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The cell adhesion molecule M-cadherin is specifically expressed in developing and regenerating, but not denervated skeletal muscle

Robert Moore and Frank S. Walsh*

Department of Experimental Pathology, UMDS, Guy's Hospital, London Bridge, London SE1 9RT, UK

*Author for correspondence

SUMMARY

The spatiotemporal distribution of M-cadherin mRNA has been determined by in situ hybridization in the mouse embryo and in adult skeletal muscle following experimental regeneration and denervation. M-cadherin mRNA is highly tissue specific and is found only in developing skeletal muscle. In contrast, N-cadherin mRNA has a broader tissue distribution in the embryo, being found on both neural elements and skeletal and cardiac muscle. M-cadherin is expressed in the myotomes shortly after they form, along with the myogenic regulatory factor myogenin. M-cadherin is expressed in muscles derived from the myotomes and is detected in forelimb bud precursor cells at embryonic day 11.5. In the latter case M-cadherin expression appears co-ordinately with that of myogenin and cardiac α-actin. Shortly before birth, M-cadherin expression is down regulated. M-cadherin can, however, be re-expressed following experimental regeneration of skeletal muscle. Here M-cadherin is transiently expressed on regenerating myoblasts but not myotubes. Following muscle denervation no evidence was found for re-expression of M-cadherin under conditions where there was strong expression of the nicotinic acetylcholine receptor on myofibres.

The highly specific tissue distribution and unique developmental profile distinguishes M-cadherin from other cadherins and suggests a role in cell surface events during early myogenesis.

Key words: cadherin, myogenesis, in situ hybridization, mouse

INTRODUCTION

The cadherins are a family of calcium-dependent cell adhesion molecules (CAMs; Takeichi, 1988, 1990, 1991). Four cadherins have been characterized at the molecular level in considerable detail. These are neural (N-), epithelial (E-) and placental (P-) cadherins and the liver cell adhesion molecule (L-CAM; Takeichi, 1991; Gallin et al., 1987). The number of members of the cadherin family has increased dramatically with the characterization of retinal (R-; Inuzuka et al., 1991a), truncated (T-; Ranscht and Dours-Zimmermann, 1991), brain (B-; Napolitano et al., 1991), *Xenopus* (EP-; Ginsberg et al., 1991) and muscle (M-; Donalies et al., 1991) cadherins and the partial identification of others by the polymerase chain reaction (Suzuki et al., 1991). Each cadherin is encoded by a specific gene present at a distinct locus in the genome with little evidence of alternative splicing. In this respect they are distinct from other CAM families such as the CAMs in the immunoglobulin superfamily where alternative splicing is often used to generate diversity. For instance over twenty different forms of the neural cell adhesion molecule (NCAM) protein, many of which have distinct functions, can be generated by alternative splicing of a single gene (Walsh and Doherty, 1991).

All members of the cadherin family share a number of characteristic features, particularly a high degree of amino acid sequence homology. Despite this, transfected cells expressing similar levels of cadherins such as P- and E-cadherin, when mixed in vitro, segregate from one another (Nose et al., 1988). It is likely that they segregate because each cadherin binds strongly in a homophilic manner. However some of the recently characterised cadherins may have the capacity to bind heterophilically with other cadherins (Inuzuka et al., 1991a; Takeichi, 1991). All cadherins are composed of a number of repeating extracellular domains which contain putative Ca^{2+} binding sites. The most membrane distal domain, termed EC1, is believed to be involved in homophilic interactions in some cadherins as neutralizing antibodies have been mapped to this region (Takeichi, 1990). Furthermore, oligopeptide sequences from EC1 can block the function of cadherins (Blaschuk et al., 1990) such as N-cadherin (Doherty et al., 1991). A single hydrophobic transmembrane region connects the extracellular domains with a short intracellular domain, although in T-cadherin this transmembrane sequence is absent. The T-cadherin protein is thought to be attached to the cell membrane surface via a glycosylphosphatidylinositol linkage (Ranscht and Dours-Zimmermann, 1991). The sequence of the cytoplasmic domain is highly conserved between cadherins and is thought to connect to cytoskeletal associated proteins

termed catenins (Ozawa et al., 1989; Takeichi, 1991). This cytoskeletal linkage appears to be essential for cell-cell adhesion as a truncated E-cadherin protein lacking the cytoplasmic domain is ineffective at cell-cell binding (Nagafuchi and Takeichi, 1988). Cadherins are also posttranslationally processed, by both the proteolytic cleavage of an amino-terminal peptide and glycosylation, to generate the functionally active protein. However, although proteolytic processing is necessary for cadherin function (Ozawa and Kemler, 1990) glycosylation of the polypeptide backbone, unlike that for the NCAM protein (Walsh and Doherty, 1991), appears not to influence adhesion (Shirayoshi et al., 1986).

In order to determine in which tissues cadherins are functional it is important to assess the distribution of individual cadherins throughout embryogenesis. The expression patterns of some cadherins has been examined using specific antibodies and/or nucleic acid blotting procedures (e.g. Hatta et al., 1987). Although the spatiotemporal patterns of expression differ for each cadherin, several unifying features have emerged. First, it appears that each cadherin is expressed in a variety of embryonic cell types and tissues. Second, the expression patterns of different cadherins often overlap such that several cadherins are expressed simultaneously within a tissue. For example, although R-cadherin was initially characterized from retina, the protein is expressed in both central and peripheral nervous systems and the developing musculature, in patterns similar to that of N-cadherin (Inuzuka et al., 1991b).

Myogenesis is a unique event during embryogenesis as it involves the fusion of mononucleate myoblasts with one another to form multinucleate myotubes (reviewed by Knudsen, 1991). Myoblast fusion is a spontaneous process that can occur in vitro, is highly specific and occurs between myoblasts only (Knudsen, 1991). Prior to fusion myoblasts withdraw from the cell cycle and align with one another. Mutual recognition and adhesive steps are believed to be important for this alignment and several studies have implicated calcium-dependent and -independent cell adhesion molecules in the process (Gibralter and Turner, 1985; Pizzey et al., 1988). For example high concentrations of N-cadherin antibodies (Knudsen et al., 1990a), or synthetic peptides modelled around the cadherin recognition sequence (D. Peck and F. S. W., unpublished observations), partially block myoblast fusion. However, a number of distinct adhesion molecules such as NCAM (Dickson et al., 1990; Knudsen et al., 1990b) or the integrin receptors (Menko and Boettiger, 1987) are also important in fusion. Another adhesion molecule that may be functionally important is M-cadherin (Donalies et al., 1991) which is specifically expressed at the cell surface of muscle cells in culture.

In this paper we compare, by in situ hybridization in sections of mouse embryos, the appearance of M-cadherin and N-cadherin mRNA relative to transcripts that have known temporal patterns of expression in skeletal muscle such as cardiac α-actin, myogenin and the nicotinic acetylcholine receptor. Additionally we analyzed the expression pattern of M-cadherin in the adult following muscle regeneration and denervation. Using ^{35}S-labelled cRNA probes we show that like cardiac α-actin (Sassoon et al., 1988) and myogenin (Wright et al., 1989), M-cadherin is expressed within the embryonic myotome and myogenic cells within the limb buds. However unlike N-cadherin, and all other cadherins described to date, M-cadherin appears to be a tissue-specific transcript being uniquely expressed in skeletal muscle. M-cadherin is found at high levels during early stages of myogenesis but by embryonic day 17.5 (E17.5) levels were barely above the threshold level of detection. M-cadherin was, however, re-expressed in activated satellite cells following the induction of muscle regeneration but was not found on denervated myofibres. This pattern of expression is unique for a muscle cell surface protein and suggests a role for M-cadherin in myoblast interactions during myogenesis rather than nerve-muscle interactions.

