Patterns of N-CAM expression during myogenesis in *Xenopus laevis*

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Summary

The neural cell adhesion molecule (N-CAM) is seen in the membrane of nerves and muscles from several vertebrate species. Using indirect immunofluorescence, we have examined the expression of this protein during embryonic and postembryonic myogenesis in the African clawed frog, *Xenopus laevis*. While good staining for N-CAM was seen in neuronal tissues at all stages examined, no staining of embryonic muscle was observed, including both mononucleated and polynucleated myoblasts. In contrast, limb muscles formed at metamorphosis showed strong expression of N-CAM. The developing limb muscles eventually lose their N-CAM, but will reexpress it dramatically when denervated. These observations suggest that myogenesis programs executed at different stages of development can display distinct patterns of N-CAM expression.

Key words: N-CAM, myogenesis, *Xenopus*, expression.

Introduction

The neural cell adhesion molecule (N-CAM) is thought to play a critical role in neurogenesis and myogenesis in all vertebrate species examined to date (Edelman, 1984; Rutishauser, 1984). In chicken embryos, N-CAM is expressed over a significant proportion of the surface of the gastrula embryo, although as development progresses, N-CAM becomes restricted to both developing muscles and nerves (Edelman *et al.* 1983; Edelman, 1986). In mouse embryos, N-CAM can be found on developing muscles and nerves (Rieger *et al.* 1985). The expression of this molecule on differentiating muscle cells is thought to be a requisite component of synaptogenesis (Grumet *et al.* 1982; Rutishauser *et al.* 1983; Covault & Sanes, 1985).

In the amphibian, *Xenopus laevis*, N-CAM expression has been shown to be unusually restricted to neural cells during embryogenesis. Immunofluorescence staining of frog embryos with rabbit polyclonal antibodies directed against *Xenopus* N-CAM has shown that the protein is not expressed until late gastrula stage (Balak *et al.* 1987). During development of the early embryo, N-CAM is detectable only on developing neural cells, with a transient expression on somitic cells. Due to its exquisite neural specificity, expression of the N-CAM protein is an early marker for the process of neural induction (Jacobson & Rutishauser, 1986). Recently, several cDNAs for *Xenopus* N-CAM have been identified and used in evaluating the levels of N-CAM mRNA during development (Kintner & Melton, 1987). From RNA blots and *in situ* hybridization experiments, it appears that N-CAM mRNA accumulates in neural tissue but not in developing somites. Thus from published reports it initially appeared that *Xenopus* development differs from that of birds and mammals in not expressing N-CAM in myotomal muscles.

In this paper, we follow the expression of N-CAM during both embryonic and postembryonic myogenesis in *Xenopus*. We find that no embryonically derived muscle cell expressed N-CAM. In contrast, limb muscles formed at metamorphosis displayed strong N-CAM expression.

Materials and methods

Embryos and antibodies

Tadpoles at various stages of development were fixed, embedded in paraffin and sectioned as described elsewhere (Kay *et al.* 1987). Embryos were staged according to Nieuwkoop & Faber (1967). Sections (10 μm) were stained with either the 4D mouse monoclonal antibody (Frelinger

& Rutishauser, 1986; Watanabe *et al.* 1986) or a rabbit polyclonal antibody (Balak *et al.* 1987) directed against frog N-CAM and then visualized with a rhodamine-labelled, goat secondary antibody (Jackson Laboratories, Avondale, PA). Whole-mount preparations were fixed, permeabilized, stained and examined according to Schwartz & Kay (1988). For some experiments, rhodamine-labelled phalloidin was used to stain all musculature (Molecular Probes, Inc., Eugene, OR).

Immunoblots

Various tissues were homogenized with a Tissuemizer (Tekmar, Cincinnati, OH) in PBS with 0·5 % NP40, 1 mM-EDTA and 100×10^3 i.u. ml^{-1} aprotinin. The extracts were then boiled in the presence of loading buffer and resolved by electrophoresis in a 7% SDS–polyacrylamide gel (Laemmli, 1970). The proteins were then transferred electrophoretically to nitrocellulose filters, and the N-CAM detected with a polyclonal rabbit antibody and ^{125}I-labelled protein A (Towbin *et al.* 1979).

Preparation of in vitro cultures

Myotomal muscle cells, which develop into tail musculature

in vivo, were isolated from stage-20 to -22 embryos and cultured according to published protocols (Peng & Nakajima, 1978). Dissociated muscle cells were also prepared from the tails of stage-50 tadpoles by anaesthetizing the animals in tricaine methylsulphonate (MS222), excising the tails, removing the skin and treating the tissue with 3 mg ml^{-1} collagenase in frog Ringer's solution for 2–3 h. Single muscle cells were then isolated by gentle trituration with a Pasteur pipette.

