THE FINE STRUCTURE OF EMBRYONIC CHICK SKELETAL MUSCLE CELLS DIFFERENTIATED IN VITRO

Y. SHIMADA, D. A. FISCHMAN, and A. A. MOSCONA

From the Department of Zoology, the University of Chicago, Chicago, Illinois 60637, Dr. Shimada's permanent address is the Department of Anatomy, School of Medicine, Chiba University, Chiba, Japan. Dr. Fischman's present address is the Armed Forces Institute of Pathology, Washington, D. C. 20012

ABSTRACT

Dissociated myoblasts from 12-day chick embryos were cultured in monolayer, and the differentiation of skeletal muscle cells was studied by electron microscopy. The results have revealed a striking ultrastructural similarity between the in vivo and the in vitro developing muscle, particularly with respect to the myofibrils and sarcoplasmic reticulum. This study demonstrates that all the characteristic organelles of mature skeletal muscle can develop in vitro in the absence of nerves.

INTRODUCTION

Under the appropriate experimental conditions embryonic myoblasts differentiate in cell culture (Konigsberg, reference 20) to form multinucleate, cross-striated myofibers that are capable of contraction. Although this is a most useful system for cytological, biochemical, and physiological investigations of in vitro myogenesis, there have been no electron microscopic studies of skeletal muscle differentiation in monolayer culture. Two pertinent, prior studies are the investigation by Firket (9) of the fine structure of embryonic muscle cultured in plasma clot and the electron microscopic examination of myocardial cell differentiation in cell culture by Cedergren and Harary (4, 5). In the present report some of the cytological features of embryonic chick skeletal muscle which develop in monolayer culture of myogenic cells are presented. The results demonstrate not only a striking parallel between the developmental sequence in vitro and that in situ (Allen and Pepe, reference 1; Dessouky and Hibbs, reference 7; Fischman, reference 10; Hay, reference 14; Przybylski and Blumberg, reference 29), but also a similar end result in both cases, particularly in the structure of the myofibrils and sarcoplasmic reticulum.

MATERIALS AND METHODS

Suspensions of embryonic skeletal muscle cells were obtained from thigh muscles of 12-day chick embryos by using the standard trypsinization procedure of this laboratory (Moscona, reference 24). The cells were dispersed in culture medium: Eagle's basal medium (Microbiological Associates, Inc., Bethesda, Md.) with 1% glutamine solution (Microbiological Associates, Inc.), 10% horse serum (sterile, unfiltered; Colorado Serum Co., Denver, Colo.), 10% embryo extract, and 1% penicillin-streptomycin mixture (Microbiological Associates, Inc.). For cultivation in monolayers the cells in suspension were inoculated at the dose of 10^5 cells in 2 ml culture medium, into 35-mm plastic culture dishes each with a round (15 mm diameter) glass cover slip on the bottom. These cover slips had been precoated with silicone, then shadowed with carbon,

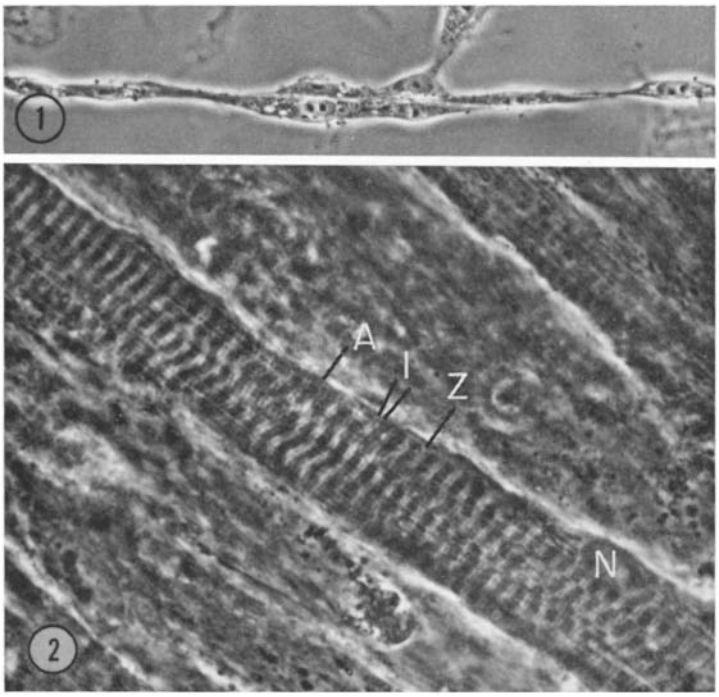

FIGURE 1 A phase-contrast photomicrograph of clustered myoblasts in a 2-day monolayer culture of dissociated embryonic muscle cells. Cells in such aggregates undergo cytoplasmic fusion to form multicellular, elongated myotubes. × 450.

