POLARITY OF ACTIN FILAMENTS AT THE INITIAL STAGE OF MYOFIBRIL ASSEMBLY IN MYOGENIC CELLS IN VITRO

YUTAKA SHIMADA and TAKASHI OBINATA. From the Departments of Anatomy and Biology, Chiba University, Chiba 280, Japan

In developing skeletal muscle, the contractile proteins, myosin and actin, are synthesized and polymerized into filamentous forms, thick and thin filaments, respectively. These newly formed myofilaments exhibit an axial alignment parallel to the long axis of the cell. They are then packed into the hexagonal myofibrillar lattice with other components, e.g., troponin, tropomyosin, and α-actinin. These aggregates of filaments increase in both girth and length and soon exhibit the sarcomeric band pattern of the myofibril (3, 4, 8).

Thin filaments of well-developed striated muscle possess a directional polarity, as demonstrated by Huxley (9) in negatively stained preparations and by Ishikawa et al. (11) in thin sections with the use of "decoration with heavy meromyosin (HMM)" technique. This finding raises the question that is potentially important for an understanding of the mechanisms of myofibril assembly: what polarity do thin filaments exhibit in regard to thick filaments at the initial phase of myofibril assembly? To answer this question, we have examined the polarity of thin filaments in developing muscle cells grown in cell culture.

MATERIALS AND METHODS

Muscle Cultures

Suspensions of embryonic skeletal muscle cells were obtained from thigh muscles of 12-day chick embryos by the standard procedure of dissociation with trypsin (15). Cell suspensions enriched in myogenic cells, prepared by a differential cell adhesion procedure (17), were used for the present experiment. The cells were plated at a concentration of 10^6 in 1 milliliter of culture medium within 35-mm plastic dishes (Lux Scientific Corp., Thousand Oaks, Calif.), each with round cover slips (15 mm in diam) on the bottom. These cover slips were precoated with silicone, then covered with evaporated carbon, and finally overlaid with collagen (15) or gelatin (14).

The culture medium consisted of Eagle's minimal essential medium with glutamine, 15% horse serum, 5% embryo extract, and penicillin-streptomycin in concentration of 50 U and 50 μg/ml, respectively. After 2–4 days in vitro, the cultures were maintained either in a medium containing only 2% embryo extract but retaining the other components at the same concentration, or in a conditioned medium prepared by the procedure described by Buckley and Koningsberg (1). Cultures were kept at 37°C in an atmosphere of 5% CO_2 in air, at saturation humidity. 4–10-day old cultures were used for the present experiment.

HMM Binding

The cultures were treated with 0.1% saponin in standard buffer (0.1 M KCl, 1 mM $MgCl_2$, and 10 mM Tris maleate buffer) or 0.1 M cacodylate buffer, pH 7.0, for 30 min at room temperature according to the method of Ohtsuki (13). The cultures were then incubated overnight at 4°C with HMM (4.5 mg/ml in standard buffer) prepared according to the method of Szent-Györgyi (16). They were rinsed with standard buffer, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 25°C, and postfixed in 1% OsO_4 in the same buffer at 4°C. After *en bloc* staining with 0.5% uranyl acetate in 0.1 M *S*-collidine buffer for 1 h at 25°C, they were dehydrated in ethanol and embedded in Epon 812. After polymerization, cover slips were removed from the cells that were embedded in Epon. Thin sections showing gray to silver interference colors were cut parallel to the substrate side of the cell, stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 or HU-11E electron microscope operated at 100 kV.

