Postnatal Development of the M-Band in Rat Cardiac Myofibrils

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SUMMARY Cardiac muscle fibers in rats at 1 and 5 days after birth showed little evidence of M-bands. An ultrastructural analysis of myofibrils failed to demonstrate dense M-band material in longitudinal sections or M-bridges in transverse sections of sarcomeres. M-bands began to increase in number after 5 days of postnatal life and were present in 60% of all sarcomeres at 11 days of age. Polypeptides with molecular weights of 190,000 (Mα) and 175,000 (Mβ) were obtained by polyacrylamide-gel electrophoresis of myofibril preparations. These two proteins were found in both 1- and 11-day-old rats and were present in 60% of all sarcomeres at 11 days of age. The M-band is a distinctive structural feature of mature myofibrils, characterized electron microscopically as a dense line across the center of each sarcomere. Mα and Mβ polypeptides increased approximately 2-fold during the interval from 1 to 11 days after birth. These structural and biochemical changes of the M-band material in myocytes appear to be related to the maturation of contractile function in the young heart. Circ Res 48: 561-568, 1981

THE tissue, cellular, and subcellular changes that occur in the early period of postnatal development of the rat heart have been described recently (Anversa et al., 1980; Olivetti et al., 1980). Maturation of cardiac myocytes from 1 to 11 days of age is characterized by an increase in the volume percentages of myofibrils, mitochondria, smooth endoplasmic reticulum and by an increase in mean size and number of mitochondria per cell (Olivetti et al., 1980). These quantitative changes are accompanied by a progressive organization of contractile proteins into characteristic cross-striated myofibrils distributed along the major axis of the cell and separated by rows of mitochondria.

The M-band is a distinctive structural feature of mature myofibrils, characterized electron microscopically as a dense line across the center of each sarcomere (McNutt and Fawcett, 1974). A number of protein subunits, ranging in molecular weight from approximately 40,000 to 190,000, have been identified in M-band material from skeletal muscle of several adult animal species (Eppenberger et al., 1967; Eaton and Pepe, 1972; Morimoto and Har-
rington, 1972; Turner et al., 1973; Masaki and Takai, 1974; Mani and Kay, 1978a; 1978b; Porzio et al., 1979). Structural and biochemical development of the M-band in cardiac myocytes has not been described previously. The present study is an ultrastructural analysis of several intermediate stages in the formation of M-bands, correlated with quantitative changes seen in the electrophoretic pattern of high molecular weight M-band polypeptides.

**Methods**

**Electron Microscopy**

Hearts of normal Wistar strain rats were examined at 1, 5, and 11 days of age, six animals at each age (Anversa et al., 1980; Olivetti et al., 1980). Each rat was anesthetized with ether and the abdominal aorta cannulated with a glass micropipette attached to a perfusion apparatus. The heart was arrested in diastole by intravenous injection of KCl, the right atrium opened, and the myocardium perfused for 3 minutes with 0.1 M phosphate buffer (pH 7.2) containing 100 IU heparin per ml. Subsequently, each heart was perfused for 15 minutes with a glutaraldehyde paraformaldehyde mixture diluted 1:1 with phosphate buffer. All perfusions were done at a pressure of 30 mm Hg, the minimal level at which a reliable flow through the tissue could be attained.

The hearts were excised rapidly and the free wall of the left ventricle was sliced transversely into several thin arcs from which blocks extending from the endocardial to epicardial surface were obtained. The blocks were fixed in 1% OsO4, dehydrated with acetone, and embedded in Araldite using flat embedding molds. Semi-thin sections were cut first to determine the orientation of myofibers. Then, thin sections of tissue areas representing various levels through the ventricular wall were prepared from at least four different blocks from each animal. Sections were stained with uranyl acetate and lead citrate. Micrographs of longitudinally and transversely oriented myofibers were taken with a Philips EM200 electron microscope.