FIGURE 2 A micrograph similar to that in Fig. 1, but of a muscle fiber in a 12-day culture. Myofibrils with characteristic cross-striations (*A*, *I*, and *Z* bands) fill almost the entire cytoplasm of the myofiber. A nucleus (*N*) is seen at the cell periphery, typical of advanced differentiation. × 1,200.

over which a collagen layer (Hauschka and Konigsberg, reference 13) was placed. The culture dishes were put in a humidified chamber, gassed with a 5% CO_2–air mixture, and incubated at 38°C. The cells that settled down and developed on the cover slips were used in this study. Cover slip cultures were fixed in phosphate-buffered 2.5% glutaraldehyde and postfixed in similarly buffered 1% osmium tetroxide (pH 7.4). After rapid dehydration, the cultures were embedded in Araldite, split off the cover slip at the carbon layer level, and cut with a Porter-Blum MT-2 microtome. Thin sections were stained sequentially with phosphotungstic acid, uranyl acetate, and lead citrate (Fischman, reference 10) and were examined with an AEI EM 6B electron microscope. Cover slip cultures fixed in 2.5% glutaraldehyde were also examined as whole mounts by phase-contrast microscopy for evidence of myofiber formation.

RESULTS

Excellent descriptions of myogenesis in vitro as observed by light microscopy have been presented by other investigators (Capers, reference 3; Konigsberg, reference 20), and only a brief summary of this material pertinent to the electron microscopy will be reviewed here. Following trypsinization of 12 day embryonic chick leg muscle, the dissociated cells assume a spherical shape. Within the first few hours in vitro the viable cells settle to the cover slip, attach to the substrate, flatten appreciably, and begin to elongate. Multiplication, migration, and side-to-side alignment of myoblasts are the most prominent visible features of the first 3 days in culture (Fig. 1). Cytoplasmic fusion of the mononuclear cells into syncytial, multinucleate forms usually begins in the next 3–4 days. During the following week, these multinucleate cells continue to grow by repeated cytoplasmic fusion and assume the shape of long, branched tubes. Cross-striated myofibrils within the cytoplasm of these myotubes generally can be recognized by the end of the first week in vitro. Myofibrils increase in both number and di-

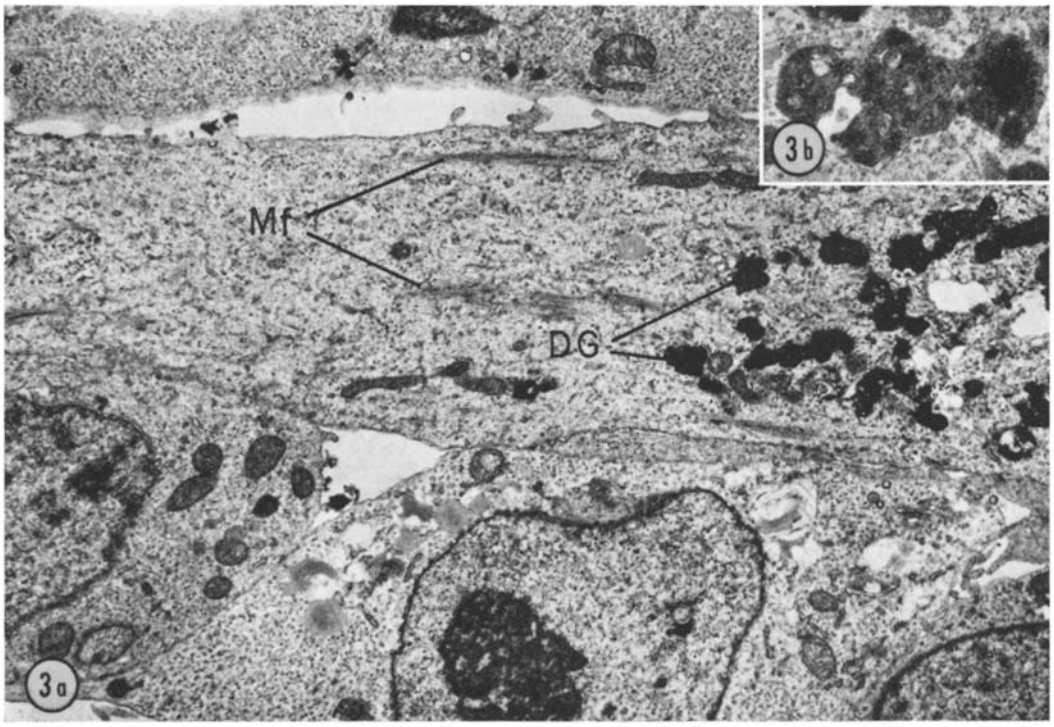

FIGURE 3 *a* A low magnification electron micrograph of myogenic cells in a 3-day culture. The plane of section was parallel to the cover slip. The elongated, centrally positioned myotube containing a few, poorly organized myofibrils (*Mf*) is surrounded by three myoblasts. All of these cells contain numerous free ribosomes, although the myoblasts tend to have more ribosomes per unit area than the myotubes. Dense granules (*DG*) are seen in the myotube (see Fig. 3 *b*). × 7,000.

