Regulation and Activity-dependence of N-Cadherin, NCAM Isoforms, and Polysialic Acid on Chick Myotubes during Development

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Abstract. Muscle development in vivo involves a complex sequence of cell–cell interactions in which secondary myotubes first form in association with primary myotubes and subsequently separate from them. We show here that during this process N-cadherin and the different structural forms of NCAM are regulated in a pattern that involves both temporal changes in expression and localization to particular regions of the muscle cell surface. In particular, levels of N-cadherin on maturing myotubes are decreased, and the form of NCAM synthesized by the muscle changes from a transmembrane non-polysialylated to a lipid-linked polysialylated membrane protein. Moreover, while NCAM was distributed on all myotube surfaces, the polysialylated form of NCAM was restricted to regions of the myotube surface that had recently separated from neighboring cells. We previously found that blockade of nerve-induced activity by d-Tubocurarine perturbed muscle cell interactions, resulting in a failure of myotubes to separate. We now show that this activity blockade also alters adhesion molecule expression. First, N-cadherin was no longer down-regulated in maturing myotubes, and its persistence on the surfaces of mature myotubes may partly explain their failure to separate. Secondly, the developmental switch from transmembrane to lipid-linked NCAM did not occur, and polysialylated NCAM was no longer formed. As the unusual physical properties of PSA have been proposed to impede cell–cell interactions, this alteration would also be expected to compromise cell separation. Together, these results suggest that the regulated expression of both N-cadherin and NCAM isoforms including their polysialylation, is an essential mechanism for the normal separation of secondary myotubes from primary myotubes.

THE formation of skeletal muscles occurs in a series of highly orchestrated cellular interactions involving several populations of cells. After migrating into the limb bud, myogenic cells condense into dorsal and ventral muscle masses which then cleave to form individual muscles (Christ et al., 1977; Chevallier et al., 1977). Within the masses, muscle precursor cells divide, differentiate, and fuse to form multinucleated myotubes. Although the myogenic precursors that give rise to all of the adult myofibers are present in the limb bud at the onset of muscle formation, they differentiate asynchronously giving rise to two populations of myotubes that are classified as primary or secondary myotubes, according to the time at which they form (Ashmore et al., 1972; Kelly and Zacks, 1969; Ross et al., 1987). A third population of myogenic cells known as satellite cells persists in adult muscle (Albrook et al., 1971; Ontell, 1977; Shultz, 1974).

Anatomical studies have provided detailed descriptions of cellular interactions during in vivo muscle formation of mammalian and avian species (Kelly and Zacks, 1969; Ashmore et al., 1972; Ontell, 1977; Ontell, 1977; Ontell and Kozeka, 1984; Ross et al., 1987; Duxson et al., 1989). From these studies, we know that until late in development of both mammalian and avian species (until embryonic day 13 [St 39] for chick, and during postnatal development for rats), myotubes are organized into clusters that also contain the precursors for subsequently formed myotubes. The cells within clusters share close membrane appositions, and are enclosed within a common basal lamina which surrounds the cluster, but which does not penetrate between individual cells of the cluster. The surfaces of myotubes within these clusters serve as substrates on which myoblasts fuse to form other myotubes, and thus, all secondary myotubes form on the surfaces of previously formed myotubes. As myotubes mature, they separate from the cluster and become ensheathed in their own basal lamina. Eventually, the entire

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muscle becomes populated by individual myofibers which have undifferentiated satellite cells adhering to them.