**SDS-Gel Electrophoresis**

The hearts of 18 normal Wistar strain rats at 1 and 11 days of age were used. These two groups consisted of 10 and 8 animals, respectively. Under ether anesthesia the hearts were excised rapidly and suspended in a relaxing buffer composed of 100 mM KCl, 10 mM Tris-acetate (pH 7.00), 2 mM MgCl2, 2 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N'-tetraacetic acid (EGTA), 10 mM Na2S, 0.2 mM dithiothreitol, and 2 mM Na2P04. The free wall of the left ventricle was dissected, immersed in fresh buffer, and then cut into small pieces for homogenization. Two ventricles from 1-day-old rats were used for each homogenization. Myofibrils were obtained according to the procedure described by Etlinger et al. (1976) with the following modifications introduced recently by Porzio and Pearson (1977): phenylmethylsulfonyl fluoride (25 μg/l) was added to the homogenizing buffer, and the 0.02% sodium deoxycholate extraction was replaced by 0.1% Triton X-100 extraction.

Myofibril proteins were dissolved in 8 M urea, 2.5% sodium dodecyl sulfate (SDS), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol, and 100 mM Tris-glycine (pH 8.8), heated for 5 minutes at 100°C and then dialyzed overnight against 25 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, and 0.2% SDS (Porzio and Pearson, 1977). Several drops of Pyronin Y (250 μg/ml) were added to the sample as a tracking dye. The following gel calibration standards and markers were used: myosin (200,000); RNS polymerase from *Escherichia coli* α-subunit (39,000), β-subunit (155,000), β'-subunit (165,000); β-galactosidase (130,000); phosphorylase B (94,000); bovine serum albumin (68,000); ovalbumin (45,000), and trypsin inhibitor from soybean (21,500).

SDS-gel electrophoresis was performed on 10% acrylamide gels containing a 100:1 weight ratio of acrylamide to bisacrylamide (Porzio and Pearson, 1977). Gels were 0.5 cm in diameter and 8 cm in length. Electrophoresis was carried out at a temperature of 22°C with a Bio-Rad Model 155 Electrophoresis Apparatus; the samples were loaded and run at 0.5 mA until the dye front was within 5 mm of the tube end. The concentration of solubilized proteins was measured by spectroscopy (E280/1% = 1.44) and the amount of proteins applied per gel was between 50 and 150 μg. After fixation, the gels were stained overnight in 0.01% Coomassie Brilliant Blue R250 (Weber and Osborn, 1969) and destained in 10% acetic acid and 5% methanol. Densitometric measurements of the gels were obtained at 580 nm with a Beckman Acta C III no. 2 gel scanner. Quantitative measurements were done on duplicate sets of gels, stained with Fast Green FCF (Petter, 1974), scanned at 620 nm in a photometric gel scanner (ISCO model UA-5 absorbance monitor, type 6 optical unit, equipped with gel scanner model 659). An electronic digital integrator (Autolab System 1, Spectra Physics) was employed to measure areas under selected absorbance peaks. In each of the five and eight samples obtained from the 1-day and 11-day groups of hearts, respectively, three runs were made and scans were made in duplicate. Thus, six values were averaged for each determination. Student’s t-test was used to evaluate the statistical significance of differences between the mean values from each age group.

**Results**

The myofibrils in left ventricular cardiac myocytes at 1 day of age were generally narrow, diffusely distributed in a loose cytoplasmic matrix, and frequently not well aligned with the principal axis of the cell. Clear band patterns including the Z-line, I-band, and A-band and a light region in the center
FIGURE 1 Longitudinal section of left ventricular myocardium from a 1-day-old rat. Several thin immature myofibrils are present in the uppermost cell. More mature myofibrils with a distinct band pattern in each sarcomere are seen in the other two cells. The lack of an M-band is apparent in these sarcomeres. 14,000x.