RESULTS

In order to allow HMM to diffuse into the cells, the muscle cultures are pretreated with saponin. When these cells are then incubated with HMM, virtually all the thin filaments in myofibrils are "decorated" and form characteristic arrowhead structures. The periodicity of the arrowhead complexes is 37.5 ± 1.7 nm (number of arrowhead spaces measured (n) is 119). This value is similar to the 37.1 ± 3.5 nm measured by Ishikawa et al. (11) in glycerinated myotubes in vitro; both values are in fairly good accord with the 35.5 ± 1.5 nm measured by Huxley (9) in negatively stained preparations. Thus, there is no apparent difference between HMM-actin complexes formed in saponin-treated cells and those formed in glycerol-treated cells. However, the preservation of the overall intracellular structures after saponin treatment is as good as or better than that after glycerination, yet the use of saponin reduces significantly the time required to make cells "leaky." For these reasons, this method of mild chemical treatment of membranes has been employed in the present investigation.

Myotubes grown in vitro for 4–6 days possess large numbers of myofibrils at the initial phases of their formation (Fig. 1). Thick and thin myofilaments are aggregated parallel or nearly parallel, but sarcomere structure is not well formed. Well-defined Z or M bands are absent at this stage, but occasionally darkly staining dense bodies are seen which appear to be the precursors of the Z bands (12). A somewhat more advanced stage of myogenesis (8–10 days in vitro) is presented in Fig. 2. Myofibrils have increased in number and diameter, but they have not yet filled the sarcoplasmic space within the developing myotube. Z and M bands are clearly discernible at this stage. In the present study, the polarity of thin filaments at these two selected culture stages was investigated.

In the nascent myofibrils, the thin filaments can be classified for convenience into the following three different types according to their spatial and/or directional combinations with the adjacent thick filaments (Fig. 3). And we have counted the frequency of occurrence of each type at the previously described earlier (Figs. 4 and 5) and later phases (Fig. 6) of myofibrillogenesis.

The First Type

The thin filaments whose arrowheads point toward the center of the neighboring thick filament are termed type A filaments (*A* in Figs. 3, 4*b*, 5*b*, and 6*b*). This combination of thick and thin filaments is the same as that in mature myofibrils (9). In this type, only the thin filaments that associate apparently with properly oriented cross-bridges of a thick myosin filament are included; those filaments whose end portions are likely to associate with improperly oriented cross-bridges are excluded, namely, the thin filaments whose ends go

