OCCURRENCE OF CENTRIOLES DURING SKELETAL AND CARDIAC MYOGENESIS

RONALD J. PRZYBYLSKI. From the Department of Anatomy, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106

INTRODUCTION

Skeletal muscle development of vertebrates is characterized by the formation of multinuclear muscle fibers through the fusion of uninucleate myoblasts (Holtzer et al., 1958; Capers, 1960; Konigsberg et al., 1960; Zhinkin and Andreeva, 1963; Mintz and Baker, 1967). The myogenic cells constitute a line of mitotically dividing elements which serve as the basis for the initiation of skeletal muscle development in the early embryo (Stockdale and Holtzer, 1961) and for the increase in nuclear number of myotubes and muscle fibers before and after birth (MacConnachie et al., 1964). This stem line has been postulated to remain in the growing and adult organism as the "satellite" cell (Mauro, 1961; Przybylski and Blumberg, 1966; Moss and Leblond, 1970). Marchok and Herrmann (1967) have shown that the number and mitotic frequency of the myoblasts decrease during development while fusion continues, thereby diminishing the myoblast pool. Associated with the lowered mitotic activity is a decrease in DNA polymerase activity, both in vivo (Stockdale, 1970) and in vitro (O'Neil and Strohman, 1969). Myoblast fusion occurs during G_1 (or G_0) of the cell cycle (Okazaki and Holtzer, 1966; Cox, 1968; Bischoff and Holtzer, 1969). When incorporation into the tube is complete, the nuclei do not synthesize DNA and do not divide mitotically (Lash et al., 1957). This state is not irreversible, since myotube nuclei have been induced to synthesize DNA

by virus infection (Fogel and Defendi, 1967) and enter into mitosis, but are arrested at prophase (Yaffe and Gershon, 1967). The mechanism responsible for the cessation of mitotic activity in myotube and muscle fiber nuclei is currently unclear. Consequently, we have proceeded to investigate this question by first determining whether centrioles are present in fusing myoblasts and in myotubes. Our electron microscope observations have shown that they are present in these cells but not in muscle fibers.

MATERIALS AND METHODS

Fertile eggs of the Babcock strain of white Leghorn fowl were obtained from a farm operated by the Biology Department of Case Western Reserve University and were incubated at 38°C in a forced draft incubator at a relative humidity of 87%. Skeletal muscle was obtained from the gastrocnemius on the 12th to the 20th day of incubation, and cardiac muscle was obtained from the ventricles of day 2–day 21 chick embryos. Approximately 70 embryos were used for this study. No adult tissue was used; however, six samples of skeletal and cardiac muscle from 7–10-day posthatch chicks were studied. Samples of the respective tissues were preserved with either 3% glutaraldehyde in 0.1 M Sorenson's phosphate buffer, pH 7.8, or with a combined formaldehyde-glutaraldehyde fixative as described by Karnovsky (1965), using 0.2 M cacodylate buffer, pH 7.8–8.0. Fixation for 1–10 hr at room temperature was followed by several washes of the buffer employed and a postfixation in 1% OsO_4 in the phosphate buffer. The material was dehydrated with ethanol, embedded in Epon 812 (Luft, 1961), and viewed with a Philips 200 electron microscope. Sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) to provide sufficient contrast for observation. Serial sections were not done; however, alternating thick and thin (skip) sections were obtained from about one-third of the specimens.

RESULTS AND DISCUSSION

The developing skeletal muscle aspect of this study was encumbered by two phenomena inherent to skeletal myogeneisis, namely, the brevity of the process of fusion of a myoblast with an established myotube and the small size of centrioles. These events, coupled with the small sample size inherent to thin sectioning for electron microscopy, necessitated numerous observations with a low yield. Consequently, the micrographs presented as evidence for the presence of centrioles in fusing myoblasts and myotubes constitute most of the photographic records which have been accumulated.