FIGURE 2 Myofibrils with immature sarcomeres composed of loosely organized filaments and an indistinct band pattern between the prominent Z-lines are shown in the cytoplasm of cardiac myocytes from a 1-day-old rat. 30,000x.
Figure 3 A and B: Longitudinal sections of left ventricular myocardium from 11-day-old rats. These illustrations demonstrate the asynchrony of M-band development in adjacent myofibrils (Fig. 3A) and in adjacent sarcomeres of the same myofibril (Fig. 3B). Myofibrils at the top of Figure 3A are lacking M-bands, whereas the myofibril at the bottom has an M-band evident in the center of each sarcomere. The myofibrils at the bottom of Figure 3B show three consecutive sarcomeres with M-bands followed by a sarcomere in which the M-band is not apparent. ×22,000.
FIGURE 4  Higher power micrographs showing two adjacent sarcomeres from the 1-day-old rat (Fig. 4A) and the 11-day-old rat (Fig. 4B). At 1 day of age, the central region of the A-band shows only parallel thick filaments and no evidence of dense M-band substance. At 11 days, an M-band is seen in the sarcomere on the left and an incomplete M-band in the sarcomere on the right. 48,000x. In Figure 4, C and D, transverse sections through the M-band region of sarcomeres correspond, respectively, to those shown in Figure 4, A and B. At 1 day of age (Fig. 4C), this region, in which only thick filaments are seen, shows the general absence of cross bridges (M-bridges) between filaments. At 11 days of age (Fig. 4D), thin M-bridges are evident between some of the thick filaments (circles). 65,000x.
of each sarcomere were seen in a majority of myofibrils. Characteristically, there was no evidence of the usual dense M-band in the center of the sarcomeres (Fig. 1). Some myofibrils from 1-day-old rats showed no internal band pattern in their sarcomeres other than a poorly demarcated I-band and A-band between Z-lines (Fig. 2). By 5 days after birth, myofibrils showed sarcomeres with the same band patterns seen at 1 day of age. The frequency of sarcomeres with an M-band was still less than 2% at 5 days. The cytoplasmic composition of myocytes at 11 days after birth (Fig. 3) and was more like that seen in adult myocardium with compact myofibrils interdigitated with rows of mitochondria. At this age approximately 60% of the sarcomeres viewed in longitudinal sections showed partial or complete M-bands.

The development of the M-bands was not synchronous in all myofibrils of a given cell (Fig. 3A). Furthermore, the M-bands did not form synchronously in adjacent sarcomeres of the same myofibril (Fig. 3B). In both 1- and 5-day-old rat heart sarcomeres (Fig. 4A), the light central region showed only parallel thick filaments. An extensive search of transverse sections through this region failed to show more than occasional faint M-bridges between the individual myosin filaments (Fig. 4C). An apparent initial step, in the formation of the M-band and a more mature stage are illustrated from an 11-day-old animal (Fig. 4B). Transverse sections of myofibrils at 11 days (Fig. 4D) frequently showed cross bridges between thick filaments in the M-band region. However, these bridges were seen only between some of the thick filaments, and they appeared to be less dense than those characteristic of fully mature myofibrils in the adult rat heart.

Because the morphological characteristics of sarcomeres at 1 and 5 days were essentially identical, polyacrylamide SDS-gel electrophoresis was performed on preparations of cardiac myofibrils only from 1- and 11-day-old rats. This procedure yielded a clear resolution and separation of high molecular weight subunits (Figs. 5 and 6). The electrophoretic protein profiles in both groups of animals included a band at 200,000 dalton corresponding to the heavy chain of myosin, a band at 42,000 dalton representing actin and different bands corresponding to α-actinin (100,000 dalton) and tropomyosin (35,000 dalton). Other relatively indistinct bands, not definitively identified, also were present in these preparations. The analysis of the band pattern of the gels in the region of high molecular weight polypeptides showed a characteristic doublet below the myosin heavy chain peak that has been identified with M-band protein Ma and Mb. The molecular weights of these two peptides were determined by comparing the electrophoretic mobilities of appropriate standard markers and found to be 190,000 (Ma) and 175,000 (Mb), respectively. A quantitative determination of these two bands was obtained by densitometry of the fast green-stained gels, using the actin scan area as the reference area. The changes in the relative amounts of Ma and Mb during the interval from 1 to 11 days of postnatal development are shown in Table 1. Both Ma and Mb fractions increased significantly from 1 to 11 days (P < 0.005). The ratio of the sum of Ma and Mb peaks to the actin peak was 0.074 and 0.146, respectively, in 1- and 11-day-old animals. The Ma to Mb ratio was 1:1.26 one day after birth and 1:1.56 ten days later.