FIGURE 3 *b* A higher magnification micrograph of dense granules similar to those seen in Fig. 3 *a*. The morphology of these granules suggests a tentative identification as an autophagic vacuole variety of lysosome. × 13,000.

ameter until their aggregate volume almost fills the cytoplasmic space within the developing myofibers (Fig. 2). Except for branching, by the end of 2 wk in culture, these cells closely resemble mature, skeletal muscle fibers in diameter, peripheral distribution of nuclei, location of mitochondria, and myofibrillar banding pattern.

An electron micrograph of a cluster of cells similar to that in Fig. 1 is shown in Fig. 3*a*. Grouped around an elongated myotube containing a few incompletely organized myofibrils (*Mf*) are four myoblasts. Studies in our own and other laboratories (Konigsberg, reference 20; Stockdale and Holtzer, reference 30) support the conclusion that such a multicellular cluster is either undergoing or soon to undergo cytoplasmic fusion. In addition to having numerous free ribosomes, elongated mitochondria, and occasional lipid droplets, the myotubes sometimes contain electron-opaque, membrane-bounded granules (Fig. 3 *b*) which are similar in appearance to the autophagic vacuole variety of lysosome (Ashford and Porter, reference 2; de Duve and Wattiaux, reference 6; Novikoff, reference 25; Trump and Ericsson, reference 31). However, the definitive identification of these granules within these cultured cells remains to be established.

In Fig. 4 the advancing edges of two myotubes, also at an early developmental stage, are seen. Prominent cytological features are as follows: abundant ribosomes and polysomes (*R*), many smooth-surfaced vesicles (*V*) beneath the plasma membranes, microtubules (*Mt*), and numerous free cytoplasmic filaments (*F*) measuring 60–70 A in diameter.

A somewhat more advanced stage of myo-

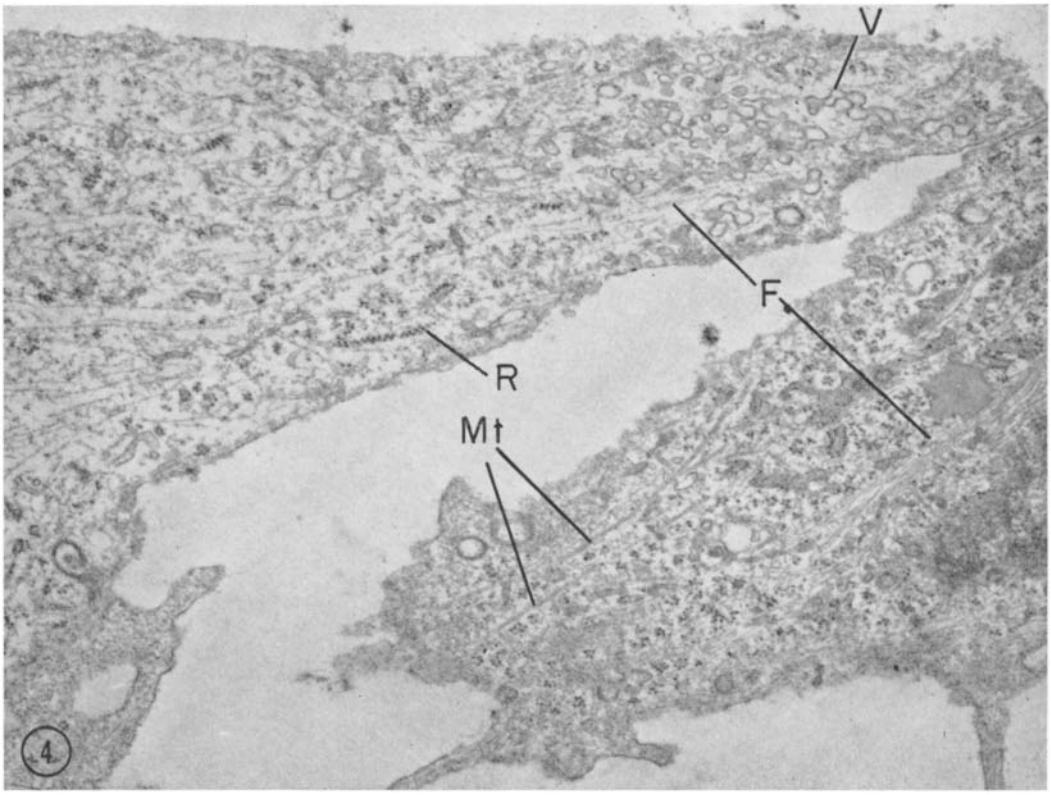

FIGURE 4 The leading edges of two myogenic cells. Ribosomes (*R*), microtubules (*Mt*), smooth-surfaced vesicles (*V*) and numerous free, thin (60–70 A) filaments (*F*) are seen. 4 day culture. × 23,000.

genesis is presented in Fig. 5. Scattered between myofibrils at varying stages of organization can be seen numerous free filaments of two characteristic sizes: one variety (*AF*) measures 60–70 A in diameter, while the other (*MF*) has a diameter of 150–170 A. Filaments of similar size and shape can be recognized in the myofibrils, the thicker variety being located in the A band, the thinner in the I band. By analogy with respect to in situ myogenesis (Fischman, reference 10), these filaments in the cytoplasm between recognizable myofibrils are termed *free myofilaments* and, presumably, are filaments which have not yet packed into the hexagonal, myofibrillar lattice (Huxley, reference 16). Support for this assumption rests on the close similarity in morphology and dimension between free filaments and the myofilaments contained in adjacent myofibrils. Furthermore, the number of free filaments varies inversely with the number of mature fibrils; this suggests an incorporation of the free filaments into the myofibrillar structure.