Myoblast cultures of developing limbs of *Xenopus* larvae were prepared as follows. Limbs of anaesthetized stage-55 tadpoles were excised in frog Ringer's solution. After the skin and bone were removed, the limbs were teased into small pieces and incubated in Ca^{2+}-, Mg^{2+}-free Ringer's solution containing 0·4 mM-EDTA, 2·5 mg ml^{-1} trypsin and 2 mg ml^{-1} collagenase for 1 h at room temperature. Then the tissue pieces were triturated and the cells dissociated from the treated tissue were recovered by gentle centrifugation in a table-top centrifuge. The cells were then plated onto coverglass squares and cultured in 50 L-15 (Leibovitz) medium (GIBCO, Grand Island, NY), containing 5 % fetal bovine serum and 10 mM-Hepes (pH 7·4) at 22 °C.
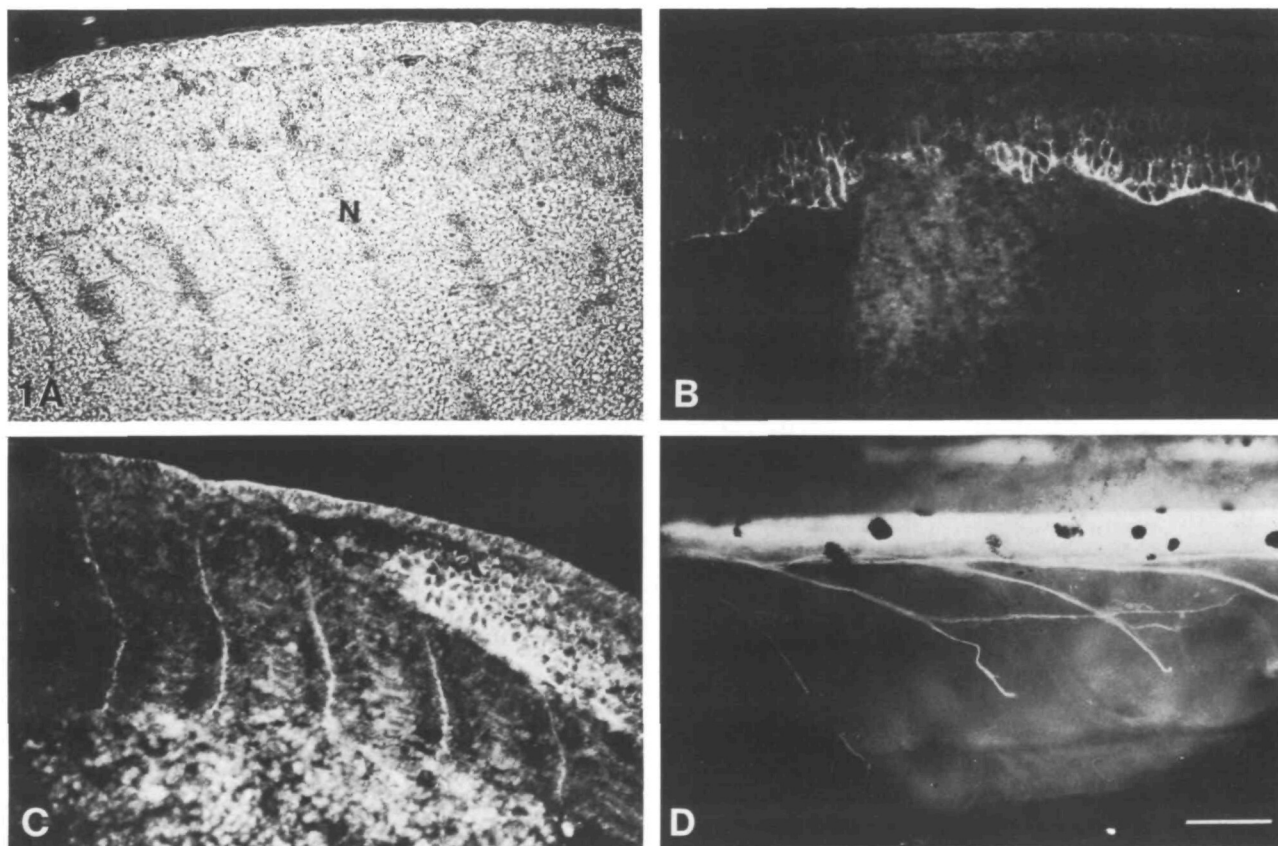
Fig. 1. During the formation of the tail, N-CAM is expressed solely on neural tissues in *Xenopus* embryos. A shows a phase-contrast image of a longitudinal cross section of a stage-22 embryo. B is the corresponding immunofluorescence image obtained with rabbit N-CAM antibodies and goat anti-rabbit Ig antibodies linked to FITC. The cell membranes of the neural tube are labelled, whereas the mononucleated muscle cells arranged in somites are not. C shows an N-CAM immunofluorescence staining of a stage-25 embryo. The level of background fluorescence is high in B and C due to the autofluorescence of yolk. D shows N-CAM immunofluorescence staining of a whole-mount strip of a stage-42 tadpole tail. The pigmented cells of the neuroepithelium do not stain, nor do the myotomal muscles separating each set of axons. N, row of nuclei. Bar, 120 μm in A–C and 100 μm in D.