Clearly cell–cell interactions play an important role in these aspects of muscle development. Accordingly, a number of cell adhesion molecules (CAMs)[1] that may mediate cell–cell interactions during myogenesis are expressed in developing muscle. These include NCAM (Covault and Sanes, 1986; Covault et al., 1986; Tosney et al., 1986), N-cadherin (Hahn and Covault, 1992), M-cadherin (Donalies et al., 1991), and integrin receptors (Bixby et al., 1987; Rosen et al., 1992; Bozyczko et al., 1989; Menko and Boetinger, 1987) and the expression of some of these CAMs has been related to particular phases of myogenesis (Tosney et al., 1986; Covault and Sanes, 1986; Covault et al., 1986). For example, a switch from the expression of the transmembrane isoform of NCAM to the low molecular weight, lipid-linked isoform occurs during secondary myogenesis (Covault et al., 1986). However, that study did not determine if the expression of the lipid-linked form was restricted to secondary myotubes and myoblasts. In fact, few studies have described differential localization of CAMs to particular muscle cell surfaces. Soler and Knudsen (1991) found that NCAM and N-cadherin were co-localized on myoblasts in culture, but did not describe their distribution on myotubes. Another study observed that, while both NCAM and N-cadherin were found on myoblasts, only NCAM persisted on myotubes after fusion (Mege et al., 1992). Recently, Rosen and co-workers (Rosen et al., 1992) found that the integrin VLA-4 was expressed on both primary and secondary myotubes, while VCAM-1, which binds to VLA-4, was restricted to secondary myoblasts and to the surfaces of myotubes apposed to these myoblasts. They also found that antibodies against either of these molecules were capable of blocking myotube formation in vitro.

Neuromuscular transmission is another form of cell–cell interaction that has been shown to influence myotube development. In a previous study (Fredette and Landmesser, 1991*b*), we observed that blocking neuromuscular transmission with d-tubocurarine during primary and secondary myogenesis resulted in striking perturbations in myotube organization. Although both primary and secondary myotubes formed, the clusters containing these cells were greatly enlarged when compared to those in control muscles. While some hypertrophy of the myotubes occurred, the increased cluster size was mostly due to an increase in the number of myotubes in each cluster. These observations suggest that following activity blockade there is an increased adhesiveness among muscle cells, resulting in a failure of myotubes to separate from their clusters. NCAM and N-cadherin have been shown to participate in myoblast aggregation, which is a prerequisite for fusion (Knudsen and Horowitz 1977; Knudsen et al., 1990*a*,*b*). Furthermore, since the expression of both NCAM and N-cadherin is down regulated in chicken muscle before hatching, but is rapidly up-regulated after denervation (Covault and Sanes, 1985, 1986; Hahn and Covault, 1992), these CAMs are good candidates to participate in the perturbations we observed following activity blockade.

1. *Abbreviations used in this paper*: CAM, cell adhesion molecule; MSD, muscle-specific domain; PI-PLC, phosphatidylinositol-specific phospholipase C; PSA, polysialic acid.

Most investigations of the mechanisms underlying muscle formation have focused on the initial events such as cell–cell recognition and membrane fusion, and have been performed in vitro with precursors of secondary myotubes or with satellite cells (Knudsen et al., 1989, 1990*a*,*b*; Entwistle et al., 1988*a*,*b*; Menko and Boetinger, 1987; Mege et al., 1992). While these in vitro studies provide important insights into these events, they do not address the cell–cell interactions occurring among the heterogeneous populations of cells that exist in vivo. Such interactions include not only the fusion of myoblasts on myotube surface substrates, but also the subsequent separation of myotubes from clusters.

We present here an in-depth study of how N-cadherin and the different structural forms of NCAM are expressed and distributed on muscle surfaces as they form and separate from clusters during primary and secondary myogenesis. A complex expression pattern was found for these two CAMs, that involved both temporal changes and localization to particular cell-surface compartments during the two phases of myogenesis. Furthermore, we provide evidence that selective glycosylation of muscle NCAM isoforms may be an important new parameter in myotube development. That is, myotubes display the polysialylated and nonpolysialylated forms on different regions of the cell surface in a pattern that, in view of the ability of polysialic acid (PSA) to regulate cell interactions, may be related to separation of myotubes from clusters. Consistent with this hypothesis we found that following activity blockade, the distribution of N-cadherin was altered and that expression of the polysialylated form of NCAM was virtually abolished. We suggest that these molecular changes may underlie the perturbations in muscle organization produced by activity blockade and therefore, that a decrease in N-cadherin expression at appositions between maturing myotubes, as well as an increase in polysialylated NCAM on muscle cell surfaces are necessary for myotube separation from clusters.