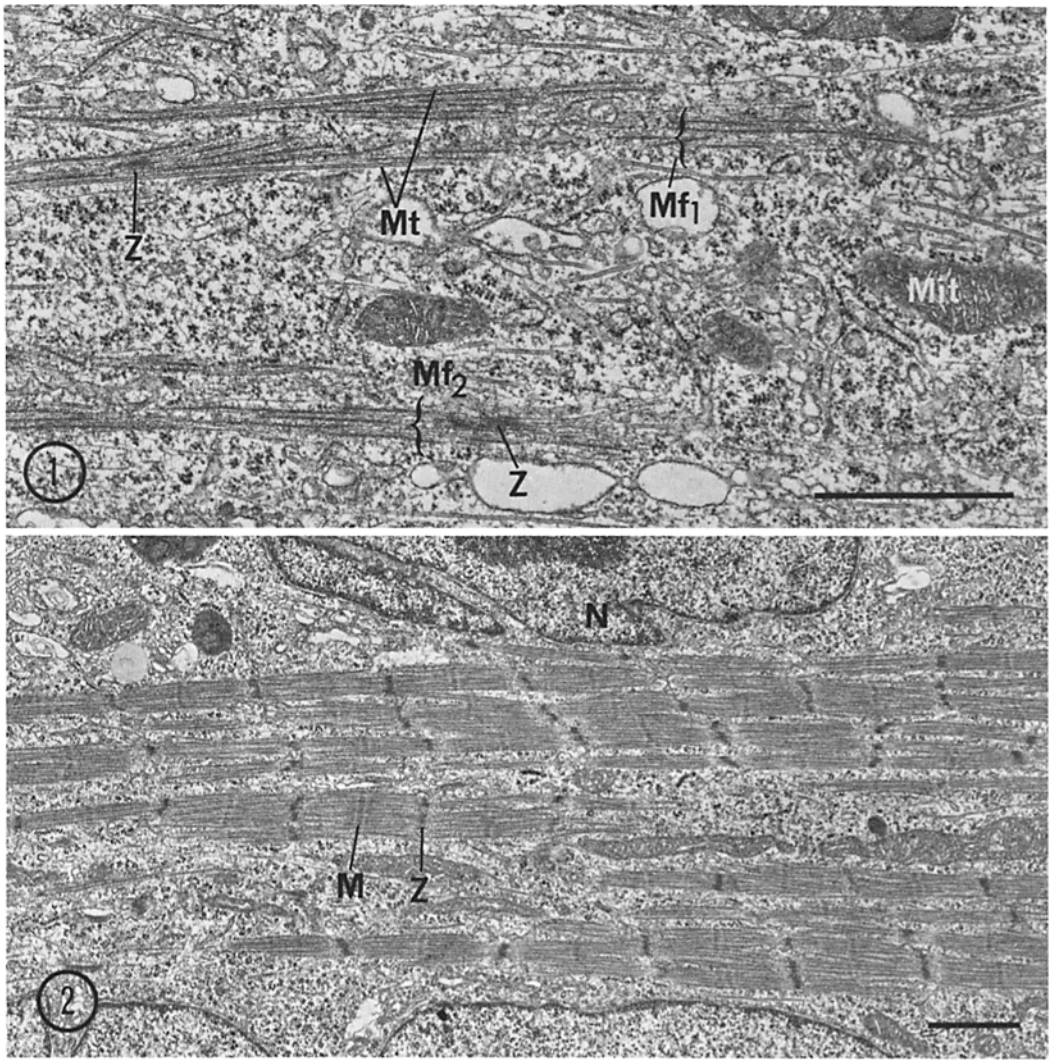


FIGURE 1 A myotube within a 6-day-old muscle culture that exhibits early phases of myofibrillogenesis. Thick and thin filaments are aligned nearly parallel. Some aggregates of filaments (Mf_1) show no definite Z-band density. Other aggregates (Mf_2) show a periodic distribution of a dense material (Z) which is assumed to be a Z-band precursor. Well-defined M bands are absent. Frequently, microtubules (Mt) are seen in close association with the myofibrils. Mitochondrion, *Mit*. × 26,000. Bar, 1 μm.

FIGURE 2 A myotube in an 8-day-old culture. Developing myofibrils can be seen, in which Z (Z) and M (M) bands are clearly discernible. Nucleus, N. × 12,000. Bar, 1 μm.

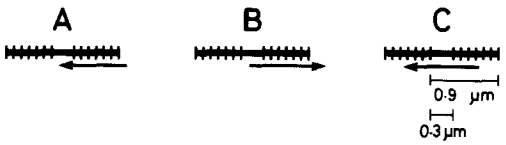
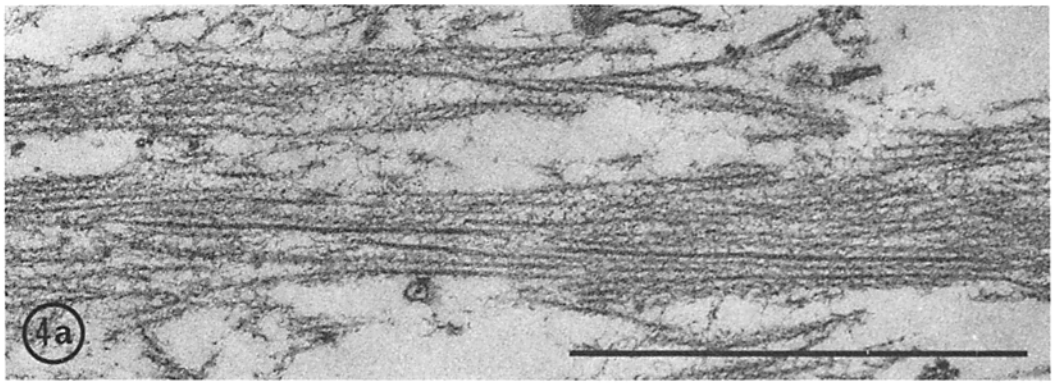