MATERIALS AND METHODS

Preparation of tissue sections prior to hybridization

Pregnant CD1 or Balb/c mice were used in this study. Mice were mated overnight and embryonic day 0.5 (E0.5) was considered to be the morning a vaginal plug was discovered. At the appropriate age of the embryos, pregnant females were killed by cervical dislocation and their embryos were removed from the uterus (if possible) and immersed in 4% (w/v) paraformaldehyde. The E17.5 embryos were perfused via the umbilicus with 4% paraformaldehyde prior to immersion.

Denervated and regenerating muscles were dissected from the animals in DMEM containing 10% FCS. The tissues were then immediately immersed in 4% paraformaldehyde at 4°C.

All tissues were left overnight in paraformaldehyde at 4°C with gentle agitation. The following morning the tissues were dehydrated and embedded in paraffin wax. Tissues were sectioned on a Biocut 2030 microtome at 6 μm with one or two sections mounted on a subbed slide. Mounted sections were dried overnight at 37°C but were subsequently stored at 4°C. Prehybridization treatment of sections was performed as detailed by Sassoon et al. (1988) although the acetylation step was omitted.

Preparation of plasmids and cRNA probes

All in vitro transcriptions were performed according to standard conditions.

The cardiac α-actin probe used in this study has been previously described (Sassoon et al., 1988). The plasmid was linearized with *Eco*RI and antisense transcripts generated using T3 RNA polymerase. This probe was labelled with [^{35}S]UTPαS (>1000 Ci/mmol; Amersham).

The myogenin plasmid (Wright et al., 1989) was linearized with *Pst*I and antisense transcripts generated using T7 RNA polymerase. The myogenin probe (approx. 700 nucleotides) was hydrolysed with alkali at 60°C for 35 minutes (Cox et al., 1984).

The muscle nicotinic acetylcholine receptor (nAChRα) probe used was used in a previous study (Lyons et al., 1992). The plasmid was linearized with *Hin*dIII and antisense transcripts generated by T7 RNA polymerase.

The sense control probe used was an NCAM probe called S1 (Lyons et al., 1992). The plasmid was linearized with *Eco*RI and the sense control was generated using T3 RNA polymerase. This probe had a G/C content of 53% and was 98 nucleotides long.

The M-cadherin and N-cadherin probes were generated using a combined reverse transcriptase/PCR protocol from 1 μg of new-

born mouse limb muscle total RNA (performed as described previously by Cavicchioli et al., 1991). The PCR products were cloned into Bluescript (BSK+) and sequenced to confirm identity.

Two M-cadherin probes were used in this study. The first spanned from nucleotide number 1465 to 1569 in the EC5 domain. This plasmid was linearized with *Bam*HI and antisense transcripts generated by T7 RNA polymerase. This probe was 106 nucleotides long with a G/C content of 63%. The second M-cadherin probe spanned from nucleotide number 6 to 452 (Donalies et al., 1991) and encompassed EC1 and EC2 domains. The plasmid was linearized with *Sac*I and antisense transcripts synthesized with T7 RNA polymerase. This probe was 447 nucleotides long with a G/C content of 58%. Sense control transcripts were synthesized from this template with T3 RNA polymerase after the plasmid was linearized with *Bam*HI.

The N-cadherin probe spanned from the nucleotide sequence encoding Arg^{194} to Arg^{227} in the EC1 domain (Miyatani et al., 1989). This plasmid was linearized with *Bam*HI and an antisense probe transcribed using T7 RNA polymerase. This probe had a G/C content of 55% and was 103 nucleotides long.

Both M-cadherin probes, nAChRα, N-cadherin, myogenin and S1 control probes were double labelled with [^{35}S]UTPαS and [^{35}S]CTPαS (>1000 Ci/mmol; Amersham).

Skeletal muscle regeneration and denervation

Adult Balb/c adult mice were anaesthetized with an i.p. injection of Hypnorm (Janssen) and Hypnovel (Roche) (mixed in the ratio 1:5).

Muscle regeneration was induced by transient ischaemia of the anterior tibialis muscle. A thick suture thread was inserted behind the anterior tibialis muscle and gently slid towards the patella. At this end of the muscle the suture thread was tied in a knot to prevent blood flow to the muscle. After two hours this suture was removed and the skin closed. The anterior tibialis muscle was removed 3 or 5 days later and processed as above.

Mice were unilaterally denervated by removal of approximately 0.5 cm of the sciatic nerve (Moore and Walsh, 1986). After 4, 8 and 14 days the denervated muscles were isolated and processed as above.

Hybridization of cRNA probes to embryo sections

Probes were applied to sections as described (Sassoon et al., 1988; Lyons et al., 1992). The antisense M-cadherin, N-cadherin, myogenin, nAChRα and S1 probes were diluted with hybridization buffer to a final concentration of 10^5 cts/minute per μl and the cardiac α-actin probe to 5×10^4 cts/minute per μl. Probes were incubated with sections overnight in a dark humidified environment at 52°C. Sections were then washed as described previously