After a culture period of 2–3 days, myotubes with 2–20 nuclei could be detected in these cultures. In some instances, the nuclei were stained in fixed preparations with 4',6-diamidino-2-phenyl-indole (DAPI; Sigma Chemical Co., St Louis, MO).

Explants of neural tubes were cultured as previously described (Peng *et al.* 1987). In short, neural tubes were removed from the excised neural tube, somite and the notochord complex of stage-22 to -25 embryos following a brief collagenase treatment. The tubes were then cut into pieces 0·55mm in length and cultured onto polylysine-coated coverglasses in Steinberg's solution (Hamburger, 1960) supplemented with 10% L-15 medium, 1% fetal bovine serum, 1% garamycin (Schering Corp., Kenilworth, NJ), and $4 \mu g \, ml^{-1}$ laminin (Bethesda Research Laboratories, Gaithersburg, MD).

Cultures were labelled with N-CAM antibody followed by fluorescein-conjugated goat anti-rabbit antibody either in the living state or after fixation. Cells were fixed either with Luther's fixative (0·25 mM-paraformaldehyde, 80 mM-cyclohexylamine, 10 mM-EGTA, 10 mM-Pipes, 10 mM-$MgCl_2$, pH 6·5; P. W. Luther, in preparation) or Bouin's fixative (Humason, 1979). Consistent results were obtained with both of these methods. The acetylcholine receptors on muscle cells were examined by labelling living cells with tetramethylrhodamine-conjugated alpha-bungarotoxin (R-BTX; Molecular Probes, Inc.).

## Results

Expression of N-CAM in developing tadpoles

Using the polyclonal antibodies to frog N-CAM, we observed strong staining of N-CAM in neuronal tissue, but none on the somites of neurula-staged *Xenopus* embryos. These observations are in good agreement with recently published observations (Balak *et al.* 1987; Kintner & Melton, 1987). Fig. 1A shows a phase-contrast micrograph of a sagittal section through a stage-22 larva, where the rows of nuclei (N) define the centre of each myotomal segment. When this same section was stained for N-CAM and examined by epifluorescence (Fig. 1B), only the cell membranes in the neural tube were stained. At stage 25, 3·5h later, coincident with the innervation of the muscles (Blackshaw & Warner, 1976), the neural tube and outgrowing neuronal processes were stained (Fig. 1C). At this level of resolution, we cannot determine if there is any postsynaptic expression of N-CAM, but this question is addressed with the *in vitro* experiments described below. The specificity of staining is revealed in Fig. 1D, where a whole mount from a stage-41 animal is examined. Here the central nervous system and the segmental nerves stain prominently, but the myotomal muscles are immunonegative.

Most vertebrate muscles are formed from myoblasts that fuse together to generate multinucleated myotubes and, subsequently, muscle fibres. In con-