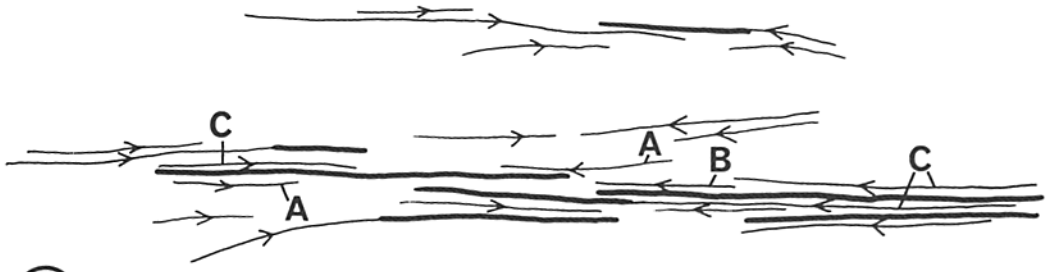
FIGURE 3 Types of spatial and/or directional combinations of thin filaments and thick filaments. Thick lines: thick filaments (cross-bridges are shown by lateral extensions from thick filaments); thin lines: thin filaments (arrows indicate polarity visualized with HMM).

over the 0.9 μm point from the adjacent end of the thick filament (beyond the bridge-free region); from this point, cross-bridges are oriented in the opposite direction (10).

The Second Type

Some thin filaments are situated at the same spatial position as the first type, but their polarity is reversed. They are classified as type B filaments





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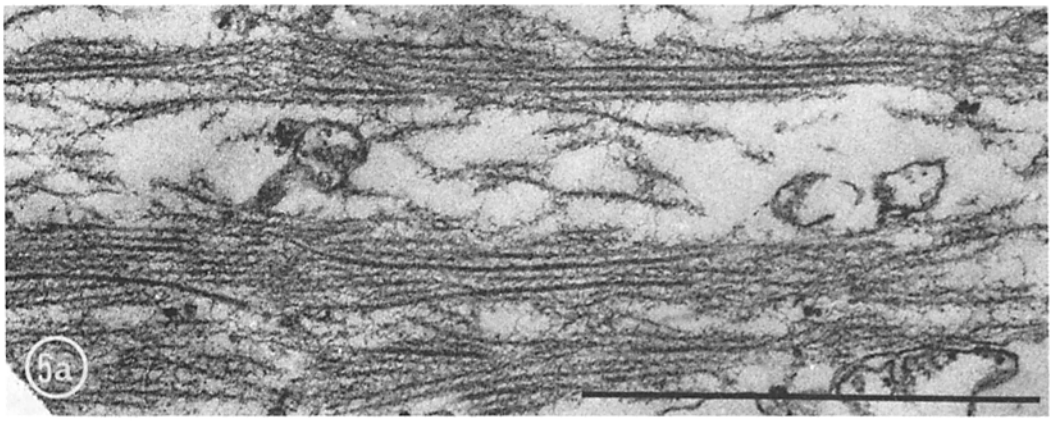