**Discussion**

This study demonstrates that the M-band in rat cardiac muscle undergoes significant development in the early postnatal period from 1 to 11 days after birth. Most myofibrils at 1 and 5 days display a light central band in the middle of which the M-band develops sometime after 5 days of age, sarcomere by sarcomere and myofibril by myofibril. Transverse sections through the M-band region at

![Figure 5](https://example.com/figure5.png)

**Figure 5** Gel pattern of cardiac myofibrillar proteins obtained from 1-day (left) and 11-day- (right) old animals, fractionated on SDS-polyacrylamide (10%) gel. The gel was stained for protein with Coomassie blue, destained, and scanned at 580 nm. M-band polypeptides were designated Ma and Mb, and their estimated molecular weights were 190,000 and 175,000, respectively. Protein load was about 90 μg.
1 and 5 days showed occasional faint cross bridges between the myosin filaments. At 11 days, thin cross bridges were seen frequently in small groups of myosin filaments. Young and adult mammalian myocardium typically shows thick M-bridges between every myosin filament (McNutt and Fawcett, 1974; Hagopian et al., 1975). Such ultrastructure of the M-band suggests that it functions to maintain the lateral and longitudinal positioning of the myosin filaments during the contraction cycle (Knappeis and Carlsen, 1968).

SDS-gel electrophoresis of cardiac myofibrillar proteins was carried out to determine whether the morphological evidence for the absence of the M-band in sarcomeres could also be confirmed at the molecular level. The molecular weights of the two proteins, $M_a$ and $M_b$, found in our preparation of cardiac muscle myofibrils are in good agreement with values recently reported for M-band components in adult rabbit skeletal muscle (Etlinger et al., 1976; Porzio et al., 1979). The 175,000 fraction is likely to be the glycogen debranching enzyme (Trinick and Lowey, 1977), whereas the 190,000 fraction may be the only true M-band protein.

The observation that M-band proteins are present in the rat cardiac myofibrils at an age when M-bands were not found by electron microscopic examination can be explained by several possibilities. The relatively low concentration of M-band proteins at 1 day of age could be a limiting factor in the formation of a clear M-band in myofibrils at this time. Alternatively, M-band proteins, although present, may not yet be fully organized in the center region of the sarcomeres. It cannot be excluded, however, that incompletely organized M-bridges may not be preserved in tissue preparations for electron microscopy. At 11 days of age, M-band components were detected in both morphological and biochemical preparations. The corresponding quantitative analysis demonstrated an approximately 2-fold increase in $M_a$ and $M_b$ peptides in this growth interval.

The structural proteins and the enzyme components of the M-band have been shown to interact with each other (Mani and Kay, 1978a) and with different heavy meromyosin subfragments of the myosin molecule (Botts et al., 1975; Mani and Kay,
1976, 1978b). In addition, it has been suggested that M-band polypeptides may have a dual function as enzymes and as structural elements in the contractile apparatus (Wallimann et al., 1977). The presence of creatine kinase in the M-band in physical association with myosin ATP-ase provides a potential cyclical mechanism for rephosphorylating ADP from creatine phosphate (Ottaway, 1967; Houk and Putnam, 1973). Such a mechanism may play a crucial role in the efficiency of both cardiac and skeletal muscle contraction.

The significance of microscopically visible M-band in the contraction phenomenon is questionable, since cardiac muscle of both chicken and frog are able to function without this structure (Sommer and Johnson, 1969). The gradual formation of M-bands, however, may correspond and contribute to the maturation of physiological properties in the developing mammalian heart. Contractile properties of the heart improve rapidly in the early postnatal period (Hopkins et al., 1973; Davies et al., 1975), reaching functional maturity by about 16 days of age in the rat (Hopkins et al., 1973). During this time, cardiac muscle of rodents also undergoes marked augmentation of the major systems involved in ATP synthesis and degradation (Baldwin et al., 1977; Nayler and Fassold, 1977). In addition, total cardiac mass is increasing rapidly by hyperplasia and hypertrophy of the myocytes (Katzberg et al., 1977; Rakusan et al., 1978; Anversa et al., 1980). Rapid myocardial growth also includes proliferative changes in the capillary vasculature of the tissue and its relationship to the population of contractile cells (Olivetti et al., 1980). Thus, assembly of the M-band represents only one aspect of the complex integrated changes occurring in cardiac muscle shortly after birth.

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