

# Myogenesis in the Mouse Embryo: Differential Onset of Expression of Myogenic Proteins and the Involvement of Titin in Myofibril Assembly

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**Abstract.** Antibodies to muscle-specific proteins were used in immunofluorescence to monitor the development of skeletal muscle during mouse embryogenesis. At gestation day (g.d.) 9 a single layer of vimentin filament containing cells in the myotome domain of cervical somites begins to stain positively for myogenic proteins. The muscle-specific proteins are expressed in a specific order between g.d. 9 and 9.5. Desmin is detected first, then titin, then the muscle specific actin and myosin heavy chains, and finally nebulin. At g.d. 9.5 fibrous desmin structures are already present, while for the other myogenic proteins no structure can be detected. Some pre-fusion myoblasts display at g.d. 11 and 12 tiny and immature myofibrils. These reveal a periodic pattern of myosin, nebulin, and those titin epitopes known to occur at and close to the Z line. In contrast titin epitopes, which are present in mature myofibrils along the

A band and at the A-I junction, are still randomly distributed. We propose, that the Z line connected structures and the A bands (myosin filaments) assemble independently, and that the known interaction of the I-Z-I brushes with the A bands occurs at a later developmental stage. After fusion of myoblasts to myotubes at g.d. 13 and 14 all titin epitopes show the myofibrillar banding pattern. The predominantly longitudinal orientation of desmin filaments seen in myoblasts and in early myotubes is transformed at g.d. 17 and 18 to distinct Z line connected striations. Vimentin, still present together with desmin in the myoblasts, is lost from the myotubes. Our results indicate that the putative elastic titin filaments act as integrators during skeletal muscle development. Some developmental aspects of eye and limb muscles are also described.

**S**KELETAL muscles of the vertebrate body develop primarily from lateral plate and paraxial somitic mesoderm (Boyd, 1960). The developing embryo reveals a gradient of myogenic differentiation, which starts dorsally in the somites. It traverses the lateral mesoderm in the dorso-ventral direction and leads to the development of limb and eye muscles (Strauss and Rawles, 1953; Konigsberg, 1965). With the availability of muscle cultures, derived from already far developed embryonic tissue, a large number of studies have tried to analyze the transition of "undifferentiated" fibroblast-like cells to highly specialized muscle cells with their typical myofibrillar apparatus (reviewed in Bischoff, 1978; Fischman, 1986; Holtzer et al., 1984; Pearson et al., 1980). Currently the first recognizable step in myogenesis seems to be the initiation of desmin in replicating presumptive myoblasts, which still contain vimentin. Other muscle-specific proteins such as sarcomere-specific actins, myosins, titin, and myomesin first appear in postmitotic mononucleated myoblasts (Grove et al., 1985; Hill et al., 1986) but thus far the relative order of expression of these proteins has not been defined in detail. Myofibrillar assembly, possibly using pre-existing stressfiber-like structures as a template, starts before

the fusion of these myoblasts into multinucleated myoblasts or myotubes (Dlugosz et al., 1984; Wang et al., 1988).

One important but as yet unclarified aspect of myofibrillogenesis concerns the integration of the giant titin molecules, which seem to measure up to 1  $\mu\text{m}$  in length (Maruyama, 1986; Wang, 1985) into the myofibril. Using distinct monoclonal antibodies in immunoelectron microscopy of adult chicken breast muscle we have earlier provided a map of 10 nonrepetitive titin epitopes, which starts at the Z line and extends close to the M line (Fürst et al., 1988). Thus per half sarcomere, titin filaments are polar units, which are arranged parallel and in register. Since the epitope positions of these titin antibodies are defined, it is possible to use them to follow the process of myofibrillogenesis. To obtain knowledge of the spatial and temporal aspects of expression of myogenic proteins in different muscle groups we chose the mouse embryo *in vivo*. We found that the expression of muscle proteins starts in the cervical somites at gestation day (g.d.)<sup>1</sup> 9. The order of the onset of expression is desmin, titin, muscle-specific actin and myosin heavy chains, nebulin. Our results

1. Abbreviation used in this paper: g.d., gestation day.

also imply that titin assembles with the I-Z-I brushes independent from the A bands and that the known interaction of the two structural units occurs at a later developmental stage, i.e., g.d. 14.

## Materials and Methods

### Embryos

Embryos were from spontaneous matings of mice (Naval Medical Research Institute). The presence of a vaginal plug indicating a successful mating was regarded as gestation day 0. Defined developmental stages in the g.d. 9 to 10 intervals were from matings which had occurred within 3 h rather than within the usual overnight period. To establish precisely the order in which the muscle-specific proteins were expressed, the embryos had to be staged by their somite counts at the early time points (g.d. 8 to 10). At later time points embryos of different sizes from the same uterus were sectioned. This is because the speed of embryonic development in utero is known to vary by around half a day when embryos from the same uterus are compared. Pregnant mice were killed by cervical dislocation on day 8 or on subsequent days. Uterine horns were removed and dissected. Day 9 embryos were freed from extraembryonic tissues, staged according to their somite counts, embedded in Tissue-Tek (Miles Scientific Div., Naperville, IL) and frozen by immersion in isopentane cooled with liquid nitrogen to  $-140^{\circ}\text{C}$ . All other embryos were placed on dry ice and directly frozen. Day 8-10 embryos were frozen together with their decidua. Embryos were stored at  $-70^{\circ}\text{C}$  until use. Embryonic development was staged according to Theiler (1972).

### Antibodies

All antibodies used in this study have been previously described and characterized: monoclonal mouse anti-desmin, clone DE-B-5 (Debus et al., 1983), monoclonal rat anti-keratin 8, clone Troma 1 (Brület et al., 1980), kindly provided by Dr. R. Kemler (Max Planck Institute for Immunology, Freiburg), polyclonal guinea pig anti-vimentin (Osborn et al., 1980), monoclonal mouse anti-titin clones T3 (Hill and Weber, 1986), T12, T20,

and T22 (Fürst et al., 1988), polyclonal rabbit anti-titin (Fürst et al., 1988), and monoclonal mouse anti-nebulin, clones Nbl and Nb2 (Fürst et al., 1988). Monoclonal mouse anti-myosin, clone BF-47, specific for heavy chains of sarcomeric myosins was a gift from Dr. Schiaffino (University of Padua, Italy). Polyclonal rabbit anti-actin antibodies, specific for muscle actins, were kindly provided by Dr. H.-D. Mannherz (University of Marburg, FRG) and Dr. U. Groeschel-Stewart (University of Darmstadt, FRG). Monoclonal antibodies were used as hybridoma supernatants; polyclonal antibodies were affinity purified on their respective antigens and used at 0.1 mg/ml antibody concentration.