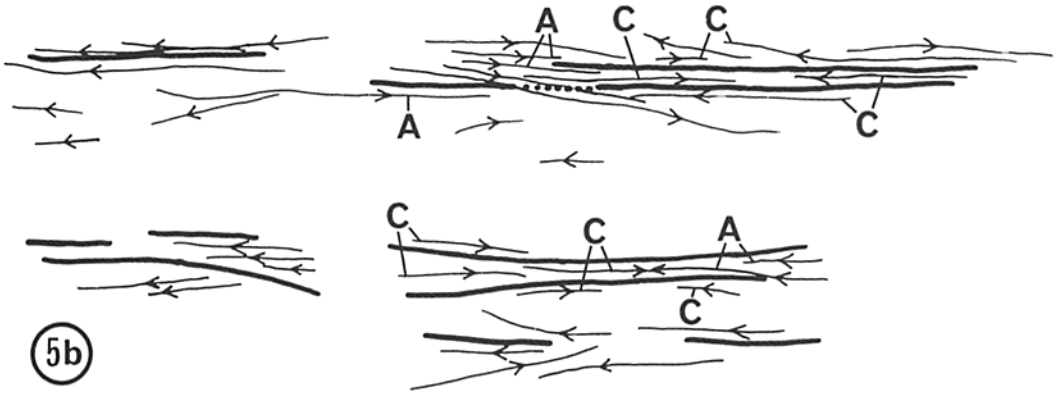

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(*B* in Figs. 3 and 4*b*). This type of spatial and directional association between the thin filaments and the thick ones does not occur naturally in mature myofibrils.

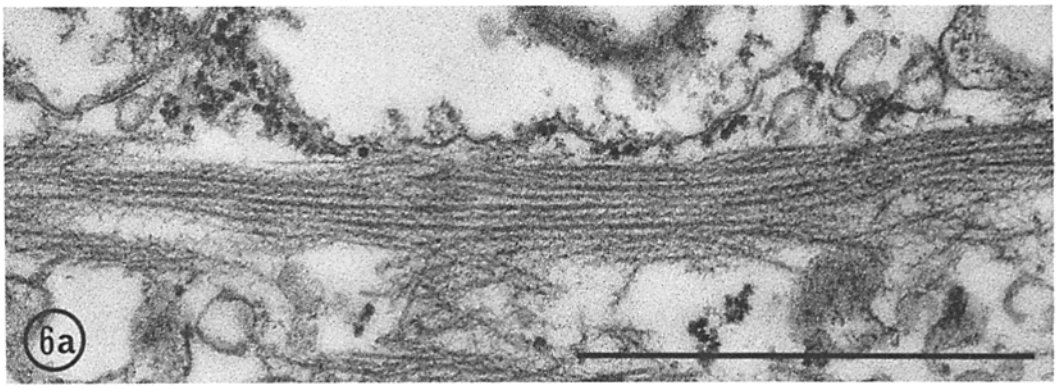
The Third Type

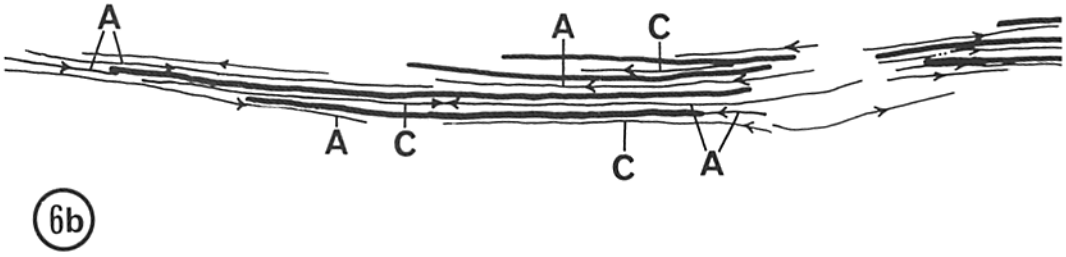
The thin filaments that appear to exceed the 0.9 μm point are called type C filaments (*C* in Figs. 3, 4*b*, 5*b* and 6*b*). Thus, this category is comprised of the filaments whose polarity cannot be expressed as either the first or second type, i.e., undetermined, because these filaments are not shifted toward one end of the thick filaments.

Embryonic myofilaments, especially the thin variety, often appear as wavy or curved lines in longitudinal section (2), and, hence, their entire length is difficult to trace in the plane of a given section. Thus, when the thick and thin filaments in a pair measure less than 1.5 and 1.0 μm, respectively, the determination of the type has been made, for convenience, by extending each end equally until a final apparent length, characteristic of the particular filament, is attained. For this counting, however, we have recorded only those thin filaments of any length that are associated with the thick filaments which are longer than 0.75 μm, i.e., the half length of a thick filament in mature myofibrils (the full length of a thick filament is approx. 1.5 μm [2, 10]), because analysis with short thick filaments is less reliable.