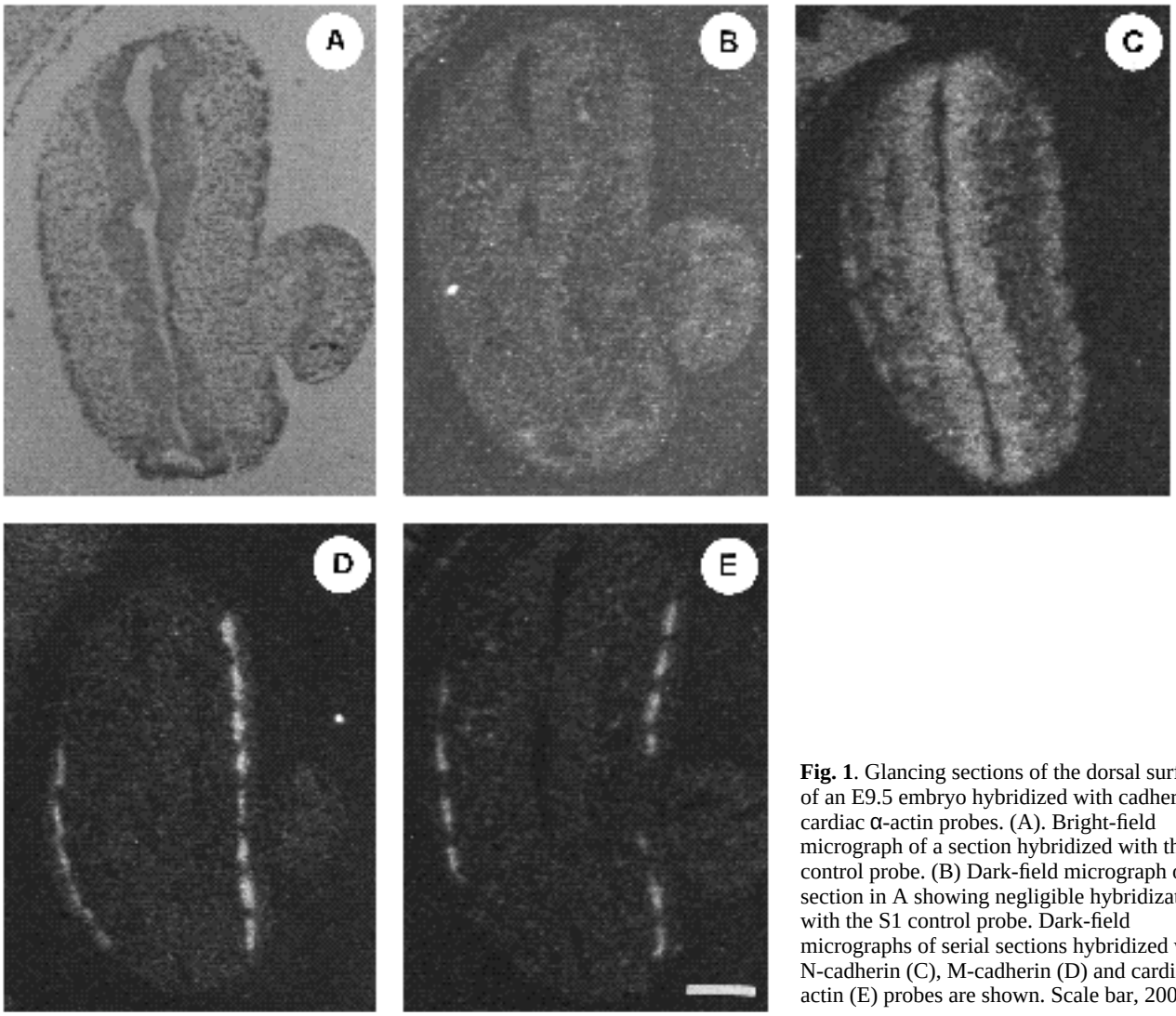

Fig. 1. Glancing sections of the dorsal surface of an E9.5 embryo hybridized with cadherin and cardiac α-actin probes. (A). Bright-field micrograph of a section hybridized with the S1 control probe. (B) Dark-field micrograph of section in A showing negligible hybridization with the S1 control probe. Dark-field micrographs of serial sections hybridized with N-cadherin (C), M-cadherin (D) and cardiac α-actin (E) probes are shown. Scale bar, 200 μm.

(Sassoon et al., 1988; Lyons et al., 1992) and all slides were collectively treated under identical washing conditions. Slides were processed for standard autoradiography by dipping in a mixture of Ilford K5 photoemulsion and 1.7% glycerol (2:3 vol/vol) at 42°C. After the slides dried they were stored in the dark at 4°C with excess desiccant.

Development of autoradiographs

After 1 week of exposure the slides were developed by immersion at room temperature in full strength D19 developer for 3 minutes, in 1% acetic acid/1% glycerol (v/v) for 1 minute and in a 30% (w/v) sodium thiosulphate solution for 5 minutes. They were washed under running tap water for 15 minutes. Sections were counterstained with either 0.02% (w/v) toluidine blue or Mayer's haematoxylin/eosin, dehydrated and mounted under DPX. Sections were examined under the bright- and dark-field optics of a Zeiss STEMI SV8 microscope.

RESULTS

M- and N-cadherin and cardiac α-actin are found in the myotome

The majority of skeletal muscles within the body develop from the myotomes and each myotome is formed within a somite shortly after somite formation. Fig. 1 shows a series of hybridizations performed on serial sections of an E9.5 embryo. The embryo was sectioned such that both neural tube and myotomes were present in a glancing section at the dorsal surface. The bright-field picture (Fig. 1A) shows the somites as discrete thickenings on either side of the embryo surrounding a centrally located neural tube. All the somites in Fig. 1 have differentiated such that each includes a myotome. Fig. 1B shows the dark-field view of Fig. 1A hybridized with a sense control probe called S1. Negligible hybridization was detected in the embryo. However, an adjacent section hybridized with a cardiac α-actin probe showed discrete and strong staining of the developing myotomes (Fig. 1E). No other component of the somite was stained with the cardiac α-actin probe. The pattern of M-cadherin hybridization (Fig. 1D) was superimposable with that of cardiac α-actin (Fig. 1E). All the myotomes in the section were positive for M-cadherin and the neural tube was clearly negative. We could not detect either cardiac α-actin or M-cadherin expression in the undifferentiated somite and the pre-somitic mesoderm (data not shown). The M-cadherin hybridization was in sharp contrast to that observed with an N-cadherin probe (Fig. 1C). The entire somite was positive for N-cadherin as was the neural tube.

The restricted distribution of the M-cadherin transcript was evident in more frontal sections of E9.5 embryos (Fig. 2). Due to the curvature of the embryo at this stage of development the neural tube was sectioned twice, in the transverse plane. No specific signal was detected in the embryo with the control probe (Fig. 2B) but all of the myotomes, on either side of the neural tube at both the rostral and caudal levels, expressed cardiac α-actin (Fig. 2C). Weak hybridization was also detected in the lining of the dorsal aorta (Fig. 2C) but no hybridization was detected in the forelimb buds at this stage. The M-cadherin probe also hybridized to the myotomes in a pattern similar to cardiac α-actin (Fig. 2D). There was however one major difference: cardiac α-actin, but not M-cadherin, was found in the dorsal

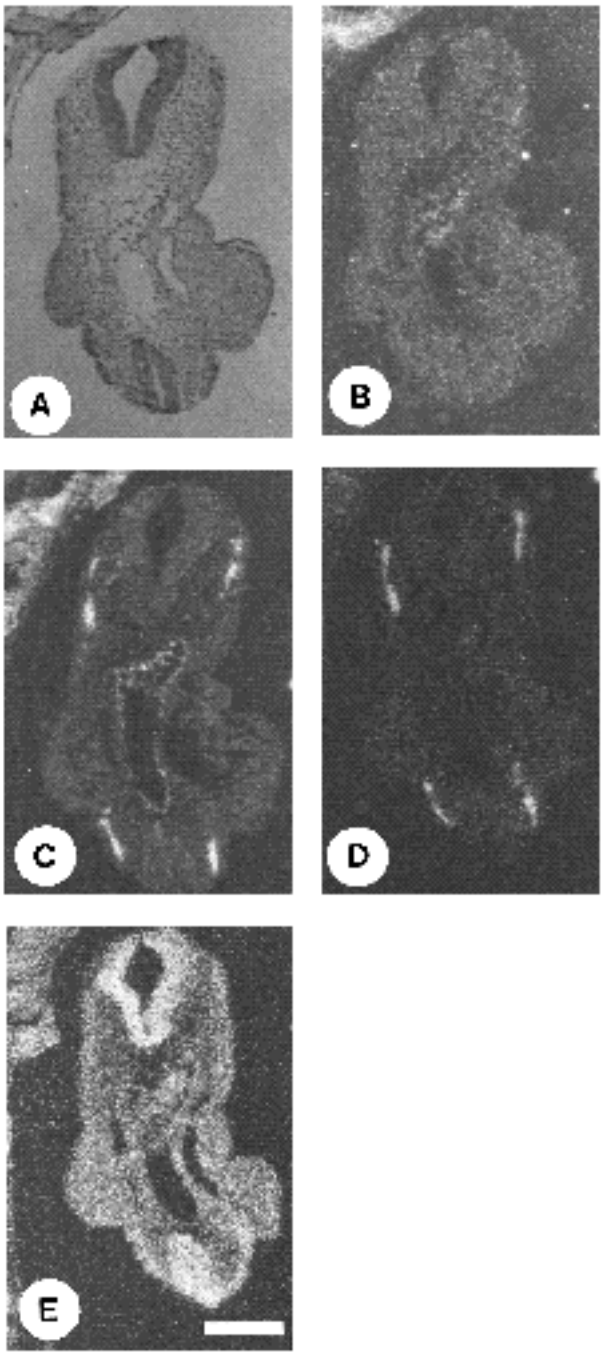

Fig. 2. Frontal sections of an E9.5 embryo showing hybridization with cadherin and cardiac α-actin probes. (A) Bright-field micrograph of a section hybridized with the S1 control probe. (B) Dark-field micrograph of section in A showing hybridization pattern with the S1 control probe. Some signal was detected in the uterus (top left corner of micrograph). Dark-field micrographs of serial sections hybridized with cardiac α-actin (C), M-cadherin (D) and N-cadherin (E) probes are shown. Scale bar, 300 μm.