Materials and Methods

D-Tubocurarine Treatment of Embryos

Embryos (obtained from the University of Connecticut Poultry Department, Storrs, CT) were raised from fertilized white leghorn chicken eggs in a forced-draft incubator at 38°C. Developmental stages of the embryos were determined according to the criteria of Hamburger and Hamilton (1951). At St 27 (day 5 in ovo), square openings were made in the egg shells, and coverslips were sealed over the openings with paraffin to serve as replaceable windows for drug treatments. Beginning at St 27, one group of embryos received daily applications of d-tubocurarine (dTC) (Sigma Immunochemicals, St. Louis) (2 mg in sterile 0.9% NaCl) onto the chorioallantoic membrane, a procedure previously shown to be sufficient for blocking nerve-evoked muscle contractions (Pittman and Oppenheim, 1979; Dahm and Landmesser, 1988). A second group, consisting of untreated embryos and embryos receiving saline instead of d-tubocurarine, served as controls.

Immunofluorescence

Three antibodies to NCAM were used: 5E, a monoclonal IgG antibody that recognizes all molecular weight forms of NCAM (Frelinger and Rutishauser, 1986); 5A5, a monoclonal IgM which recognizes the polysialylated form of NCAM (Dodd et al., 1988; Acheson et al., 1991), and α735D4, a monoclonal IgG also against polysialic acid (Frosch et al., 1985; Finne et al., 1987). Two antibodies against N-cadherin were used and gave identical results: a mouse monoclonal antibody GC-4 (Volk et al., 1990) obtained

from Sigma Immunochemicals, and a rabbit polyclonal antibody kindly provided by M. Takeichi (Kyoto University, Japan).

Hindlimbs of the dTC-treated embryos were embedded and frozen along with stage-matched control hindlimbs in OCT (Miles Scientific, Elkhart, IN) either directly after dissection in Tyrode buffer, or after being fixed for 30 min in 3.7% formaldehyde in PBS (pH 8.0). Cryosections, 10-μm thick, were cut transverse to the hindlimb throughout the thigh, and dried onto gelatin-coated slides. The sections from the freshly frozen hindlimbs were fixed for 15 min before immunostaining. All sections were then processed for immunocytochemistry by first incubating for 2 h at room temperature with one of the following primary antibodies diluted in PBS and 2% bovine serum albumin: 5E; 5A5; α735D4; GC-4; rabbit polyclonal anti-N-cadherin. After rinsing, appropriate fluorophore-conjugated second antibodies in PBS and 2% BSA were applied for 1 h followed by rinsing in PBS. The slides were coverslipped with a glycerol and PBS mixture (1:2) containing *p*-phenylenediamine (0.3 mg/ml) to prevent fading. All comparisons in staining intensity were made on tissue treated in the same manner and stained with identical dilutions of antibody, and were repeated at least three times. The sections were viewed and photographed with Kodak Tri-X Pan film with a Nikon Microphot microscope equipped with epifluorescence.

Immuno-Electron Microscopy

Dorsal thigh muscles and attached femur and pelvic girdles were dissected from dTC-treated and control embryos at St 32, 35–36, and 38, rinsed quickly in calcium-free Tyrode buffer, and fixed while pinned in an extended position for 1 h with 4% formaldehyde and 0.2% glutaraldehyde in 0.13 M phosphate buffer, pH 7.4. The muscles were removed, rinsed in 0.13 M phosphate buffer and embedded in 30% albumin and 3% gelatin. The embedded muscle was Vibratome sectioned transverse to the limb at 50–100 μm, and the sections were collected in TBS, pH 7.6, and immunostained for NCAM, PSA, or N-cadherin as floating sections.