trast, the myotomes of *Xenopus* are produced from large mononucleated myocytes which become fully functional both in receiving innervation and in contractility in the absence of cell fusion (Hamilton, 1969). They do undergo fusion with satellite cells and enlarge later in development (Muntz, 1975). These cells degenerate ultimately during metamorphosis when the tail is reabsorbed. We sought to determine whether the lack of N-CAM staining is due to these unique developmental features of the myotomal muscles. To answer this question, another muscle in the *Xenopus* larva, the mylohyoid (intermandibular) muscle was examined. Embryonically, it is formed by the fusion of myoblasts (Deuchar, 1975). This muscle is a flattened sheet of multinucleated fibres, which resides at the base of the throat and survives through metamorphosis (Deuchar, 1975). In whole mounts of tadpole heads, this muscle was easily discernible by staining with rhodamine-labelled phalloidin
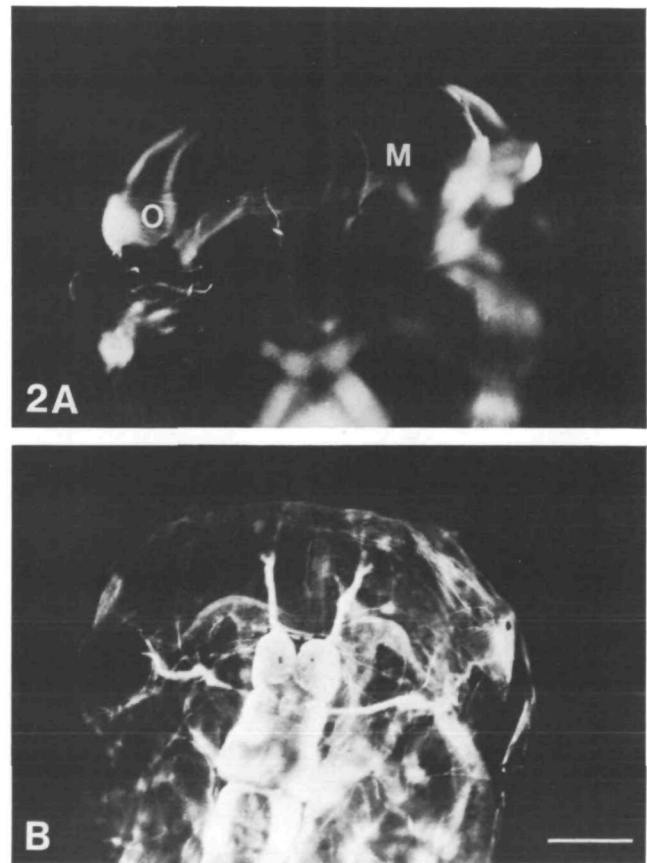
Fig. 2. N-CAM expression is neural-specific in tadpole heads. A is the fluorescent image of a stage-41 tadpole stained with rhodamine-labelled phalloidin. In this ventral view, the mylohyoid muscle (M) and ocular muscle (O) groups stain strongly. B displays the N-CAM antibody immunofluorescence staining of a comparably staged tadpole whole-mount preparation. The ventral side of the head is shown, with bright staining of the brain, and the optic and olfactory nerves. Bar, 500 μm.
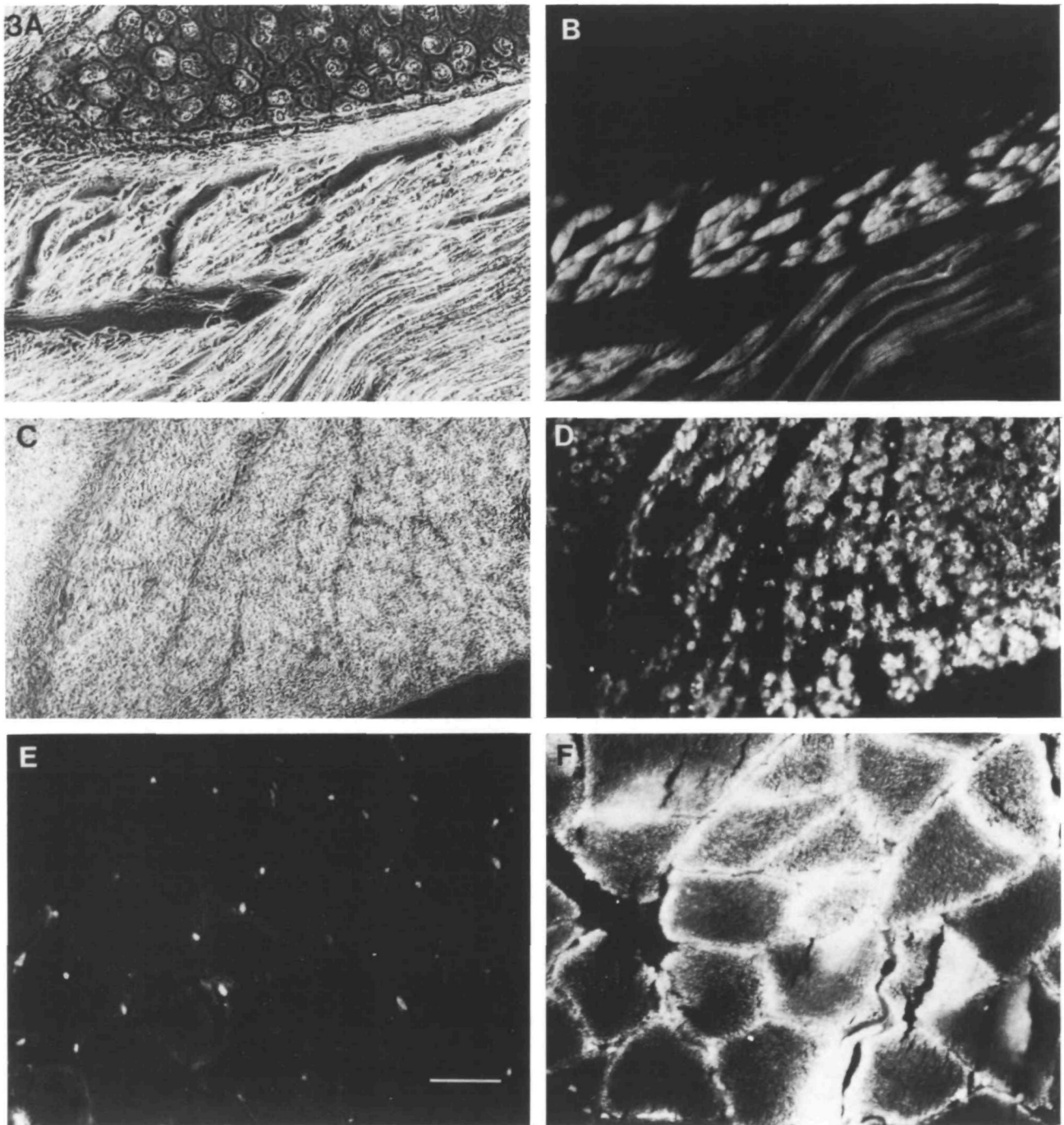
Fig. 3. N-CAM is expressed on the hindlimb muscles of tadpoles and on adult leg muscles after denervation. A shows a cross section of a hindlimb of a stage-55 tadpole in phase contrast, and B shows the immunofluorescence staining of the same section obtained with the monoclonal antibody 4D. Note the strong staining of the muscle fibres with N-CAM antibodies. C is the phase-contrast image of a hindlimb muscle of a comparably staged tadpole, and D is the staining pattern obtained with polyclonal anti-frog N-CAM antibodies. E–F display the N-CAM immunofluorescence staining of gastrocnemius muscles from the same adult frog with polyclonal anti-frog N-CAM antibodies; E is from the intact limb and F is the contralateral leg 4 days after denervation. The bright spots in E are autofluorescing red blood cells.
Bar, 135 μm.

