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α-actin proteins and gene transcripts are colocalized in embryonic mouse muscle

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Summary

The α-actins are among the earliest muscle-specific mRNAs to appear in developing cardiac and skeletal muscle. To determine if there is coexpression of the α-actin proteins at early stages of myogenesis, we have used an α-actin-specific polyclonal antibody and *in situ* hybridization with specific cRNA probes to cardiac and skeletal α-actin transcripts on serial slides of mouse embryo sections. As soon as we can detect α-actin mRNAs in embryonic striated muscle, we also detect the protein suggesting that α-actin transcripts are translated very rapidly after transcription during myogenesis. In skeletal muscle, this colocalization of α-actin mRNA and protein was observed both in the myotomes of somites and in developing muscles in the limbs. In cardiac muscle, α-actin transcripts and proteins are abundantly expressed as soon as a cardiac tube forms.

Key words: sarcomeric actins, mouse embryo, myotome, antibody staining, *in situ* hybridization, muscle development.

Introduction

Cardiac and skeletal α-actin gene transcripts are known to be early markers for myogenesis in mouse embryos (Sassoon *et al.* 1988). Both of these isoforms are expressed in developing cardiac and skeletal muscle. The cardiac isoform is predominant in the heart throughout development. During embryonic skeletal muscle development (which we define as 8–15 days *p.c.*), cardiac α-actin is initially predominant, but skeletal α-actin accumulates very rapidly (Sassoon *et al.* 1988). In fetal skeletal muscle (between 15 days *p.c.* and birth), approximately 30 % of the α-actin transcripts are cardiac (Minty *et al.* 1982). The smooth α-actin gene is also expressed transiently in developing striated muscle: in the embryonic chick heart (Ruzicka and Schwartz, 1989), and in fetal rat cardiac and skeletal muscles (Woodcock-Mitchell *et al.* 1988). Both skeletal and cardiac α-actin protein is known to be expressed in fetal and postnatal mammalian muscles (Vandekerckhove *et al.* 1986), but little is known about its expression in embryonic muscle.

Earlier studies of muscle-specific gene expression with muscle cells in culture have suggested that there is a close correlation between the amounts of mRNAs coding for contractile proteins and the rate of synthesis of these proteins (Shani *et al.* 1981). Recently, however, Lawrence *et al.* (1989) have shown that cardiac α-actin mRNA can accumulate in individual postmitotic myogenic cells without coexpression of the corresponding protein. This observation suggests that as myoblasts terminally differentiate *in vivo* there may be a delay between the appearance of α-actin mRNA and its protein product. Since cardiac and skeletal α-actin transcripts can be detected at very early stages of myogenesis by *in situ* hybidization (Sassoon *et al.* 1988), we investigated whether the proteins encoded by these mRNAs could also be localized. We found that, at each developmental stage where cardiac and skeletal α-actin transcripts are detected in embryonic skeletal and cardiac muscle, α-actin protein is also present.

Materials and methods

Preparation and prehybridization of tissue sections

The protocol that was used to fix and embed mouse embryos is described in detail in Sassoon *et al.* (1988). Briefly, embryos were fixed in 4 % paraformaldehyde in phosphate-buffered saline, dehydrated and infiltrated with paraffin. 5–7 μm serial sections were mounted on subbed slides (Gall and Pardue, 1971). 1–3 sections were mounted on each slide, deparaffinized in xylene and rehydrated. The sections were digested with proteinase K, post-fixed, treated with triethanolamine/acetic anhydride, washed and dehydrated.

Probe preparation

Bluescribe+ (Stratagene) was grown in *E. coli* TG1. The following probes were used:

1) 5′ UTR of mouse cardiac α-actin mRNA (Sassoon *et al.* 1988). This plasmid was linearized with *Eco*RI and the antisense probe was generated using T3 polymerase.
2) 5′ UTR of mouse skeletal α-actin mRNA (Sassoon *et al.* 1988). This plasmid was linearized with *Eco*RI and the antisense probe was generated using T3 polymerase.
The cRNA transcripts were synthesized according to manufacturer's conditions (Stratagene) and labelled with ^{35}S-UTP (>1000 Ci mmol^{-1}; Amersham).

