluorescence images showing localization of myosin (a, c, e, g) and titin (b, d, f, h) in EMS-treated cultures. (a, b) Myosheet after 2 d of recovery in normal medium; both myosin and titin are localized in diffuse patterns in the cytoplasm and along fiber-like structures that run parallel to the long axis of the myosheet. (c, d; e, f) Myosheets after 3 d of recovery, showing arrangement of both proteins in longitudinal fibers. Fiber indicated by arrow in (e, f) shows organization of muscle myosin into A-bands (e), while titin (f) remains in a more filamentous arrangement. (g, h) Myosheet after 4 d of recovery, demonstrating bright, diffuse staining around the nuclei characteristic for all antibodies used, especially at early times of recovery from EMS treatment. Bar, 10 μm.

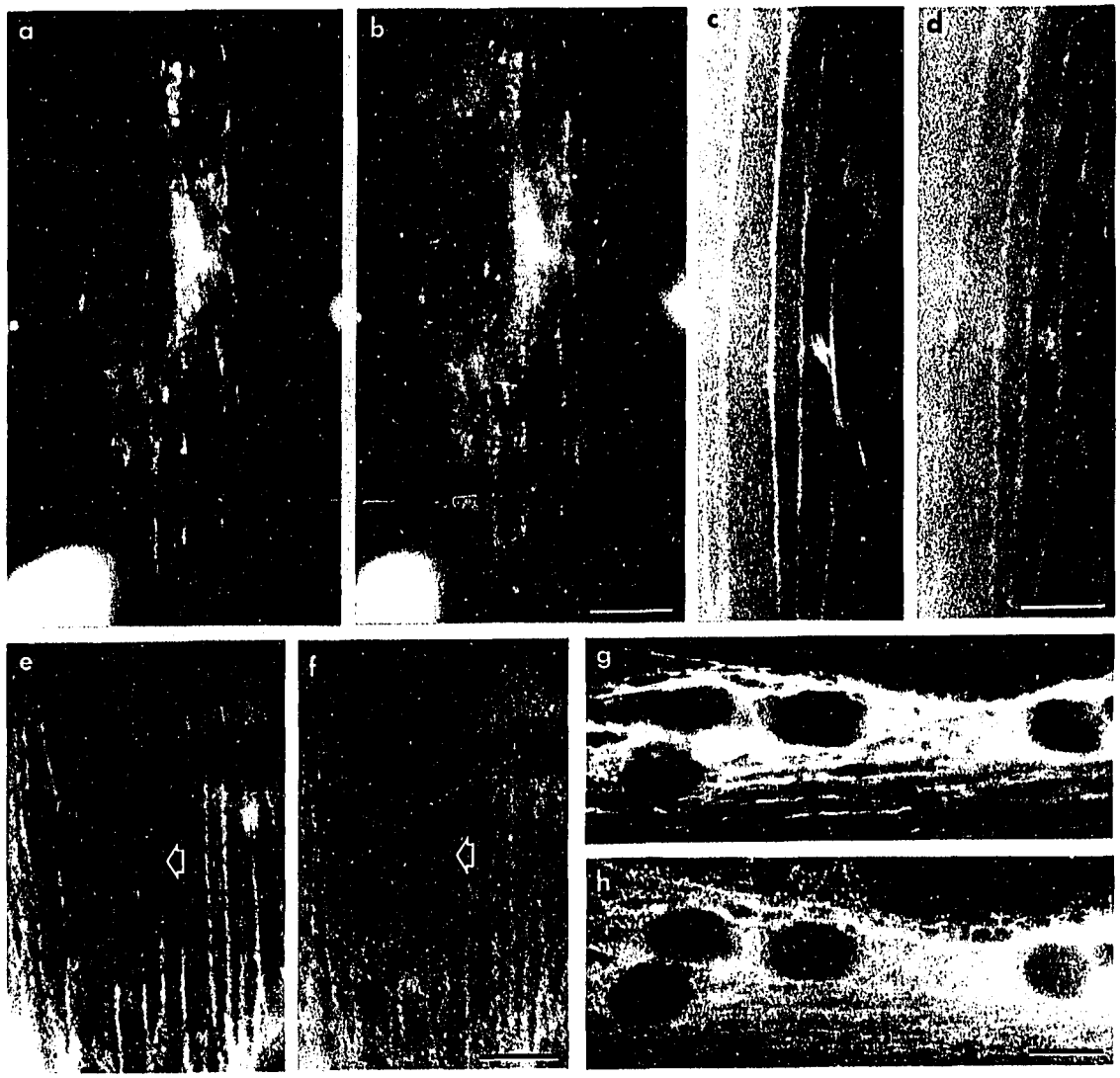
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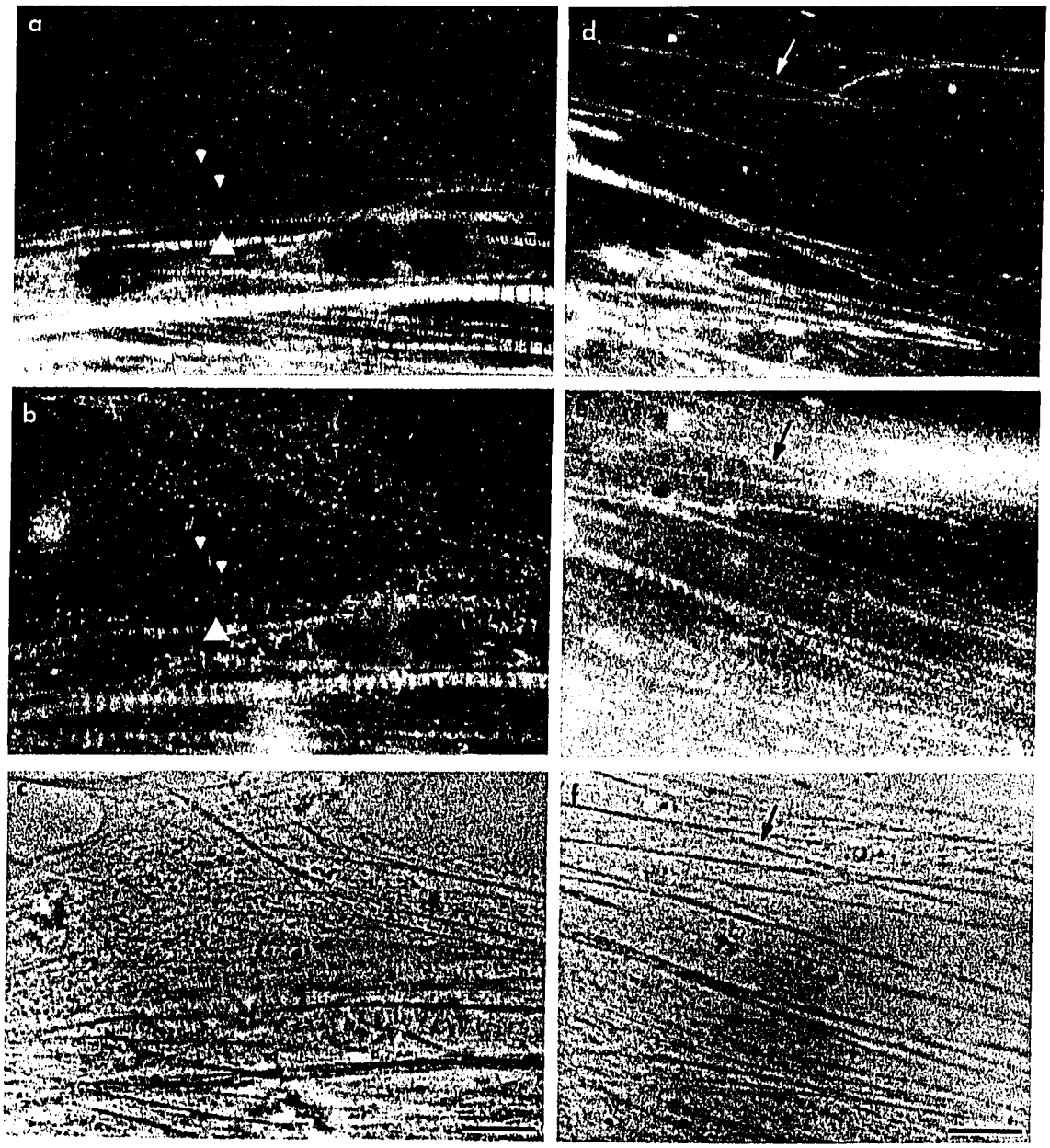
Fig. 11. Double-immunofluorescence localization of myosin and titin in EMS-treated chick myogenic cell cultures at later stages of recovery in normal medium
Fluorescence images of anti-myosin (a, d) and anti-titin (b, e) localization and corresponding phase-contrast images (c, f). (a-c) Myosheet allowed to recover from EMS treatment for 6 d in normal medium, showing many apparently mature, banded myofibrils as well as some myofibrils at early stages of assembly. The smaller diameter, nascent myofibrils indicated by the small arrowheads show organization of myosin into discreet A-bands (a), while the anti-titin labeling exhibits either a punctate pattern or a series of evenly-spaced bands (b). The larger myofibril indicated by the large arrowheads exhibits both anti-myosin and anti-titin labeling in a pattern identical to that seen in adult myofibrils (Fig. 2). (d-f) Myosheet after recovery for 5 d in normal medium, showing several stress-fiber-like structures, one of which is indicated by the arrow, as well as several more mature myofibrils. Bar, 10 μm.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(Shimizu et al., 1985). At the small arrowheads in Fig. 11b, anti-titin antibodies label the nascent myofibril in a punctate pattern or in a series of evenly spaced bands. The larger myofibril at the large arrowhead, however, does show adult-like titin banding.

As described earlier, titin organization seems to progress through a series of four stages. This does not seem to be the case for organization of myosin into A bands. In our normal cultures and EMS recovering myosheets, the only organizational states detected are either a nonstriated filamentous one (Fig. 10c; Fig. 11d, arrows) or a distinct, adult-like banded one (Fig. 10e; Fig. 11a; Fig. 11d).

DISCUSSION

Double-label immunofluorescence localization of muscle-specific myosin, actin and titin using normal and EMS-treated embryonic chick myogenic cell cultures has allowed us to determine the organizational states of titin, and its temporal sequence of assembly with respect to thick and thin filament organization in the striated muscle myofibril. There appears to be four distinct organizational states of titin as determined by antibody labeling patterns. Anti-titin first localizes in continuous patterns along narrow diameter SFLS, and then as a series of dots or broad bands along narrow diameter fibers. As the diameter of nascent myofibrils increases, the localization pattern changes to a series of evenly spaced narrow bands, and finally to the double-banded pattern seen in adult myofibrils.

Titin (Hill and Weber, 1986), myosin, and actin (Emerson and Beckner, 1975; Devlin and Emerson, 1978) are expressed in apparently postmitotic, mononucleated myoblasts. In both normal and EMS-treated cultures of embryonic chick skeletal muscle, however, we detected a definite temporal sequence of assembly of these three proteins. Titin organization appeared to occur in close coordination, and probably slightly after the organization of myosin into A bands. In the vast majority of cells examined, titin banding was not seen in the absence of myosin banding. Titin and myosin organization are then followed by actin organization into I bands. Hill and Weber (1986) labeled chick embryo skeletal muscle cell cultures with a panel of monoclonal antibodies specific for titin. They found that titin first localized as

longitudinal fibers in postmitotic, mononucleated myoblasts before mature myofibrils could be seen. In mature myotubes, titin was found at the A-I junction of myofibrils. Immunofluorescence analysis of cultured rat leg muscle with monoclonal antibodies to titin reveals that titin periodicity sometimes precedes that of myosin and actin (Wang et al., 1984). A more detailed study by Hill et al. (1986) suggests, however, that titin appears simultaneously with myosin heavy chain, for in both normal and Colcemid- or taxol-treated, postmitotic, mononucleated myoblasts, titin staining was not seen in the absence of myosin heavy chain staining. Titin and myosin heavy chain first localized to nonstriated, then to striated myofibrils. They could not exclude the possibility that titin did organize before myosin, but in such a short time frame that it was not detected by light microscope techniques. In EMS-treated cultures, myofibrils assemble over a longer period of time than in regular cultures. In this culture system, we also did not see evidence of titin organization preceding that of myosin. Although titin and myosin organization appeared to be coupled, myosin organization normally occurred before titin. In cultures double-labeled with anti-titin and anti-actin, we have consistently observed the appearance of titin double bands before the appearance of I bands, suggesting that titin organization occurs before organization of actin-containing thin filaments into I bands.

One proposed role for titin has been that of a scaffold, or template, for myofibril assembly (Hill and Weber, 1986). It is becoming clear, through our work and the work of Hill et al., that titin does not

serve such a role, at least in the case of myosin filament assembly. It is possible that myosin- containing thick filaments and titin-containing filaments bind and are organized together into mature A bands, but our studies demonstrate that titin filaments are not aligned first, with subsequent thick filament interdigitation into the titin filament network. Titin filaments may play a scaffolding role for thin filament assembly, since we found that titin organization precedes that of actin.

One putative role for titin filaments is the attachment of thick filaments to Z lines (Wang, 1985; Maruyama, 1986; Horowits and Podolsky, 1987). The close temporal coordination of myosin and titin assembly might fit with this hypothesis. Indeed, evidence exists for an *in vitro* association between titin and myosin (Kimura and Maruyama, 1983) and titin and actin (Kimura et al., 1984). Connectin (titin) was found to cause aggregation of myosin filaments at 50-120mM KCl at pH 7.0, and to cause actin filament bundles in the presence of KCl up to 0.15M. Recently, it has been suggested that a large fraction of newly synthesized titin is in direct association with myosin heavy chain, even early in development (Issacs et al., 1987). It is possible, then, that titin could aid in the alignment of myosin-containing thick filaments in the A band.

We also occasionally observed titin labeling at the Z line when myosin is organized into A bands. This may be due to exposure of a particular epitope for a very brief time at this stage of myofibrillogenesis. Alternatively, this could be an artifact due to specimen preparation.

We have found that myosin, titin, and actin initially localize to narrow, nonstriated filaments, which, by phase contrast microscopy, are morphologically identical to the SFLS described by Holtzer and co-workers (Dlugosz et al., 1984; Antin et al., 1986). The precise nature of the relationship between developing myofibrils and SFLS in skeletal muscle cells has been a source of controversy. Peng et al. (1981) studied myofibril assembly in cultured myotome cells from Xenopus embryos. They observed the formation of sarcomeres on preexisting thin filament bundles, suggesting that microfilaments are myofibril precursors. Sanger et al. (1986) have examined myofibrillogenesis by using antibodies to alpha-actinin and have concluded that fibrils found in young myotubes, at the ends of older myotubes, and near the periphery of cardiac myocytes that exhibit a stress-fiber-like punctate alpha-actinin labeling pattern are, in fact, nascent myofibrils.