Sections were processed for NCAM or N-cadherin immunocytochemistry with the indirect enzyme method of Sternberger, as previously described (Fredette and Landmesser, 1991*a*). All of the immunoreagents were diluted in TBS. Briefly, the reaction steps were: (*a*) blocking for 1 h in 5% normal rabbit serum; (*b*) incubation for 48–72 h at 4°C with 1:100 mAb 5E or 1:50 mAb GC-4 in 1% normal rabbit serum; (*c*) incubation for 1 h in rabbit anti-mouse (1:50) with 1% normal rabbit serum; (*d*) incubation for 1 h in mouse clono-PAP (1:100) with 1% normal rabbit serum; (*e*) steps 3–4 were repeated to enhance the signal. Some sections were processed for N-cadherin immunocytochemistry with a rabbit polyclonal antibody according to the same protocol with the following exceptions: rat serum was used as the blocking serum, rabbit anti-N-cadherin was used as the primary antibody, goat anti-rabbit (Sigma Immunochemicals) was used as the second antibody and the PAP was raised in rabbit. Immunocytochemistry for PSA was performed by the direct enzyme method with rabbit serum as the blocking agent, 1:200 mAb 5A5 ascites as the primary antibody and goat anti-mouse IgG (Sigma Immunochemicals) as the second antibody. Immunostaining in all three groups of sections was made visible and electron dense by reacting the sections in 0.05% diaminobenzidine and 0.001% hydrogen peroxide for 10–15 min. The immunostained sections were osmicated, dehydrated and embedded in a TAAB/EPON resin mixture according to standard electron microscopy protocols, and thin sections were cut, collected on Formvar-coated slot grids, stained with 0.2% lead citrate, and viewed with a Philips 301 transmission electron microscope.

Enzymatic Removal of GPI-linked NCAM

St 38 thigh muscles were homogenized in 50 mM Hepes buffer, pH 7.4, with protease inhibitors (100 μg/ml leupeptin, 1 mM EDTA, 900 TIU aprotinin, 2 mM PMSF) and 15 mM NaCl, were pelleted and washed to remove soluble proteins, and then separated into three equal portions. One sample received 5 U/ml of phosphatidylinositol-specific phospholipase C (PI-PLC, from *Bacillus cereus*; Sigma Immunochemicals), and the other two samples served as controls containing either no enzyme or enzyme plus 5 mM $ZnCl_2$ which specifically blocks phosphatidylinositol hydrolysis by PLC (Sundler et al., 1978). The samples were incubated for 2 h at 37°C, the soluble (supernatant) and insoluble (pellet) fractions were separated by centrifugation, and the pellets were rinsed and resuspended in Hepes extraction buffer containing 1% NP-40 and protease inhibitors. The protein concentrations of the pellets and corresponding supernatants were measured by the Pierce BCA method (Pierce, Rockford, IL), and when necessary, both pellet and corresponding supernatant samples were equally diluted. The resulting samples were then analyzed by either ELISA or SDS-PAGE immunoblotting.

ELISA Analysis of PSA-NCAM

Polysialic acid on PI-PLC-solubilized and PI-PLC-resistant NCAM was analyzed in St 38 muscle by an ELISA sandwich assay in two separate experiments, each consisting of tissue pooled from four embryos. Anti-NCAM antibody was absorbed onto the wells of Pro-Bind plates by overnight incubation of 2 μg/ml of 5E antibody in 50 mM sodium bicarbonate. The wells were rinsed with PBS, and blocked with 1% BSA in PBS. Following PI-PLC treatments (above), pellet and corresponding supernatant samples were serially diluted to between 2 and 200 μg/ml. In parallel sets of wells, the samples were assayed for either NCAM with a rabbit anti-NCAM polyclonal antibody and HRP-conjugated goat anti-rabbit (Sigma Immunochemicals) as second antibody, or for PSA with 5A5 ascites and HRP-conjugated goat anti-mouse IgM (Sigma Immunochemicals) as second antibody. Both 5A5 and 5E were used at concentrations that ranged from 1:500 to 1:5,000, and both second antibodies were used at 1:10,000 dilutions. Wells lacking muscle extract, but processed with either 5A5 or rabbit anti-NCAM immunoreactions served as controls for non-specific staining. Immunostaining was detected by 0.04% *o*-phenylenediamine and 0.01% hydrogen peroxide in 0.1 M citrate buffer. The results from two separate experiments are presented in Table I where the values of NCAM are expressed in absorbance units. PSA absorbance values are expressed in relation to the amounts of NCAM measured in parallel wells (PSA/NCAM) and these values were used to calculate the percentage of polysialylated NCAM released from the muscle extracts of PLC treatment according to the following formula: $100 \times (PSA_{pellet}/NCAM_{pellet})/(PSA_{pellet}/NCAM_{pellet}) + (PSA_{supernatant}/NCAM_{supernatant}))$.