Skeletal and cardiac myogenic cells undergoing mitosis contain a typical mitotic apparatus including centrioles. During mitotic interphase these cells often possess a cilium which appears to be derived from the existing centrioles. Numerous formative stages have been observed in both developing heart and skeletal muscle during all stages of *in ovo* development. Fig. 1 depicts a skeletal myoblast situated at the periphery of a myotube. The myoblast contains a cilium, numerous closely packed ribosomes, and no myofibrils. In contrast, the myotube contains fewer ribosomes per unit area, and these are often found as the large polysomes implicated in contractile protein synthesis (Heywood et al., 1967). Myofibrils and numerous free filaments are evident in the myotubes. It is unclear whether this cell was binucleate or whether the nucleus was sufficiently lobulated to permit both a transverse and a tangential section in a single plane. Generally, myoblast nuclei are fairly uniform in outline; this norm would suggest that the myoblast in this micrograph was binucleate. In this plane of section, the cell membranes of the myoblast and the myotube were intact and thus fusion of the two cell types was not suspected.

Designating myoblasts as fusing or nonfusing is difficult; however, two criteria can be utilized for a tentative identification until more precise ones can be established. During fusion the cell membranes separating myoblasts from myotubes are replaced by a series of vesicles in OsO_4-fixed material (Przybylski and Blumberg, 1966). Such fusion vesicles are rarely seen in aldehyde-fixed tissue; instead, a loss of structural integrity indicative of membrane dissolution is represented by varied degrees of diffuseness. Admittedly, this criterion is encumbered by the possibility that a tangential section of the membrane has been photographed. Greater assurance is provided if the first criterion is used in conjunction with the general state of the myoblast cytoplasm. As evidenced in Figs. 1–3, a progressive change in the number of cytoplasmic organelles per unit area exists as the myoblasts fuse with existing myotubes. This hydration or solation appears to occur concomitantly with the synthesis of contractile proteins as indicated by the presence of thick, myosin-like filaments (Przybylski and Blumberg, 1966).