Holtzer and co-workers, however, suggest that SFLS serve as a scaffold for assembly of nascent myofibrils, which disappear as myofibrils mature (Dlugosz et al., 1984; Antin et al., 1986). They base this view on the facts that SFLS and mature, striated myofibrils are mutually exclusive in both cardiac and EMS-treated skeletal muscle cells in culture. Also, both non muscle and muscle-specific forms of myosin co-localize along fibers during myogenic cell maturation. Others have reported co-localization of nonmuscle and muscle isoforms of myofibrillar proteins along microfilaments (Lin and Lin, 1986) and stress fibers (Fallon and Nachmias, 1980).

Since we did not employ antibodies that recognized nonmuscle

isoforms of myosin or actin and nonmuscle forms of titin do not exist, we cannot confirm or dispute existing reports of co-localization of nonmuscle and muscle isoforms along SFLS in cultured myogenic cells. We have observed, however, that the appearance of SFLS and mature, striated myofibrils within a given myogenic cell are mutually exclusive. The initial event of myofibril assembly appears to be the coalescence of myofibrillar proteins along SFLS, evidenced in this report by the observation that muscle myosin and titin first organize along nonstriated filamentous structures. Many times, antibodies localized in both striated and nonstriated patterns along the same SFLS (Fig. 3g-i; Fig. 4a-c; Fig. 5e-g). We saw no evidence of the existence of a series of mini-sarcomeres which subsequently expand to form sarcomeres of adult size (Sanger et al., 1984). Thus, our observations on the assembly of muscle myosin, titin and actin appear most compatible with those of Holtzer and co-workers, who suggest that myofibrillar protein organization and the appearance of discreet bands takes place along SFLS.

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SECTION II. IMMUNOFLUORESCENCE LOCALIZATION STUDIES ON THE ROLE OF NEBULIN IN MYOFIBRIL ASSEMBLY IN EMBRYONIC CHICK SKELETAL MUSCLE CELLS GROWN *IN VITRO*

IMMUNOFLUORESCENCE LOCALIZATION STUDIES ON THE ROLE OF NEBULIN IN MYOFIBRIL ASSEMBLY IN EMBRYONIC CHICK SKELETAL MUSCLE CELLS GROWN *IN VITRO*

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FOOTNOTES

[1] Abbreviations used in this paper: SFLS, stress-fiber-like-structures; EMS, ethyl methanesulfonate.

ABSTRACT

Nebulin is a high-molecular weight (M_r = 6 x 10^5) myofibrillar protein that constitutes 5-8% of the total myofibrillar protein in skeletal muscle. Nebulin has been localized in the I band in adult muscle myofibrils in the region of the N_2 line, where it may function in maintaining the structural organization of the thin filaments. Alternatively, nebulin may be attached to, or be a component of, the elastic, titin-containing filaments in myofibrils. In this study, immunofluorescence was used to examine the expression and organization of nebulin during myofibrillogenesis in embryonic chicken skeletal muscle. To determine the time course of organization of nebulin in relation to the organization of A bands, I bands, and titin filaments, a series of double-label immunofluorescence experiments were done to localize simultaneously nebulin and myosin, nebulin and actin, and nebulin and titin in embryonic chick myogenic cell cultures. Localization studies were also done using myogenic cultures that had been treated with ethyl methanesulfonate (EMS) to delay the synthesis and assembly of myofibrils until after fusion of the myoblasts to form large myosheets. Nebulin was expressed at early stages of differentiation in normal cultures, as evidenced by a diffuse localization of nebulin in myoblasts and early myotubes. In later myotubes, nebulin was associated with actin-containing stress-fiber-like-structures (SFLS), where it was localized either in a continuous distribution along the SFLS or as a series of dots or broad bands. Organization of nebulin into the adult myofibril-like pattern occurred

after the assembly of myosin into A bands and the organization of titin into the normal adult myofibrillar pattern. Nebulin organization was correlated with the later organization of actin into I bands. The final, adult pattern of nebulin organization was predominant only in myofibrils that exhibited Z lines by phase contrast. These results suggest a possible role for nebulin in the organization of actin filaments into mature I bands.

INTRODUCTION

Nebulin is an approximately 600,000 dalton protein first identified, along with the even larger protein titin, as a structural component of vertebrate skeletal muscle myofibrils by Wang et al. (1979). This protein, initially referred to as "band-3", was first isolated by Wang and Williamson (1980) and was later named "nebulin" to denote its unknown function (Wang, 1981). Nebulin has been estimated to constitute 3-5% of total myofibrillar protein in vertebrate skeletal muscle (Wang, 1985). Because this protein has been purified only in denatured form by gel filtration chromatography of myofibril extracts in SDS (Wang, 1982), the biophysical and biochemical properties of nebulin have not been well characterized.

By indirect immunofluorescence on isolated myofibrils, polyclonal nebulin antibodies have been shown to label a pair of bands within the I band, one on either side of the Z line, that coincide with the N_2 line (Wang and Williamson, 1980). N_2 lines are transverse structures located in the I band on both sides of the Z line (Franzini-Armstrong, 1970; also see Locker (1984) or Wang (1985) for review of N lines). The exact distance of N_2 lines from the Z line depends on sarcomere length of the myofibril (Page, 1968; Franzini-Armstrong, 1970). When sarcomeres are lengthened by stretching of the muscle, the N_2 line moves away from both the M line and Z line, but maintains the same proportional distance (Page, 1968; Wang and Williamson, 1980). This stretch-dependent movement suggests that N_2 lines and their associated proteins are not strongly bound to either thick or thin filaments (Locker and Leet, 1976;

Wang, 1985). The N_2 line is also located in the I band region where the thin filament array changes from the square lattice found at the Z line to the hexagonal lattice found in the overlap of the A and I bands (Bennett and Porter, 1953; Franzini-Armstrong, 1970; Locker and Leet, 1976; Traeger et al., 1983).

The movement of the N_2 line and nebulin with changes in sarcomere length has led to the suggestion that nebulin may be associated with elastic filaments. Although significant evidence exists to demonstrate that titin is a major component of an elastic filament network in striated muscle myofibrils, conflicting models exist for the arrangement of this set of elastic, longitudinal filaments in the sarcomere. One model, proposed by Maruyama and colleagues (Maruyama, 1986) suggests that filaments composed of only titin (connectin) act to link thick filaments to Z lines. An alternative model, proposed by Wang (1985), includes nebulin as a component of these filaments. In this model, titin filaments are anchored at or near the M line, run parallel to thick filaments in the A band, and connect to nebulin in the I band at the N_2 line. Nebulin filaments then traverse the remaining portion of the I band to the Z line. It is not known if attachment of the elastic filaments to the M line, Z line, and other possible attachment sites is direct or mediated through other proteins.

While the location of nebulin in the adult myofibril has been established, the role of nebulin is still unclear. Little information exists concerning the developmental role of nebulin, and the temporal sequence of assembly of nebulin with respect to other myofibrillar

proteins into the myofibril has not been established. To examine the role of nebulin in striated muscle myofibrillogenesis, normal and ethyl methanesulfonate (EMS) treated chick skeletal muscle cell cultures were examined using double-label immunofluorescence. Polyclonal nebulin antibodies were employed in conjunction with monoclonal myosin antibodies to follow thick filament organization, monoclonal actin antibodies to follow thin filament organization, and monoclonal titin antibodies to follow elastic filament organization. Cultures were examined after exposure to EMS because this mutagenic agent results in large, flat, myosheets which are easily examined in the light microscope (Dlugosz et al., 1984; Antin et al., 1986). Also, myofibril assembly takes place over a period of a few days, allowing for a closer examination of the temporal sequence of assembly of individual myofibrillar proteins.

In this paper, we present evidence that assembly of nebulin into adult-like double bands occurs after myosin, titin, and actin assembly into their respective adult banded arrays. The appearance of nebulin banding appears to coincide with the appearance of phase dense Z lines in mature, striated myofibrils. Some of these results have been presented previously in abstract form (Kurpakus and Huiatt, 1986).

MATERIALS AND METHODS

Antibody production and characterization

Denatured titin and nebulin were prepared by gel filtration of SDS-extracts of adult chicken breast muscle myofibrils on Biogel A50 (BioRad Laboratories, Richmond, CA) according to Wang (1982). Myofibrils were prepared according to the method of Dayton et al. (1976). Monoclonal antibodies to titin were prepared by modification of the method described by van Deusen and Whetstone, (1981). Two Balb/c 3T3 mice were injected intraperitoneally with 200 μg of purified, SDS-denatured titin. Mice were boosted (also intraperitoneally) with 200 μg of the same titin sample one month later, and again three days before fusion. Spleen cells from both mice were mixed and fused with Sp2/0 myeloma cell line using polyethylene glycol (PEG). Hybridomas were initially screened for titin antibody production by ELISA. Positive clones were further screened by indirect immunofluorescence labeling of isolated adult chicken myofibrils. Positive clones were subcloned twice by limiting dilution. The clone showing the highest anti-titin antibody titer in hybridoma culture supernatants was then selected for use in labeling experiments. Antibody isotype was determined to be IgG1 using a commercial idiotyping kit (HyClone Laboratories, Logan, UT). Hybridoma antibodies were partially purified from culture supernatants by ammonium sulfate precipitation as described by Dennis et al. (1984).

Polyclonal antibodies were produced in New Zealand white rabbits against electrophoretically purified SDS-denatured adult chicken breast nebulin by the method of Richardson et al. (1981). IgG was purified

from serum by salting out 0-50% with ammonium sulfate and dialysis against phosphate buffered saline (PBS, 0.01M Na Phosphate, 0.15M NaCl, pH 7.2). To eliminate antibodies that cross-reacted with titin, the IgG fraction was incubated with pieces of nitrocellulose (Bio Rad) onto which purified titin had been absorbed (Talian et al., 1983; Gassner, 1986). The resulting affinity-purified nebulin antiserum was used for antibody characterization and immunofluorescence experiments. Characterization of antibodies by immunoblotting and immunofluorescence labeling of adult chicken breast muscle myofibrils was done as described previously (see Section I herein).

Hybridoma cells from clone MF20 (Bader et al., 1982) were a generous gift of Dr. Donald Fischman (Cornell Univ. Medical College, New York). Myosin monoclonal antibody was partially purified from culture supernatant by ammonium sulfate precipitation according to Dennis et al. (1984). Actin monoclonal antibody culture supernatant from clone JLA20 was purchased from the Developmental Studies Hybridoma Bank (Johns Hopkins Univ. School of Medicine, Baltimore, MD).

Double-label immunofluorescence of myogenic cell cultures

Myogenic cells were isolated from mixed leg and thigh muscles of 12 d chick embryos and cultured as previously described (see Section I herein). Cells were double-labeled for nebulin and myosin, nebulin and actin, or nebulin and titin. Cultures were rinsed with PBS, then fixed on the plastic culture dishes in -20°C methanol containing 50mM EGTA for 6 min. Dishes were washed in PBS and stained with a mixture of: a) 100

µl of 1:10 polyclonal anti-nebulin IgG and 100 µl of 1:20 monoclonal anti-myosin IgG, b) anti-nebulin and 200 µl undiluted monoclonal anti-actin IgM, or c) anti-nebulin and 100 µl undiluted monoclonal anti-titin IgG. After incubation at 37°C for 30 min in a humid chamber, cells were washed in several changes of PBS, pH 8.6, for 15 min. Cells were then incubated with a mixture of 100 ul of 1:200 rhodamine-labeled goat-anti-rabbit IgG (ICN ImmunoBiologicals, Lisle, IL) and 75 ul undiluted FITC-labeled goat-anti-mouse IgG (nebulin-myosin and nebulin-titin pair), or rhodamine and 100 ul 1:200 FITC-labeled goat-anti-mouse IgG, u chain specific (Cappel) (nebulin-actin pair). After washing in PBS, coverslips were mounted in a 9:1 solution of glycerol:PBS containing 1% n-propyl gallate (Giloh and Sedat, 1982). Cells were examined in a Zeiss Photomicroscope III equipped with epifluorescence optics, using a 63X oil immersion objective. Images were recorded on Kodak Tri-X Pan film developed in D-76.