For indirect immunofluorescence microscopy the following second antibodies from Cappel Laboratories (Malvern, PA) were used: FITC goat anti-mouse, rhodamine goat anti-mouse, rhodamine goat anti-guinea pig, FITC sheep anti-rabbit, and rhodamine goat anti-rat. For double labeling experiments second antibodies were cross-absorbed on unlabeled IgGs from the other species coupled to a solid support.

### Immunocytochemistry

5- $\mu\text{m}$ -thick transverse or longitudinal cryostat sections were treated with acetone for 6 min at  $-10^{\circ}\text{C}$  and air-dried before antibody labeling. Indirect immunofluorescence staining using the antibodies listed above was as described (Hill and Weber, 1986). After the incubation with the second antibody some sections were treated with Hoechst dye No. 33258 to allow visualization of the nuclei. All samples were mounted in 80% glycerol in PBS with *p*-phenyldiamine (Janssen Pharmaceutica, Beerse, Belgium) added as an antibleach agent.

Immunoperoxidase labeling followed the same protocol except that peroxidase-labeled rabbit anti-mouse or anti-guinea pig IgGs (DAKO, Glostrup, Denmark) were used as second antibodies.

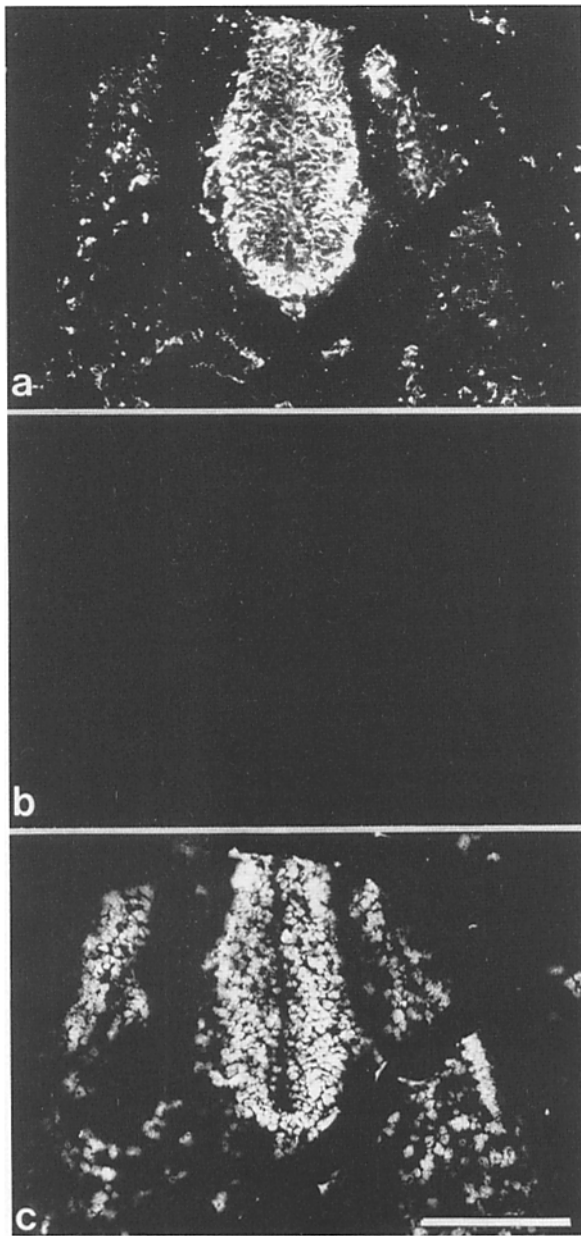
## Results

### Onset of Myogenic Protein Expression in Mouse Somites (g.d. 9.0-9.5)

Although the different striated muscles derive from the meso-

Table I. Summary of Immunofluorescence and Morphological Data

Embryonic day	Morphological data from literature	Immunofluorescence data
8	Formation of the first somites in the cervical region	Presumptive myotome region still negative for all muscle-specific proteins; vimentin stains filaments in somites
9	13-20 somites, myotomes begin to differentiate	In embryos with >16 somites one layer of cells in the cervical somites begins to express muscle proteins in the order: desmin, titin, myosin and actin (punctate and homogeneous distribution); these myoblasts are also positive for vimentin
9.5	21-29 somites	Myoblasts express nebulin; desmin present as cytoplasmic filaments
10	30-34 somites	Number of myoblasts increases
11	Myoblasts now longitudinally oriented (ventral processes)	Myoblasts elongate; formation of tiny myofibrils in a few cells (periodic stain for nebulin, myosin, and Z line epitopes of titin)
12	Further myoblast proliferation	Increase in myoblast number; only a few more immature myofibrils have formed; for the first time positive stain for all muscle proteins in developing eye and limb muscles
13	Myoblasts show spindle-shaped morphology; fusions occur	Myoblasts become spindle shaped; more myofibrils are formed; fusion of myoblasts begins
14	Myotube formation	Myotubes show mature myofibril periodicities with all muscle proteins; desmin filaments still longitudinally oriented; negative for vimentin; nuclei remain in central position
15-16	Cross-striated myofibrils in all myotubes; muscles become contractile	Fiber number and fiber size increase; muscle nuclei are at the edge of the fibers
17-18	Secondary fibers increase; tertiary fibers appear	Fiber number increases; muscle masses get more compact; desmin pattern changes from mainly longitudinal orientation to a mainly Z line connected pattern
Newborn mouse	Secondary fibers increase in size; intercellular space is reduced; tertiary fibers become more dominant	Diameter of fibers increases; different fibers cannot be discerned by the staining patterns obtained with the antibodies used in this study



**Figure 1.** Immunofluorescence micrographs of a frozen section through a cervical somite of a 12-somite mouse embryo, triply stained with antibodies to vimentin (*a*) and desmin (*b*) to show intermediate filaments, and with H $\ddot{o}$ chst dye to reveal nuclei (*c*). Note that the early somites are desmin negative but vimentin positive. In *c* the DNA stain reveals the organization of the section. Bar, 70  $\mu$ m.