Well-developed myofibrils (Figs. 6 and 7) possess a sarcomere structure typical of vertebrate skeletal muscle (Huxley, 16). Sarcomere and I-band length vary depending upon the degree of contraction, but A-band length consistently measures about 1.6 μ. Most myofibrils possess an M band bisecting the A band, but no attempt was made to correlate fiber type with the presence or absence of an M band (Page, reference 27). In very thin sections an H band, which varies in width depending upon the extent of contraction, can be observed. As in most developing muscle (Fischman, reference 10; Hay reference 14), the Z bands present a jagged or irregular appearance when viewed in longitudinal section. This irregularity of Z-band structure decreases with increasing differentiation of the myofibers. In cross-section (Fig. 7) the dual filament make-up of the myofibrils is clearly evident. Thick and thin filaments interdigitate in the double hexagonal array described by Huxley (16).

Extensive development of both the sarcoplasmic

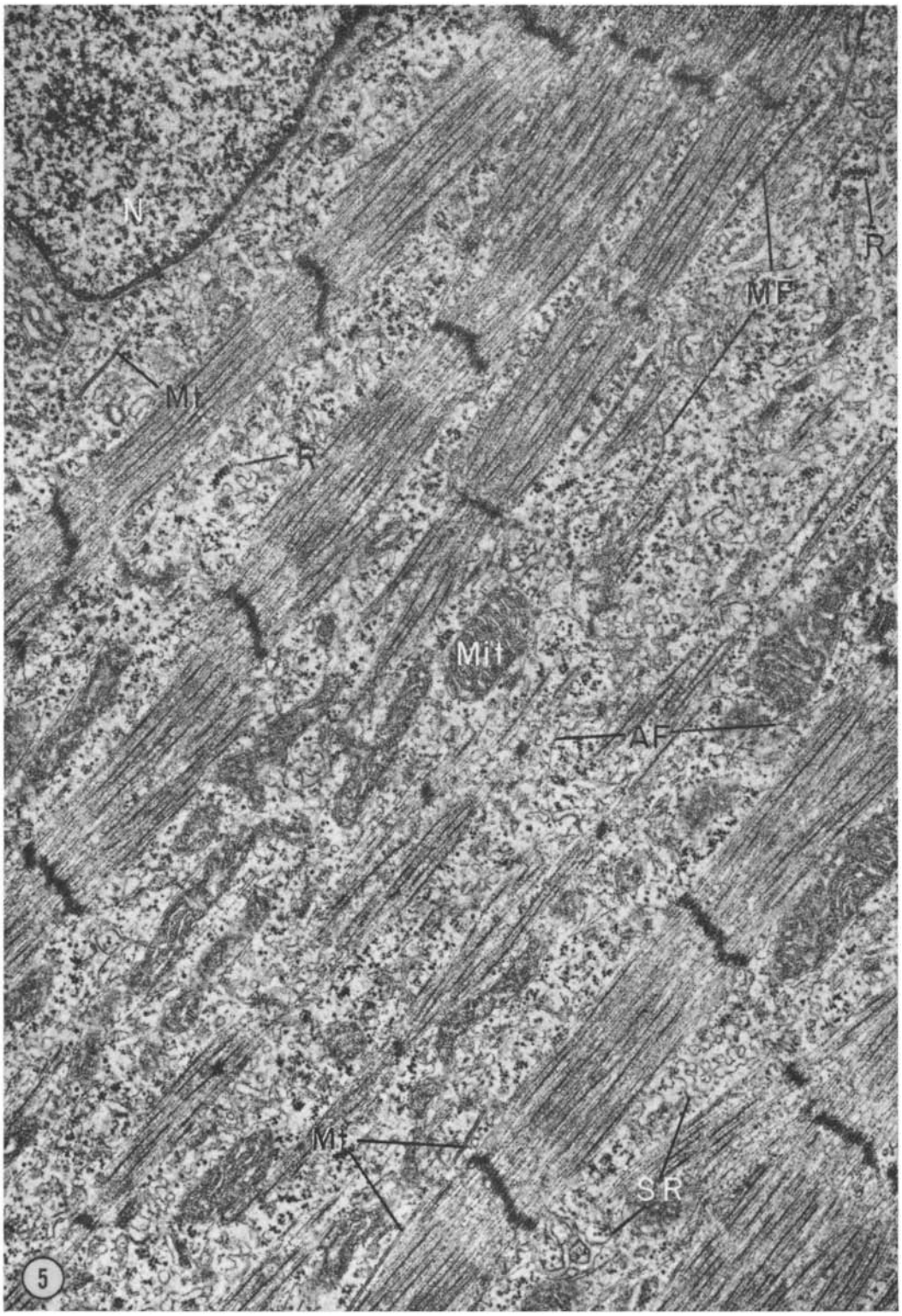

FIGURE 5 Longitudinal section of a myotube from a 14-day culture illustrating stages of myofibril formation ranging from free myofilaments to complete myofibrils. Free, thin (actin) filaments (*AF*) and free, thick (myosin) filaments (*MF*) which have not aggregated into myofibrils are clearly distinguishable. Polyribosomes (*R*), sarcoplasmic reticulum (*SR*), mitochondria (*Mit*), and microtubules (*Mt*) also are visible. × 21,000.

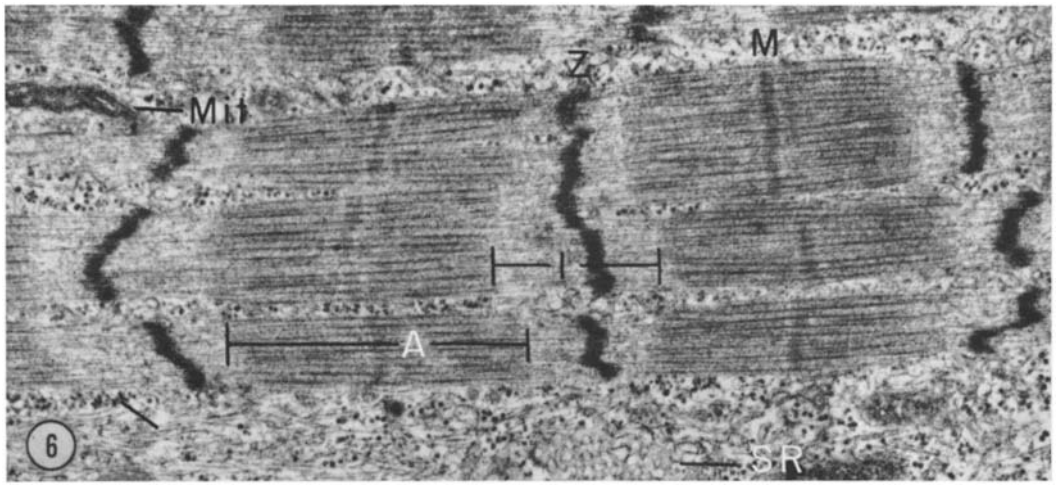

FIGURE 6 An electron micrograph of fully developed myofibrils within a myofiber cultured for 14 days. The cross-striations (*A*, *I*, *Z*, and *M*) of adjacent myofibrils are closely aligned. The sarcoplasmic reticulum (*SR*) shows a honeycomb-like appearance. Glycogen granules (marker in lower left corner); mitochondria (*Mit*). × 24,000.