Figs. 2 and 3 portray myoblasts which appear

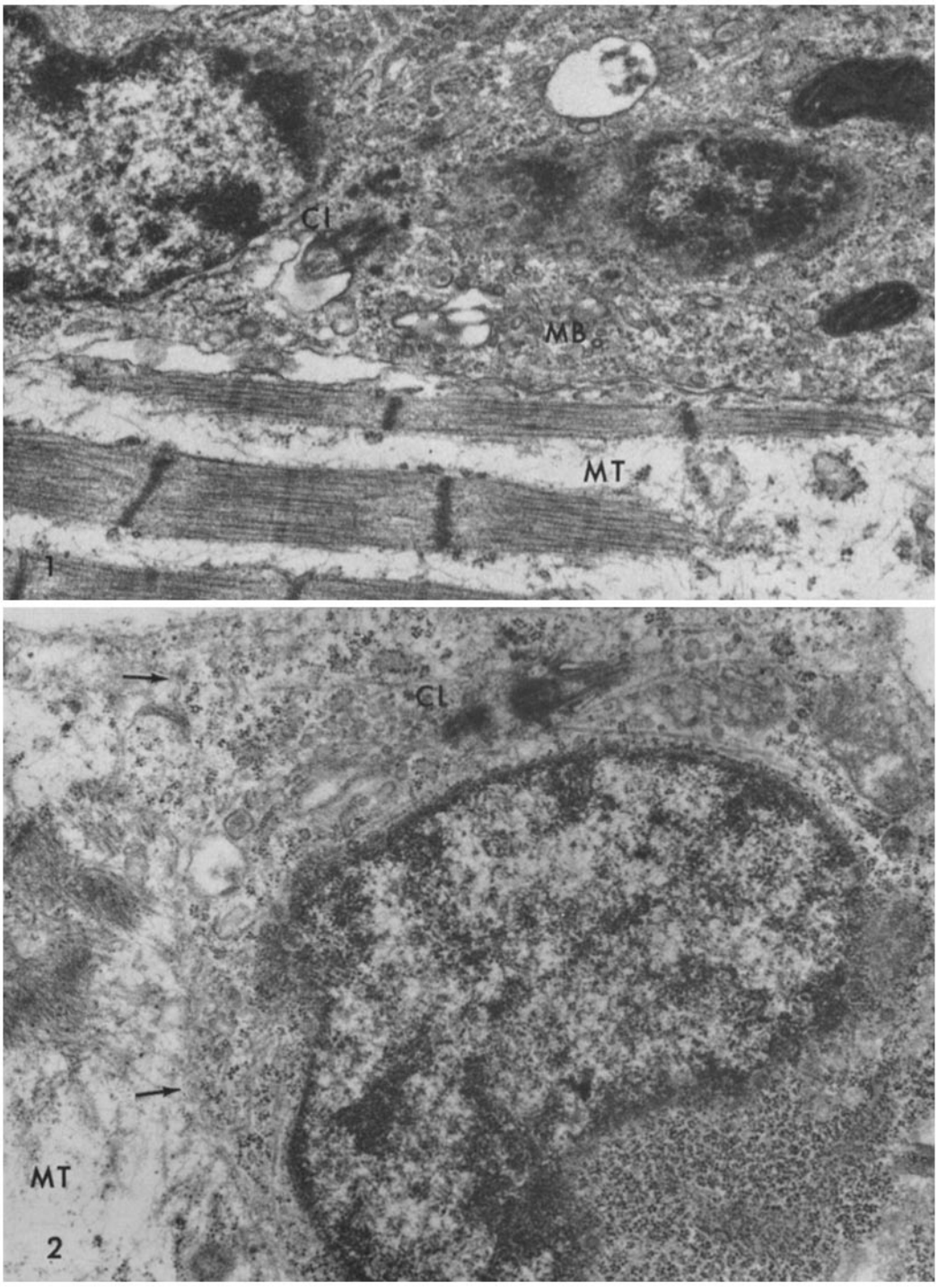

FIGURE 1 An electron micrograph of a myoblast (*MB*) and a myotube (*MT*) obtained from day 12 embryonic skeletal muscle. The myoblast is characterized by its numerous ribosomes, two nuclei, and a cilium (*CI*). The myotube cytoplasm contains myofibrils and many fewer ribosomes per unit area than the adjacent myoblast. × 27,000.

FIGURE 2 An electron micrograph of a skeletal myoblast in the process of fusing with an adjacent myotube (*MT*). The myoblast contains a cilium (*CI*) sectioned tangentially and its accompanying basal body. Numerous microtubules are present in the region of the cilium. The locus of fusion (arrows) is situated near myotube myofibrils. Note the difference in the number of ribosomes at this point as compared to the cytoplasm farther from the point of fusion. × 20,000.