RESULTS

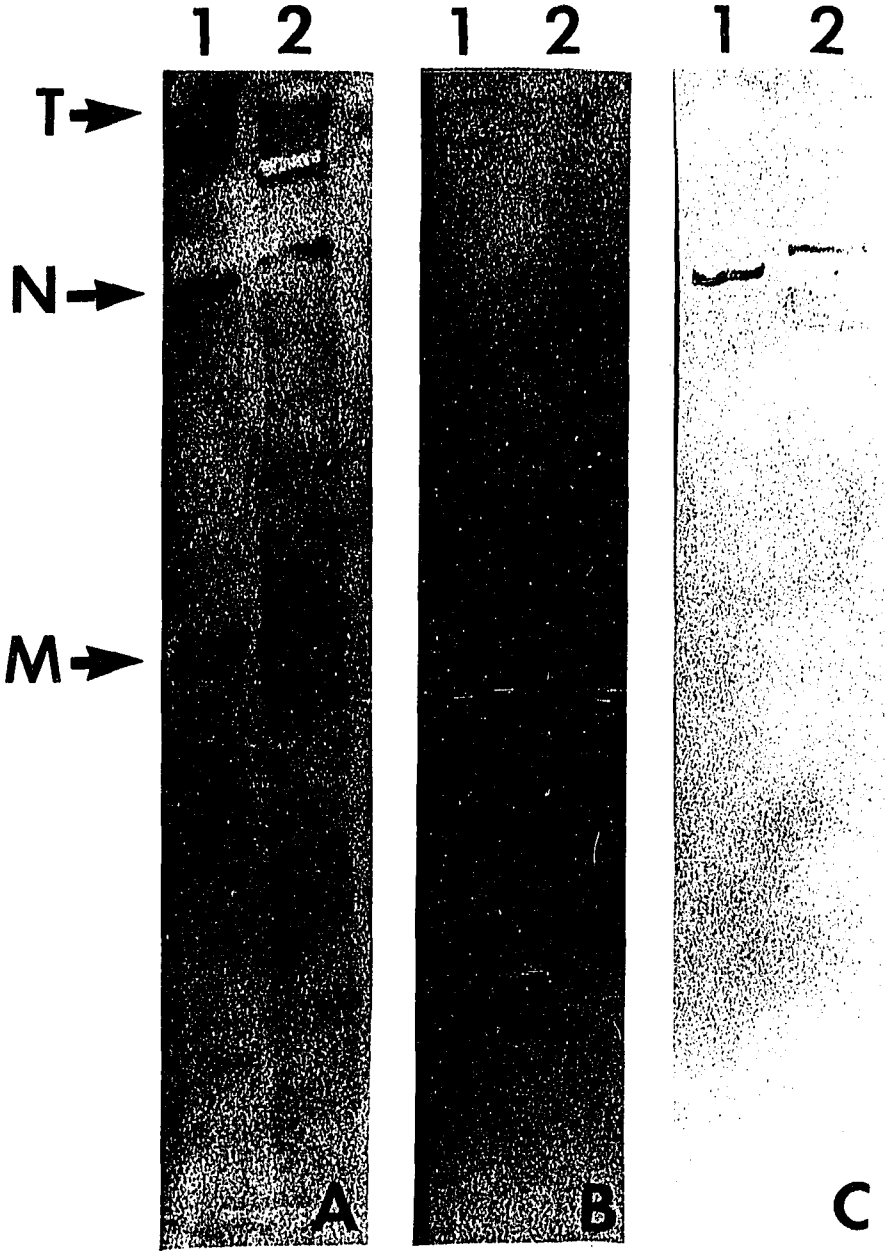
Characterization of antibodies

The specificity of all of the antibodies used for localization was examined by Western blotting (Fig. 1). To improve the transfer efficiency of titin and nebulin from the gel to the Zeta-Probe blotting matrix, methanol was omitted from the transfer buffer, and the electrophoretic transfer was done for 16-18 h. This method results in transfer of significant amounts of both titin and nebulin to the blots (Fig. 1a). On the blots, monoclonal titin antibodies labeled only titin (Fig. 1b) and polyclonal nebulin antibodies labeled only nebulin (Fig. 1c) in both embryonic myogenic cell culture and adult myofibril homogenates.

The myosin monoclonal antibody used in these studies, MF20, is an IgG_{2b} prepared against adult chicken pectoralis myosin heavy chain. MF20 binds to all chicken muscle myosin isoforms, but does not cross-react with cytoplasmic myosin isoforms (Bader et al., 1982). It binds to a site in the light meromyosin portion of the myosin molecule (Shimizu et al., 1985). The actin monoclonal antibody used, JLA20, is an IgM prepared against a cytoskeletal protein preparation from chicken gizzard. This antibody reacts with all actin isoforms and has been shown to label the I bands of mammalian myofibrils by indirect immunofluorescence (Lin et al., 1982). The specificity of the myosin and actin monoclonal antibodies were checked by immunoblotting as described above for anti-titin and anti-nebulin antibodies (results not shown).

Fig. 1. Immunoblot characterization of monoclonal titin and polyclonal nebulin antibodies

Triplicate samples of a 96-h embryonic chick myogenic cell culture homogenate (lane 1) and adult chicken thigh muscle myofibrils (lane 2) were separated by SDS-PAGE on a 5% acrylamide gel and transferred electrophoretically to Zeta-Probe membrane. Blot A was stained for total protein using a biotin-avidin-HRP staining procedure, blot B was labeled with anti-titin antibodies and HRP-goat-anti-mouse IgG, and blot C was labeled with anti-nebulin antibodies and HRP-goat-anti-rabbit IgG. The titin monoclonal antibodies reacted only with titin, and the polyclonal nebulin antibodies reacted only with nebulin in both samples. Positions of titin (T), nebulin (N), and myosin (M) are indicated.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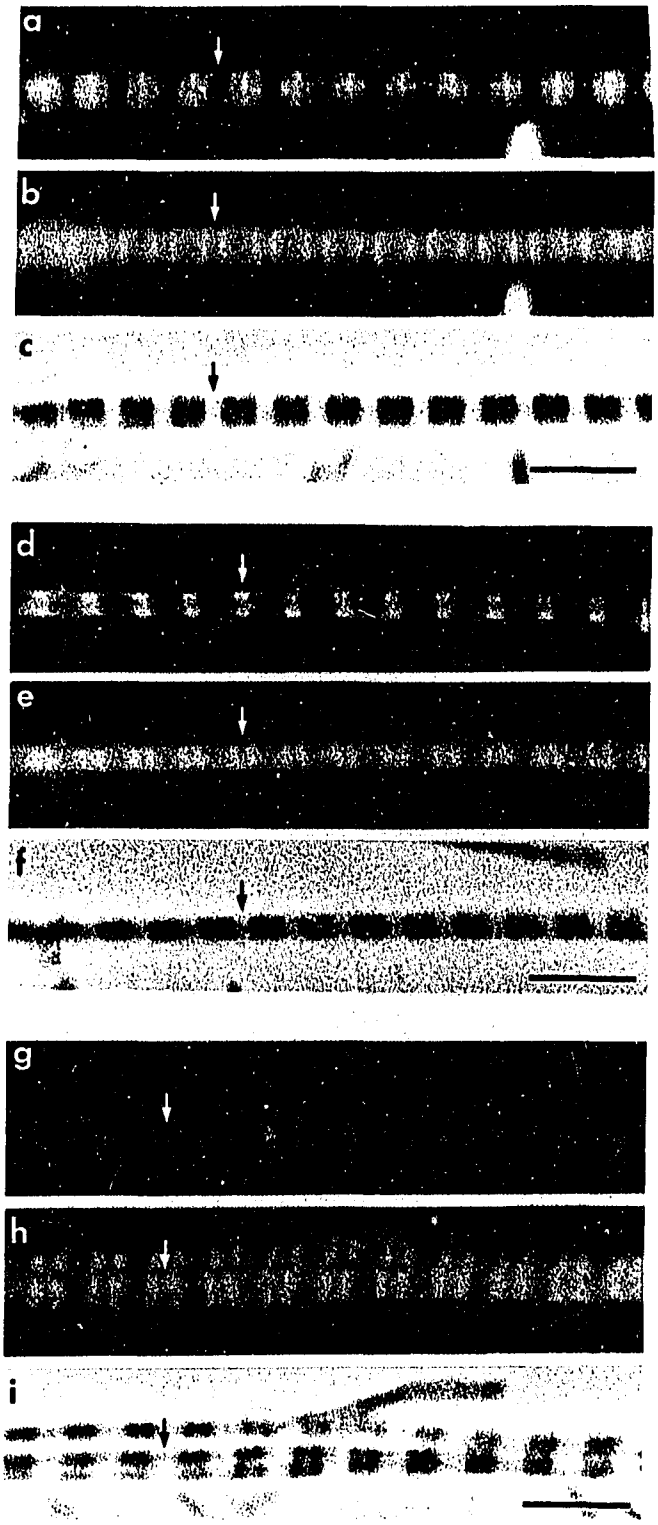
The antibodies were also examined by double-immunofluorescence labeling of adult chicken muscle myofibrils (Fig. 2). Myofibrils were fixed with -20°C methanol to ensure that the antibodies would recognize myofibrillar proteins in methanol-fixed cell cultures. No difference in labeling patterns of methanol fixed myofibrils as compared to unfixed myofibrils was seen, except that a higher percentage of intact myofibrils remained after labeling and washing of the methanol fixed myofibrils (results not shown). The typical labeling pattern obtained with the MF20 myosin monoclonal antibody is shown in Fig. 2a for a myofibril double labeled with MF20 and anti-nebulin. MF20 labels the entire myofibrillar A band with a more intense line of fluorescence through the middle of the A band at the level of the M line as described previously by Shimizu et al. (1985). The monoclonal actin antibody used in this study labels the non-thick-filament-overlap region of the I band (Fig. 2d). The lack of a prominent, dark Z line in I bands labeled with this anti-actin antibody has also been observed previously by Lin et al. (1982). The characteristic double-band pattern of anti-titin antibodies can be seen with the titin monoclonal antibody as shown in Fig. 2g. This monoclonal antibody labels the A-I junction, with some fluorescence extending into the I band. In myofibrils double-labeled with polyclonal nebulin antibodies and each of the three monoclonal antibodies (myosin, actin, and titin, Figs. 2b, 2e, and 2h, respectively) the nebulin antibodies label two bands within the I band, one on each side of the Z line. These two nebulin bands are symmetrical with respect to the distance from the Z line, as for the monoclonal anti-titin, but the

Fig. 2. Double-label immunofluorescence labeling of adult chicken skeletal muscle myofibrils

(a-c) Paired immunofluorescence images of a myofibril double-labeled with monoclonal anti-myosin (a) and polyclonal anti-nebulin (b) antibodies, and phase-contrast image of the same myofibril (c) shows anti-myosin staining in the A band and anti-nebulin staining in the I band. (d-f) Paired immunofluorescence images of a myofibril double-labeled with monoclonal anti-actin (d) and polyclonal anti-nebulin (e) antibodies, and phase-contrast image of the same myofibril (f) illustrates that anti-actin staining is localized in the I band. Anti-nebulin staining is found in the I band. (g-i) Paired immunofluorescence images of a myofibril double-labeled with monoclonal anti-titin (g) and polyclonal anti-nebulin (h), and phase-contrast image of the same myofibril (i) demonstrates that anti-titin antibodies label at the A-I junction, and anti-nebulin antibodies label in the I band. Arrows indicate the position of Z lines. Bar, 5 μm.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nebulin labeling appears closer to the A-I junction than the titin labeling (c.f. Figs. 2h and 2g). The bands of fluorescence seen with anti-nebulin are also broader than the bands obtained with the titin antibody.

Myosin and nebulin localization in normal cultures

Myogenic cell cultures were double-labeled with anti-nebulin and anti-muscle myosin in order to follow the temporal organization of nebulin with respect to the organization of myosin-containing thick filaments into A bands. Results of labeling studies on early, intermediate, and late stages of *in vitro* myogenic cell differentiation are shown in Figs. 3, 4, and 5 respectively. Both muscle-specific myosin and nebulin are expressed in postmitotic, mononucleated myoblasts (Fig. 3a-c). Both proteins were localized in a diffuse pattern in these myoblasts and early myotubes. Fig. 3 also demonstrates that initiation of myofibril formation is not synchronized throughout fusing and immature fused myotubes, as several nascent fibrils can be seen in an immature myotube that shows predominately diffuse, cytoplasmic myosin and nebulin distribution. The small arrows in Fig. 3 point to a myoblast, in the process of fusing to the immature myotube, that is positive for anti-nebulin label, but negative for anti-myosin (also see Fig. 5a-c at the arrowhead).

The initial event in myofibril assembly appears to be the arrangement of the myofibrillar proteins into an array of nonstriated, fiber-like structures that run approximately parallel to the long axis

Fig. 3. Double-label immunofluorescence localization of myosin and nebulin in normal embryonic chick myogenic cell cultures Cells in a 24-h normal embryonic chick myogenic cell culture labeled with monoclonal anti-skeletal myosin (a) and polyclonal anti-nebulin (b). Corresponding phase-contrast image is shown in (c). Both proteins are localized primarily in a diffuse pattern in these early cultures. Small arrows indicate a myoblast that appears to be in the process of fusing with the adjacent myotube; this cell is labeled with the anti-nebulin (b), but not with the anti-skeletal myosin (a). Large arrows indicate a portion of the myotube where both proteins are located in a filamentous pattern. Bar, 10 μm.

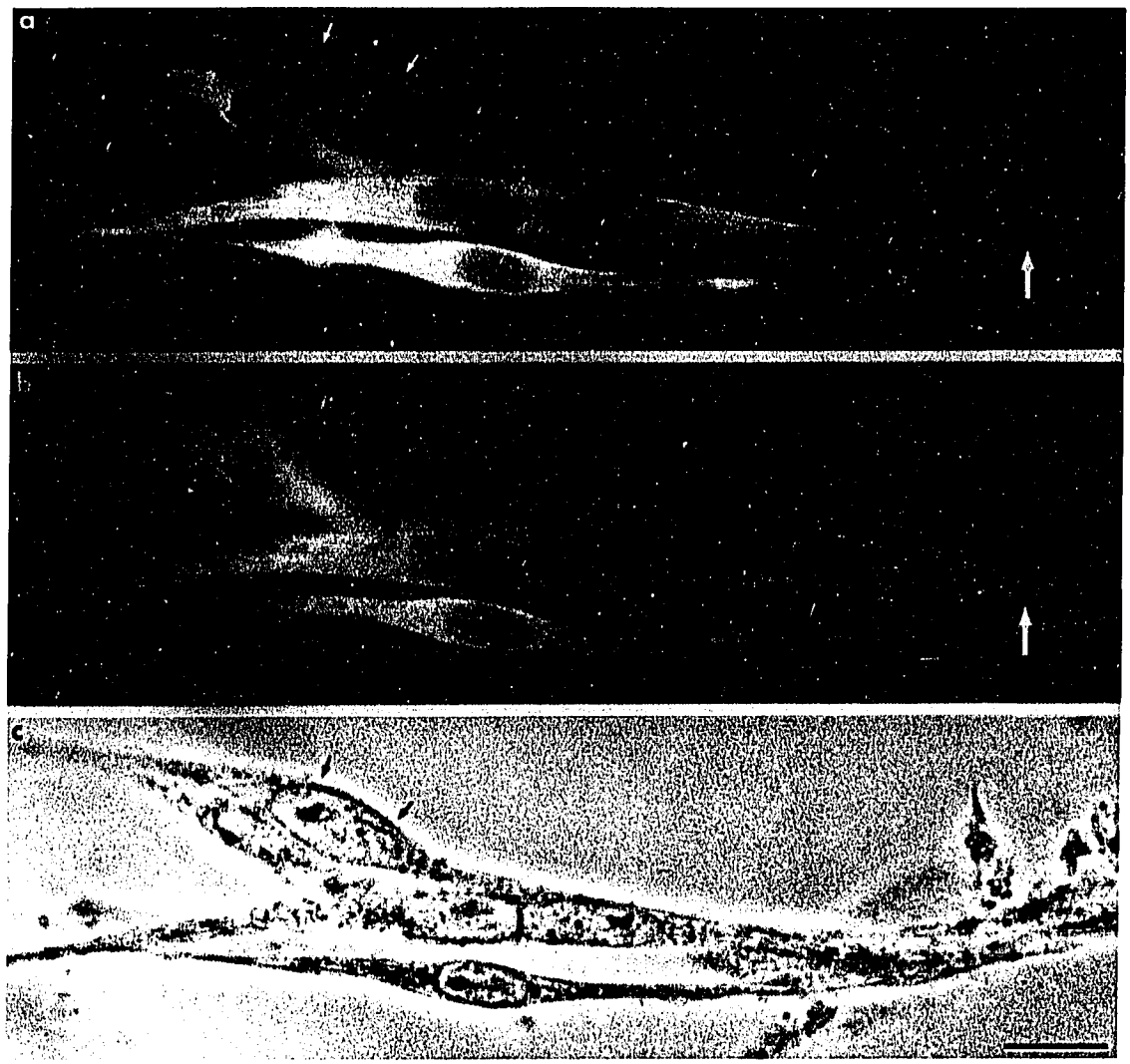
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Fig. 4. Double-immunofluorescence localization of myosin and nebulin in 2- and 3-d normal chick embryonic cell cultures
Paired fluorescence images show localization with monoclonal anti-skeletal myosin (a, d, f) and polyclonal anti-nebulin (b, e, g); phase contrast images of (a, b) and (f, g) are shown in (c) and (h) respectively. (a-c), (d,e), 48-h cultures; (f-g), 72-h culture. Myotube in (a-c) shows nascent myofibrils located along the cell periphery. Myosin appears to be in a more advanced stage of organization than nebulin. Arrows indicate a nascent myofibril in which myosin in (a) is organized into A-bands, while nebulin in (b) shows a fiber-like pattern in the same nascent myofibril. In the myotube in (d, e) myosin is beginning to show organization into nascent A-bands (d), while nebulin remains in a filamentous pattern; this is particularly evident in the nascent myofibril indicated by the arrows. In the myotube in (f-g), both proteins are arranged primarily in a filamentous pattern along SFLS. Bar, 10 μm.