reticulum and transverse tubular system (T system) was observed in some cells. In young myotubes many smooth-surfaced vesicles and tubules are interspersed among free myofilaments. Membranes with apposed ribosomes (rough-surfaced endoplasmic reticulum) are not a prominent feature of these cells. In certain areas of the sarcoplasm complex, patterned (often honeycombed) arrangements of the intracellular membranes are found adjacent to formed myofibrils (*SR* in Figs. 5 and 6). These membrane networks resemble the fenestrated collar arrangements of certain regions of the sarcoplasmic reticulum (Franzini-Armstrong, reference 11; Peachey, reference 28), albeit at an imperfect or early stage of development. In more mature fibers (Fig. 8) the extensive network surrounding the Z and I bands of the myofibril can be well seen. Characteristic triads (Fawcett and Revel, reference 8) composed of two terminal cisternae apposed to an intermediary T tubule are located at the A–I junctional level of the sarcomere.

Aggregations of ribosomes (*R*) often in long chains which seem helical (Waddington and Perry, reference 32) are prominent in the myotubes (Fig. 5). No clear structural connection between these polyribosomes and the free myofilaments has been observed. As cellular differentiation progresses, the number of ribosomes and polyribosomes decreases.

Earlier histological observations (Moscona, 23) on cultured myoblasts demonstrated relatively large cytoplasmic inclusions which decreased in size, while fine fibrils became discernible. The inclusions may have been similar to the dense granules in Fig. 3 *b* or may have resulted from the culture conditions which were different from those used in this study; on the basis of the present observation, it would appear that these inclusions represented a side effect rather than a normal feature of myodifferentiation.

On the surface of the mature myofibers (Fig. 7) a typical basement membrane can be seen. In addition, collagen and fibroblasts are often encountered in the interspaces between muscle cells. The developmental sequence of basement membrane formation, as it relates to the stages of myogenesis described here, remains to be investigated.