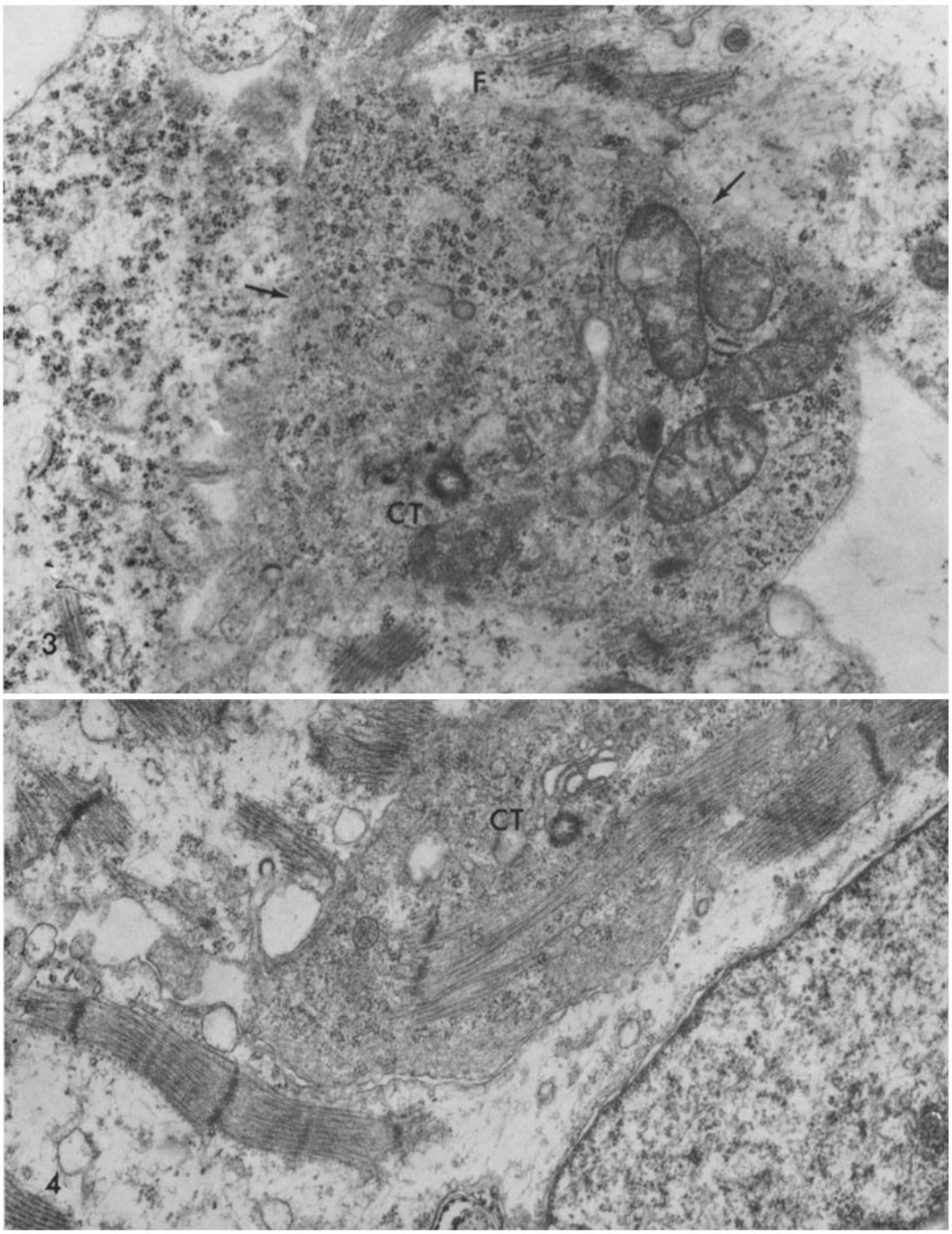

FIGURE 3 An electron micrograph of a skeletal myoblast containing a centriole (*CT*). This myoblast appears to be in the process of fusion (arrows) with a myotube containing well-organized myofibrils. One region of fusion (*F*) contains a series of vesicles which may have been derived from the cell membranes of the respective cells. × 27,000.

FIGURE 4 An electron micrograph of a centriole (*CT*) in a skeletal myotube containing well-organized myofibrils situated in a cytoplasmic region which is relatively rich in ribosomes and free filaments. A Golgi region is evident. × 18,000.