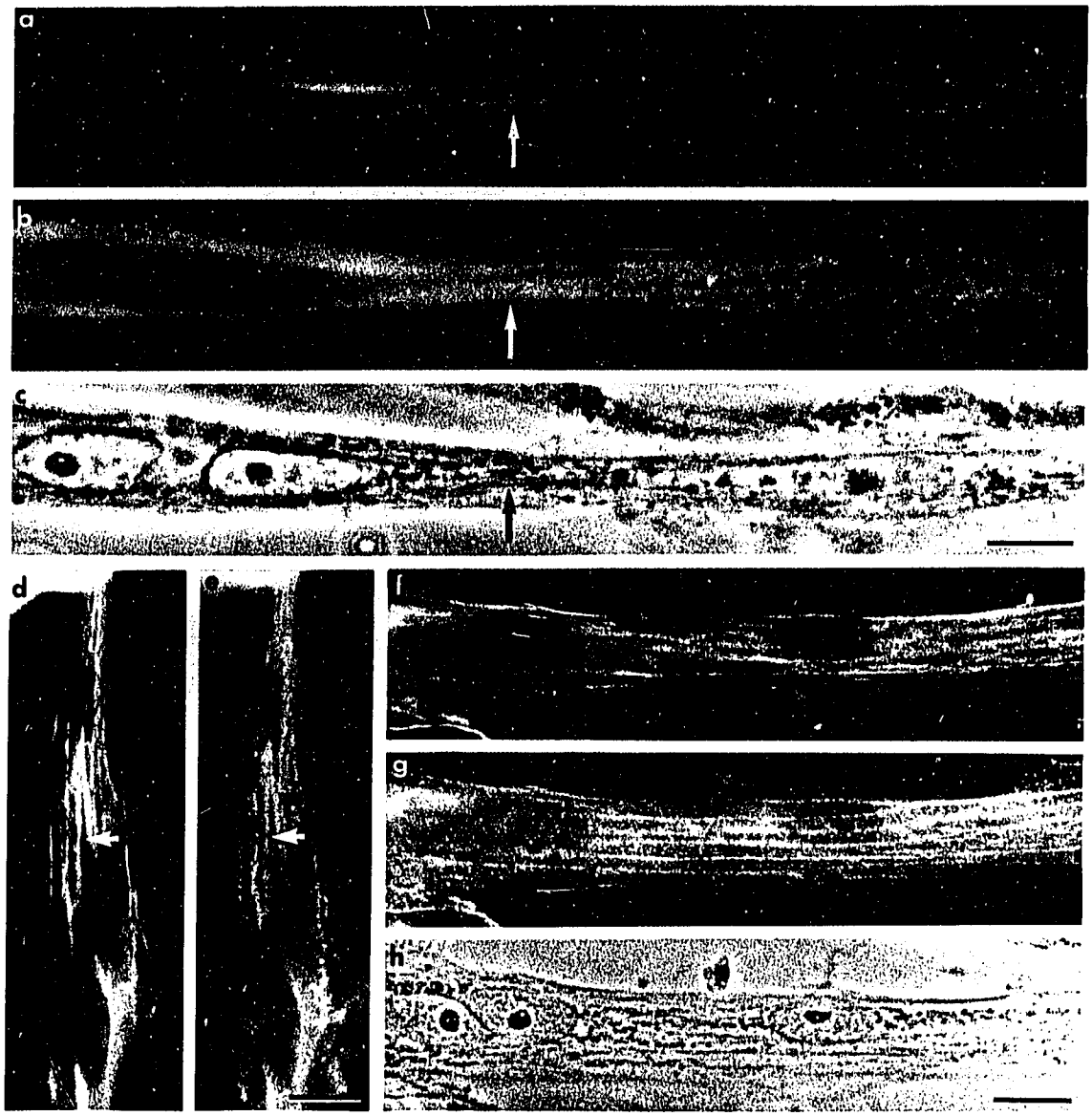
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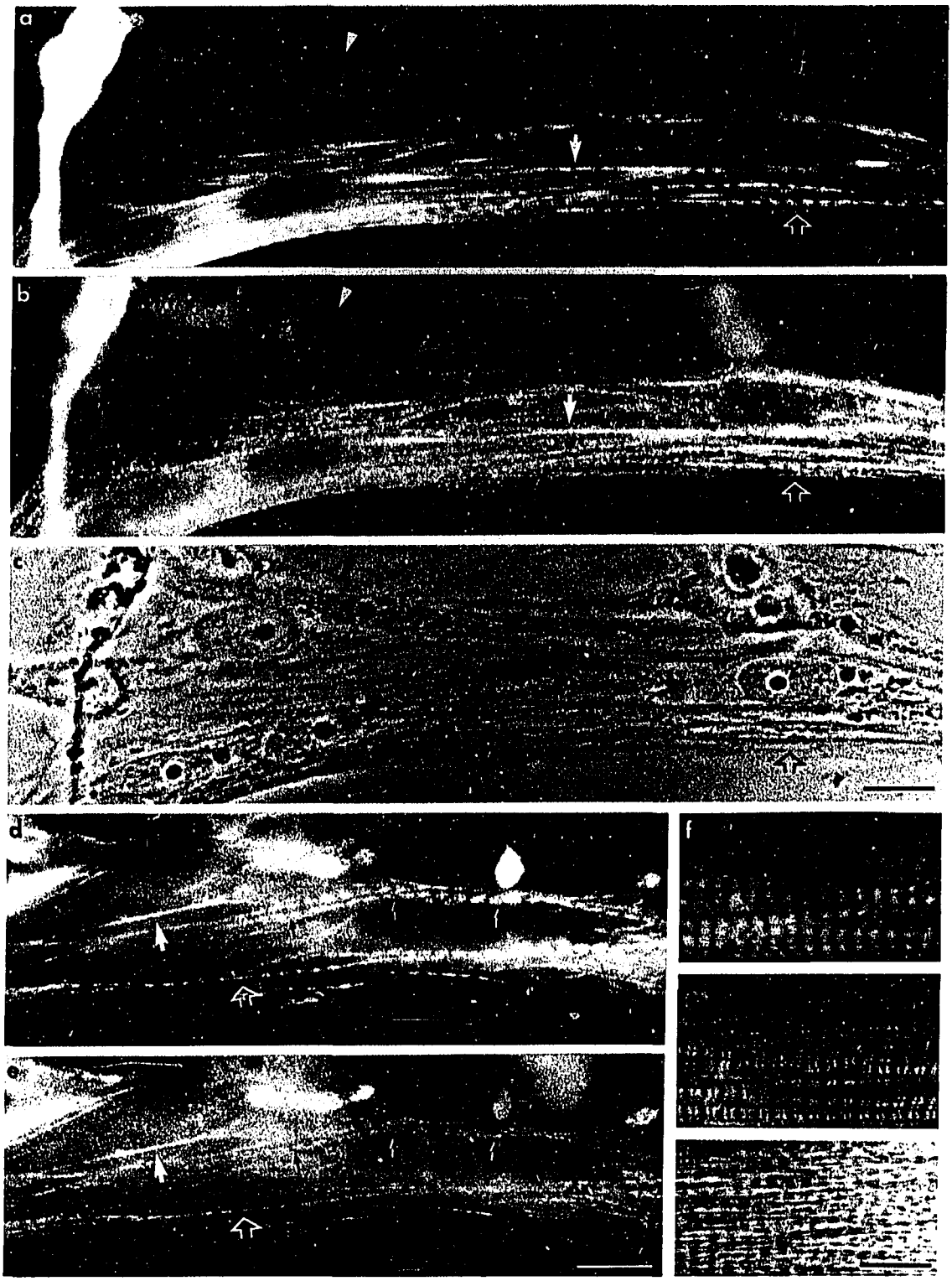
Fig. 5. Double-immunofluorescence localization of myosin and nebulin in normal embryonic chick myogenic cell cultures

Fluorescence images show localization with monoclonal anti-skeletal myosin (a, d, f) and polyclonal anti-nebulin (b, e, g); phase contrast images are shown in (c) and (h) respectively. (a-c), (d,e), 72-h cultures; (f-h), 96-h culture. Arrowheads in (a-c) show a mononucleated myoblast that is not labeled with anti-skeletal myosin (a), but shows a diffuse pattern of nebulin staining (b). (a-c) and (d,e) show several stages of myofibril assembly within the same myotube. At very early stages, both proteins show a continuous, fiber-like arrangement (large, closed arrows in d, e). Closed arrows in (a-c) show a nascent myofibril that exhibits uneven myosin labeling (a) suggesting the beginning of organization into A-bands (a). The nascent myofibril indicated by the open arrows in (a-c) shows myosin arranged in an adult-like banding pattern (a), nebulin appears to be at a much earlier stage of organization. In the myotube in (d,e), the nascent myofibrils indicated by both the small, closed arrows and the open arrow show arrangement of myosin in A-bands, while nebulin appears as a series of broad, diffuse bands. In the more mature myotube in (f-g), the labeling for both myosin (f) and nebulin (g) shows a banding pattern similar to that seen in adult myofibrils. Bar, 10 μm.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of the myotube (Fig. 4f-h; large closed arrow in Fig. 5d-e). These fiber-like structures are similar to the stress-fiber-like-structures (SFLS) previously described in cultured myogenic cells by Holtzer and co-workers (Dlugosz et al., 1984; Holtzer at al., 1985; Antin et al., 1986). These structures are also labeled with actin (see Figs. 8-10). Myosin clearly organizes into A bands before nebulin band organization, for many instances of putative myosin bands (arrow in Fig. 4d-e; closed arrow in Fig. 5a-c), or distinct myosin banding (arrow in Fig. 4a-c) along non-striated nebulin fibers can be detected in normal cultures. Although interrupted patches of fluorescence indicating nascent nebulin banding can be found along nonstriated fibers (open arrow in Fig. 5a-c), nebulin bands are predominantly seen in relatively mature myofibrils (open arrow in Fig. 5d-e; triple arrows in Fig. 5d-e; Fig. 5f-h).

Myosin and nebulin localization in EMS-treated cultures

In addition to normal medium, myogenic cells were cultured in medium containing the carcinogen EMS. One characteristic of myosheets recovering from EMS exposure in normal medium is the relatively protracted period of myofibril assembly. This allows for a more precise examination of the temporal sequence of myosin and nebulin incorporation into the myofibril. Fig. 6 shows myosheets allowed to recover in normal medium for 2-d, while Fig. 7 shows a 4-d recovering myosheet. Within a given myosheet at any stage of recovery, there exists a population of both SFLS, nascent myofibrils, and mature myofibrils. At early stages of recovery, however, there is a greater number of SFLS, and as the

Fig. 6. Double-immunofluorescence localization of myosin and nebulin in EMS-treated embryonic chick myogenic cell cultures Myogenic cell cultures were treated with EMS, allowed to recover in normal medium for 2 d, and double-labeled with anti-muscle myosin (a, d) and anti-nebulin (b, e). Corresponding phase images are shown in (c, f). The myosheet in (a-c) shows a progression from stress-fiber-like structures to more mature myofibrils from the periphery to the center of the myosheet. Portions of the fiber indicated by the single arrow in (a-c) show arrangement of myosin into nascent A bands (a) and with a continuous distribution of nebulin (b). Triple arrows in the lower right of (a-c) point to three Z lines of a nascent myofibril, which shows myosin labeling in the A band (a) and nebulin labeling in the I band (b). The large arrowheads in (d-f) indicate a nascent myofibril with a continuous, fiber-like distribution of nebulin labeling (e) with myosin arranged into distinct A bands (d); several other nascent myofibrils with similar relative organization of nebulin and myosin are visible at the top of the micrographs in (d-f). The myofibril indicated by the arrow in (d-f) shows myosin labeling in A-bands (d), distinct Z lines in phase contrast (e), and early nebulin organization in (e). Bar, 10 μm.