DISCUSSION

The electron micrographs presented above demonstrate the remarkable similarity between myogenesis in monolayer culture and that in situ. Our results support the view that multinucleation in myofibers arises by cytoplasmic fusion of myoblasts and myotubes (Konigsberg, et al., reference 21; Lash, et al., reference 22; Stockdale and Holtzer, reference 30); no amitosis or mitosis within multinucleated muscle cells have been seen. Since myofibrils formed in culture or in situ appeared identical under phase and polarization optics, it was not surprising to find their ultra-

structural appearance also similar. The observed changes in myofibril band pattern at different sarcomere lengths are compatible with the sliding filament hypothesis of muscle contraction (Huxley and Hanson, reference 19).

Recently, Heywood et al. (15) have isolated from embryonic chick skeletal muscle a class of polyribosomes containing 60–70 ribosomes which synthesize myosin. Presumably, many of the large aggregates of ribosomes which have been visualized in thin sections of myotubes in situ (Allen and Pepe, reference 1; Fischman, reference 10; Przybylski and Blumberg, reference 29) and in vitro are involved in myosin synthesis, but this remains to be firmly established. In our material, we could not resolve any structural connection between these polyribosomes and the thick filaments which have been shown to contain myosin (Huxley, reference 17; Huxley and Hanson, reference 18). Thus, further work is required to de-

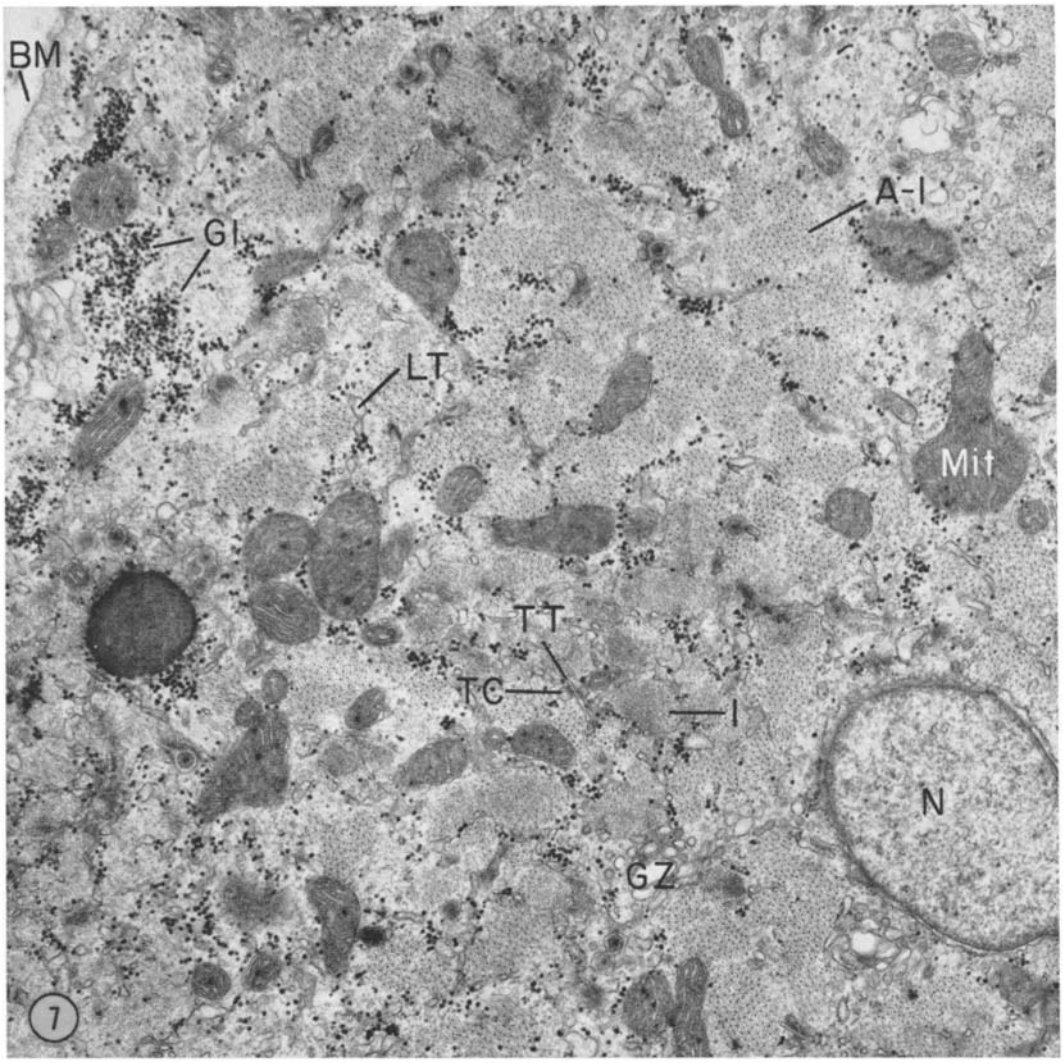

FIGURE 7 A cross-section of a highly differentiated myofiber cultured for 9 days in monolayer. Transverse sections through the I band (*I*) and A band (*A–I*) are seen. Triads with a transverse tubule (*TT*) and terminal cisternae (*TC*) are evident. Longitudinal tubules (*LT*) of the sarcoplasmic reticulum have roughly circular profiles. Mitochondria, *Mit*; glycogen granules, *Gl*; Golgi zone, *GZ*; nucleus, *N*; basement membrane, *BM*. × 17,000.

