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Cardiac myofibrillogenesis inside intact embryonic hearts

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ABSTRACT

How proteins assemble into sarcomeric arrays to form myofibrils is controversial. Immunostaining and transfections of cultures of cardiomyocytes from 10-day avian embryos led us to propose that assembly proceeded in three stages beginning with the formation of premyofibrils followed by nascent myofibrils and culminating in mature myofibrils. However, premyofibril and nascent myofibril arrays have not been detected in early cardiomyocytes examined *in situ* in the forming avian heart suggesting that the mechanism for myofibrillogenesis differs in cultured and uncultured cells. To address this question of *in situ* myofibrillogenesis, we applied non-enzymatic procedures and deconvolution imaging techniques to examine early heart forming regions *in situ* at 2- to 13-somite stages (beating begins at the 9-somite stage), a time span of about 23 h. These approaches enabled us to detect the three myofibril stages in developing hearts supporting a three-step model of myofibrillogenesis in cardiomyocytes, whether they are present *in situ*, in organ cultures or in tissue culture. We have also discovered that before titin is organized the first muscle myosin filaments are about half the length of the 1.6 μm filaments present in mature A-bands. This supports the proposal that titin may play a role in length determination of myosin filaments.
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Introduction

The first muscle to assemble in avian and mammalian embryos appears in cells destined to form the heart. Early in embryogenesis, muscle-specific structural proteins are expressed in precardiac mesoderm cells, and in a series of reactions, the proteins are assembled into a contractile apparatus composed of myofibril arrays that supply the force needed to direct blood throughout the forming embryo. The steps in this assembly process are not understood in detail, but in broad terms they involve filament formation, association of binding proteins with filaments, and integration of filaments into contractile subunits that are linked in series and in parallel to create myofibril arrays (Sanger et al., 2004, 2005; Stout et al., 2008). In vertebrates each contractile subunit or sarcomere, is about 2 μm at rest length, bounded by Z-bands that serve as embedding sites for thin filaments and titin molecules, both of which interact with thick filaments, that are aligned laterally in uniform elongated blocks termed A-bands. The thin and thick filaments consist of filament-forming proteins, actin and myosin, respectively, and proteins that bind to them: nebulins, tropomyosin and troponins bind actin; C-Protein, myomesin, and creatine kinase bind myosin. The giant molecule, titin, spans half the sarcomere from Z-band to the middle of the A-band. Proteins of the Z-band, the most prominent of which is α-actinin, provide a scaffold for integrating the sarcomeres with the plasma membrane and with the signaling molecules localized at this sarcomere-membrane juncture (Clark et al., 2002).

In the search to understand how these protein complexes are assembled into contractile units in cardiomyocytes, substantial effort has been focused on detecting the subcellular sites where individual proteins are initially expressed, and determining the initial localization of the proteins with respect to one another. The picture that has emerged from these studies is one of coordinate expression of the major sarcomeric proteins of the forming myofibril with temporally and spatially distinct association of particular proteins into sub-sarcomeric complexes that become integrated into full sarcomeres (Sanger et al., 2004, 2005; Wang et al., 2007; Stout et al., 2008). Resolving the temporal sequence of sarcomeric protein interactions has been a long-term challenge that has relied on antibody specificity and sensitivity, selection of myofibril-forming cells for observations, and microscopic resolution of the assembling complexes. In the selection of cells, avian material has been the most widely studied vertebrate model system for its ease of manipulation *in situ*, in explant cultures, and in cultures of dissociated embryonic cardiac tissue. The widespread similarity in composition and structure of myofibrils across vertebrate species suggests that the basic assembly process in avian cells is likely to be universal in other vertebrates (Sanger et al., 2005).

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Currently there is a lack of consensus on how sarcomeric proteins assemble into myofibrils in avian cardiomyocytes (Sanger et al., 2005). In broad terms, there is disagreement on whether the two main sarcomeric subunits, A-bands and I–Z–I-bands, assemble in tandem on a temporary stress fiber-like template (Dlugosz et al., 1984); or whether they assemble independently of one another and subsequently interdigitate to create a sarcomere (Schultheiss et al., 1990; Holtzer et al., 1997; Gregorio and Antin, 2000); or whether myofibrillogenesis proceeds as a transition through three categories of fibrils: premyofibrils containing non-muscle myosin II, nascent myofibrils containing both non-muscle myosin II and muscle specific myosin II, and mature myofibrils containing the muscle specific myosin II and no non-muscle myosin II (Rhee et al., 1994; Du et al., 2003; Wang et al., 2005b). The strongest data currently against the premyofibril model for myofibrillogenesis are the immunostaining results obtained from hearts of avian embryos fixed *in situ* at the eight- to twelve-somite stages (Ehler et al., 1999) in which no evidence for premyofibrils or even localized non-muscle myosin II was found in