Hybridization and washing procedures

The hybridization and posthybridization procedures are as described by Wilkinson *et al.* (1987). Sections were hybridized overnight at 52°C in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl pH 7.4, 5 mM EDTA, 10 mM $NaPO_4$, 10% dextran sulfate, 1× Denhardt's, 50 μg ml^{-1} total yeast RNA, and 50–75 000 cts min^{-1} μl^{-1} ^{35}S-labelled cRNA probe. The tissue was subjected to stringent washing at 65°C in 50% formamide, 2×SSC, 10 mM DTT and washed in PBS before treatment with 20 μg ml^{-1} RNAse A at 37°C for 30 min. Following washes in 2×SSC and 0.1×SSC for 15 min at 37°C, the slides were dehydrated and dipped in Kodak NTB-2 nuclear track emulsion and exposed for one week in light tight boxes with desiccant at 4°C. Photographic development was carried out in Kodak D-19. Slides were analyzed using both light- and dark-field optics of a Zeiss Axiophot microscope.

Antibody staining of paraffin sections

Paraffin sections were stained using a polyclonal, affinity-purified anti-α-actin antibody described earlier (Polzar *et al.* 1989) using the alkaline phosphatase anti-alkaline phosphatase procedure (Cordell *et al.* 1984) with some modifications. Briefly, after treating paraffin sections sequentially with xylene, methanol and methanol/PBS buffer (1:2) for 10 min each, they were incubated for 30 min with the polyclonal anti-actin at room temperature. After 3 PBS washes, the sections were treated with mouse anti-rabbit IgG (Dakopatts (M 737)) at a dilution of 1:250 for 30 min, followed by a rabbit anti-mouse IgG (Dakopatts (Z 259)) at a dilution of 1:25 for 30 min. After 3 PBS washes, the preformed APAAP complex (Dakopatts) was added at a dilution of 1:150 for 30 min. Thereafter the last two incubations were repeated for 10 min each with extensive washing in between. Visualization of the bound alkaline phosphatase was achieved as detailed by Cordell *et al.* (1984).

Results

Cardiac muscle, which is the first striated muscle to form in the mouse embryo between 7.5 and 8 days *post coitum* (*p.c.*) (Rugh, 1990) expresses α-actin transcripts at high levels (Sassoon *et al.* 1988). Cardiac α-actin is the main isoform expressed in the heart throughout development. In an 18-somite mouse embryo (at 9.25 days *p.c.*, Rugh, 1990), cardiac actin transcripts and α-actin proteins are both expressed abundantly in cardiac myocytes (Fig. 1A,B). The levels of cardiac α-actin transcripts and α-actin protein at 8 days *p.c.* (data not shown) are comparable to that seen in Fig. 1A,B.

In contrast to the heart, α-actin transcript and protein levels in the myotomes of somites at 9.25 days *p.c.* (arrowheads Fig. 1A,B) are very low but detectable. The myotomes are the first skeletal muscles to form in the embryo. Myotomes develop in a rostrocaudal gradient, and the α-actins are expressed in a similar fashion. Thus, myotomes of somites more caudal to those shown in Fig. 1A,B are negative for cardiac action mRNAs and protein (data not shown). An earlier report on myogenesis in the mouse embryo (Fürst *et al.* 1989) showed positive α-actin antibody staining in the cervical somites of a 20-somite embryo. Our results show that α-actin protein synthesis in myotomes has begun by the 18-somite (9.25 days *p.c.*) stage.

The antibody we used also cross-reacts with smooth α-actin (Polzar *et al.* 1989), but it is clear that the level of cross-reactivity is low in paraformaldehyde-fixed sections. In Fig. 1A, there is an abrupt transition in staining between the smooth muscle in the blood vessel leading to the heart and the cardiac muscle. Also, the smooth muscle in the primitive gut between the neural tube and the heart does not stain positively with our antibody.

As myotomes mature and enlarge to form the premuscle masses around the developing vertebrae (arrowheads Fig. 1C,D), both α-actin transcripts and protein continue to be expressed at high levels. Interestingly, the antibody staining in Fig. 1C suggests that the α-actin protein expression is uniform throughout the myotome, whereas the *in situ* hybridization results show discrete areas of cardiac actin mRNA production concentrated in the central region of the myotome. However, this observation may reflect the different sensitivities of the two experimental techniques.