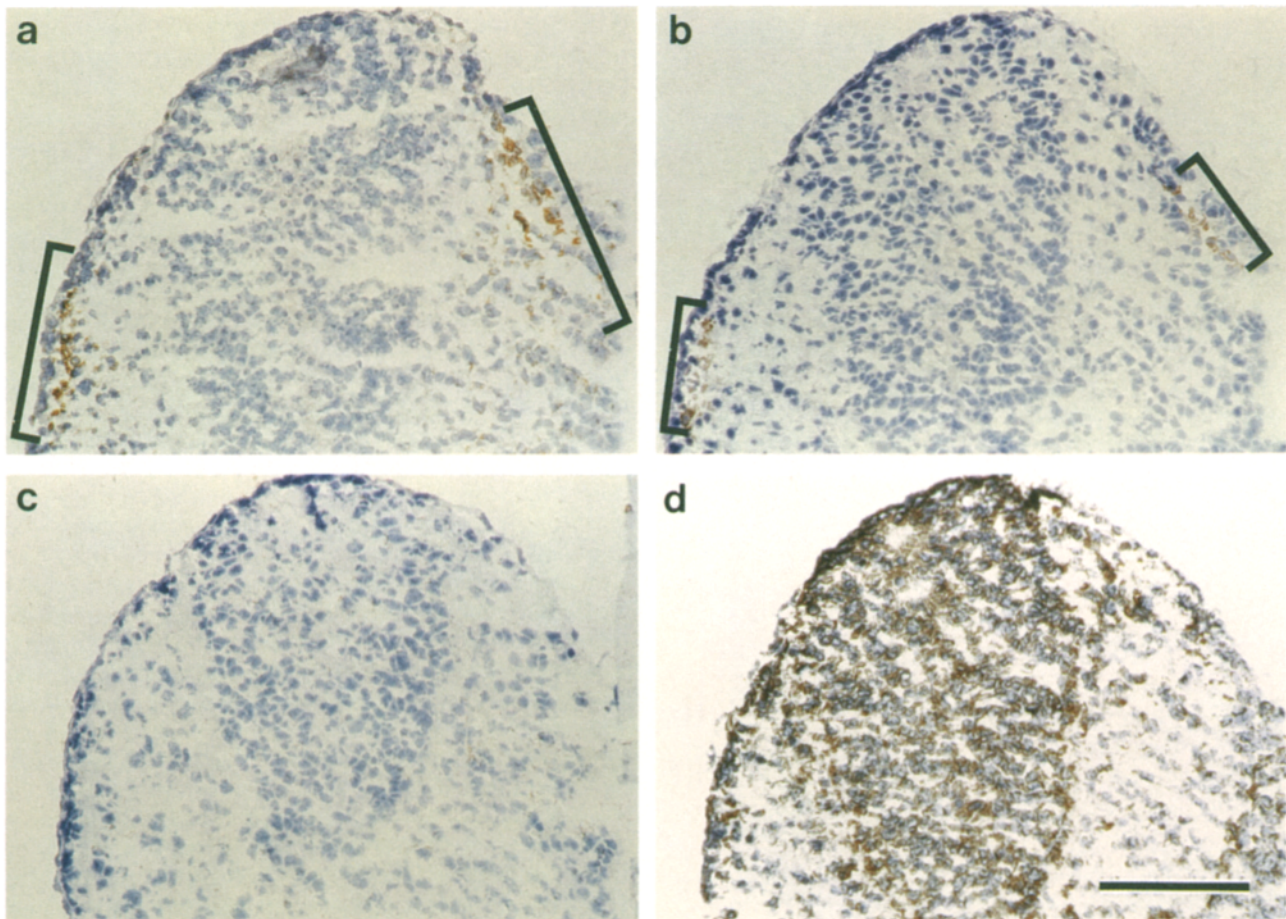
dermal germ layer, they develop independently at different times and positions. For example, myogenic processes begin in the mouse at g.d. 9 in the trunk and at g.d. 12 in the forelimbs. Cephalic muscles, best illustrated by the eye muscles, begin to arise at g.d. 12 from the head mesoderm (Boyd, 1960; Hopper and Hart, 1985; Rugh, 1968). We performed immunofluorescence studies on cryostat sections of whole mouse embryos from g.d. 8 until birth, since this period covers all important stages of tissue differentiation of the

above mentioned sarcomeric muscle groups. Onset and progress of myogenesis were monitored by antibodies to different muscle proteins. Our results are shown in Figs. 1-7 and summarized in Table I.

The first somites form at g.d. 8 in the cervical region of the embryo. These groups of undifferentiated cells express vimentin filaments in line with their mesodermal derivation but lack keratin 8, as well as all muscle proteins used in this study as markers of myogenesis (desmin, titin, nebulin,  $\alpha$  actin, and muscle specific myosin heavy chain) (Fig. 1).

At g.d. 9 between 13 and 20 somites have formed and differentiation into three cell types is usually distinguishable (Rugh, 1968). The cells of the sclerotome, which are situated close to the neural tube, migrate out in ventral direction. Subsequently the ventral part of the residual dermamyotome differentiates into the myotome (Boyd, 1960; Hopper and Hart, 1985). By purely morphological criteria the myotome cells cannot be distinguished from their neighbors. The "presumptive myoblasts" exhibit a rounded cell shape. They are mononucleated and lack myofibrils. In embryos of the 16 somite stage one layer of cells in the myotome region of the cervical somites begins to stain with antibodies to myogenic proteins. Onset of expression of the different proteins is not simultaneous. Instead a very specific order of events can be observed. The first muscle protein detected is desmin (Figs. 2 *a* and 3 *a*). Antibodies to desmin specifically stain one narrow layer of cells in the myotomes of the cervical somites (an overview of an immunoperoxidase stained specimen is shown in Fig. 2 *a*). In immunofluorescence microscopy no fibrous material is observed at this stage. Instead desmin labeling of these cells shows a spotty distribution throughout the cytoplasm (Fig. 3 *a*). Double labeling experiments document that these early myoblasts still reveal vimentin, which is organized as fibrous structures (Fig. 4, *a-c*). More caudally situated somites are still undifferentiated and lack desmin-positive cells. Within a very short time after the onset of desmin expression myotome cells begin to reveal titin staining. Fig. 2 compares on serial sections the staining patterns obtained with antibodies to desmin, titin, myosin, and vimentin using the immunoperoxidase procedure. Titin staining is detected in fewer cells than desmin staining and is generally weaker than the desmin stain. Immunofluorescence micrographs show an obvious lack of titin organization. The titin stain at this early stage occurs in a punctate or speckled manner in the cytoplasm (Fig. 3 *b*). Interestingly, as shown in Fig. 3, *c* and *d* these cells still lack the muscle-type isoforms of the contractile proteins actin and myosin. They are first detected in embryos with 19 to 20 somites, i.e., in myoblasts, which have already expressed desmin and titin for several hours. The two proteins show a punctate or more homogeneous distribution as noted for desmin and titin (Fig. 3, *h* and *i*).

Expression of nebulin in the myotomes is first noted at g.d. 9.5 and is thus clearly delayed versus the other myogenic proteins. Like all other muscle proteins nebulin expression starts in the cervical somites and its first appearance is again characterized by a speckled distribution (Fig. 3 *o*). A second major event at this stage concerns the display of desmin. The previously noted punctate display becomes organized and a meshwork of distinct fibrous structures is clearly discerned (Fig. 3 *k*).