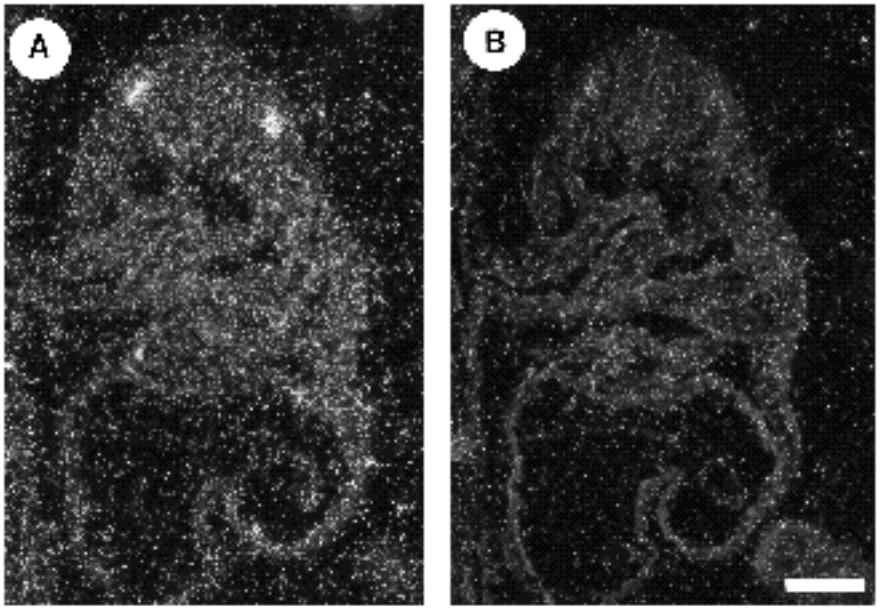

Fig. 3. Hybridization pattern observed with myogenin and M-cadherin probes at E8.5. (A) Dark-field micrograph showing hybridization pattern observed with the myogenin probe. (B) A dark-field micrograph of an adjacent section to that in A, hybridized with the M-cadherin probe. Scale bar, 100 μm.

aorta. In contrast N-cadherin hybridization was much more widespread and a signal was detected from many tissues (Fig. 2E). The entire neural tube at both rostral and caudal levels was uniformly and strongly stained with the N-cadherin probe.

Myogenin and M-cadherin are found in the myotome

We wished to determine the temporal pattern of expression of M-cadherin in the myotome in relation to that described for myogenic regulatory factors such as myogenin (Wright et al., 1989). Fig. 3 shows two serial sections of an E8.5 embryo which include some of the most rostral somites in the embryo. Myogenin transcripts were found in two myotomes (Fig. 3A), one on either side of the neural tube. However, an adjacent section hybridized with a M-cadherin probe (Fig. 3B) showed very weak staining in this myotomal region. The staining was nevertheless just above background levels but was certainly less intense than that of myogenin. In more caudal somites no staining with either probe was detected. When more rostral myotomes were examined the M-cadherin signal was no stronger than that shown in Fig. 3B but the myogenin staining seen in Fig. 3A persisted. Thus, the expression of the M-cadherin transcript in the myotome occurs with roughly the same temporal pattern as myogenin confirming that M-cadherin is a very early gene product in myogenesis.

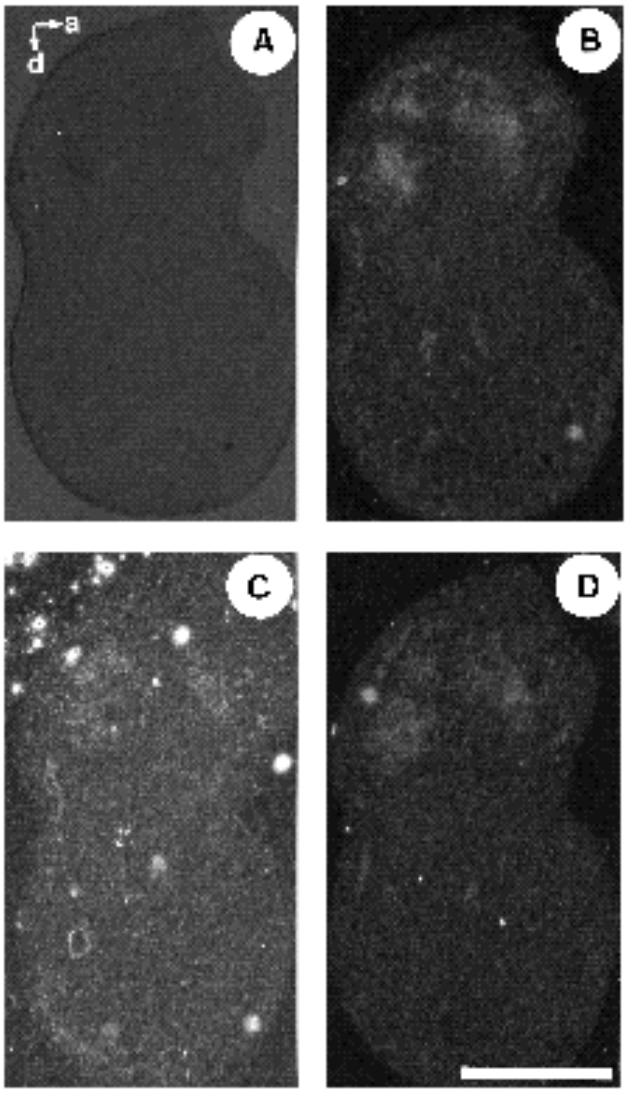

Fig. 4. Serial sections of a forelimb from an E11.5 embryo hybridized with cardiac α-actin, M-cadherin and myogenin probes. (A) A bright-field micrograph of a parasagittal section of an E11.5 forelimb hybridized with the cardiac α-actin probe. a, anterior; d, distal. (B) The dark-field micrograph of section in A showing the presence of cardiac α-actin transcripts in the proximal forelimb. (C) The dark-field micrograph of an adjacent section to that in B hybridized with the myogenin probe. (D) The dark-field micrograph of an adjacent section to that in C hybridized with the M-cadherin probe. Scale bar, 500 μm.

M-cadherin is first detected in the forelimb buds at E11.5

The myogenic cells within the limb buds are also derived from the somite, specifically they are thought to migrate from the ventrolateral somite edge prior to myotome formation (Jacob et al., 1979; Ede and Al-Gadi, 1986). These cells also express M-cadherin. However, hybridization within the forelimb bud was not detected with either the M-cadherin, cardiac α-actin or myogenin probes until E11.5. At E11.5 distinct staining was detected in the proximal region of the forelimb bud (Fig. 4). The staining pattern obtained with cardiac α-actin (Fig. 4B), myogenin (Fig.