The results are summarized in Table I. The first type (type A), in which both kinds of filaments are arranged like those in myofibrils of mature skeletal muscle fibers (9), has been found to occur predominantly at any phase of myofibrillogenesis. Type B thin filaments are extremely rare. There are fewer undetermined type C filaments than type A filaments.

In some areas of myogenic cells, sheets or bundles of thin filaments approx. 5–6 nm in diam are





FIGURES 4–6 Higher magnifications of the HMM-filament complexes formed in myotubes from 6-day (Figs. 4*a* and 5*a*) and 8-day (Fig. 6*a*) cultures. The Z bands become less prominent by HMM treatment as noted previously (11). × 60,000. Bars, 1 μm. Traces of these electron micrographs are presented in Figs. 4*b*, 5*b*, and 6*b*. Most thick and thin filaments that are associated in pairs are shown; not all free filaments are drawn. Thick lines: thick filaments; thin lines: thin filaments (polarity of the thin filaments is indicated by arrows). *A*, *B*, and *C*: types A, B, and C thin filaments, respectively. See text for details.

seen (Fig. 7). Filaments of this type, which are termed cortical filaments (4) because they occur preferentially beneath the plasma membrane, also bind HMM (Fig. 8). The interaction of these cortical thin filaments with HMM is indistinguishable from that of actin filaments within myofibrils: the HMM-filament complexes here are also polarized along the length of any given filament, and the arrowheads have a periodicity similar to that of the thin myofilaments (average periodicity: 37.7 ± 1.5 nm [n = 156]). These filaments are positioned almost parallel to one another, but are not arranged in order longitudinally. Therefore, alternation of polarity in neighboring filaments is difficult to analyze precisely. However, it appears that the polarity of these thin filaments is not uniform and, thus, that there seems to be no directional relation between neighboring thin filaments. We have counted the numbers of thin filaments polarizing in the one direction and those polarizing in the other on five areas from randomly selected electron micrographs (Table II). As one can expect in such bipolar cells, no preferential directionality has been found between the oppositely polarized filaments.

TABLE I

Polarity of Thin Filaments in Developing Myofibrils

	4–6 days in vitro		8–10 days in vitro	
Types of combination	No. of thin filaments		No. of thin filaments	
		%		%
A	59	53.2	60	53.5
B	2	1.8	3	2.7
C	50	45.0	49	43.8
Total	111	100.0	112	100.0

DISCUSSION

The mechanism by which the filaments are arranged into the mature sarcomere pattern is still incompletely understood, and a number of hypotheses have been proposed (see review by Fischman [3]). In the present study, almost as soon as thick filaments can be identified within myotubes, clusters of both thick and thin filaments are observed. As early as this phase of myofibrillogenesis, the vast majority of the thin filaments that lie parallel or nearly parallel to the thick filaments is found to exhibit the right polarity and spatial position in regard to the thick filaments (type A), as seen in adult muscle (9). The filaments with the same spatial position as the type A filaments but with the reversed polarity are extremely rare (type B). From this result, it seems reasonable to assume that the appearance of the thick filaments in myotubes plays an important role in the regular disposition of thin filaments, and further that the interaction between both types of filaments already exists at the initial phases of myofibrillogenesis. Since cross-bridges contain the actin-combining sites of each myosin molecule (10), it is