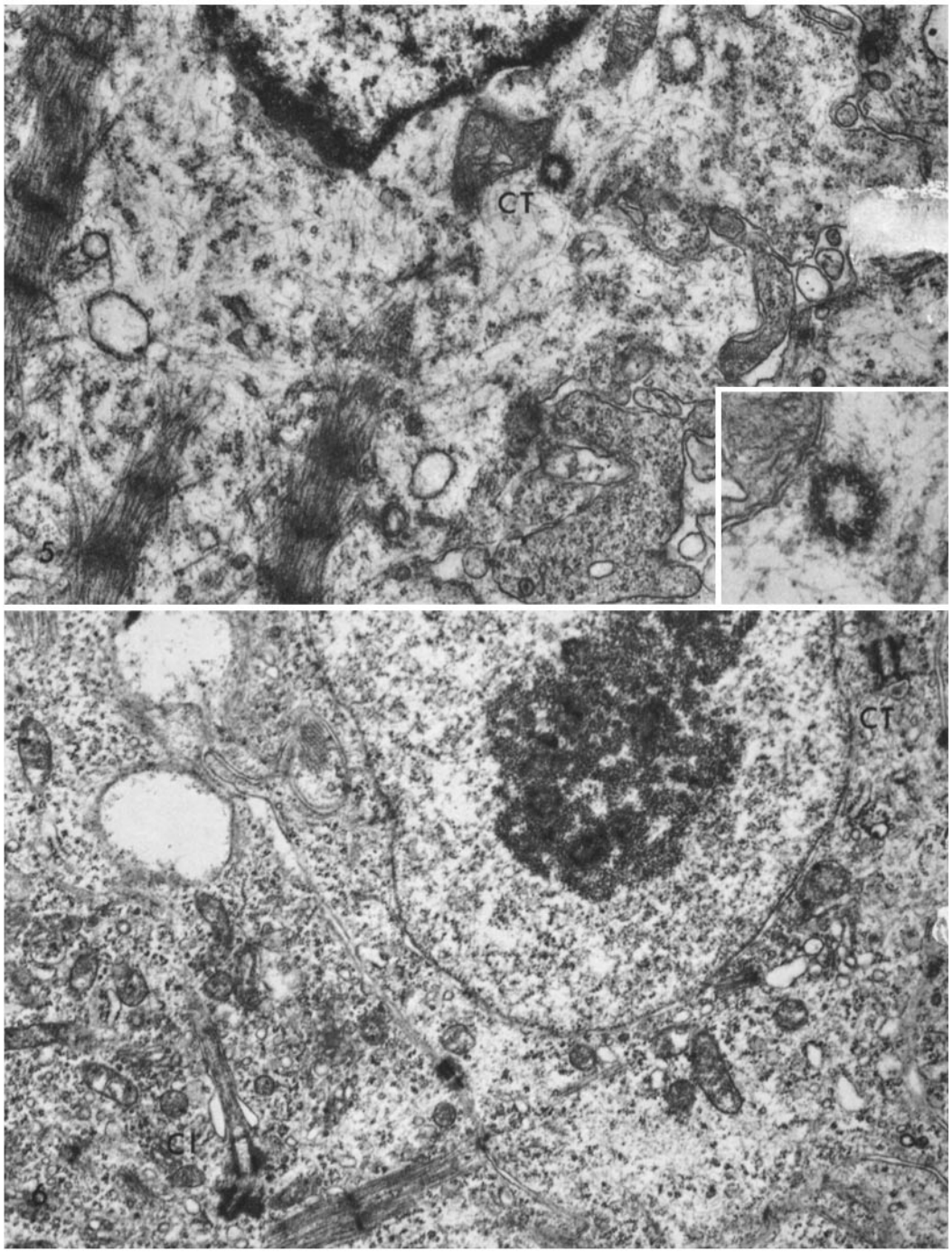

FIGURE 5 An electron micrograph of a centriole (*CT*) situated within an established myotube containing myofibrils. The centriole does not appear to be associated with a Golgi region as were those in previous figures. The *insert* shows that myotube centrioles contain the usual structural complex of peripheral triplets of microtubules arranged in pinwheel fashion. × 20,000; *Insert*, × 60,000.