SDS-PAGE Immunoblotting

Thigh muscles of either control or dTC-treated embryos between St 28 and 39 were dissected and tissue from each stage was pooled until about 500 μL was obtained (usually three to five embryos at st 28–32, 3 at st 35, and 1–2 at st 38–40). The tissue was homogenized in Hepes extraction buffer containing 1% NP-40 and protease inhibitors (see above). The solubilized protein concentrations were determined (BCA method; Pierce) and adjusted to 1–2 μg/ml. Some of the extracts were divided into two sets, and one of these sets was adjusted to pH 5.5 and incubated for 1 h at 37°C with neuraminidase (0.08 U/ml, type X from Clostridium perfringens; Sigma Immunochemicals) to remove sialic acid while the other was incubated with buffer alone. In other cases, a third portion of the extract was incubated for 1 h at 37°C in 600 U/ml endoneuraminidase purified from phage infected bacteria according to previous procedures (Rutishauser et al., 1985) to remove polysialic acid. SDS sample buffer containing dithiothreitol was added to each sample and the proteins separated by SDS-PAGE according to the Laemmli method (Laemmli, 1970) on a 6% gel. The proteins were transferred onto Immobilon P membranes, which were immunostained for either NCAM with mAb5E or for PSA with mAb5A5 and appropriate alkaline phosphatase-conjugated second antibodies, which were visualized with the BCIP/NBT method. Pyruvate kinase, fructose 6-phosphate kinase, β-galactosidase, and α_2-macroglobulin stained with Bromphenol blue served as molecular weight standards. The different isoforms of NCAM are

Table I. Polysialic Acid on PI-linked NCAM

	Control		PI-PLC		PI-PLC + $ZnCl_2$	
	Pellet	Supernatant	Pellet	Supernatant	Pellet	Supernatant
N-CAM	93.1	6.9	68.5	31.5	100	0
	87.5	12.5	73.3	26.7		
PSA	97.2	2.8	61.7	38.3	100	0
	97.1	2.9	55.3	44.7		

The quantity of PSA on PI-PLC extractable NCAM was measured by ELISA. St 38 hindlimb muscle membranes incubated with PI-PLC were separated by centrifugation into solubilized proteins in the supernatants and insoluble proteins remaining in the pelleted membranes, homogenized with non-ionic detergent, and incubated in ELISA wells precoated with 5E antibody to NCAM. In parallel plates, the quantities of NCAM and of PSA associated with the 5E-bound NCAM were measured with rabbit anti-NCAM and 5A5 antibodies, respectively, and the appropriate HRP-conjugated second antibodies. After reaction of the HRP with *o*-phenylene diamine, absorbance units were expressed as a percentage of the total absorbance measured for each sample (pellet + supernatant). Control samples were provided by incubation in carrier alone (*Control*) or by inhibition of PI-PLC with $ZnCl_2$ ($ZnCl_2$ + PI-PLC).

indicated throughout the text, except where indicated, by their apparent molecular weights after desialylation with neuraminidase.

For the experiments presented in Fig. 13, non-enzyme-treated St 38 muscle detergent extracts were run on 6% polyacrylamide slab gels. The gels were cut into two portions at the 165-kD position, as determined by NCAM immunoblotting of flanking regions of the gel, and the proteins were electroeluted out of the two portions. The samples were concentrated about 100-fold, 0.1% bovine serum albumin was added, and SDS was removed by passing the samples over Extracti-gel D columns (Pierce). The protein concentrations were determined, split into two aliquots, one of which was desialylated with neuraminidase, and then both samples were processed for NCAM and PSA immunoblot analysis as described above. The experiment was performed twice on different tissue samples and both gave identical results.