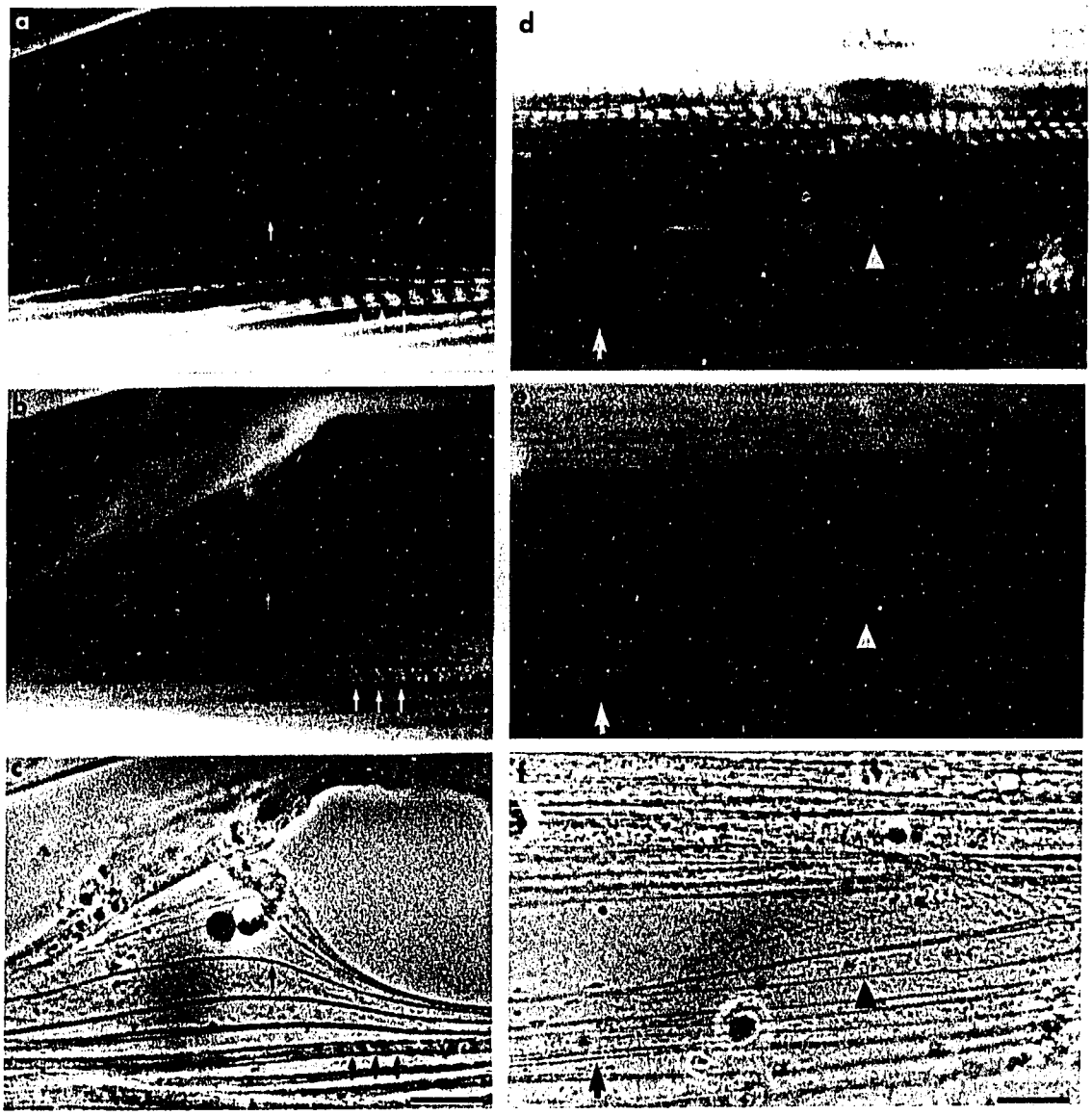
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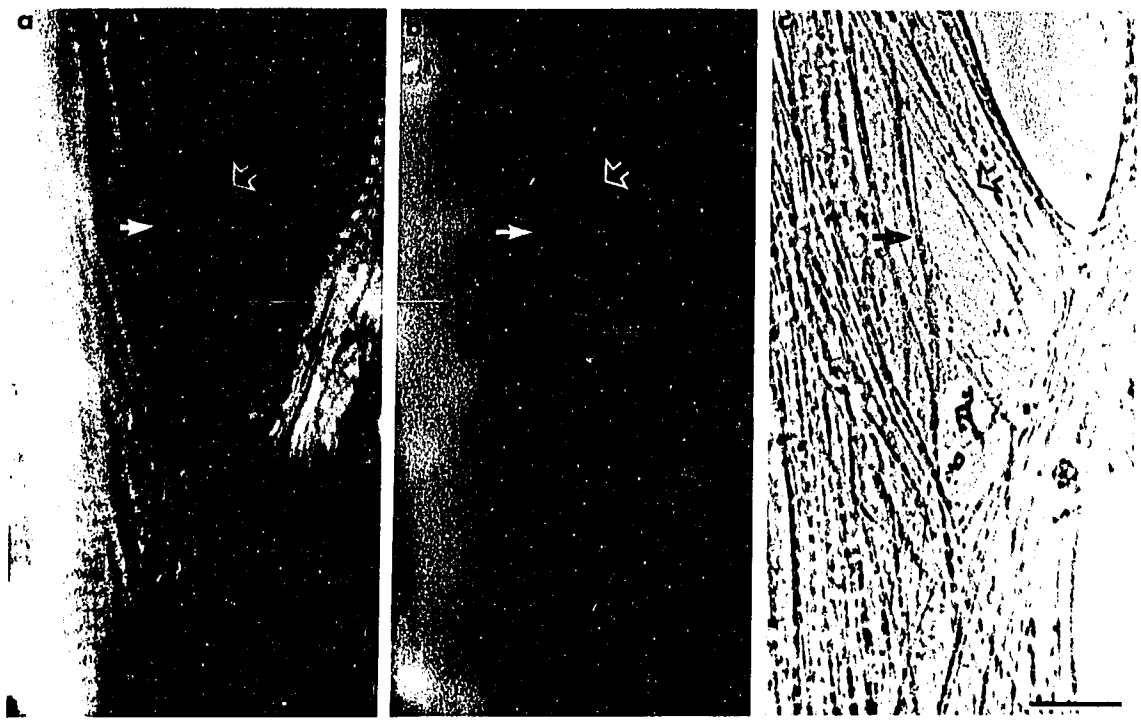
Fig. 7. Double-immunofluorescence localization of myosin and nebulin in EMS-treated embryonic myogenic cell cultures
EMS-treated culture after 4 days of recovery, labeled with anti-myosin (a) and anti-nebulin (b). (c) Corresponding phase contrast image. The double-banding pattern characteristic of nebulin labeling of adult myofibrils can be seen in the myofibrils in this myosheet that show a distinct Z-line in phase-contrast; an example is indicated by the closed arrow. The nascent myofibril indicated by the open arrow shows a periodic distribution of myosin (a), indicating early organization of the myosin into A bands, but a nearly continuous distribution of nebulin. Bar, 10 μm.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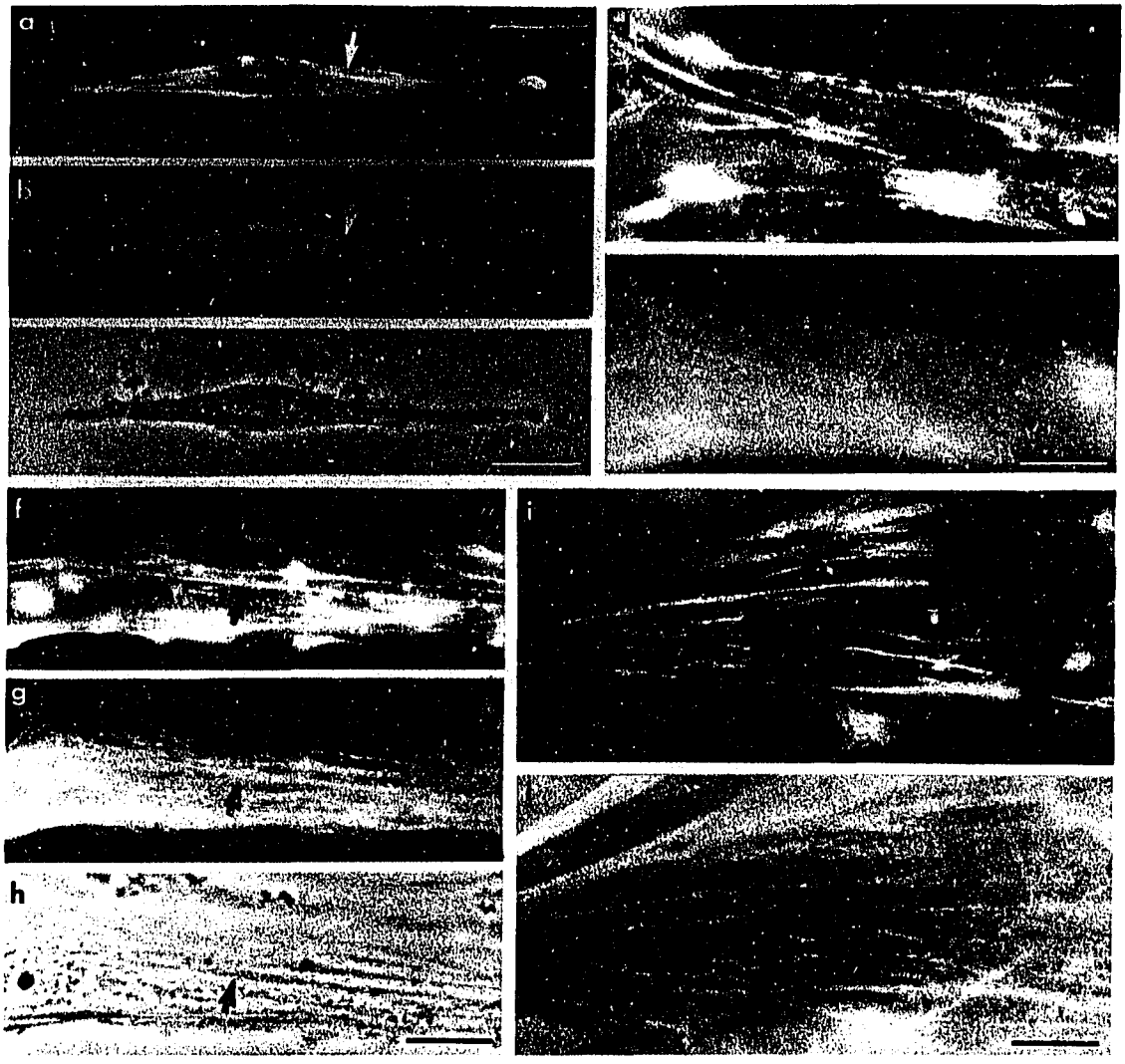
recovery time increases, the number of nascent and more mature myofibrils increases. Fig. 6a-c shows one such myosheet. At the single small arrow, putative myosin bands can be seen along a fiber with a continuous nebulin distribution. Two small diameter nascent myofibrils, in which both myosin and nebulin bands are discernible, are visible immediately below this fiber. Three adult-like sarcomeres are indicated by the triple small arrows. Not only is myosin organized into A bands in these three sarcomeres, but nebulin is also organized into discreet double bands. It is interesting to note the possible correlation between the appearance of adult-like nebulin double bands (Fig. 6b) and the presence of Z lines in phase contrast images (Fig. 6c).

Several other examples of advanced organization of myosin along nebulin-containing fibers are shown in Figs. 6 and 7. In those myofibrils showing nebulin banding in addition to myosin banding, phase contrast images indicate a relatively advanced state of organization (Fig. 6d-f, arrow; Fig. 7, arrow).

Actin and nebulin localization in normal and EMS-treated cultures

Normal and EMS-treated cultures were double-labeled with anti-actin and anti-nebulin antibodies to follow the temporal sequence of organization of actin and nebulin into the I band of adult striated muscle myofibrils. Fig. 8 shows results of double-label experiments of normal cell cultures, while Figs. 9 and 10 show results on EMS-treated cultures. Initially, actin antibodies label in fiber-like patterns both in mononucleated myoblasts (arrow in Fig. 8a-c), and in developing

Fig. 8. Double-immunofluorescence localization of actin and nebulin in normal embryonic chick myogenic cell cultures
Double-labeling of normal myogenic cell cultures with anti-actin (a, d, f, i) and anti-nebulin (b, e, g, j) showing early stages of myofibrillogenesis. Phase contrast images corresponding to (a, b) and (f, g) are shown in (c) and (h) respectively. (a-c) A mononucleated myoblast in a 24-h culture exhibits a diffuse distribution of both actin (a) and nebulin (b); SFLS near the cell periphery (arrow) are labeled by both antibodies in a continuous distribution. (d, e) Early myotube present in a 48-h culture. Numerous actin cables (SFLS) are visible (d) while nebulin (e) exhibits primarily a diffuse distribution. (f-h) and (i-j) Myotubes in 72-h cultures. In (f-h) several actin cables (f) are positive for nebulin (g) (example indicated by arrow); nebulin is also distributed diffusely throughout the cytoplasm. In the myotube in (i, j) the majority of the actin cables (i) are also positive for nebulin. Bar, 10 μm.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myotubes (arrow in Fig. 8f-g; Fig. 8i-j), confirming the identification of these structures as SFLS. Actin appears to localize to these structures more quickly than nebulin. Cytoplasmic nebulin fluorescence persists even after all of the actin has localized to fibers (Fig. 8d-e; Fig. 9d-f). Later, actin is organized into I bands. Temporally, actin assembles into adult-like I bands before nebulin.

The temporal sequence of actin and nebulin assembly can be more easily discerned in EMS-treated cultures. The recovering myosheet (Fig. 9a-c) shows several stages of myofibril assembly. Actin appears to progress from a nonstriated, fiber-like array directly to an adult-like banded pattern (compare upper portion of fiber at arrow in Fig. 9a to area at arrow point). Nebulin assembly, however, progresses from a nonstriated, fiber-like arrangement, to a punctate, or dotted array along fibers (arrow in Fig. 9b). Organization progresses to a series of seemingly evenly-spaced bands along nascent myofibrils. Discreet nebulin bands are seen only in mature, striated myofibrils (arrowhead in Fig. 9b). The recovering myosheets in Fig. 10 demonstrate that distinct nebulin bands are most likely to be found along mature myofibrils containing discreet Z lines (open arrow in Fig. 10b; arrow in Fig. 10c). Note that the majority of the nascent myofibrils in Fig. 10a already show distinct actin bands, again indicating that actin organization preceded that of nebulin.

Fig. 9. Double-immunofluorescence localization of actin and nebulin in EMS-treated embryonic chick myogenic cell cultures Paired immunofluorescence images of EMS-treated cultures labeled with anti-actin (a, d) and anti-nebulin (b, e). Phase contrast images corresponding to (a, b) and (d, e) are shown in (c) and (f) respectively. (a-c) Myosheet in EMS-treated culture after 2 d of recovery in normal medium. The nascent myofibril indicated by the arrow shows organization of actin into I-bands (a), although no banding is apparent in the phase contrast image (c) and nebulin exhibits a nearly continuous, fiber-like distribution. The relatively mature myofibril indicated by the arrowhead shows arrangement of both actin and nebulin in an adult-like pattern. Between these two nascent myofibrils, an apparent progression in nebulin organization, from a fiber-like pattern to a series of evenly spaced single bands and finally to a mature, double-banding pattern, can be seen in (b). (d-f) Myosheet in an EMS-treated culture allowed to recover for 3 d in normal medium. This particular cell appears to be at an earlier stage of myofibrillogenesis than that shown in (a-c); actin is localized primarily in a stress-fiber-like pattern (d), and nebulin is located both along the SFLS or diffusely in the cytoplasm. Bar, 10 μm.