(Fig. 2A). However, this muscle was immunonegative when stained for N-CAM (Fig. 2B). Mylohyoid muscles were examined at several stages of larval development, and only the nerve running over its surface and the neuromuscular junctions were ever stained (data not shown). Thus, the lack of N-CAM expression in the myotomal muscle is shared by a very different embryonically derived muscle.

One attractive feature of amphibian development is that the animals undergo a second phase of *de novo* muscle differentiation at metamorphosis with the formation of the limbs. The section shown in Fig. 3A and B shows muscle and bone from the thigh of a stage-55 tadpole. For this experiment, the 4D mouse monoclonal antibody (IgG), which recognizes a cytoplasmic determinant of the chicken $180 \times 10^3 M_r$ N-CAM species (Frelinger & Rutishauser, 1986; Watanabe *et al.* 1986), was used to stain the musculature. In cross sections, the staining with the monoclonal antibody was restricted to the periphery of the muscle fibres, suggesting that the antigen may be membrane bound. Polyclonal antibodies to frog N-CAM likewise stained many muscle fibres (Fig. 3C,D). Interestingly, not all muscle fibres stained equally well, suggesting either a spatial or temporal pattern of regulated N-CAM expression by muscle cells (Fig. 3D). Developing muscles in the forelimbs of tadpoles revealed a comparable pattern of N-CAM expression (data not shown).

After metamorphosis to the adult, the amount of N-CAM staining in the limb musculature was greatly reduced (Fig. 3E). To determine if the expression of N-CAM was under neuronal influence, as has been described for mouse and rat (Covault & Sanes, 1986; Sanes, 1986), we severed the sciatic nerve of an adult frog, thus denervating the gastrocnemius muscle. We examined by immunofluorescence the denervated muscle after 4 days (Fig. 3F) and compared it to the contralateral, unmanipulated gastrocnemius muscle from the same individual (Fig. 3E). As can be readily observed, the degree of N-CAM immunoreactive material increased dramatically after denervation.

Molecular characterization of the N-CAM antigens

As stated in the Materials and methods, most of the indirect immunofluorescence data shown in this study were generated with a rabbit polyclonal antibody generated against frog N-CAM (Balak *et al.* 1987). To validate that the antigens recognized in these photographs reflect the distribution of authentic N-CAM, a representative Western blot is shown in Fig. 4. In lane A, a chicken embryo brain extract was used as a control to reveal the previously described N-CAM polypeptide species of 180, 140 and $120 \times 10^3 M_r$, respectively (Rutishauser, 1984; Edelman, 1986). Lane B displays the presence of the same three
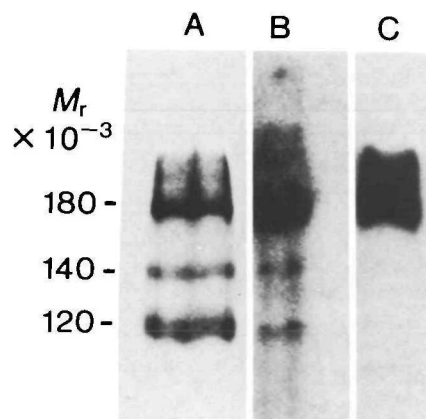

Fig. 4. Western blots of *Xenopus* embryonic tissues with N-CAM antibodies. Various samples were homogenized in extraction buffer, electrophoresed in a 7 % SDS–polyacrylamide gel, blotted and reacted with the rabbit anti-frog N-CAM antibodies. Lane A contains chick embryonic brain (E15), B developing limbs of a stage-55 *Xenopus* tadpole, and C brain of a stage-55 tadpole. Sizes of the major reactive polypeptide chains are $M_r \times 10^{-3}$.

N-CAM species in an extract of *Xenopus* developing leg muscle. The material examined in lane C is from tadpole brain and shows that only the 180×10^3 species appears to be expressed by this tissue. This observation is in good agreement with Sunshine *et al.* (1987). No other bands above background were evident on Western blots. These data suggest that our polyclonal antibody recognized authentic N-CAM in sectioned material.