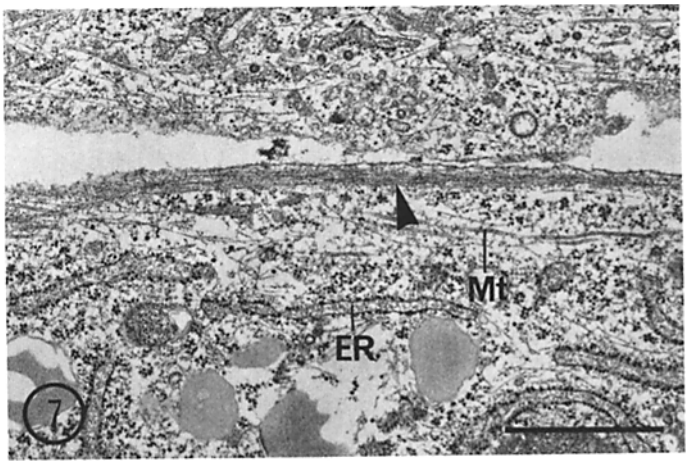


FIGURE 7 Thin section through the cortical region of a myotube after 4 days in vitro. In this region, the tightly packed sheet or skein of thin filaments (arrowhead) is seen. Granular endoplasmic reticulum, *ER;* microtubules, *Mt*. × 21,000. Bar, 1 μm.

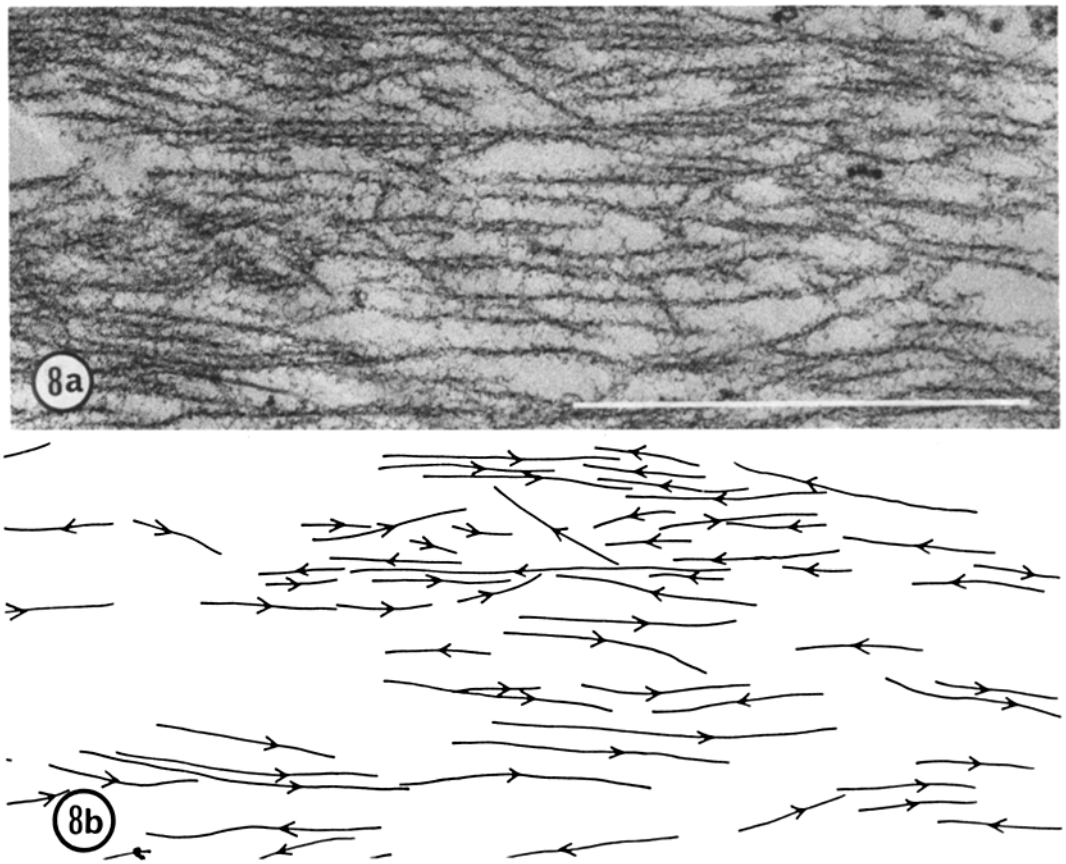


FIGURE 8 Higher magnification of cortical thin filaments. The cell in Fig. 8*a* has been treated with saponin and then reacted with HMM. × 60,000. Fig. 8*b* is a trace of Fig. 8*a*. Arrows indicate the filament polarity. Its direction is not uniform. The relationship of these cortical filaments to myofibrillogenesis is unknown. Bar, 1 μm.