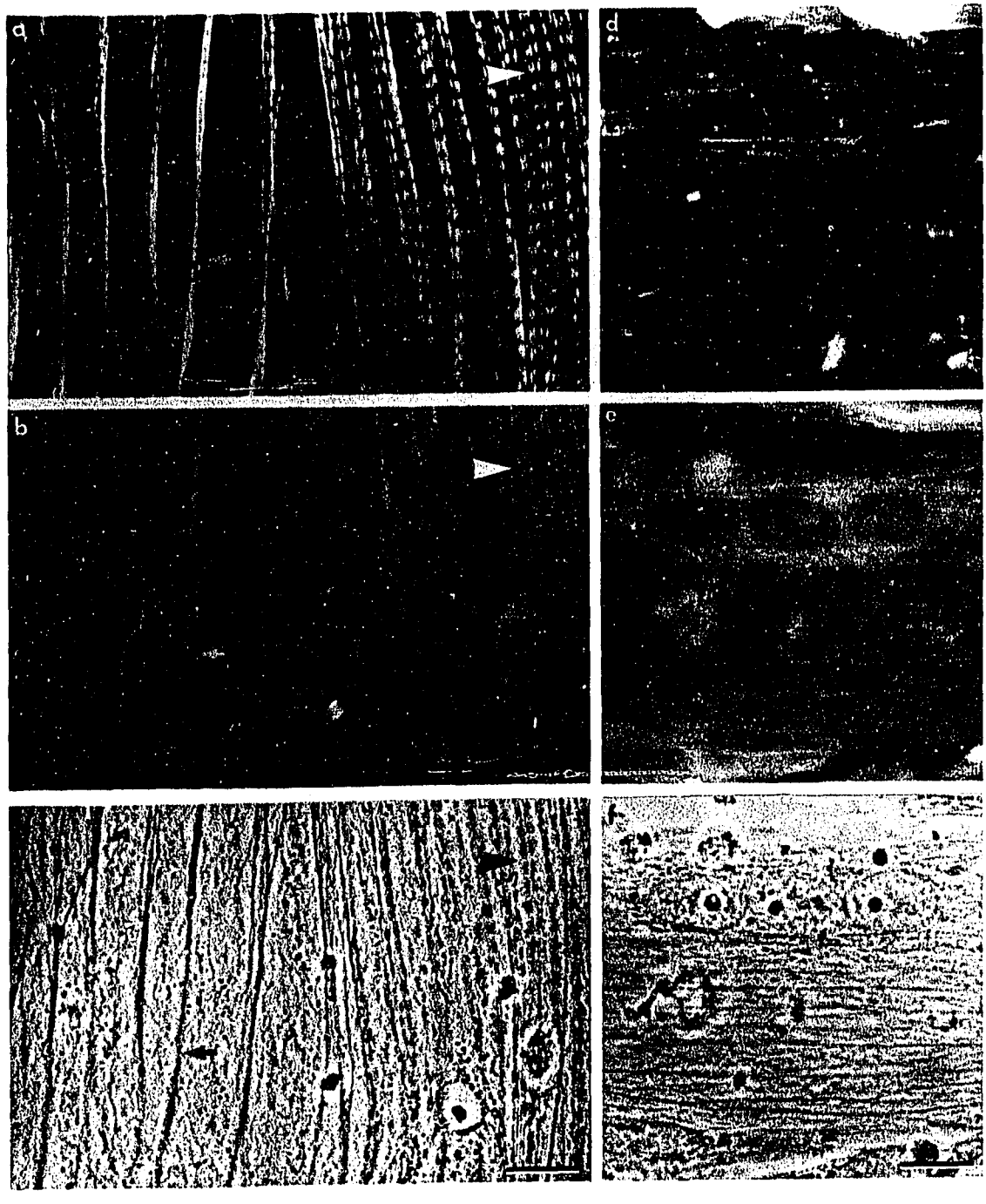
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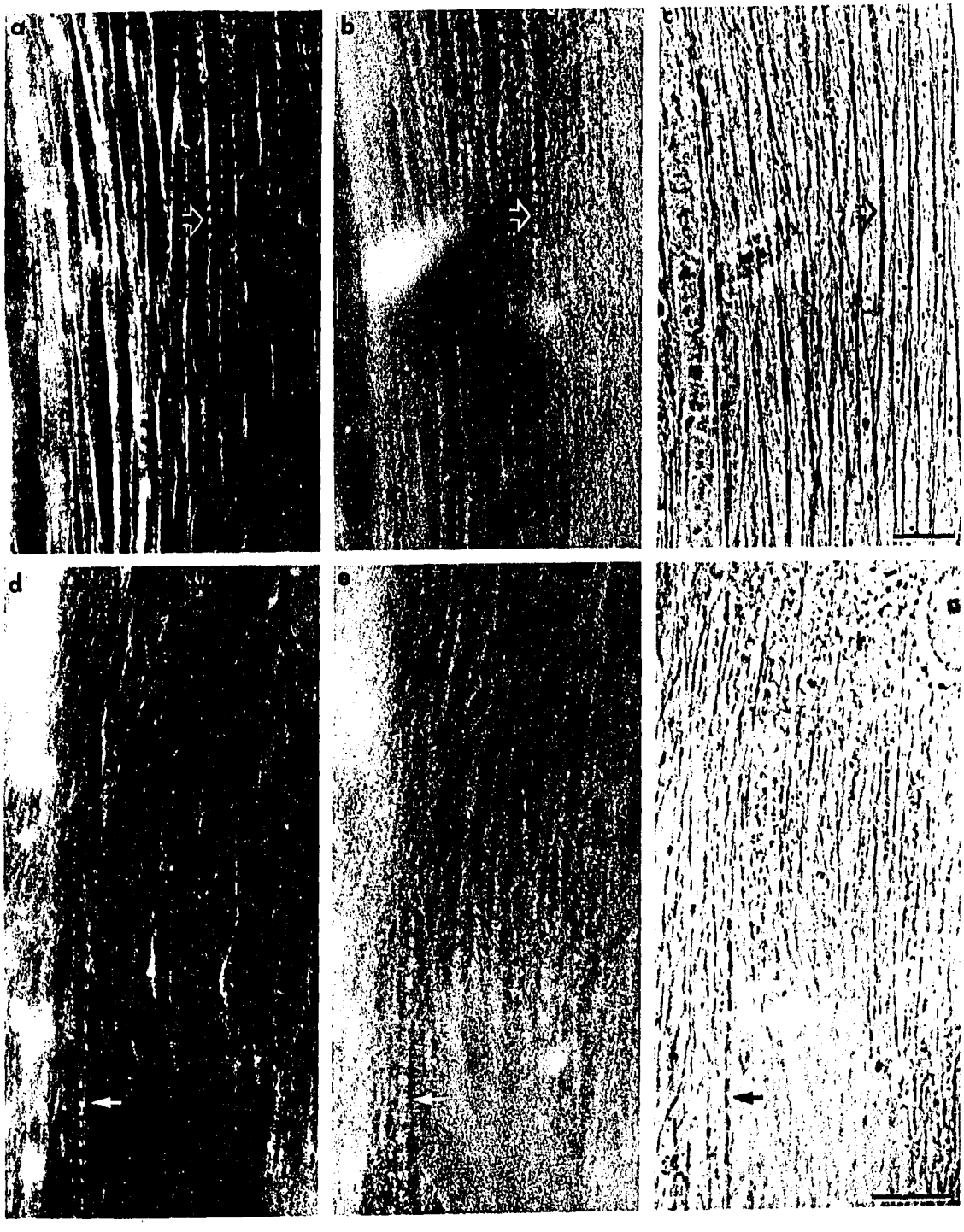
Fig. 10. Double-immunofluorescence localization of actin and nebulin in EMS-treated embryonic chick myogenic cell cultures

Paired immunofluorescence images of EMS-treated cultures labeled with anti-actin (a, d) and anti-nebulin (b, e). Phase contrast images corresponding to (a, b) and (d, e) are shown in (c) and (f) respectively. (a-c) Myosheet in a culture allowed to recover from EMS treatment for 3 d in normal medium shows several stages of myofibril assembly within the same cell. A nascent myofibril that exhibits an adult-like distribution of both actin and nebulin near the center is indicated by the open arrow; Z lines are barely visible in this portion of the myofibril in the phase contrast image (c). This same myofibril exhibits a more continuous distribution of actin as one moves towards the bottom of the micrograph, and nebulin is more diffusely distributed in this region. (d-f) In this myosheet, which had been allowed to recover from EMS treatment for 4 d, nebulin (e) is located both diffusely in the cytoplasm and along actin positive fibers (d). Nebulin is distributed both in a continuous pattern and a periodic pattern along the SFLS's. The arrow indicates a relatively mature myofibril with adult-like banding in this same cell. Bar, 10 μm.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Titin and nebulin localization in normal cultures

In a third set of experiments, cell cultures were double-labeled with anti-titin and anti-nebulin antibodies. We wished to determine the temporal relationship of titin and nebulin incorporation into the adult sarcomere, and to determine if titin and nebulin organization progressed at the same rate. In Fig. 11, localization studies on normal myogenic cells at early stages of differentiation is shown. Fig. 12 represents normal cells in relatively later stages of differentiation. As previously described for muscle myosin, actin, and nebulin, titin is expressed in postmitotic, mononucleated myoblasts (Fig. 11a-c), and typically shows a diffuse distribution (Fig. 11a-c; Fig. 11d-f). Also as noted previously, initial assembly of titin and nebulin occurs along actin-containing SFLS, although at the arrow in Fig. 11d-f, nebulin is also distributed in a series of dots along a SFLS. Temporally, titin organization, as defined by the appearance of discreet titin bands along nascent myofibrils, precedes nebulin organization. Several developing myotubes shown in Fig. 12 demonstrate this unsynchronized assembly of titin- and nebulin-containing filaments into the sarcomere (arrowhead in Fig. 12a-c; arrow in Fig. 12d-e; arrow in Fig. 12f-g). Adult-like banding patterns of both titin and nebulin are present in mature myotubes which contain striated myofibrils (Fig. 12h-i). In mature myofibrils found in myogenic cell culture, titin banding is at the level of the A-I junction, while the nebulin bands are significantly closer to the Z line.

Fig. 11. Double-immunofluorescence localization of titin and nebulin in early myotubes in normal embryonic chick myogenic cell cultures

Myogenic cell cultures double-labeled with monoclonal anti-titin (a, d) and polyclonal anti-nebulin (b, e). Phase contrast images corresponding to (a, b) and (d, e) are shown in (c) and (f) respectively. (a-c) Myogenic cell in a 24-h culture shows the diffuse distribution of both titin (a) and nebulin (b) characteristic of myoblasts and early myotubes. (d-e) Early myotubes in a 48-h culture. Both proteins are distributed primarily in a diffuse pattern. However, some titin-positive fibers are visible in (d), as indicated by the arrow, and nebulin is distributed in a series of dots along these fibers in (e). Bar, 10 μm.