Myotomal cells in culture

The results described above show that the myotomes of *Xenopus* do not express N-CAM except at regions of innervation. To determine whether or not this was a presynaptic or postsynaptic labelling, we examined the appearance of N-CAM on primary cultures of myotomal muscle cells. These cells do not undergo fusion in tissue culture and remain mononucleated. As shown in Fig. 5A and B, these muscle cells did not demonstrate detectable levels of N-CAM on their surfaces. N-CAM was likewise absent from the spontaneously formed, acetylcholine receptor clusters ('hot spots') as shown by R-BTX labelling (Fig. 5C,D). This is in sharp contrast to the bright staining of neurones and neuronal processes in explant cultures of *Xenopus* spinal cord (Fig. 5E).

To further assess the localization of N-CAM at the neuromuscular junction, we examined myotomal cells dissociated from the tail musculature of stage-50 tadpoles. The muscle cells at this stage are multinucleated, as determined by DAPI fluorescence (not shown). At the ends of these isolated cells are patches of acetylcholine receptors, which mark the sites of the postsynaptic membrane (Fig. 5F). Double staining
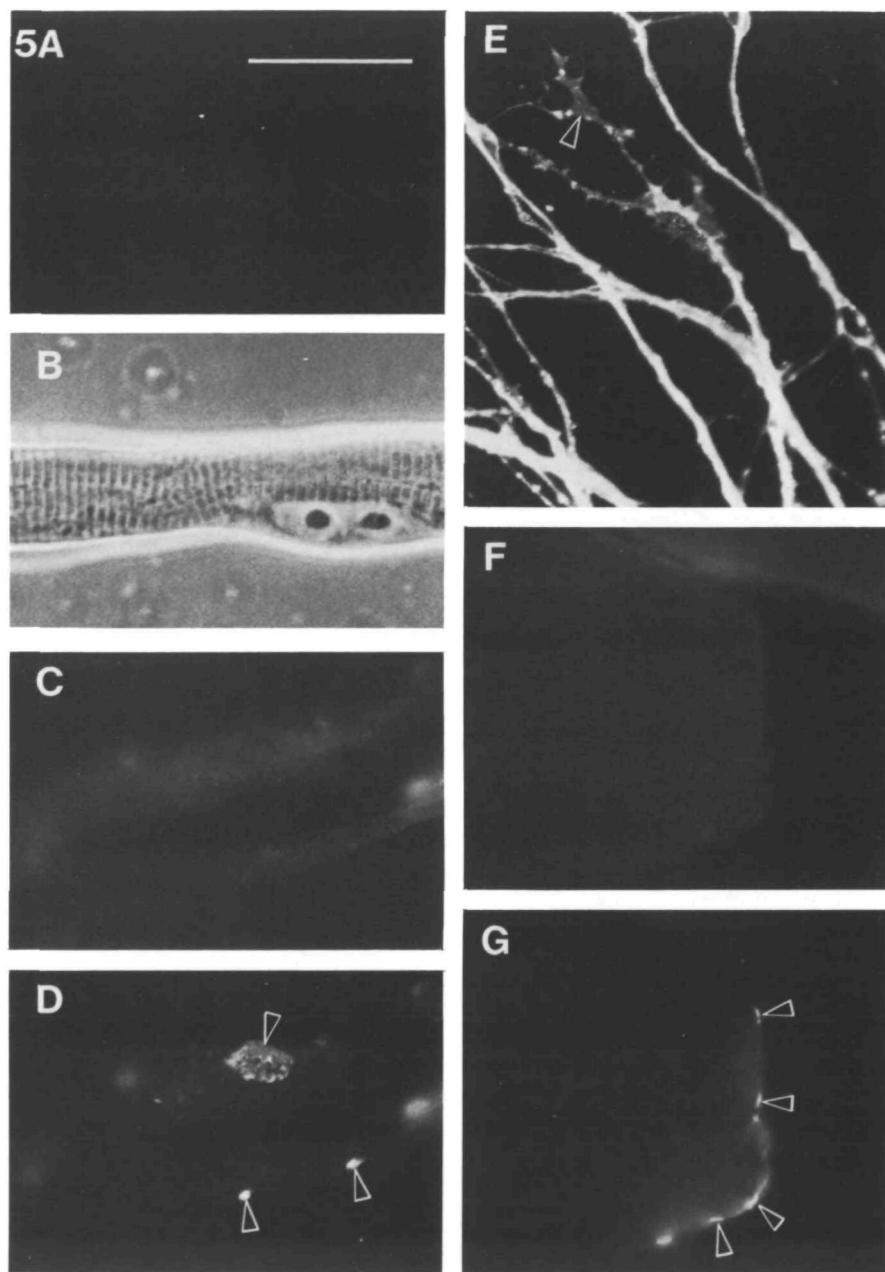
Fig. 5. Lack of N-CAM expression in cultured *Xenopus* myotomal muscle cells. Tail muscle cells from stage-22 embryos were cultured and reacted in the living state with N-CAM antibodies and FITC-conjugated secondary antibodies (A), or examined by phase-contrast microscopy (B). Cells were also double stained with N-CAM antibodies (C) and R-BTX (D); the spontaneously formed acetylcholine receptor clusters (marked with arrowheads in D) do not react with N-CAM antibodies. Neurites and growth cones (arrowhead) from neural tube explants, on the other hand, react quite well with N-CAM antibodies (E). Muscle cells from stage-50 tadpoles were also examined for N-CAM expression after dissociation from the tail musculature. After dissociation, these cells were immediately fixed and processed for binding of N-CAM antibodies (F) or R-BTX (G). Note the lack of N-CAM at the postsynaptic acetylcholine receptor clusters, marked by arrowheads in G, as well as along the extrajunctional sarcolemma. Bar, 30 μm.