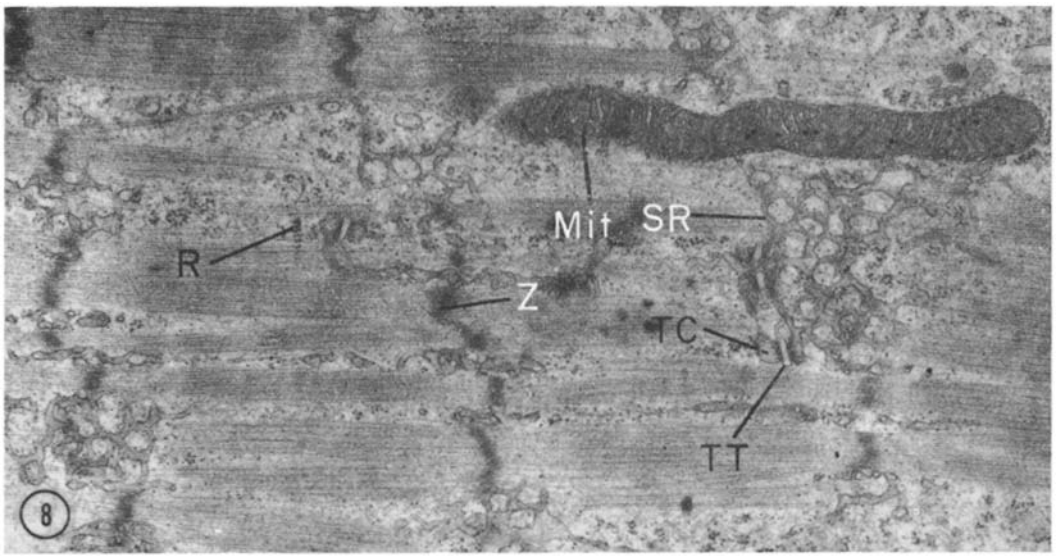

FIGURE 8 Longitudinal section of a myofiber cultured for 9 days in vitro. Triads consisting of two terminal cisternae (*TC*) and a centrally located transverse tubule (*TT*) are seen adjacent to the A–I junctional region of the sarcomere. The sarcoplasmic reticulum (*SR*) with its numerous fenestrations is well visualized. Clusters of ribosomes (*R*) are prominent. Z disc, *Z*, mitochondria *Mit*. × 20,000.

termine that the polyribosomes play a role in the actual construction of the thick filaments. It remains conjectural as to whether these polyribosomes are involved in the aggregation of myosin subunits or in the folding of the completed molecule into its enzymatically active, tertiary configuration.

In a previous study (Fischman, reference 10) free, thin filaments within the cytoplasm of myotubes were identified tentatively as actin filaments because of their morphological similarity to thin filaments within the I band (Hanson and Lowy, reference 12). Support for this concept has recently been presented by Obinata et al. (26) who have isolated these free, thin filaments from embryonic chick muscle and have identified them as actin-containing filaments.

A somewhat unexpected finding in the present study was the extensive development of the sarcoplasmic reticulum and T system in some of the cultured myotubes. In view of the fact that no functional nerve elements were included in these cell cultures, it is concluded that the development of the intracellular membrane system in muscle can proceed in the total absence of nerve influence. Furthermore, this study supports the conclusion that all intracellular structures so far described in mature skeletal muscle can develop in vitro in the absence of peripheral nerves.

This research was supported by USPHS grant HD-01253 to A. A. Moscona and USPHS grant GM-13-975-01 to D. A. Fischman. Support from the Lewis Block and Otho Sprague Funds of the University of Chicago is also gratefully acknowledged.

The authors wish to thank Mrs. Montez Williams for technical assistance.

Received for publication 17 March 1967; revision accepted 19 July 1967.

REFERENCES

1. ALLEN, E. R., and F. A. PEPE. 1965. Ultrastructure of developing muscle cells in the chick embryo. *Am. J. Anat.* **116**:115.
2. ASHFORD, T. P., and K. R. PORTER. 1962. Cytoplasmic components in hepatic cell lysosomes. *J. Cell Biol.* **12**:198.
3. CAPERS, C. R. 1960. Multinucleation of skeletal muscle *in vitro*. *J. Biophys. Biochem. Cytol.* **7**:559.
4. CEDERGREN, B., and I. HARARY. 1964. *In vitro* studies on single beating rat heart cells. VI. Electron microscopic studies of single cells. *J. Ultrastruct. Res.* **11**:428.