Both cardiac and skeletal α-actin genes are expressed in developing myotomes (Sassoon *et al.* 1988; Fig. 1D,F), and it is likely that both contribute to the α-actin protein detected with our antibody. At 10.5 days *p.c.* (34–36 somites, Rugh, 1990), α-actin transcripts and protein are expressed at much higher levels in rostral myotomes (arrowheads Fig. 1E,F) than they were at the 18-somite stage (9.25 days *p.c.*).

α-actin mRNAs and proteins are coexpressed in developing limb and body wall muscles as well as in the muscles derived from the myotome. Limb muscles form from cells that migrate out from the ventrolateral edge of somites early in development (Milaire, 1976; Jacob *et al.* 1979). The myogenic cells in limb buds do not express α-actin mRNAs or proteins as early as myotomal cells. However, when cardiac α-actin transcripts are first detected in limb buds at 11.5 days *p.c.* (Sassoon *et al.* 1989), α-actin protein is also detected (data not shown). At 13 days *p.c.*, in the developing mouse hindlimb bud, α-actin mRNAs and the corresponding proteins (Fig. 1G,H) continue to be expressed in the muscle groups. As skeletal muscle development proceeds, cardiac α-actin transcript levels decrease and skeletal α-actin mRNA levels increase (Minty *et al.* 1982; Garner *et al.* 1989).