with N-CAM antibody and R-BTX show that both the postsynaptic and the extrasynaptic membranes were devoid of N-CAM expression (Fig. 5G).

Myoblasts and myotubes of skeletal muscle in culture

We also examined skeletal muscle cells from the developing limbs of tadpoles after culturing *in vitro*. The myoblasts were identified by their long, spindle-shaped outline and uniform R-BTX staining (Fig. 6B,C). When the cells were reacted with N-CAM antibodies, the myoblasts always stained positively (Fig. 6A). In the cultures, myoblasts often fused into myotubes, as seen by DAPI staining of the nuclei (Fig. 6F). These myotubes also revealed strong staining with both N-CAM antibodies and R-BTX

(Fig. 6D,E), although non-muscle cells did not stain with either reagent (Fig. 6A–F). Control cultures stained with preimmune serum always had greatly reduced fluorescein fluorescence levels when compared to N-CAM-stained preparations (Fig. 6G–I).

Discussion

From immunofluorescence staining of both *in vivo* and *in vitro* preparations, we have shown that the expression of N-CAM in embryonically and postembryonically derived muscle cells is quite different in *Xenopus*. The pattern of N-CAM expression in the
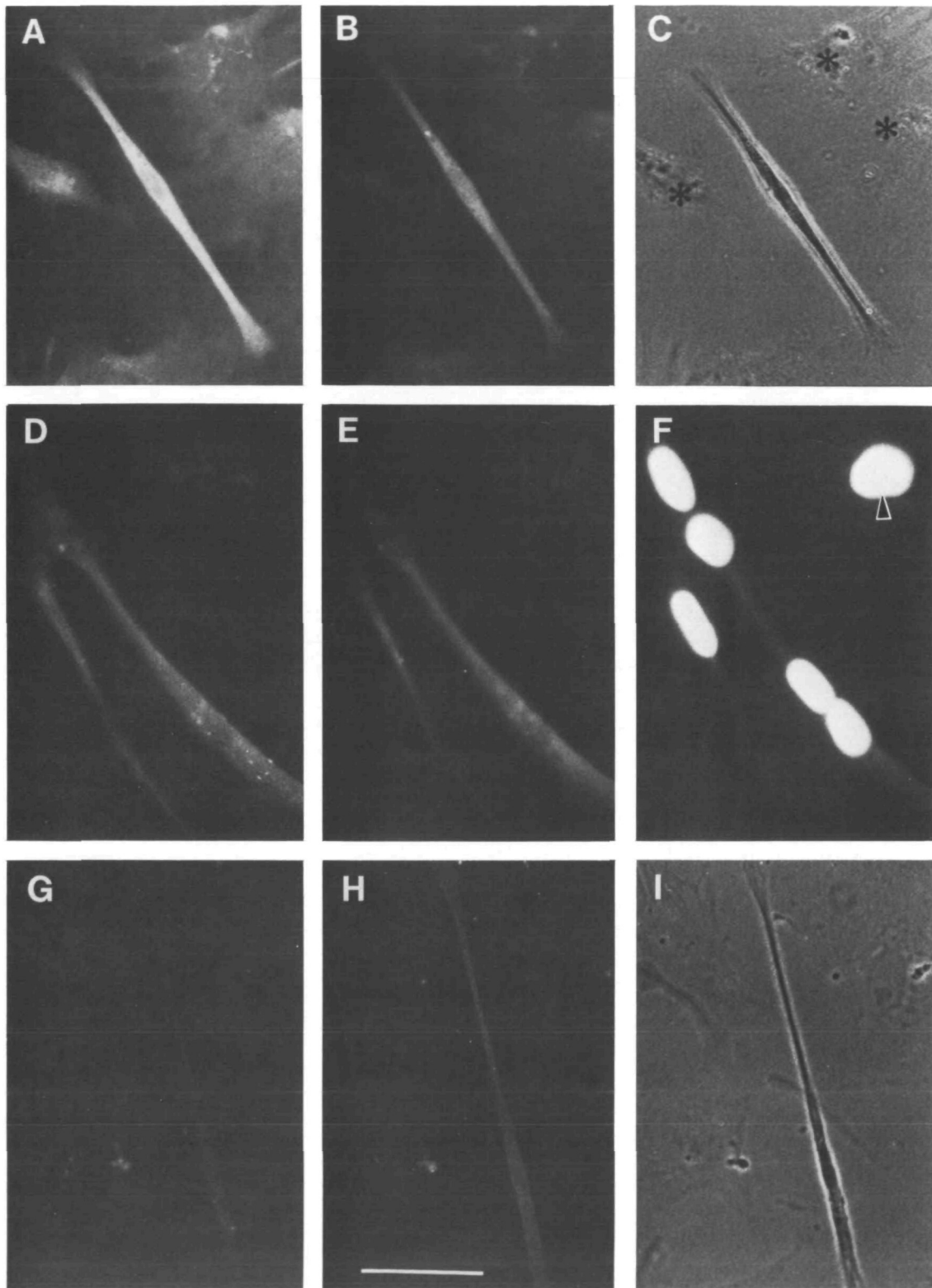
Fig. 6. N-CAM expression on cultured muscle cells from developing limb musculature of *Xenopus*. Left column: immunofluorescence staining for N-CAM; middle column: R-BTX staining to show the presence of acetylcholine receptors: right column: phase-contrast image (C,I) or DAPI staining (F). A–C shows a mononucleated myoblast and several fibroblasts (asterisks). D–F shows a developing myotube with four nuclei, a myoblast and a fibroblast (arrowhead). G–I shows a myoblast stained with preimmune serum (G) or R-BTX (H), and the corresponding phase-contrast image (I). Bar, 40 μm.