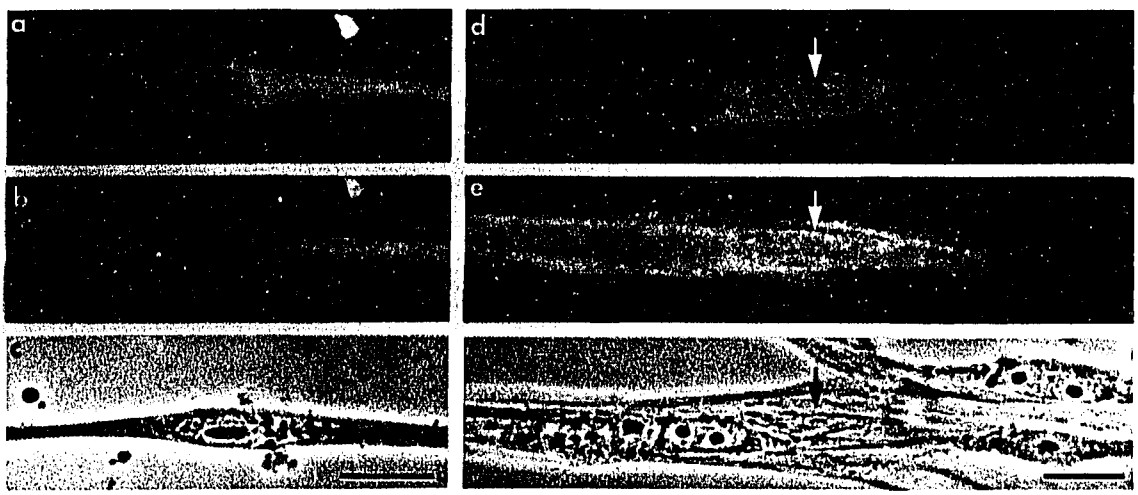
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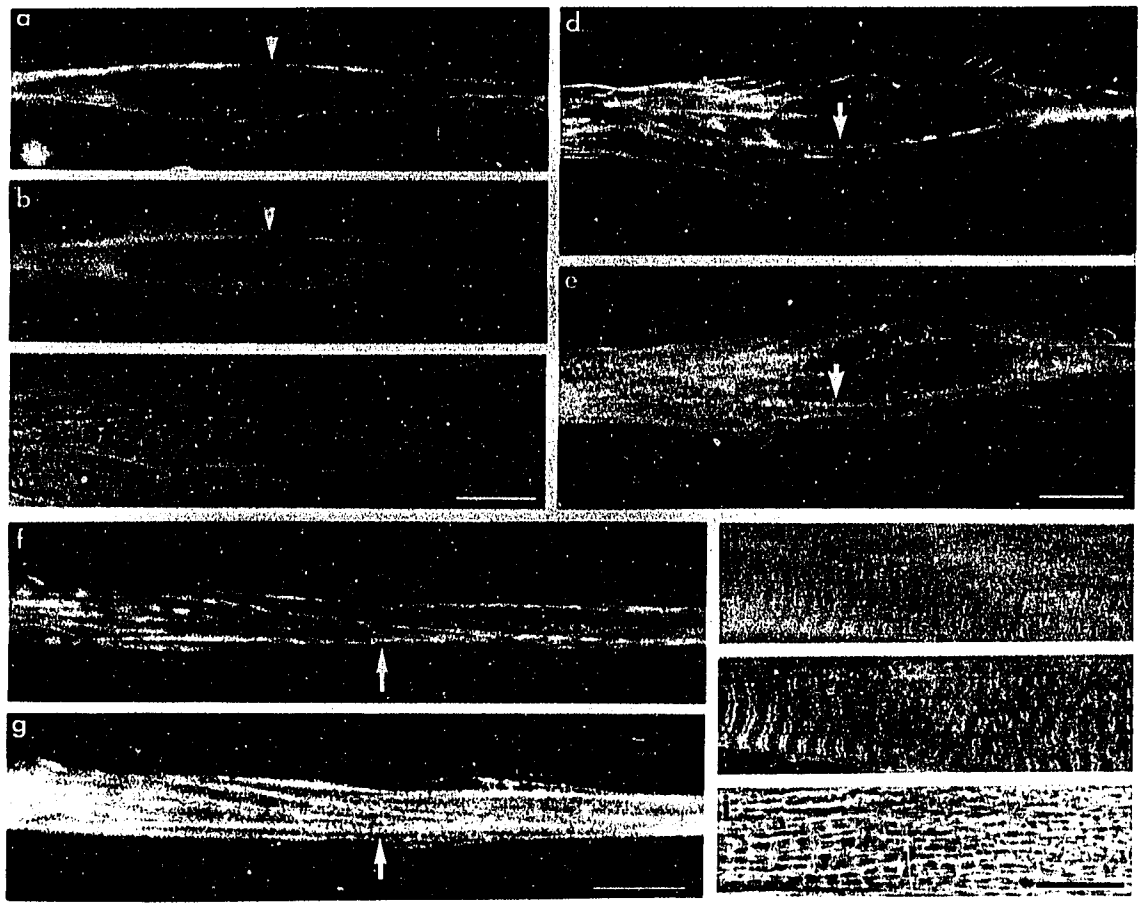
Fig. 12. Double-immunofluorescence localization of titin and nebulin in normal embryonic chick myogenic cell cultures Cultures double-labeled with monoclonal anti-titin (a, d, f, h) and polyclonal anti-nebulin (b, e, g, i) showing intermediate and late stages of myofibrillogenesis. Phase contrast images corresponding to fluorescence images in (a, b) and (h, i) are shown in (c) and (j), respectively. (a-c) Early myotube in a 72-h culture. A fiber that shows a continuous distribution of nebulin (b) with a broad, periodic distribution of titin (a) is indicated by the arrow. (d, e) Myotube in a 72 h culture demonstrates a filamentous organization of titin (d); several of these titin fibers are also positive for nebulin (e). In some fibers, titin appears to be at a more advanced stage of organization than nebulin (arrow). (f, g) Myotube in a 72 h culture. This cell shows several nascent myofibrils with titin organized in a banded pattern (f) while nebulin is organized in a continuous distribution along the same fiber (g). An example is indicated by the arrow. (h-j) Portion of a myotube in a 96-h culture, showing the organization of both titin (h) and nebulin (i) in an adult myofibril-like banding pattern. Titin banding is at the level of the A-I junction, while the nebulin bands are closer to the Z line. Bar, 10 μm.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Titin and nebulin localization in EMS-treated cultures

The results of double-label immunofluorescence of EMS-treated recovering myosheets are shown in Fig. 13 and 14. These myosheets confirm several of the observations seen in other double-label experiments. Diffuse, cytoplasmic nebulin fluorescence in addition to a few nonstriated fibers is characteristic of immature, developing myotubes (single arrows in Fig. 13a-c). As myotube development proceeds, cytoplasmic nebulin labeling is reduced, but nebulin is still present as a nonstriated array along apparently nascent myofibrils which show distinct titin banding (arrows in Fig. 13d-f). In the phase contrast image shown in Fig. 13f the fibril indicated by the arrow seems to be a cluster of many narrow fibers. In several nonstriated nascent myofibrils, illustrated in the myosheet in Fig. 13g-i titin banding has occurred (arrowhead and open arrow) but only rudimentary nebulin organization into bands has been initiated. The nascent myofibrils in Fig. 14a-c have segregated into distinct light and dark bands, but do not show distinct Z lines by phase contrast. In these myofibrils titin shows a discreet, adult-like double banded pattern. Nebulin, however, appears to be in a series of broad, single bands rather that the double banded pattern indicative of an adult state of organization. The myofibrils shown in Fig. 14d-f, however, do show Z lines by phase contrast. In this case, both titin and nebulin are organized into banded patterns characteristic of adult myofibrils.

Fig. 13. Double-immunofluorescence localization of titin and nebulin in EMS-treated embryonic chick myogenic cell cultures

Immunofluorescence images of EMS-treated cultures labeled with monoclonal anti-titin (a, d, g) and polyclonal anti-nebulin (b, e, h), and corresponding phase contrast images (c, f, i). (a-c) Portion of a myosheet in a culture that had been allowed to recover in normal medium for 2 d. Titin in this cell is localized along several SFLS and nascent myofibrils (a); arrows point to specific fibers where distinct titin double bands are apparent. Nebulin is still diffusely localized along SFLS and throughout the cytoplasm. (d-f) Portion of a myosheet allowed to recover for 2 d showing several large diameter nascent myofibrils (arrow) that demonstrate titin localization in an adult myofibril-like double banding pattern in (d). Nebulin in this cell (e) is localized in a diffuse pattern along these nascent myofibrils. Banding is not yet apparent in these myofibrils in the phase contrast image (f). (g-i) Myosheet in a culture that had been allowed to recover in normal medium for 3 d. Open arrow indicates a nascent myofibril that shows distinct titin banding (g) but a more diffuse distribution of nebulin. Myofibril indicated by closed arrow shows both titin and nebulin banding, however the nebulin banding remains much more diffuse. Bar, 10 μm.

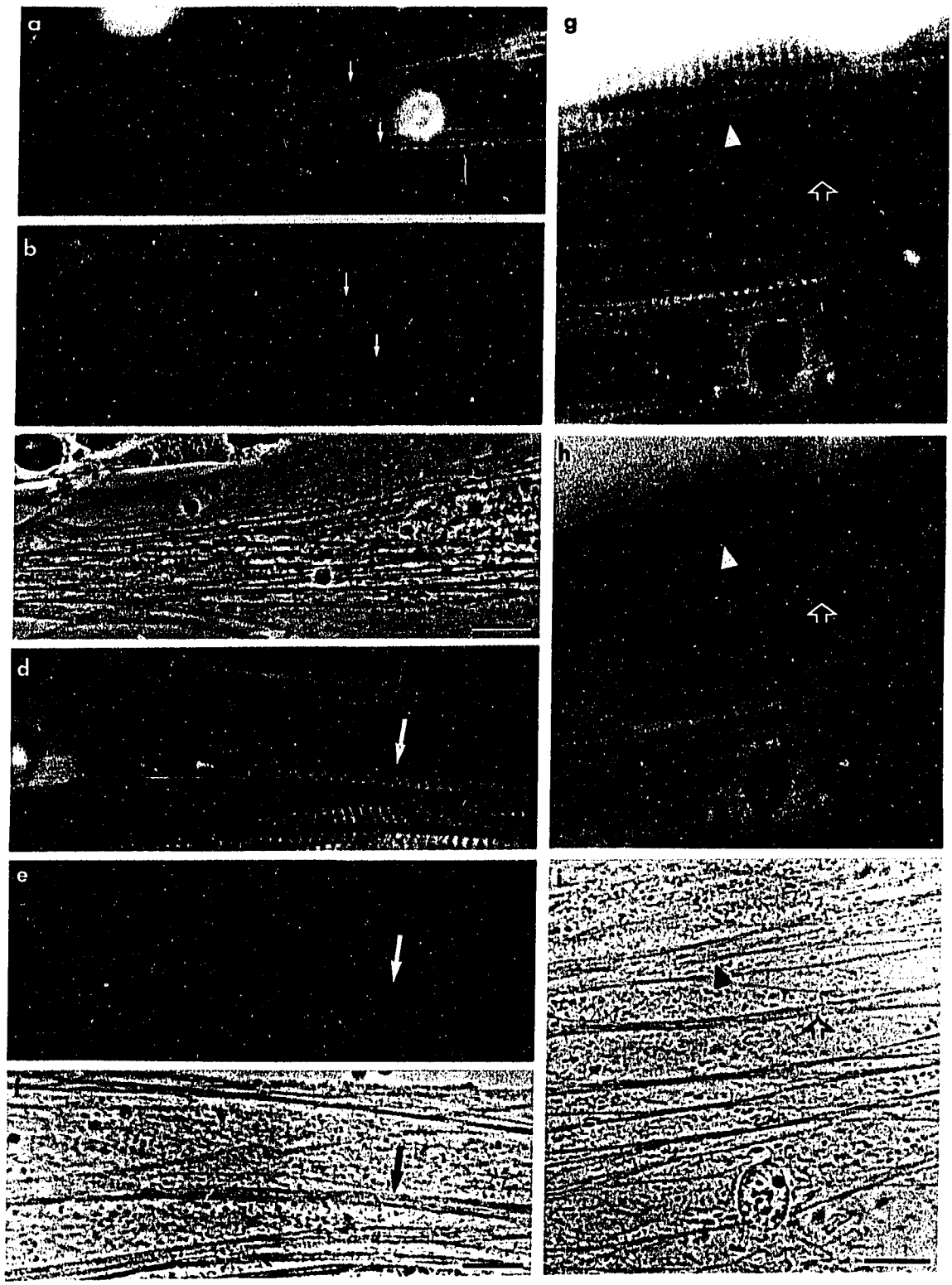
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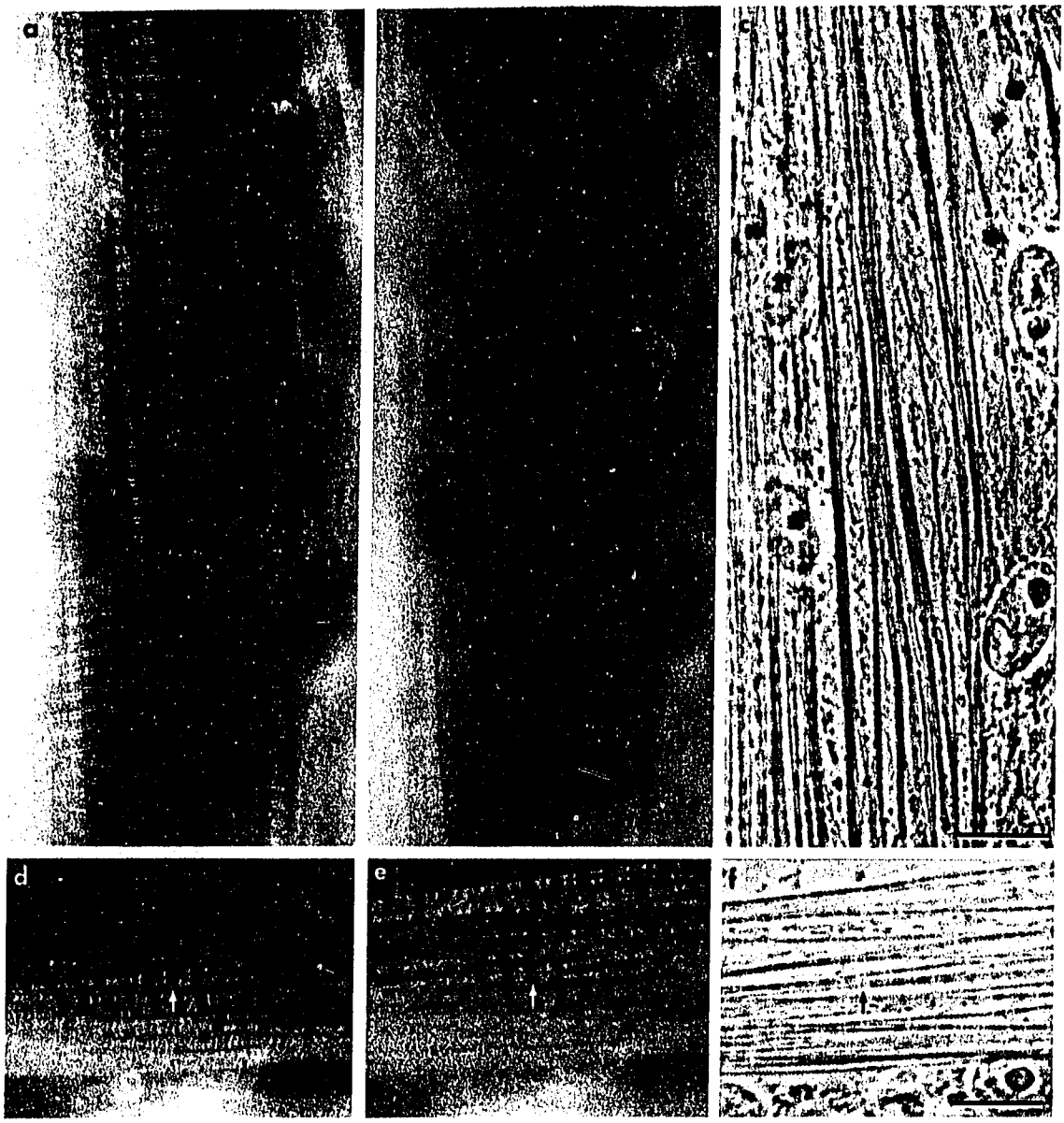
Fig. 14. Double-immunofluorescence localization of titin and nebulin in EMS-treated embryonic chick myogenic cell cultures

Paired immunofluorescence images of EMS-treated cultures that had been allowed to recover for 4 d in normal medium, labeled with monoclonal anti-titin (a, d) and polyclonal anti-nebulin (b, e). Corresponding phase contrast images are shown in (c, f). (a-c) Myosheet showing several nascent myofibrils at relatively late stages of assembly. A and I bands are visible in the phase contrast image (c), but Z lines are not readily apparent. Anti-titin labeling at the A-I junction is evident in these myofibrils (a). Nebulin labeling (b) is seen as a series of broad, single bands, rather than the double banding pattern normally seen in adult myofibrils, suggesting that nebulin organization is not yet complete relative to the organization of titin. (d-f) Portion of a myosheet showing adult myofibril-like labeling of nascent myofibrils with both anti-titin (d) and anti-nebulin (e). Z lines are clearly evident in these myofibrils in the phase contrast image. Bar, 10 μm.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DISCUSSION

The objective of this work was to establish the temporal sequence of expression and assembly of nebulin in differentiating skeletal muscle cells, in relation to that of the other myofibrillar proteins myosin, actin, and titin. Results of the immunofluorescence studies demonstrate that nebulin is initially expressed in postmitotic myoblasts, at approximately the same time as the expression of the majority of the other myofibrillar proteins begins (Devlin and Emerson, 1976). In early stages of differentiation in myogenic cell cultures (myoblasts and early myotubes), nebulin is localized diffusely throughout the cell cytoplasm. In developing myotubes, the primary organizational pattern is a continuous distribution along actin-containing stress-fiber-like structures (SFLS). With further development, the continuous distribution of nebulin along SFLS changes first to a series of dots, and then to a series of broad bands. The mature pattern of double bands was found only in well-developed myofibrils that were visible in phase contrast. Appearance of the adult pattern of nebulin localization appeared to correlate with the appearance of phase-dense Z lines.