**Figure 2.** Onset of myogenic protein expression in the somites, visualized by the immunoperoxidase method. Serial sections through the region of cervical somites of an 18-somite mouse embryo stained with antibodies to desmin (*a*), titin T12 (*b*), myosin (*c*), and vimentin (*d*). Nuclei were counterstained with Hematoxylin. Positive staining in the myotome region is indicated by the arrows. Note that more cells are positive for desmin (*a*) than for titin (*b*), and that at this time point no cells are positive for myosin (*c*). Vimentin positivity is found not only in the myotome region but is also seen in many cells of the neural tube. Bar, 45  $\mu\text{m}$ .

### **Differentiation of Myoblasts (g.d. 10–12)**

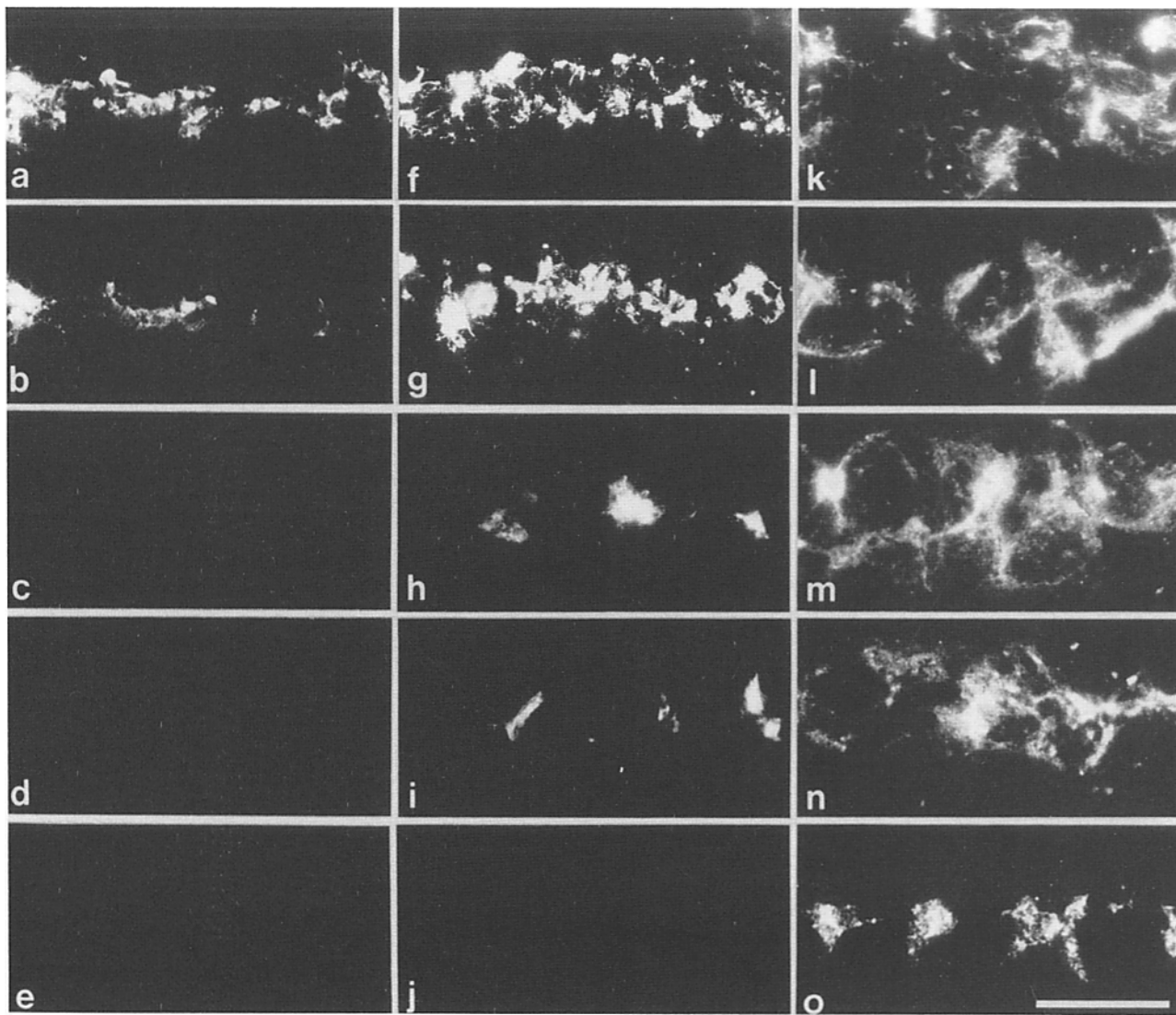
The number of myoblasts in the myotomes increases on g.d. 10, but no change in cellular shape and in the labeling patterns can be observed. The next obvious developmental step does not occur until g.d. 11. Now myoblasts lose their round morphology and achieve an elongated shape by the development of ventral processes (Fig. 5). In a few of these cells the formation of tiny myofibrils is detected. Over short stretches myosin and nebulin are now visualized by periodically aligned spots, which occur in the peripheral cytoplasm (Fig. 4, *c* and *d*).

Also titin antibodies such as T12 and T20, which recognize epitopes at or close to the Z line (Fürst et al., 1988), show a periodic pattern (Fig. 4 *a*). In contrast titin antibodies, which bind in mature muscle either at the A–I junction (T3) or within the A band (T22) do not reveal periodicities along the immature myofibril. Instead a continuous staining pattern is still observed (Fig. 4 *b*). We conclude from these patterns that the Z line–connected structures and the A bands (myosin filaments) assemble as separate units and that the interaction of the I–Z–I units with the A bands is an event of myofibril maturation, which occurs at a later stage (see below and Discussion).

In cells revealing immature myofibrils desmin still forms a filamentous meshwork without any preferential orientation versus the emerging myofibrils. No indication for an incorporation of desmin filaments into the Z line can be seen (Fig. 4 *f*). These events seem to occur rather slowly (see below) since at g.d. 12 only a few more immature myofibrils have been formed. At this stage of embryonic development one also observes for the first time myoblasts both in the limbs and around the eyes. They are readily identified by staining with the various antibodies to myogenic proteins (Fig. 6).