postembryonically derived skeletal muscle is consistent with the previously published results for mammalian and avian systems (Thiery et al. 1982; Tosney et al. 1986; Covault & Sanes, 1985; Duband et al. 1987). Moreover, the presence of both N-CAM-expressing and -nonexpressing muscle cells in the developing hindlimb is similar to the pattern seen in musculature for developing chick hindlimbs (Tosney et al. 1986).

The general lack of N-CAM staining of embryonic muscles in Xenopus is remarkable. It cannot be due to the fact that the myotomal cells are mononucleated (Hamilton, 1969; Muntz, 1975) or that these cells degenerate during metamorphosis, since the mylohyoid muscle, which is composed of multinucleated cells and survives to adulthood, is immunonegative as well. The absence of the N-CAM molecule on larval muscles indicates that N-CAM is not likely to have a prominent function in their formation or innervation. Moreover, we cannot detect N-CAM at the postsynaptic membrane of Xenopus embryonic muscles where acetylcholine receptors are clustered, in contrast to the muscles of other animal systems which have N-CAM at their postsynaptic surfaces (Covault & Sanes, 1985; Rieger et al. 1985). Nevertheless, we cannot rule out a minor role for N-CAM on Xenopus myotomal muscles, as a minor, transient expression of N-CAM has been reported on developing Xenopus muscle cells (Balak et al. 1987).

Several lines of evidence suggest that the patterns of immunofluorescent staining described in Results reflect the true distribution of N-CAM. First, neuronal tissue stained in all histological preparations, independent of developmental stage. Therefore, the lack of staining by embryonic muscles is not an artifact due to tissue preparation. Second, the polyclonal and monoclonal antibodies against N-CAM reacted with developing limb muscle. Given that the polyclonal antibody recognized the well-characterized 180, 140 and $120 \times 10^3 M_r$ N-CAM species on Western blots, we feel that the antigen recognized in our immunofluorescence experiments is authentic N-CAM.

The significant expression of N-CAM in the developing limb muscles suggests that this molecule may mediate the nerve–muscle interaction in postembryonically derived muscles of Xenopus. In adults, these muscles have very little N-CAM and the limb bud muscles appear to have diminished expression shortly after their innervation (Jacobson, 1987). Recently, it has been shown that rodent adult muscles can reexpress N-CAM to high, embryo-like levels after denervation or paralysis (Covault & Sanes, 1986; Sanes et al. 1986). Presumably, this reexpression is important in reestablishing proper nerve–muscle interactions. The adult frog gastrocnemius muscle also appears

capable of such a response after denervation. This is in contrast to the tadpole myotomal muscles which do not express N-CAM, even when grown alone in tissue culture or after tubocurare-induced paralysis (Schwartz & Kay, data not shown).

Recently, Bixby et al. (1987) have shown that axonal growth on muscle surfaces depends on the presence of cell adhesion molecules (CAMs), such as N-CAM and neural Ca^{2+}-dependent CAM, as well as molecules in the extracellular matrix. Suppression of any one molecule with antibodies had only a small effect on neurite outgrowth, but a suppression of all these molecules inhibits growth. Thus, N-CAM is but one of a class of molecules used by the developing nerve and muscle in their mutual interaction. Our study suggests that during embryonic development in Xenopus, this interaction is dominated by molecules other than N-CAM. This differential expression of N-CAM during the formation of Xenopus muscles makes the animal particularly suitable for examining the comparative role of this molecule in myogenesis and synaptogenesis.

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