TABLE II

Polarity of Cortical Thin Filaments

Area	Direction of arrowheads		
		No.	*%*
1	Left	37	54
	Right	31	46
2	Left	10	50
	Right	10	50
3	Left	11	48
	Right	12	52
4	Left	13	39
	Right	20	61
5*	Left	60	59
	Right	41	41

* Area 5 is a part of Fig. 8.

likely that thin filaments aligned parallel with the thick filaments and attached to these filaments at cross-bridges will of necessity be disposed in six-fold symmetry.

Many thin filaments belong to neither type A nor B (type C or undetermined). In our counting, the thin filaments with arrows pointing toward the center of a thick filament and with their ends probably extending past the central bare zone are not included in type A. In mature muscle in vivo, the thin filaments can slide until their ends bypass one another; at this state of contraction, their ends are interacting with improperly oriented cross-bridges (10). Thus, it is highly likely that some or many of the undetermined type filaments actually belong to type A and, therefore, that the number

of type A filaments is estimated rather conservatively.

However, some of the type C filaments are located to the side of the oppositely oriented cross-bridges beyond the position that can be assumed physiologically *in situ*. There are also a few filaments of the reversed polarity (type B). It is possible that such myofilaments will later acquire the right position and directionality as development progresses. Support of this supposition comes from the work of Hayashi and his coauthors (6, 7) in which it was demonstrated that the association of myosin and actin filaments in vitro will produce the hexagonal arrangement of actomyosin units and that this protein system can generate tension, indicating that there is relative motion of filaments so that actin filaments are facing the effective cross-bridges. It is not clear, however, whether these filaments of incorrect polarity and/or position disappear as their constituent proteins turn over because of lack of the correct associations with the thick myofilaments or whether this represents a superposition artifact due to a viewing of the thin filaments that are associated with the thick filaments in the next neighboring section, or finally whether this represents an artifact produced by movement of the myofilaments during specimen preparation.

Concerning the question of how the thick and thin filaments become oriented in the longitudinal axis of the cells before they occupy the right spatial and directional position, the following two possibilities can be considered: the first is, in analogy with the reconstruction in vitro of contractile units demonstrated by Hayashi and his coauthors (5, 7), that after the thick filaments have been synthesized polymerization of actin (G → F transformation) occurs under the influence of the thick filaments, thus assuring that the two types of filaments will be nearly parallel. The other possibility is that thick and thin filaments are polymerized mutually unrelated or independently and that they later become oriented in the longitudinal axis of the cells. In the electron micrographs of developing muscle, thick filaments are always seen to be associated with thin filaments; thick and thin filaments alone do not appear to exist scattered within the cytoplasm (except cortical thin filaments), and both filament types exhibit a nearly parallel alignment from the very beginning of their appearance. Furthermore, both kinds of filaments are seen to align in a regular spatial and directional combination in the initial phases of myofibrillogenesis. These observations suggest that the former possibility is more likely. However, other additional or alternative factors can not be ruled out.

Beneath the plasma membrane, particularly near the ends of the myotube, bundles or sheets of thin filaments approx. 5–6 nm in diam are seen. These cortically located microfilaments, which are probably not involved in specialized muscle function (4), have their polarities arranged randomly. This is probably due to the lack of myosin filaments at the cortical region.

SUMMARY

The polarity of thin filaments in relation to thick filaments in developing muscle cells in vitro was investigated. The majority of thin filaments exhibited the right polarity and spatial position similar to that seen in mature myofibrils. It appears that the interaction between thick and thin filaments exists in the initial phases of myofibrillogenesis. Cortical microfilaments are found to have their polarities arranged randomly.

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