FIGURE 6 An electron micrograph of myocardial cells obtained from the ventricle of a stage 13 embryo. Note the numerous free filaments and well-organized myofibrils of these differentiated cells. One myocardial cell contains a centriole (*CT*) and the other a cilium (*CI*). These structures were frequently found in myocardial cells during all stages of *in ovo* development. × 16,000.

to have been fixed during fusion with myotubes. In both figures, the cell membranes of the myoblasts and myotubes are evident except at the suspected points of fusion (arrows). In these regions, the membranes appear to be in a process of dissolution as evidenced by their diffuseness. Tangential sections of the membrane at the points of fusion are not evident but cannot be completely excluded. Similar morphological evidence of cell fusion has been observed previously in developing muscle cells (Przybylski and Blumberg, 1966; Firket, 1967). Note that the centriole in Fig. 2 is involved in cilium formation, whereas the centriole of the myoblast in Fig. 3 is not. The latter centriole is situated in the Golgi region and has microtubules in close proximity. The myoblast in Fig. 2 shows a diffuse cytoplasm near the point of fusion with the myotube and a dense cytoplasm in the nonfusing portion.

Figs. 4 and 5 represent sections through myotubes with their characteristic cytoplasmic filaments, myofibrils, and ribosomes, and a very pronounced centriole. In Fig. 4, the centriole is adjacent to a Golgi region with myofibrils in close proximity. In Fig. 5, the centriole is adjacent to a mitochondrion. Myofibrils containing sarcomeric components, i.e. A bands, I bands, and Z lines, are obvious. Structurally, the centriole is similar to centrioles of other cells (Brinkley and Stubblefield, 1970) and contains nine triplets of tubules at its periphery arranged in a "pinwheel" pattern with a small amount of granular material in its core. No microtubules are present. No pericentriolar satellites have been observed in any skeletal myoblast or cardiac cell. Firket (1967) has observed a centriole in a "sarcoblastic ribbon," and Fischman (1967) has similarly observed one in a chick myotube.

In the chick heart ventricle, centrioles and cilia have been observed frequently in myocardial cells in all developmental stages. Fig. 6 represents myocardial cells from a stage 13 embryo. In one plane of section, one cell contains a centriole while the adjacent cell contains a cilium. Similarly, centrioles and formative stages of cilia have been observed in myocardial cells up to day 20 of incubation. Those cells containing centrioles always contained myofibrils. The myocardial cells appeared to be actively engaged in contractile protein synthesis and assembly as indicated by large polysomal aggregates, numerous "myofilaments," and well-organized myofibrils.

It is apparent from these observations that skeletal muscle and cardiac muscle differ in the presence of centrioles and centriolar derivatives during embryogenesis. Skeletal muscle myoblasts contain centrioles and cilia in all stages of embryogenesis observed (stage 12 to hatching). Centrioles continue to be present in the myoblasts during their fusion with myotubes or with other myoblasts. In myotubes, the centriole can remain as a distinct organelle for some undetermined time; however, it is surmised that some process of degradation ensues. This supposition is based on the fact that centrioles are not generally found in embryonic myotubes and, to my knowledge, have not been reported in mature muscle fibers and have not been observed in chicken or mouse muscle fibers in our laboratory. These observations are consonant with the inability of normal myotube and muscle nuclei to undergo mitosis. Cox (1968) and Bischoff and Holtzer (1969) have shown that myoblast fusion occurs during G_1 or G_0 of the mitotic cycle. Similarly, DNA polymerase activity decreases as myoblast proliferation decreases and the number of myotube nuclei increases (O'Neil and Strohman, 1969; Stockdale, 1970). If the mitotic and DNA synthetic machinery is inactivated before fusion, the presumed absence of centrioles in skeletal muscle fibers may be a secondary consequence of the loss of the mitotic capacity of their nuclei.