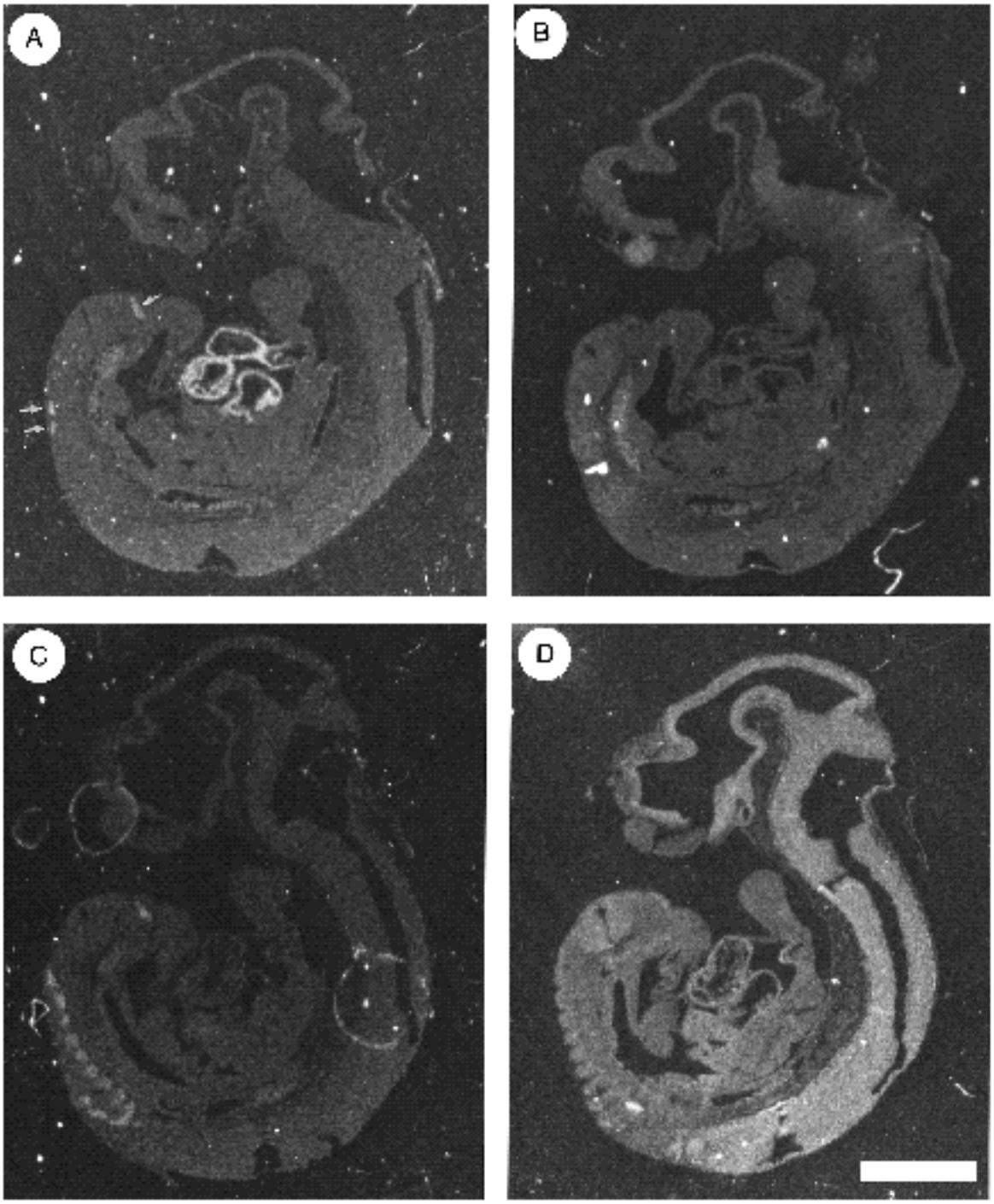

Fig. 5. Serial parasagittal sections of an E10.5 embryo hybridized with cadherin and cardiac α-actin probes. (A) Dark-field micrograph of a parasagittal section hybridized with the cardiac α-actin probe. (B) A section adjacent to that in A which has been hybridized with the S1 control probe. This micrograph shows some refractile blood cells in the dorsal aorta. Dark-field micrographs of serial sections hybridized with M-cadherin (C) and N-cadherin (D) probes are shown. Scale bar, 1 mm.

4C) and M-cadherin (Fig. 4D) appeared to overlap in two separate masses, one anteriorly the other posteriorly located in the limb. This suggests that the same cells expressed all three transcripts co-ordinately.

M-cadherin is found in muscles derived from the myotome

Parasagittal sections of an E10.5 embryo further illustrate the unique distribution of M-cadherin (Fig. 5). In some of