Discussion

Our results show that early in the process of striated

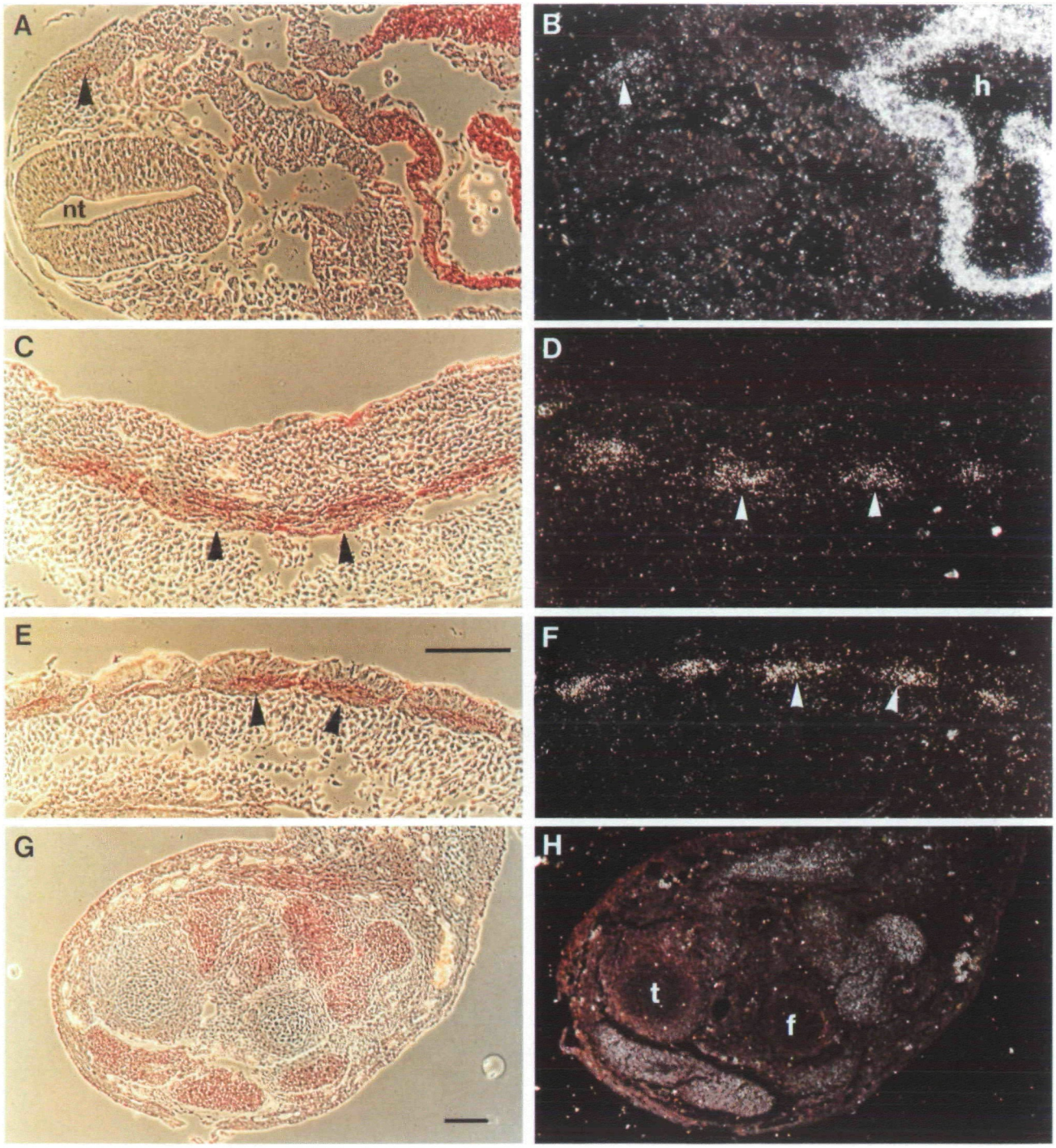

Fig. 1. α-actin proteins and mRNAs are colocalized in developing myotomes and muscle masses. (A,B) Parallel transverse sections of an 18-somite (9.25 days *p.c.*) mouse embryo at the level of the cervical somites and the heart. The section in B was hybridized with the cardiac actin-specific probe. (C,D) Serial slides of 11.5 day *p.c.* embryonic parasagittal sections in the cervical region where somites have matured and distinct dermatomes are no longer present. D was hybridized with the cardiac actin-specific probe. (E,F) Rostral somites in a 10.5-day *p.c.* embryo. F was hybridized with the skeletal actin specific probe. (G,H) Serial transverse sections of a hindlimb of a 13-day *p.c.* embryo. H was hybridized with the cardiac actin-specific probe. At this stage, subdivision of the limb muscle masses into the mature muscle groups is not yet complete. h=heart, t=tibia, f=fibula. Bars A–F=100 μm. Bar G,H=100 μm.

muscle formation in developing mouse embryos, both α-actin transcripts and their corresponding proteins are expressed. In somites, this coexpression in rostral myotomes occurs by the 18-somite stage and probably earlier. Myotomal cells are elongated, mononucleated cells, which are known to express myosin heavy chain (MHC), another muscle-specific protein, prior to fusion (Holtzer *et al.* 1957; Vivarelli *et al.* 1988). However, α-actin mRNA and protein accumulation appears to occur before that of MHC mRNA and protein (Lyons *et al.* 1990). The MHCs and α-actins are assembled into functional myofibrils because myotomal myocytes have been observed to contain cross-striations and to contract (Holtzer *et al.* 1957). Other muscle structural proteins such as tropomyosin, α-actinin (Jockusch *et al.* 1984), titin and nebulin (Fürst *et al.* 1989) have also been detected in embryonic mouse skeletal muscle. These proteins appear to be expressed in a specific sequence (Fürst *et al.* 1989).

The use of paraffin sections of paraformaldehyde-fixed mouse embryos for antibody staining gives significantly better morphology when compared to frozen sections. Most previous reports of localization of muscle-specific proteins in embryos have used immunocytochemistry on frozen sections, and the spatial relationships of the different cell types were difficult to discern. Our results show that fixed, embedded sections can be compatible with antibody staining. Further improvements of the techniques used in this paper, i.e. the use of nonradioactive *in situ* probes and antibodies on the same sections will provide definitive evidence for the colocalization of mRNAs and proteins in embryonic myocytes.

The results presented here suggest that there is no lag time between α-actin gene transcription and mRNA translation in embryonic mouse muscle. Translational controls of certain muscle-specific mRNAs may occur in embryonic chick muscle (reviewed in Roy *et al.* 1984) and in embryonic mouse muscle, in which perinatal MHC transcripts appear to accumulate prior to perinatal MHC protein (Lyons *et al.* 1990). Recently, Lawrence *et al.* (1989) have shown that *in vitro* mononucleated chick myocytes that have withdrawn from the cell cycle make high levels of cardiac α-actin mRNA but little or no α-actin protein. These authors suggest that the α-actin transcripts may not be translated until some time after these cells fuse. The differences between our results and those of Lawrence *et al.* (1989) may be explained in several ways. First, they may result from *in vitro versus in vivo* growth conditions. Second, Lawrence *et al.* (1989) were able to colocalize mRNA and protein in single cells, but embryonic mouse sections always contain a population of cells and one cannot conclusively localize autoradiographic signal to a specific cell. A third more interesting possibility is that the 12-day chick embryonic pectoral muscle cells that were grown *in vitro* represent a different myogenic cell population from that found in myotomes.

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