Cardiac myoblasts are similar to the skeletal myoblasts in that they also contain centrioles and cilia; indeed, our observations on various types of embryonic chick cells suggests that this is a common phenomenon. However, unlike the skeletal muscle cell which loses its centriole, differentiated myocardial cells retain the centrioles, and these centrioles are still capable of forming cilia. This basic difference is enhanced by the observations that differentiated cardiac cells have been shown to synthesize DNA and divide mitotically in vivo (Overy and Priest, 1966; Manasek, 1968; Rumyantsev and Snigerevskaya, 1968; Przybylski and Chlebowski, 1970) and in vitro (Rumery and Rieke, 1967).

Under abnormal situations, i.e. induced viral infection, skeletal myotube nuclei have been shown to be capable of DNA synthesis and do attempt to undergo mitosis. Fogel and Defendi (1967) were able to detect by radioautography DNA synthesis in myotubes if fusing myoblasts were infected with polyoma or SV40 virus. However, in the absence of fusion DNA synthesis could not be induced in myotube nuclei. Yaffe

and Gershon (1967) have shown that myotube nuclei will undergo the nuclear changes associated with mitosis after long-term infection with polyoma virus, but appear to be arrested at metaphase. This arrest may be associated with the absence or minimal number of centrioles in the myotubes. Centrioles may be a prerequisite for mitosis in muscle cells but are not a universal prerequisite as evidenced by the numerous protozoan and plant species that survive without them. In light of the retention of centrioles by myocardial cells and their absence in skeletal myotubes, it would be interesting to see if a hybrid myotube formed by the fusion of myocardial cells and skeletal myoblasts could undergo DNA synthesis and mitosis in the absence of oncogenic viruses. Such hybrids have recently been produced in our laboratory (Przybylski and Chlebowski, 1970), and these studies are underway.

SUMMARY

Embryonic skeletal and cardiac muscles have been studied with the electron microscope to determine if centrioles are present during development. It was found that skeletal myoblasts, before fusion with myotubes and during fusion, did contain centrioles and cilia. Upon incorporation of myoblasts into the myotube, centrioles were observed but only rarely; muscle fibers never contained them. In contrast, myocardial cells contained centrioles and cilia during all stages of differentiation. These results coincide with the inability of skeletal muscle nuclei to divide mitotically and the retention of this ability by myocardial cells.

This study was supported by a grant from the Heart Association of Northeastern Ohio, Inc.

Received for publication 14 August 1970, and in revised form 17 November 1970.

REFERENCES

BISCHOFF, R., and H. HOLTZER. 1969. Mitosis and the process of differentiation of myogenic cells *in vitro*. *J. Cell Biol.* **41**:188.

BRINKLEY, B. R., and E. STUBBLEFIELD. 1970. Ultrastructure and interaction of the kinetochore and centriole in mitosis and meiosis. *In* Advances in Cell Biology. D. M. Prescott, L. Goldstein, and E. McConkey, editors. Appleton-Century-Crofts Inc., New York. **1**:119.

CAPERS, C. R. 1960. Multinucleation of skeletal muscle *in vitro*. *J. Biophys. Biochem. Cytol.* **7**:559.

COX, P. G. 1968. *In vitro* myogenesis of promuscle cells from the regenerating tail of the lizard, *Anolis carolinesis*. *J. Morphol.* **126**:1.

FIRKET, H. 1967. Ultrastructural aspects of myofibril formation in cultured skeletal muscle. *Z. Zellforsch. Mikrosk. Anat.* **78**:313.

FISCHMAN, D. A. 1967. An electron microscope study of myofibril formation in embryonic chick skeletal muscle. *J. Cell Biol.* **32**:557.

FOGEL, M., and V. DEFENDI. 1967. Infection of muscle cultures from various species with oncogenic DNA viruses (SV40 and polyoma). *Proc. Nat. Acad. Sci. U.S.A.* **58**:967.