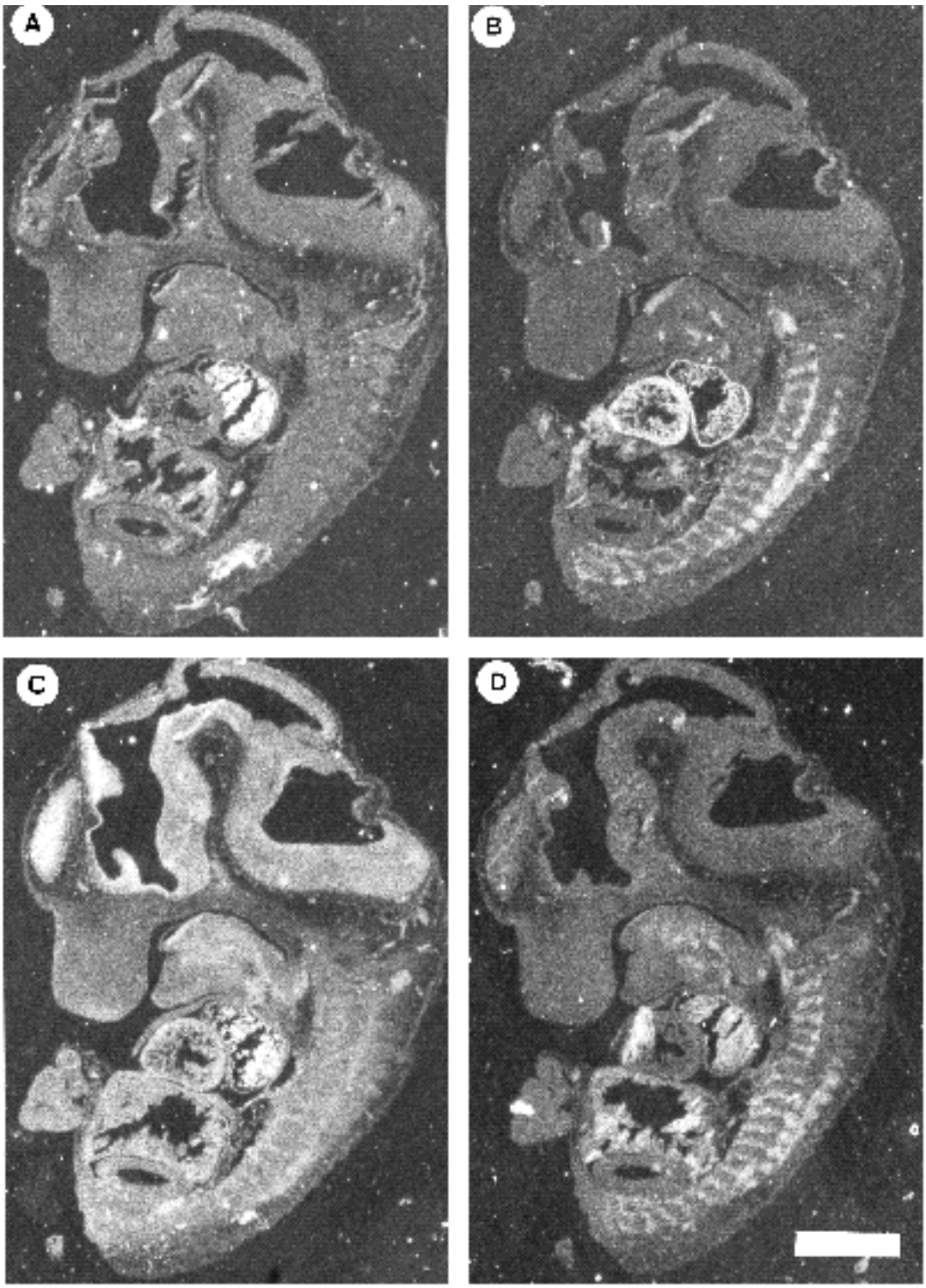

Fig. 6. Serial parasagittal sections of an E12.5 embryo hybridized with cadherin and cardiac α-actin probes. (A) A dark-field micrograph of a section hybridized with the S1 control probe. Refractile particles were present in the atria and above the liver which tore during sectioning. (B) A dark-field micrograph of an adjacent section to that in A hybridized with the cardiac α-actin probe. Adjacent sections hybridized with N-cadherin (C) and M-cadherin (D) probes are shown. Scale bar, 1 mm.

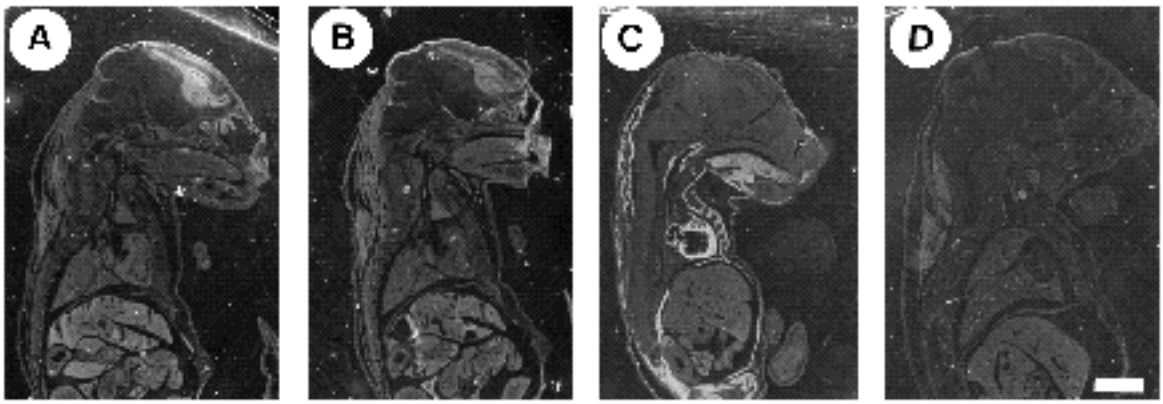

Fig. 7. Serial parasagittal sections of an E17.5 embryo hybridized with cadherin and cardiac α-actin probes. (A) Dark-field micrograph of a parasagittal section hybridized with the N-cadherin probe. Dark-field micrographs of sections hybridized with M-cadherin (B), cardiac α-actin (C) and S1 sense control (D) probes are shown. Scale bar, 2 mm.

the sections refractile blood cells were present in the dorsal aorta and generated a false positive signal. The positions of such cells were clearly seen in the section hybridized with the sense control probe (Fig. 5B). The heart and myotomes were clearly delineated with the cardiac α-actin probe (Fig. 5A). In an adjacent section only the myotomes hybridized with the M-cadherin probe (Fig. 5C). In Fig. 5, due to the twisting of the neural tube, the myotomes were sectioned in two planes, both transverse and longitudinal (the positions have been marked with arrows in Fig. 5A). They hybridized with the M-cadherin probe at both orientations (Fig. 5C). No M-cadherin hybridization was detected in the atria and ventricles of the heart (Fig. 5C). In fact no M-cadherin transcript was detected in the heart at any prior or subsequent date (data not shown and Figs 3B, 6D and 7B). Fig. 5C,D also shows that the M-cadherin and cardiac α-actin transcripts, respectively, were absent from the hindlimb bud. Again M-cadherin was absent from the entire nervous system at this stage. This was in contrast to N-cadherin which was present in the nervous system and heart with only weak hybridization in the myotome (Fig. 5D).

At later stages of embryogenesis the myotomes enlarge and migrate throughout the embryo. The myotomes form the muscles surrounding the vertebrae. These muscles were stained at E12.5 with a cardiac α-actin probe (Fig. 6B). The heart, with a clearly defined ventricle and atrium, was also stained together with several discrete populations of myogenic cells within the tongue. Although M-cadherin was not found in the heart (Fig. 6D) its pattern of expression was very similar to cardiac α-actin. The sense control probe identifies refractile blood cells in the heart and refractile particles in the liver (Fig. 6A). In sharp contrast to the M-cadherin hybridization the N-cadherin transcript was almost exclusively found in neural tissue at this age with only low levels in skeletal and cardiac muscle (Fig. 6C). Dorsal root ganglia and the olfactory epithelium also hybridized strongly with the N-cadherin probe (data not shown). M-cadherin was never detected in smooth muscles, such as those lining the gut (shown below the liver in Fig. 6D).

Down regulation of M-cadherin transcription occurs near birth

By E17.5 the N-cadherin transcript could only be detected in the ventricular zone of the telencephalon (Fig. 7A). The M-cadherin transcript was below the threshold level of detection in the musculature (Fig. 7B) and no signal in the embryo (Fig. 7B) above that from the control probe (Fig. 7D) was found. These negative results were not due to extensive RNA degradation in the embryos of this age as cardiac α-actin transcripts were still abundant in skeletal and cardiac muscle (Fig. 7C).

M-Cadherin is re-expressed during skeletal muscle regeneration but not denervation

We wished to determine whether the down regulation of M-cadherin transcription that we observed during embryogenesis was reversible. Although we were unable to detect any hybridization signal from the normal adult anterior tibialis muscle (data not shown), adult muscle induced to regenerate did show positive hybridization with the M-cadherin probe. This was found in muscle analyzed 3 days after the induction of regeneration by transient ischaemia. Positive hybridization was found in discrete areas throughout the muscle (Fig. 8A) and was qualitatively very similar to that seen with the nicotinic acetylcholine receptor probe (Fig. 8C). Analysis at higher magnification than in Fig. 8 showed that the hybridization obtained from both probes was similar in that the hybridization signal was generated from activated satellite cells (data not shown). In fact each of the punctate spots of staining in Fig. 8A overlies an activated satellite cell. The sense control hybridization of an adjacent tissue section is shown in Fig. 8E.

The hybridization of the M-cadherin probe to the regenerating muscle sections was transient. 5 days after ischaemia, M-cadherin hybridization was still present but was much weaker (Fig. 8B). In contrast, however, hybridization with the nicotinic acetylcholine receptor probe was still widespread and found in immature myotubes (Fig. 8D). Again little signal was detected in tissue sections hybridized with the sense control probe (Fig. 8F).