5. CEDERGREN, B., and I. HARARY. 1964. *In vitro* studies on single beating rat heart cells. VII. Ultrastructure of the beating cell layer. *J. Ultrastruct. Res.* **11**:443.
6. DE DUVE, C., and R. WATTIAUX. 1966. Functions of lysosomes. *Physiol. Rev.* **28**:435.
7. DESSOUKY, D. A., and R. G. HIBBS. 1965. An electron microscope study of the development of the somite muscle of the chick embryo. *Am. J. Anat.* **116**:523.
8. FAWCETT, D. W., and J. P. REVEL. 1961. The sarcoplasmic reticulum of a fast acting fish muscle. *J. Biophys. Biochem. Cytol.* **10**:89.
9. FIRKET, H. 1967. Ultrastructural aspects of myofibril formation in cultured skeletal muscle. *Z. Zellforsch. Mikroskop. Anat.* **78**:313.
10. FISCHMAN, D. A. 1967. An electron microscope study of myofibril formation in embryonic chick skeletal muscle. *J. Cell Biol.* **32**:557.
11. FRANZINI-Armstrong, C. 1963. Pores in the sarcoplasmic reticulum. *J. Cell Biol.* **19**:637.
12. HANSON, J., and J. LOWY. 1963. The structure of F-actin and of actin filaments isolated from muscle. *J. Mol. Biol.* **6**:46.
13. HAUSCHKA, S. D., and I. R. KONIGSBERG. 1966. The influence of collagen on the development of muscle clones. *Proc. Natl. Acad. Sci. U. S.* **55**:119.
14. HAY, E. D. 1963. The fine structure of differentiating muscle in the salamander tail. *Z. Zellforsch. Mikroskop. Anat.* **59**:6.
15. HEYWOOD, S. M., R. M. DOWBEN, and A. RICH. 1967. The identification of polyribosomes synthesizing myosin. *Proc. Natl. Acad. Sci. U. S.* **57**:1002.
16. HUXLEY, H. E. 1957. The double array of filaments in cross-striated muscle. *J. Biophys. Biochem. Cytol.* **3**:631.
17. HUXLEY, H. E. 1963. Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. *J. Mol. Biol.* **7**:281.
18. HUXLEY, H. E., and J. HANSON. 1957. Quantitative studies on the structure of cross-striated myofibrils. *Biochim. Biophys. Acta.* **23**:229, 250.
19. HUXLEY, H. E., and J. HANSON. 1960. The molecular basis of contraction in cross-striated muscles. *In* Structure and Function of Muscle. G. H. Bourne, editor. Academic Press Inc., New York. 183.
20. KONIGSBERG, I. R. 1963. Clonal analysis of myogenesis. *Science.* **140**:1273.
21. KONIGSBERG, I. R., N. MCELVAIN, M. TOOTLE, and H. HERRMANN. 1960. The dissociability of ribonucleic acid synthesis from the development of multinuclearity of muscle cells in culture. *J. Biophys. Biochem. Cytol.* **8**:333.
22. LASH, J. W., H. HOLTZER, and H. SWIFT. 1957. Regeneration of mature skeletal muscle. *Anat. Record.* **128**:679.
23. MOSCONA, A. A. 1955. Cytoplasmic granules in myogenic cells. *Exptl. Cell Res.* **9**:377.
24. MOSCONA, A. A. 1961. Rotation-mediated histogenetic aggregation of dissociated cells. *Exptl. Cell Res.* **22**:455.
25. NOVIKOFF, A. B. 1963. Lysosomes in the physiology and pathology of cells: contribution of staining methods. *In* Ciba Foundation Symposium on Lysosomes. A. V. S. de Reuck and M. P. Cameron, editors. Little, Brown and Company, Boston. 36.
26. OBINATA, T., M. YAMAMOTO, and K. MARUYAMA. 1966. The identification of randomly formed thin filaments in differentiating muscle cells of the chick embryo. *Develop. Biol.* **14**:192.
27. PAGE, S. G. 1965. A comparison of the fine structure of frog slow and twitch fibers. *J. Cell Biol.* **26**:477.
28. PEACHEY, L. D. 1965. The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. Cell Biol.* **25**:209.
29. PRZYBYLSKI, R. J., and J. M. BLUMBERG. 1966. Ultrastructural aspects of myogenesis in the chick. *Lab. Invest.* **15**:836.
30. STOCKDALE, F. E., and H. HOLTZER. 1961. DNA synthesis and myogenesis. *Exptl. Cell Res.* **24**:508.
31. TRUMP, B. F., and J. L. E. ERICSSON. 1965. Some ultrastructural and biochemical consequences of cell injury. *In* The Inflammatory Process. B. W. Zwerfach, L. Grant, and R. T. McCluskey, editors. Academic Press Inc., New York. 80.
32. WADDINGTON, C. H., and M. M. PERRY. 1963. Helical arrangement of ribosomes in differentiating muscle cells. *Exptl. Cell Res.* **30**:599.