### **Myotube Formation and Maturation of Myofibrils (g.d. 13 and 14)**

At g.d. 13 myoblasts have clearly acquired a spindle-shaped morphology. Considerably more and longer myofibrils have now been formed. They are no longer limited to the periphery of the cell but instead fill a considerable part of the cytoplasm (Fig. 4, *h–l*). At the same time the previously mononucleated myoblasts begin to show cytoplasmic fusion resulting in the formation of multinucleated syncytia. This process is known as “formation of myotubes” (Bischoff, 1978; Holtzer et al., 1957; Fischman, 1986). The myotube nuclei still remain in a central position within the cytoplasm



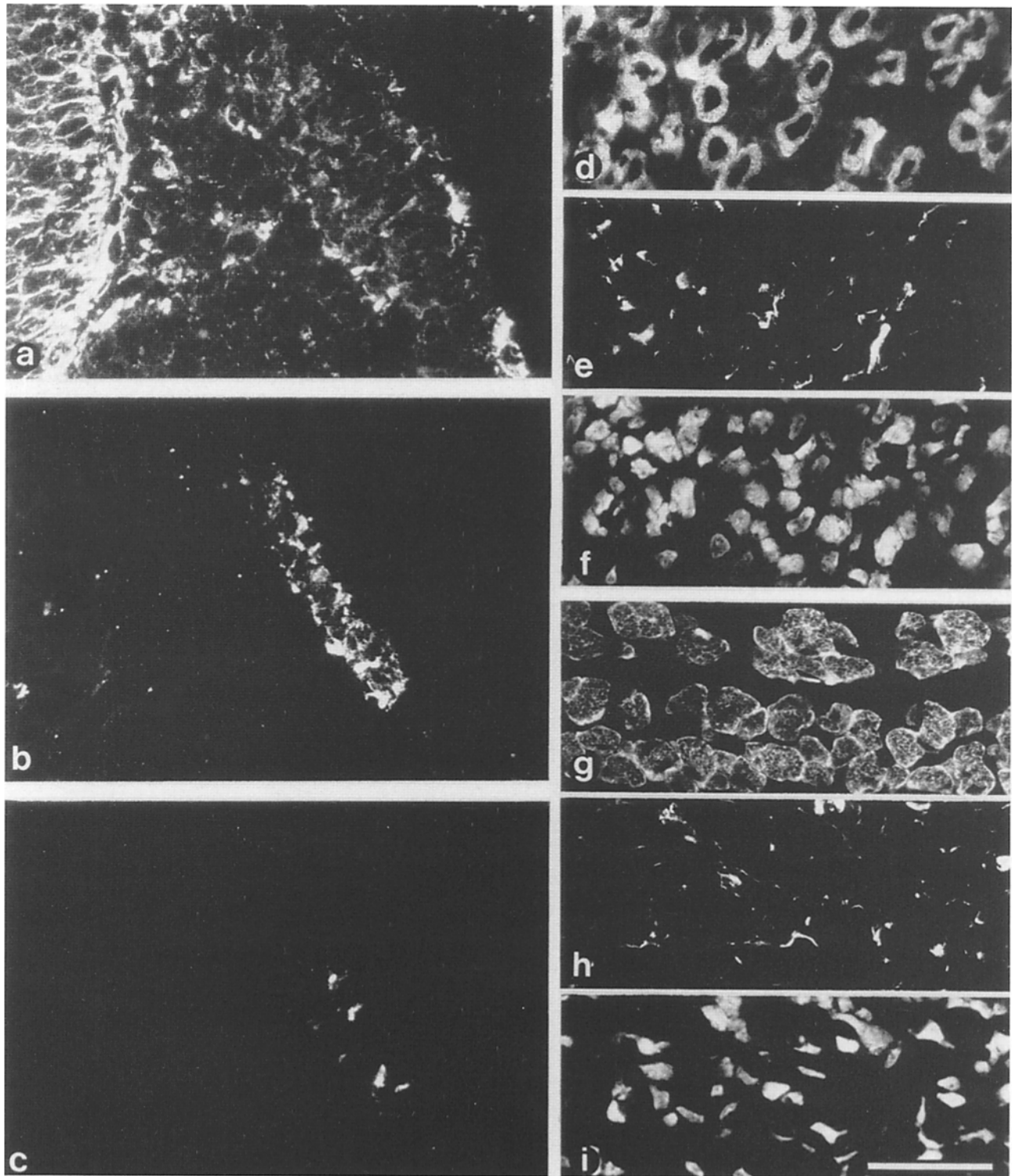
**Figure 3.** Comparison of myogenic protein expression in embryos with different somite numbers in immunofluorescence microscopy. Serial cross sections through cervical somites of an 18-somite (*a-e*), a 20-somite (*f-j*), and a 24-somite mouse embryo (*k-o*) stained with antibodies to desmin (*a, f, and k*), titin T20 (*b, g, and l*), skeletal muscle-specific myosin heavy chain (*c, h, and m*), skeletal muscle  $\alpha$  actin (*d, i, and n*), and nebulin (*e, j, and o*). Note that the order in which the muscle-specific proteins are expressed is desmin, titin,  $\alpha$  actin and skeletal muscle myosin, and finally nebulin. Bar, 25  $\mu$ m.

(Fig. 4, *d-f*). At 14 g.d. myotube formation seems completed in most muscles of the trunk, the limbs, and the head (Rugh, 1968). Myotubes now display the same myofibrillar banding patterns as adult muscle (Fig. 5, *m-q*). All titin antibodies give the well-known periodicities of mature myofibrils (Fig. 5, *m and n*). The sole exception to the full appearance of mature myofibrils is seen by staining with desmin antibodies. Desmin is still found in longitudinal fibers parallel to the cell axis (Fig. 5 *r*). Interestingly vimentin reactivity has become drastically reduced at this stage and is totally lost in multinucleated myotubes (Fig. 4, *d-i*).