We also investigated whether M-cadherin was re-

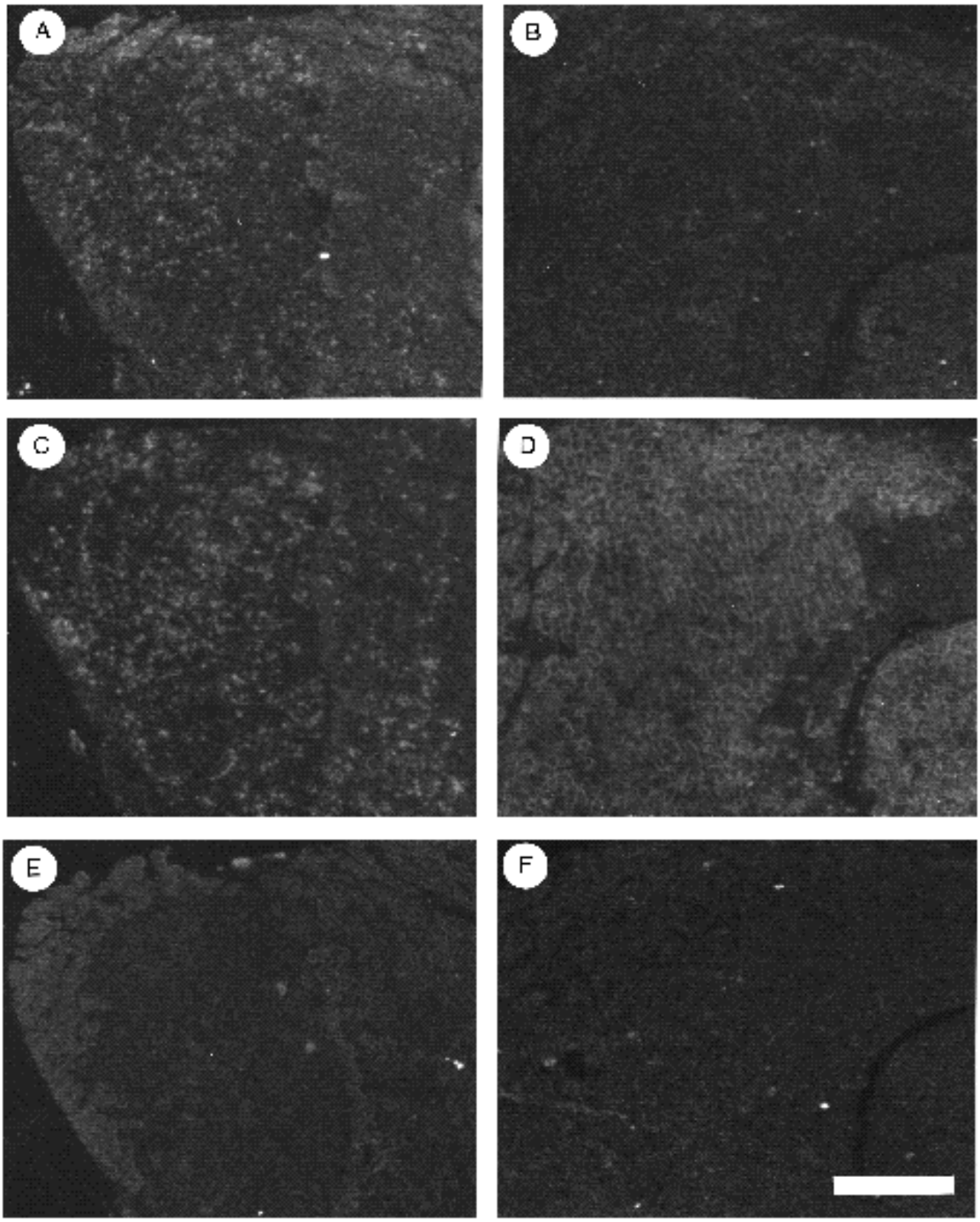

Fig. 8. M-cadherin and the nicotinic acetylcholine receptor are re-expressed in regenerating anterior tibialis muscle. Dark-field micrographs show the hybridization pattern obtained on serial tranverse sections of regenerating adult anterior tibialis muscle hybridized with M-cadherin (A,B), nicotinic acetylcholine receptor (C,D) and the S1 sense control (E,F) probes. The hybridization patterns obtained 3 days (A,C and E) and 5 days (B,D and F) after ischaemia are shown. Scale bar, 500 μm.

expressed in acutely denervated skeletal muscle. Although we could detect hybridization to denervated myofibres with the nicotinic acetylcholine receptor probe (Fig. 9A) no signal above background levels could be detected from the sections hybridized with the M-cadherin probe (Fig. 9B). This is clear when Fig. 9B is compared to the sense control hybridization in Fig. 9C. These results were observed at all three postoperative time points.

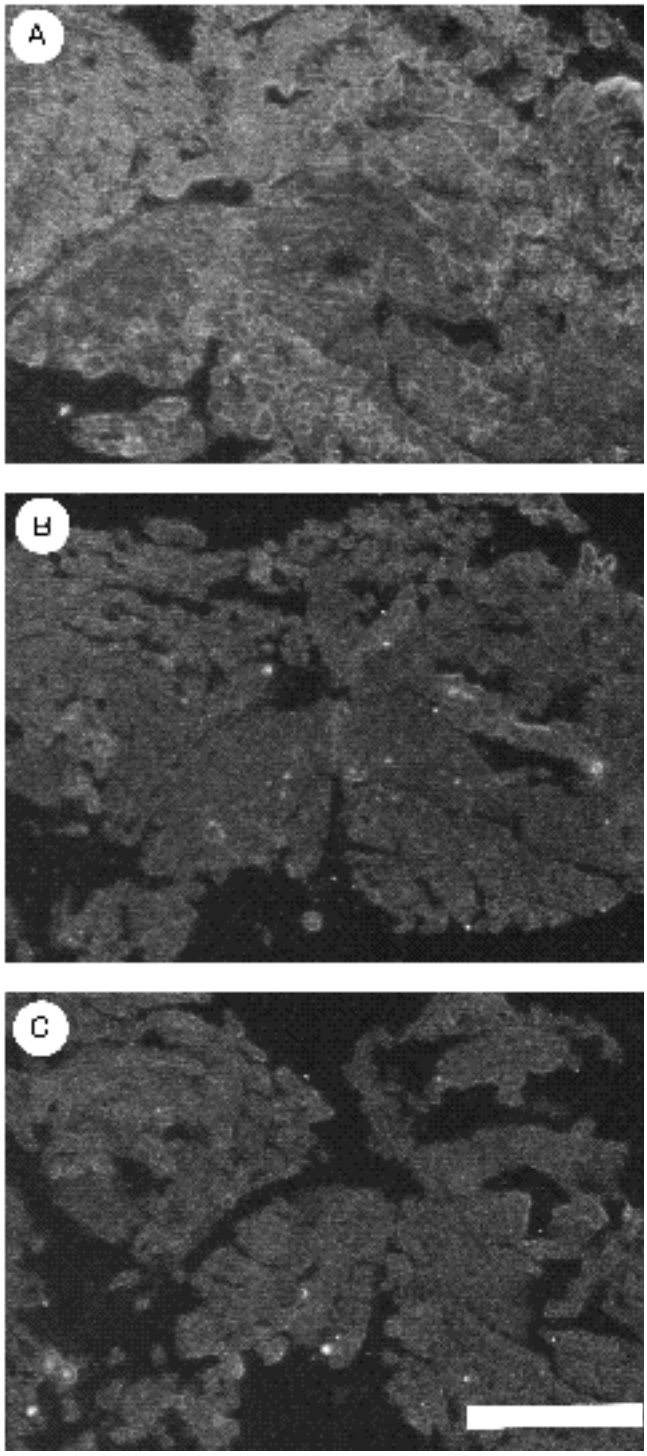

Fig. 9. M-cadherin is not expressed in denervated skeletal muscle. The muscle shown here was material isolated 8 days after the sciatic nerve was cut. Serial sections were hybridized with the nicotinic acetylcholine receptor (A), M-cadherin (B) and M-cadherin sense control (C) probes. Scale bar, 500 μm.