Our results also provide evidence for a sequence of organization of the various proteins examined during myofibrillogenesis. While the initial localization of nebulin along the actin-containing SFLS occurred at the same time as the incorporation of myosin and titin into these structures, the subsequent organization of nebulin into the adult-like pattern lagged behind the organization of titin, myosin and actin into myofibrillar patterns. Assembly of myosin into A bands occurred first,

followed closely by the organization of titin. A close correlation between the assembly of myosin into A bands and the appearance of an adult-like localization pattern for titin has been reported previously (Hill et al., 1986; see Section I herein). Appearance of discreet I bands as determined by actin localization occurs later than the organization of myosin and titin, and appears to be accompanied by the arrangement of nebulin into a series of dots or broad bands along the nascent myofibril. The final event in this sequence is the appearance of nebulin labeling in the adult-like double-banded pattern. This later event appears to be associated with assembly of the Z-line, but this correlation cannot be made with certainty due to the fact that Z lines are not always easily seen in myofibrils in cultured cells on plastic plates by phase-contrast microscopy. This sequence of events was confirmed by the use of myosheets recovering from EMS treatment. The assembly of myofibrils in myosheets is prolonged in comparison to that in normal cultures, and this allows the temporal sequence to be more easily determined.

Unfortunately, the exact role of nebulin in the myofibril structure is not known. On the basis of immunofluorescence labeling patterns of isolated myofibrils using polyclonal antibodies to nebulin, Wang (1985) initially proposed that nebulin is a component of a third set of elastic, longitudinal filaments in the myofibril, in addition to the thick and thin filaments. In this model, titin-containing filaments span the sarcomere from near the M line to the N_2 line, while nebulin-containing filaments run from the N_2 line to the Z line and connect the

titin filaments to the Z-line. The organization of nebulin at late stages of de novo myofibril assembly observed herein would correlate with the final attachment of titin to the Z-line.

More recently, on the basis of preliminary results reported in abstract form, Wang and Wright (1987) have stated that nebulin constitutes a distinct set of filaments in parallel, but not in series, with titin filaments. They propose a four-filament sarcomere model consisting of thick filaments and thick filament (A segment) linked titin filaments, and thin filaments plus thin filament (I segment) linked nebulin filaments. In this model, nebulin might act as an organizing template and as a length-determining factor for the assembly of I-segments during muscle development. This model would also suggest a characteristic, temporal sequence of organization of nebulin with respect to A band and I band assembly much like that observed in our immunofluorescence studies.

We have shown previously (see Section I herein) that actin organization into I bands occurs after A band organization. Nebulin organization seems to be correlated with this later organization of actin into I bands, as the appearance of discreet nebulin bands along nascent myofibrils occurred at the same time, or after, localization of actin into I bands. Thus, nebulin does not appear to have a role in the organization of the A band, in contrast to preliminary results reported by Miranda et al. (1987), who stated that nebulin and myosin heavy and light chains become organized into sarcomeres simultaneously in regenerating clonal human muscle cell cultures. On the other hand,

nebulin might play a role in the assembly of either the I band or the Z line. In agreement with this latter possibility, Miranda et al. (1987) found that nebulin and actin or nebulin and alpha-actinin did not co-localize prior to sarcomere formation, but appeared simultaneously in organized sarcomeres.

Finally, our results add support to the hypothesis that SFLS are the site for myofibril assembly in myogenic cells grown in culture, as suggested by Holtzer and coworkers (Dlugosz et al., 1984; Antin et al., 1986). In agreement with their results, we have observed that the existence of SFLS and striated myofibrils is mutually exclusive within a given myotube (in normal cultures) or recovering myosheet (in EMS-treated cultures). In addition, the ratio of SFLS to striated myofibrils appears to decrease with increasing time in culture. For all of the muscle-specific proteins examined in this study (myosin, titin, and nebulin), the first identifiable state of organization was a continuous distribution along fibers. Double-labeling with antibodies to nebulin and actin demonstrated that these fibers contained actin, and previous double-immunofluorescence localization of titin and actin (see Section I herein) also demonstrated titin localization along actin-containing fibers. While positive identification of these structures as SFLS would require labeling with antibodies specific for cytoplasmic isoforms of actin, the present results leave little doubt that these structures are identical to the SFLS identified by previous investigators (Dlugosz et al., 1984; Antin et al., 1986). This initial localization of muscle-specific proteins along SFLS is followed by

organization of these proteins into bands to form an adult, striated myofibril, according to the temporal sequence described previously.

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OVERALL SUMMARY AND CONCLUSIONS

The precise nature of events leading to the formation of the myofibril in striated muscle is still not clear, despite many previous ultrastructural and immunofluorescence studies. Possible new myofibrillar proteins are still being isolated and characterized. Two muscle specific high molecular weight proteins, titin and nebulin, were first isolated in the late 1970s (Wang et al., 1979; Wang and Williamson, 1980). My studies have concentrated on the role of titin and nebulin in myofibril assembly in embryonic chick skeletal muscle cell cultures.

Although the exact function of titin and nebulin in the myofibril is unknown, both proteins are striated muscle specific, suggesting that they may play a role in myofibril organization. Furthermore, previous immunofluorescence studies from this laboratory showed an early appearance and incorporation of nebulin into myofibrils in myogenic cell culture. To examine the organization of these proteins during myofibrillogenesis, I used double-label immunofluorescence techniques to follow the temporal sequence of incorporation of titin and nebulin into the myofibrillar structure with respect to actin (thin filament) and myosin (thick filament) assembly. Preliminary studies by other investigators suggested that titin filaments assembled first in the striated muscle myofibril structure, to form a scaffold for subsequent thick and thin filament interdigitation (Hill and Weber, 1986).

Polyclonal antibodies to SDS-denatured adult chicken breast titin and nebulin were available for use in immunofluorescence labeling

experiments. Because immunoblots and myofibril staining demonstrated monospecificity, whole antiserum to titin was used in immunofluorescence labeling studies. Antiserum to nebulin was affinity purified by absorption with titin-coated nitrocellulose pieces to remove contaminating titin antibody. I produced a monoclonal antibody to SDS-denatured adult chicken breast titin in order to perform double-labeling experiments with the polyclonal nebulin antibody. Western immunoblot analysis showed that the titin monoclonal antibodies were specific for titin. Monoclonal antibodies to actin were obtained from the Developmental Studies Hybridoma Bank, Johns Hopkins Univ. Medical School, Baltimore, MD (Lin, 1981). Monoclonal antibodies to muscle-specific myosin were a gift of Dr. Donald Fischman, Cornell Univ. Medical College, New York (Shimizu et al., 1985). Adult chicken skeletal myofibrils were isolated and double-labeled with the four antibodies described above in order to further characterize the antibodies and to determine the banding patterns of the anti-titin and anti-nebulin antibodies on adult myofibrils.

The model system used in these studies was primary skeletal muscle cell cultures derived from mixed leg and thigh muscles of 12 day chick embryos, using standard laboratory methods (Ridpath, 1983). With this cell culture system I could follow the assembly of the contractile apparatus during the development of myogenic cells from mononucleated myoblast to multinucleated myotube. In addition to the use of normal culture techniques, myogenic cell differentiation in culture was manipulated by the addition of the drug ethyl methanesulfonate (EMS) to

the culture medium. I chose the mutagenic alkylating agent EMS because exposure of myoblast cultures to EMS results in the formation of large, flat, multinucleated myosheets which are ideal for light microscope examination. Synthesis of myofibrillar proteins, and thus myofibril assembly, is arrested in myosheets cultured in medium containing EMS. Myosheets recovering in normal medium assemble myofibrils at a relatively protracted rate as compared to normal myogenic cells so the time course of myofibrillar protein incorporation could be further investigated. Normal embryonic chick myogenic cell cultures were incubated for 2, 3, or 4 d, then processed for double-label immunofluorescence. EMS-treated cultures were incubated in the presence of the drug for 2 d, then allowed to recover in normal medium for 2, 3, or 4 d before processing for immunofluorescence. In one set of experiments, normal and EMS-treated myogenic cell cultures were double-labeled with polyclonal antibodies to titin and monoclonal antibodies to muscle-specific myosin, or with the titin antibodies and monoclonal antibodies to actin. In a second set of experiments, cultures were double-labeled with polyclonal antibodies to nebulin, and monoclonal antibodies to either muscle-specific myosin, to actin, or to titin.

The main conclusions which can be drawn from the results described in this dissertation are:

1. Postmitotic, mononucleated myoblasts synthesize titin, nebulin, and muscle-specific myosin as shown by the labeling of mononucleated myoblasts, but not fibroblasts, in early cultures. This is additional evidence that myoblast fusion is not a prerequisite for the initiation

of myofibrillar protein synthesis (Allen et al., 1979). This also shows that the synthesis of titin and nebulin appears to be coordinated with the synthesis of other myofibrillar proteins. At this stage of development, the proteins analyzed were predominantly found spread diffusely throughout the myoblast cell cytoplasm.

2. Results of immunofluorescence labeling experiments suggest that although myofibrillar proteins are expressed simultaneously in myogenic cell culture, organization into the myofibril is not simultaneous. Thus, there is evidence for a temporal sequence of protein incorporation into myofibrils.

3. Titin, nebulin, muscle-specific myosin and actin distribution along filamentous structures is seen in some mononucleated myoblasts and in all multinucleated, developing myotubes. These filaments are morphologically similar to nonmuscle stress fibers when viewed in the phase microscope, and are morphologically identical to the stress-fiber-like-structures (SFLS) described by Holtzer and co-workers (Antin et al., 1986). An initial event of myofibril assembly, then, appears to be the coalescence of myofibrillar proteins along SFLS. At this stage, no segregation of individual proteins into their respective adult bands could be detected.

4. Controversy exists in the literature on the subject of whether SFLS are templates for myofibril assembly, or are actual myofibril precursors. I could not address this question directly because I did not compare antibodies that recognize nonmuscle and muscle isoforms of myosin and actin, and to date, no nonmuscle forms of titin and nebulin

have been described. I have observed, however, that the existence of non-striated SFLS and mature, striated myofibrils within a given muscle cell are mutually exclusive. In other words, myotubes with many SFLS have few striated myofibrils, and vice versa. I have also shown that muscle myosin, titin and nebulin first organize along SFLS, and that a given antibody could label in both a striated and non-striated pattern along SFLS. I saw no evidence of the existence of a series of mini-sarcomeres which subsequently expand to form sarcomeres of adult size. My observations appear compatible with those of Holtzer and co-workers in that myofibrillar protein organization and the appearance of discreet bands takes place along SFLS.

5. During myofibril assembly, the organization of titin and myosin appear to be linked. The two proteins co-localize at all stages of myogenic cell development. In the vast majority of cells examined, titin banding is not seen in the absence of myosin banding, and vice versa. These studies were extended by following titin and myosin localization in EMS-treated cultures, in the event that initial titin or myosin organization was simply not seen in the time frame of myofibril assembly in normal cell culture. The results of double-label experiments in the EMS system confirmed the results obtained with normal cultures. It has recently been suggested that a large fraction of titin is in direct association with myosin heavy chain, even early in development (Isaacs et al., 1987). Thus, these results suggest that titin may serve to align myosin-containing thick filaments into A bands.

6. In contrast to titin and myosin, titin and nebulin or titin and

actin do not appear to be linked during myofibril assembly. Titin organization into adult-like double bands precedes the organization of both nebulin and actin. This observation raises the possibility that titin filaments may serve a scaffolding role in I band assembly.

7. During myofibrillogenesis, the localization of nebulin into a punctate type of labeling pattern correlates with the initial organization of actin into I bands. However, nebulin organization into discreet, adult-like double bands occurs after the organization of actin into I bands. The appearance of adult-like nebulin banding correlates well with the presence of phase-dense Z lines in mature, striated myofibrils. Of the four proteins studied, nebulin appears to be the last to organize by the criterion of adult-like banded labeling patterns along myofibrils. Possible roles of nebulin in the sarcomere may involve Z line organization or the regulation of the insertion of thin-filaments into Z lines.

8. Both titin and nebulin progress through four stages of organization. The initial stage is a filamentous form along SFLS. In relatively narrow diameter SFLS, titin and nebulin antibodies localize to a series of dots, or "beads on a string". Along nascent myofibrils, a series of evenly spaced bands is seen. Double-banded patterns of titin and nebulin labeling are found along mature, striated myofibrils. As mentioned previously, all four stages of titin organization normally precede those of nebulin.

9. Double-label immunofluorescence labeling of EMS-treated myosheets allowed to recover in normal medium confirms the results of

labeling experiments in normally cultured myogenic cells. The general temporal sequence of organization that can be derived from both culture systems appears to be myosin and titin, followed by actin, and finally nebulin.

10. An interesting observation of labeling studies using the muscle-specific myosin monoclonal antibody is that a population of cells in our cultures do not stain with this antibody. These cells do stain, however, with the other three antibodies used. The nature of this lack of staining was not investigated in this dissertation.

In summary, it appears that titin and nebulin are expressed simultaneously with muscle-specific myosin and actin in embryonic chick myogenic cell cultures. There is, however, a temporal sequence of assembly of these four proteins into the adult, striated myofibril. Titin and myosin are organized at the same time, followed by actin and nebulin. Titin may play a scaffolding or template role for the organization or alignment of thick filaments in the A band. Nebulin may have a similar organizational function in the assembly of I bands.

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