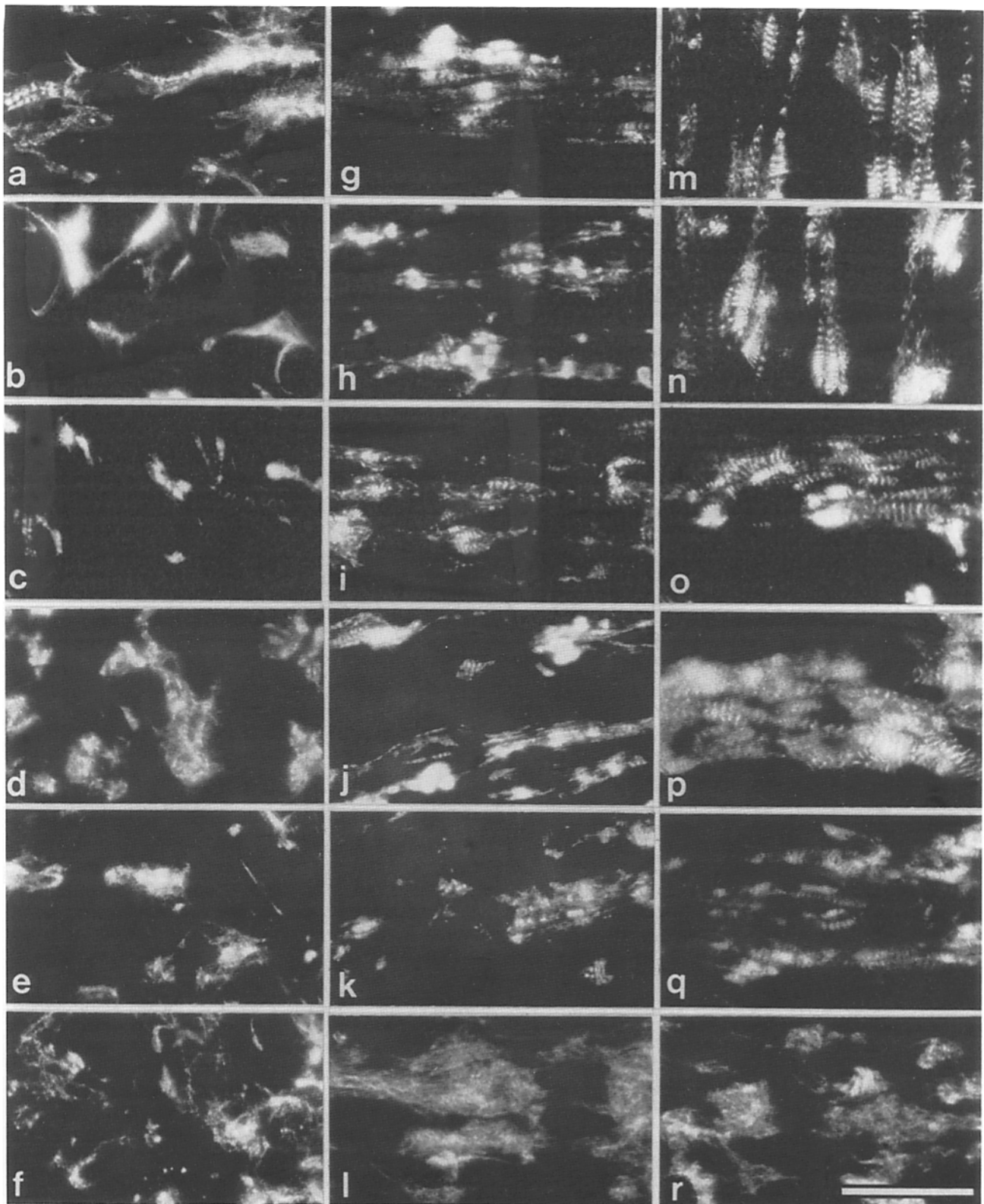
#### **Maturation of Skeletal Muscle Fibers (g.d. 15 until Birth)**

The first sign indicating the further maturation of muscle fibers concerns the position of the nuclei. At g.d. 15 and 16 nuclei are pushed to the periphery of the myotubes (Fig. 4,

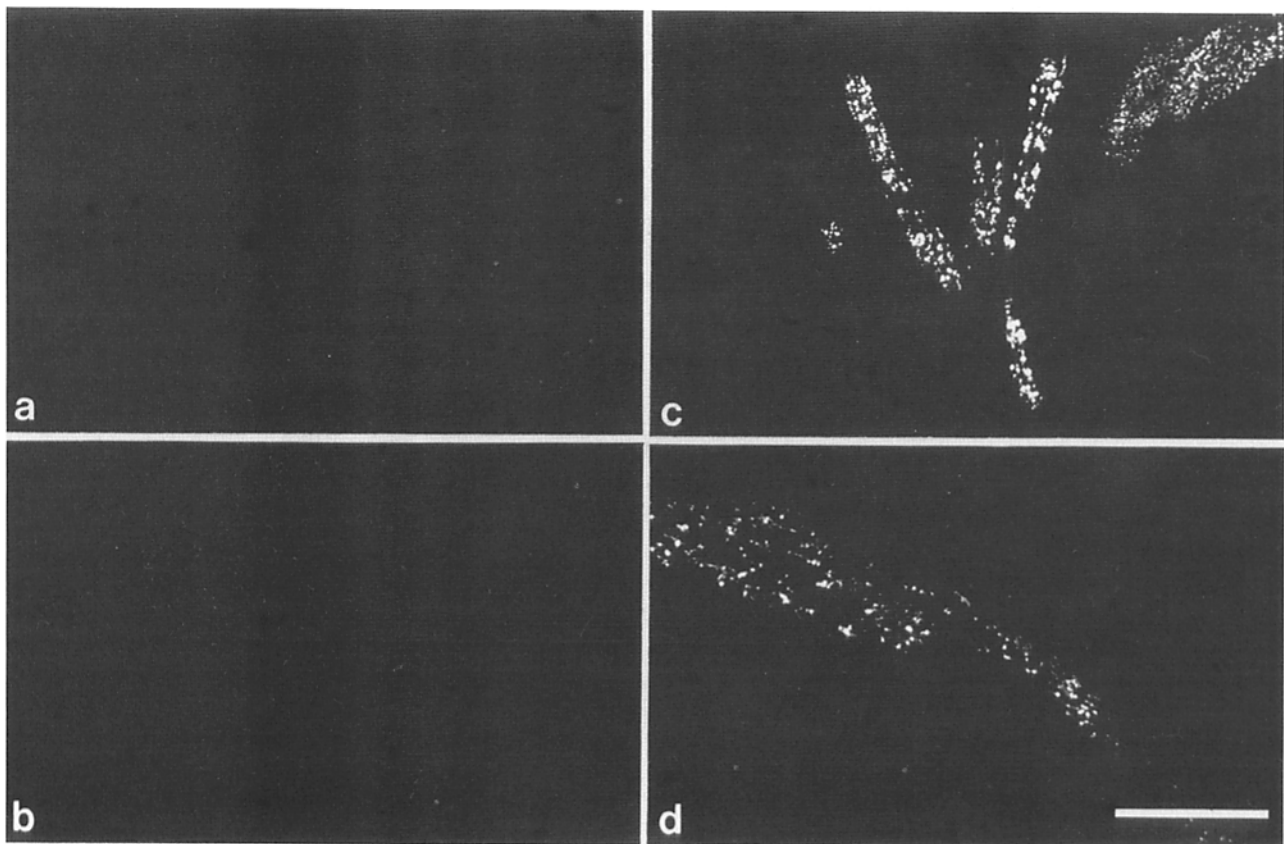
*g-i*) and the number and the size of the fibers now increases considerably (see also Fig. 7). This process and a more compact development of the muscle mass continues till birth. At g.d. 17 and 18 the last conspicuous change in myogenesis is noted, i.e., the transformation of desmin organization. Desmin antibodies emphasize distinct striations at the level of the consecutive Z lines. These striations, well-established for mature muscle, are particularly obvious in longitudinal sections (Fig. 7, *i and k*). Cross-sectional patterns give the impression that individual myofibrils are caged by desmin arrays (Figs. 4 *g and 7, j and l*) (see also Granger and Lazarides, 1979). The well-known development of secondary and tertiary fibers (Kelly and Zachs, 1969; McLennan, 1983; Miller et al., 1985), which finally leads to the characteristic appearance of adult skeletal muscle was not pursued as fiber-specific antibodies were not used in this study. Although keratin filaments are transiently expressed in em-