DISCUSSION

The cadherins are a large family of morphoregulatory molecules that, in the presence of Ca^{2+}, promote cell adhesion (Takeichi, 1988, 1990, 1991). They are believed to bind in a homophilic manner and as a result may be responsible for cell sorting phenomena during embryogenesis. A polymerase chain reaction strategy was used by Donalies et al. (1991) to isolate a partial cDNA of 2.56 kb in length from C2 myoblasts. The cDNA was classed as a new cadherin since the translated amino acid sequence showed extensive sequence similarity with other cadherins (Donalies et al., 1991). Although the cDNA was lacking the signal and prepeptide sequences, it did contain five extracellular domains, a transmembrane region and a cytoplasmic domain. Subsequently the gene for M-cadherin was mapped to mouse chromosome 8 and human chromosome 16q24.1, in both species to regions near the E-cadherin gene (Kaukman et al., 1992). The sequence homology of M-cadherin with the mouse E-, P- and N-cadherins was greatest in the cytoplasmic domain and in the extracellular domain EC1 but least in the EC5 domain. However, M-cadherin was different from the other cadherins in that E-, P- and N-cadherins were more similar to each other than M-cadherin was to any one cadherin.

By in situ hybridization we examined the spatiotemporal pattern of expression of M-cadherin. Although we did not examine any embryos aged less than E8.5, we found that after this age M-cadherin transcripts were expressed in a tissue-specific manner. Spatially the M-cadherin transcript was restricted to developing skeletal muscle and its temporal distribution is summarized in Table 1. The M-cadherin transcript was found in the skeletal muscle anlage, the myotomes, shortly after they formed. This early expression suggests that M-cadherin may be involved in the segregation of myotome cells from the remainder of the somite. M-cadherin was expressed specifically for several days in skeletal muscle but at later stages of embryogenesis we observed a down regulation of the transcript. N-cadherin expression in skeletal muscle was also down regulated below the threshold level of detection prior to birth. A similar pattern, where expression of a cadherin is restricted to embryogenesis, has also been described for B-cadherin (Napolitano et al., 1991).

M-cadherin was also found in myoblasts in the developing forelimb bud but these cells did not start to express M-cadherin co-ordinately with the myotomal cells. The M-cadherin transcript was first detected in the proximal forelimb at E11.5. This was precisely the time that cardiac α-actin and several other genes were first detected here (Sassoon et al., 1989). As suggested by Sassoon et al. (1989) it appears that the myogenic cells originating from the ventrolateral edge of the somite (Ede and Al-Gadi, 1986) do not express muscle-specific genes until they have entered the limb bud.

A role for M-cadherin during myogenesis is suggested

Table 1. Summary of expression of M-cadherin in mouse myotomes and skeletal muscles*

Embryo age in days	8.5 (3)	9.5 (4)	10.5 (3)	11.5 (2)	12.5 (4)	13.5 (1)	14.5 (1)	15.5 (2)	17.5 (2)
	+/−	+++	++	++	++	++	++	+	−

*The numbers in brackets refer to the number of embryos examined at each age.

from the transient re-expression we observed during skeletal muscle regeneration. We found that at early stages of the regenerative response the cells responsible for the regeneration of the myofibre, activated satellite cells, expressed M-cadherin. The hybridization of adjacent sections with the nicotinic acetylcholine receptor probe was quantitatively similar. As this probe appears to hybridize with all activated satellite cells, the coincidence of staining suggests that *all* activated cells express M-cadherin. Thus M-cadherin, like the myogenic regulatory factors MyoD1 and myogenin (Fuchtbauer and Westphal, 1992), is a marker of skeletal muscle regeneration. There are however differences in the temporal pattern of expression between these different muscle genes. As the satellite cells fuse to form myotubes they appear to lose M-cadherin whereas they retain MyoD1 and myogenin (Fuchtbauer and Westphal, 1992) and the nicotinic acetylcholine receptor. In view of the fact that there is a distinct temporal pattern of gene expression during muscle regeneration, (analogous to that described by Buckingham (1992) for myogenic differentiation during embryogenesis), we class M-cadherin as an 'early marker' of the regenerative response. In this sense M-cadherin is similar to other cell surface markers such as the 24.1D5 antigen. This is a marker for mononucleated myoblasts (Blau et al., 1985; Gower et al., 1989). M-cadherin is, however, different from the nicotinic acetylcholine receptor which is found in immature myotubes as well as myoblasts. The results suggest that M-cadherin has a dynamically regulated and unique pattern of expression in regenerating skeletal muscle.

We were unable to detect any re-expression of M-cadherin following muscle denervation. In this case M-cadherin differs from MyoD1 and myogenin. Both of these molecules are transiently re-expressed in denervated muscle, although whether they are found in denervated myofibres or transiently activated satellite cells is not known (Eftimie et al., 1991). Thus the transcriptional regulation of M-cadherin appears, at least in the adult, to be independent of electrical activity. Although many developmentally regulated gene products found in muscle such as the nicotinic acetylcholine receptor (Fontaine et al., 1988) and NCAM (Moore and Walsh, 1986) are re-expressed in denervated muscle, the control of M-cadherin expression is much more tightly regulated.

It is likely that the restriction of expression to skeletal muscle, which has not been described for any other cadherin to date, suggests that M-cadherin may have a function unique to developing skeletal muscle. Skeletal muscle development, whether in the embryo or during regeneration, is unique in one respect. It involves the fusion of mononucleate myoblasts with one another and results in the formation of multinucleate myotubes. The process is spontaneous and appears to result from the intrinsic morphogenetic capacity of myoblasts. It is well known that myoblast fusion occurs only between myoblasts and not between myoblasts and other cell types (Knudsen, 1991). Interspecies mixtures of myoblasts will fuse in vitro to form chimeric myotubes but myoblasts and non-muscle cells (of any species) will not fuse with one another. This phenomenon is an example of specific cell sorting and suggests the presence on the myoblast cell surface of unique components. It is possible that, based on its pattern of expression during development and re-expression during regeneration, the M-cadherin protein plays a role in events associated with myoblast fusion such as myoblast recognition and/or adhesion. Other surface components of myoblasts such as NCAM and N-cadherin may also play a role in myoblast recognition and/or adhesion (Knudsen et al., 1990a,b). However, these molecules are less tissue specific as they are also expressed by neural cells. Hence M-cadherin may be the, or one, of the cell surface molecules that control interactions prior to myoblast fusion.

M-cadherin is expressed in the myotome prior to MyoD1 (Sassoon et al., 1989) but with roughly the same temporal pattern of expression as myogenin. Thus in vivo the M-cadherin transcript is one of the earliest markers of skeletal muscle and is, to date, the most specific cell surface marker. Clearly the identification of transcription factors controlling the expression of M-cadherin will be of particular interest, specifically with regard to myogenin. Whether M-cadherin has a homophilic and/or heterophilic mechanism of binding is not known at present. It is likely that this and other features will be revealed by gene transfer experiments similar to those that have defined a role for NCAM during myoblast fusion (Dickson et al., 1990).

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