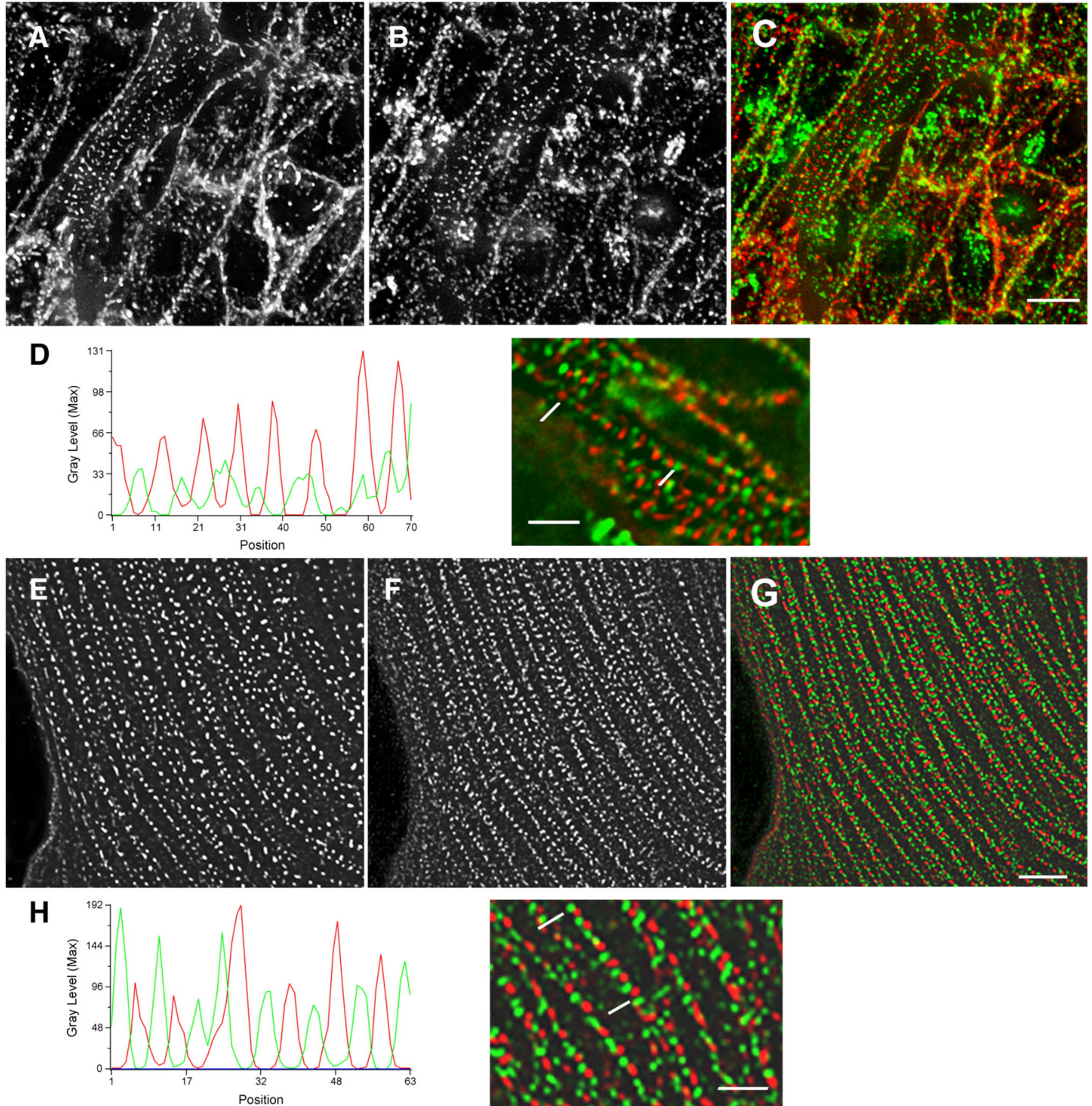

Fig. 1. Arrangement of sarcomeric alpha-actinin (A, E, red in C, D, G, H) and non-muscle myosin IIB (B, F, green in C, D, G, H) early in myofibrillogenesis in cardiomyocytes fixed *in situ* (6-somite stage, HH stage 8+) (A–D), and in the spreading margin of cells cultured for 3 days after isolation from 10-day embryonic hearts (E–H). Graph and high magnification view of premyofibrils below each set illustrate the alternating pattern of the two proteins that can be resolved in cells *in situ* (D) and that is clearer in the flat cultured cardiomyocytes (H). The sizes and spacing of the two different types of bands are the same in the two types of cardiomyocytes. Bars = 5 μm A–C and E–G. Bars = 2 μm in D and H.

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