**Figure 4.** Redistribution of intermediate filament proteins during muscle development, visualized by immunofluorescence microscopy. *a* and *b* are a cross section through the region of cervical somites of an 18-somite mouse embryo stained with antibodies to vimentin (*a*) and desmin (*b*). *c* is the subsequent section stained with titin antibody T12. Note coexpression of vimentin (*a*) and desmin (*b*) in the myoblasts and that considerably fewer cells are positive for titin (*c*). *d-f* show a triply stained cross section through the developing trunk muscles in a g.d. 14 mouse embryo, labeled with antibodies to desmin (*d*) and vimentin (*e*) and with Hoechst dye (*f*). Note that vimentin is almost exclusively expressed in the interstitial cells and not in the myotubes. Nuclei occur in a central position in myotubes. *g-i* are a triply stained cross section through the diaphragm muscle of a g.d. 17 mouse embryo, labeled with antibodies to desmin (*g*) and vimentin (*h*) and with Hoechst dye (*i*). Note that nuclei now occur in a peripheral position in the myotubes. Bars: (*a-c*) 30  $\mu\text{m}$ ; (*d-i*) 50  $\mu\text{m}$ .



**Figure 5.** Myoblast differentiation assayed with antibodies to muscle-specific proteins. Immunofluorescence micrographs of serial longitudinal sections of developing muscle cells in the trunk muscles in g.d. 11 (*a-f*), 13 (*g-l*), and 14 (*m-r*) mouse embryos. Antibodies used are titin T20 (*a*), titin T12 (*g* and *m*), titin T22 (*b*, *h*, and *n*), nebulin Nb2 (*c*, *i*, and *o*), skeletal muscle-specific myosin (*d*, *j*, and *p*), skeletal muscle  $\alpha$  actin (*e*, *k*, and *q*), and desmin (*f*, *l*, and *r*). Note that myofibril formation is first detected with titin antibodies which recognize the Z line (*a*), and is not seen at this time with titin antibodies which recognize the A-I band (*b*). Alignment can also be seen with the nebulin (*c*) and with the myosin (*d*) antibodies. After fusion has occurred (*m-r*) the myofibril organization is visualized by antibodies to all the muscle proteins, including the titin antibody specific for the A-I junction (*n*). Bar, 30  $\mu$ m.



**Figure 6.** Titin expression in developing eye and limb muscles. Immunofluorescence micrographs of serial sections of developing eye (*a* and *c*) and limb (*b* and *d*) muscles of g.d. 11 (*a* and *b*) and 12 (*c* and *d*) mouse embryos stained with titin antibody T12. Note that titin is detected only in the day-12 embryo. Bars: (*a* and *c*) 150  $\mu\text{m}$ ; (*b* and *d*) 70  $\mu\text{m}$ .

bryonic cardiac muscle (Kuruc and Franke, 1988; van Muijen et al., 1987) we detected no staining of the developing skeletal muscles.

Table I summarizes the most important results and tries to bring them into context with the accepted morphological results found in the literature.

### Discussion

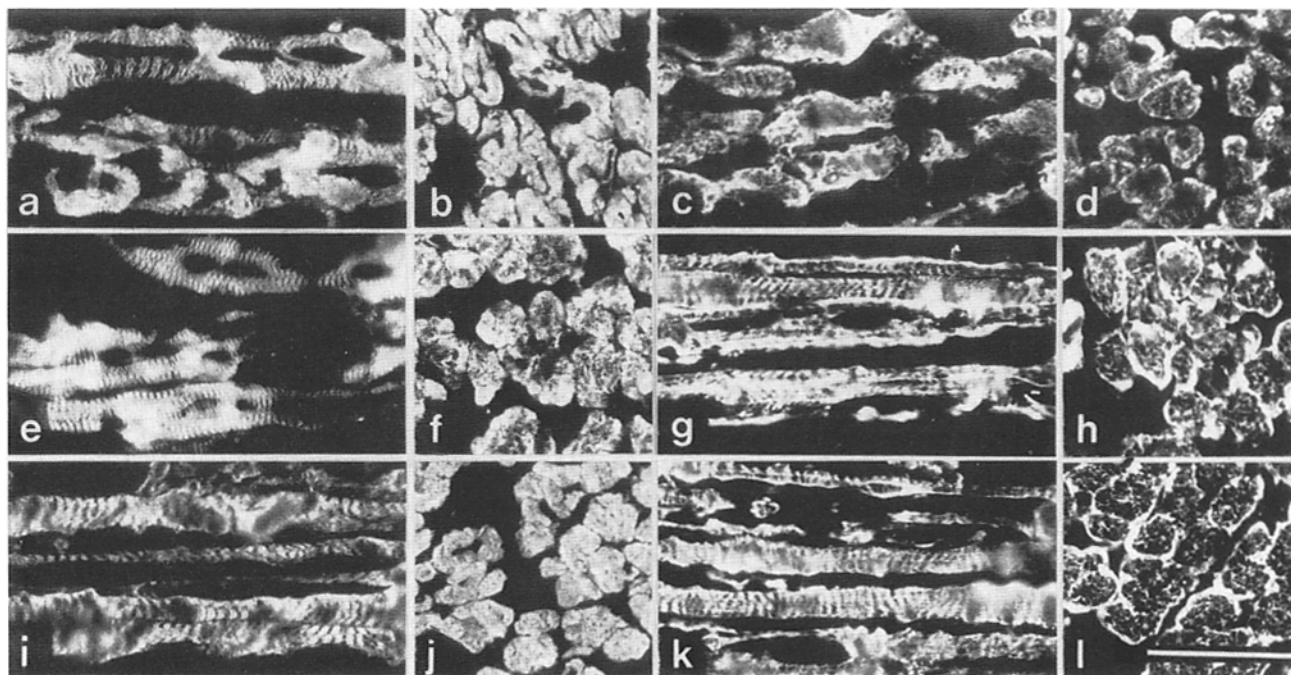
Our aim was to precisely define the sequence in which the major myogenic proteins are expressed in situ during skeletal muscle development of the mouse embryo. Thus we established the relative order of expression to be desmin, titin, muscle-specific actin and myosin heavy chains, and finally nebulin in myotome differentiation of embryos with 13 to 20 somites. In addition and unexpectedly the characterization of the tiny and immature myofibrils already present in a few elongated myoblasts of g.d. 11 embryos has provided a new look at myofibrillogenesis. Our results indicate a key role for titin in the formation of sarcomeres.

Desmin seems to be the first muscle-specific protein detected during myotome differentiation. Our results documenting desmin at g.d. 9 of the mouse embryo are probably equivalent to those of Bignami and Dahl (1984), who reported desmin positivity of g.d. 11 rat myotomes. Like us they noted that the cervical somites reveal desmin before other somites. These in situ results fit observations made on embryonic

chicken muscle in culture. Here desmin synthesis was shown to be already initiated in replicating presumptive myoblasts, while myosin was first detected in postmitotic, mononucleated myoblasts (Hill et al., 1986; Holtzer et al., 1957). In addition on embryonic material C protein positivity was reported for myotubes of 7-d-old chicken embryos (Obinata et al., 1984; Bähler et al., 1985). M protein and myomesin were also found to be expressed in myotubes of chicken embryos, beginning at day 7 (Grove et al., 1985).