HEYWOOD, S. M., R. M. DOWBEN, and A. RICH. 1967. The identification of polyribosomes synthesizing myosin. *Proc. Nat. Acad. Sci. U.S.A.* **57**:1002.

HOLTZER, H., J. ABBOTT, and J. LASH. 1958. On the formation of multinucleated myotubes. *Anat. Rec.* **13**:567.

KARNOVSKY, M. F. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**:137A. (Abstr.)

KONIGSBERG, I. R., N. MCELVAIN, M. TOOTLE, and H. HERMANN. 1960. The dissociability of deoxyribonucleic acid synthesis from the development of multinuclearity of muscle cells in culture. *J. Biophys. Biochem. Cytol.* **8**:333.

LASH, J., H. HOLTZER, and H. SWIFT. 1957. Regeneration of mature skeletal muscle. *Anat. Rec.* **128**: 679.

LUFT, J. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.

MACCONNACHIE, H. F., M. ENESCO, and C. P. LEBLOND. 1964. The mode of increase in the number of skeletal muscle nuclei in the postnatal rat. *Amer. J. Anat.* **114**:245.

MANASEK, F. J. 1968. Mitosis in developing cardiac muscle. *J. Cell Biol.* **37**:191.

MARCHOK, A. C., and H. HERMANN. 1967. Studies on muscle development. I. Changes in cell proliferation. *Develop. Biol.* **15**:129.

MAURO, A. 1961. Satellite cell of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9**:493.

MINTZ, B., and W. W. BAKER. 1967. Normal mammalian muscle differentiation and gene control of isocitrate dehydrogenase synthesis. *Proc. Nat. Acad. Sci. U.S.A.* **58**:592.

MOSS, F. P., and C. P. LEBLOND. 1970. Nature of dividing nuclei in skeletal muscle of growing rats. *J. Cell Biol.* **44**:459.

OKAZAKI, K., and H. HOLTZER. 1966. Myogenesis: fusion, myosin synthesis and the mitotic cycle. *Proc. Nat. Acad. Sci. U.S.A.* **56**:1484.

O'NEIL, M., and R. STROHMAN. 1969. Changes in DNA polymerase activity associated with cell fusion

in cultures of embryonic muscle. *J. Cell Physiol.* **73**:61.

OVERY, H. R., and R. E. PRIEST. 1966. Mitotic cell division in postnatal cardiac growth. *Lab. Invest.* **15**:1100.

PRZYBYLSKI, R. J., and J. M. BLUMBERG. 1966. Ultrastructural aspects of myogenesis in the chick. *Lab. Invest.* **15**:836.

PRZYBYLSKI, R. J., and J. CHLEBOWSKI. 1970. DNA synthesis, mitosis and fusion of differentiated myocardial cells. *J. Cell Biol.* **47**(2, Pt. 2):162 a. (Abstr.)

RUMERY, R. E., and W. O. RIEKE. 1967. DNA synthesis by cultured myocardial cells. *Anat. Rec.* **158**:501.

RUMYANTSEV, P. P., and E. S. SNIGIREVSKAYA. 1968. The ultrastructure of differentiating cells of the heart muscle in the state of mitotic division. *Acta Morphol. Acad. Sci. Hung.* **16**:271.

STOCKDALE, F. E. 1970. Changing levels of DNA polymerase activity during the development of skeletal muscle tissue *in vivo*. *Develop. Biol.* **21**:462.

STOCKDALE, F. E., and H. HOLTZER. 1961. DNA synthesis and myogenesis. *Exp. Cell Res.* **24**:508.

VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.

YAFFE, D., and D. GERSHON. 1967. Multinucleated muscle fibers: induction of DNA synthesis and mitosis by polyoma virus infection. *Nature* (*London*). **215**:421.

ZHINKIN, L. N., and L. F. ANDREEVA. 1963. DNA synthesis and nuclear reproduction during embryonic development and regeneration of muscle tissue. *J. Embryol. Exp. Morphol.* **11**:353.