The most exciting finding concerns our view of how titin is incorporated into the developing myofibril. Extending results from the laboratories of Wang (1985) and of Maruyama (1986) we have previously shown that in mature muscle, titin molecules, in line with their length of 1  $\mu\text{m}$ , span the half-sarcomere length from the Z line to the M line. In this study 10 single and distinct epitopes along the titin filament could be sequentially ordered by using 10 different monoclonal antibodies in immunoelectron microscopy (Fürst et al., 1988). In the current study the same monoclonal titin antibodies could therefore be employed to monitor the process of early myofibrillogenesis. Thus the Z line, the A-I junction, or certain positions along the A band could be independently monitored by using different monoclonal antibodies from our collection. This has greatly facilitated the interpretation of the images given by the immature myofibrils (g.d. 11). They already show a periodic alignment of fluorescence spots with antibodies to myosin and nebulin. Interestingly the images





**Figure 7.** Maturation of skeletal muscle fibers. Immunofluorescence micrographs of serial sections of trunk muscles in g.d. 15 and 16 (*a-d*) and 17 and 18 (*e-h*) mouse embryos and newborn mice (*i-l*) stained with titin antibody T3 (*a, b, e, f, i, and j*) and desmin antibody (*c, d, g, h, k, and l*). *a, c, e, g, i, and k* are examples of longitudinally sectioned trunk muscles; *b, d, f, h, j, and l* are cross sections. Note the reorganization of the desmin distribution from a longitudinal pattern at g.d. 15 and 16 (*c*) to the Z line orientation shown at g.d. 17 and 18 (*g*). Bar, 50  $\mu\text{m}$ .

provided by the titin antibodies depended on the epitope position. Two types of images could be distinguished. Periodic patterns in immature myofibrils seen by myosin and nebulin antibodies were only detected by those titin antibodies, which recognize epitopes at or close to the Z line. In contrast titin epitopes located in mature muscle at the A-I junction or along the A band are not aligned in the immature myofibrils. This process seems delayed until the early myotube stage, where all titin epitopes show the periodicities seen in adult myofibrils. Thus the Z line end of the titin molecule is brought into register before the other end of the molecule which is anchored within the A band. These results further imply that immature myofibrils assemble as two independent structural units: the thick filaments of the A bands and the I-Z-I brushes, in which one end of the titin molecule is already bound to the established Z line. This view is in line with previous experiments on cultured myocytes treated with TPA (12-tetradecanoylphorbol 13-acetate), taxol, and Colcemid (Antin et al., 1981; Toyama et al., 1982). Upon treatment with TPA myotubes lose their myofibrils. Further growth of these cells in taxol leads to the formation of "A bands," consisting of interdigitating thick filaments and microtubules in the virtual absence of thin filaments and Z lines. The reverse situation is created when the TPA-treated cells are allowed to recover in the presence of Colcemid. They exhibit patchy Z lines on to which thin filaments insert but lack thick filaments.

The proposed independent assembly of two myofibrillar structural units would obviously require a higher template structure which could be the so-called "stress fiber-like structures" postulated by others (Dlugosz et al., 1984; Wang et al., 1988). This would account for a number of electron microscopical observations on skeletal and cardiac muscle

development (Allen and Pepe, 1965; Chacko, 1974; Hiruma and Hirakow, 1985; Manasek, 1968; Markwald, 1973; Peng et al., 1981). These authors report the presence of nonstriated microfilament bundles before the appearance of nascent myofibrils. Z lines and A bands seem to assemble on these bundles. The Z line has already been earlier considered as an organizing center, because it appears before the alignment of the thick filaments into A bands (Markwald, 1973; Peng et al., 1981).

Our results on *in vivo* skeletal muscle myofibrillogenesis in the mouse can also be compared and contrasted with those of Tokuyasu and Maher (1987*a,b*) on cardiac myofibrillogenesis in the chick embryo and of Wang et al. (1988) in cultured chicken cardiac cells. While our data show an independent assembly of the thick filaments of the A bands and of the I-Z-I brushes, Tokuyasu and Maher suggested that the alignment of titin and myosin into the sarcomere occurred at the same time. This difference can probably be attributed to the fact that while we could monitor several different titin epitopes, Tokuyasu and Maher were probably limited to a single epitope. Wang et al. (1988), using a single antibody recognizing an epitope in the I band close to the A-I junction, could also not resolve the temporal alignment of titin and myosin into the myofibril. However they clearly showed that such alignment preceded the sarcomere periodicity seen with antibodies to  $\alpha$  actin and tropomyosin.

A further question previously left open by studies of the chicken culture systems can now be answered by our study of the mouse embryo. This relates to the relative onset of synthesis of the major myogenic proteins. As stated above, *in vivo* titin is expressed before the myosin heavy chains, whereas *in vitro* it was not possible to separate the turn-on

of these two proteins (Hill et al., 1986; Tokuyasu and Maher, 1987a,b; Wang et al., 1988). The precise reasons for the difference between the *in vitro* and *in vivo* results are unclear. It is however important to note that standard muscle cultures are derived from embryonic muscle tissue already highly advanced in development. For instance a culture from a 10-d-old chicken embryo breast muscle is comparable to muscles of a 16-d-old mouse embryo. Therefore muscle cultures cannot mimic the early myotome differentiation and probably reflect only events typical for stages of massive muscle increase and/or regeneration. In cultures derived from such material synchrony of events is not optimal and the rate of myogenic changes seems faster than in the early embryo. Here the myotome differentiation as well as the immature myofibrils of myoblasts offer unique advantages to study myogenic changes and myofibrillar assembly. Finally the use of different titin epitopes with the extremes located at the Z line and close to the M line has offered a better tool to follow titin integration than the previous limitation to A-I junction specific antibodies.

Our observations on desmin and vimentin during skeletal muscle differentiation of the mouse embryo confirm the reports of others made on chicken embryos (Bennett et al., 1979; Osborn et al., 1982; Tokuyasu et al., 1984; Gard and Lazarides, 1980). Thus the majority of the studies agrees that both vimentin and desmin are present in longitudinal filaments in myoblasts. With the onset of myofibril formation the amount of vimentin becomes reduced and already in early myotubes it cannot be detected by our criteria (g.d. 14). It is not until g.d. 17 that desmin is rearranged and shows the typical display at the Z line known for adult muscle (Granger and Lazarides, 1979). The mechanism of this rearrangement remains unclear but may be connected to a yet unknown process of Z line maturation.

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