Quantities of the NCAM isoforms relative to total NCAM within samples were obtained by scanning immunoblots with a Shimadzu scanner. The resulting spectral densities for each NCAM isoform were expressed as percentages of the total density measured within each gel lane. All measurements were made from gel lanes loaded with 5 μg total protein, and in order to ensure that the relative quantity of any of the isoforms was not underestimated due to saturation of the immunologic or enzymatic reactions used to detect NCAM, measurements were also made from lanes containing 2.5, 10, and 15 μg of protein obtained from St 38 muscle. In all of these cases, the relative proportions of the NCAM isoforms remained equivalent (within +/−2%).

Results

Immunocytochemical Comparison of Adhesion Molecule and Polysialic Acid Expression in Normal and Activity-blocked Muscle

N-cadherin and NCAM are known to be expressed in developing chicken muscle throughout primary and secondary myogenesis. Expression of both is down-regulated at late embryonic stages (Tosney et al., 1986; Covault and Sanes, 1986*a*,*b*; Hahn and Covault, 1992), and in both late development and adult muscle their expression can be altered by nerve activity. In addition, neuromuscular blockade late in embryonic development (St 39–42) results in an increase in N-cadherin mRNA (Hahn and Covault, 1992). However, these studies did not determine if activity plays any role during earlier developmental stages. Therefore we first evaluated the effects of nerve activity on CAM expression throughout primary and secondary myogenesis by comparing N-cadherin and NCAM immunocytochemistry in chicken hindlimb muscles that developed in the presence or absence of functional activation by nerve. Spontaneous motoneuron-evoked muscle contractions, normally beginning by stage 27, were blocked with daily in ovo injections of dTC (Dahm and Landmesser, 1988), and frozen cryosections of the activity-blocked and stage-matched control hindlimbs were immunofluorescently stained with antibodies that recognize N-cadherin, all forms of the NCAM polypeptide, or NCAM PSA. Since we had previously characterized primary and secondary myogenesis in the chicken iliofibularis muscle (Fredette and Landmesser, 1991*a*,*b*), this muscle was chosen as the focus for the present study.

While immunofluorescence microscopy allowed us to detect differences in the overall levels of expression of adhesion molecules, the resolution was inadequate to determine if expression was limited to specific cell types at certain developmental stages (myoblasts, primary myotubes, or secondary myotubes). These cells interact at precise developmental stages within clusters, and the interactions include myoblast proliferation and fusion, as well as separation of maturing myotubes from the clusters. Descriptions of these interactions during development have been provided at the ultrastructural level (Kelly and Zacks, 1969; Kikuchi and Ashmore, 1976; Ontell, 1977; Ross et al., 1982; Duxson, 1989), including those in the normal and chronically paralyzed iliofibularis muscle (Fredette and Landmesser, 1991*a*,*b*). Therefore, immunoelectron microscopy with antibodies to N-cadherin, NCAM, and PSA was performed to determine if the distribution of these adhesion molecules on cell surfaces could help to explain either the normal interactions between cells within a cluster, or the dTC-induced perturbations of these interactions (Fredette and Landmesser, 1991*b*).

N-Cadherin

N-cadherin expression was observed using two different antibodies: the monoclonal antibodies GC-4 (Volk et al., 1990) and a rabbit polyclonal antibody (kindly provided by M. Takeichi), both of which gave the same results. Intense N-cadherin immunofluorescence staining, visualized in Fig. 1 by the GC-4 antibody, was observed in the iliofibularis muscle as well as in other hindlimb muscles during primary myogenesis (Fig. 1 *A*; between St 27 and St 35). It then decreased throughout secondary myogenesis which begins at St 35 (Fig. 1 *C*). This pattern of N-cadherin immunofluorescence in control muscle confirms observations recently made by Hahn and Covault (1992) with the NC-2 antibody of Takeichi (Hatta and Takeichi, 1986). By St 40 only a few myofibers, possibly corresponding to slow tonic fibers described by Hahn and Covault (1992), remained N-cadherin positive (not shown). In dTC-treated muscles, N-cadherin immunostaining was also intense throughout primary myogenesis (Fig. 1 *B*), and decreased during secondary myogenesis (Fig. 1 *D*). However, at all stages investigated (St 30-40), staining intensity was always greater in the dTC-treated muscle than in the stage-matched control muscles, and by stage 35 was substantially more intense (compare (Fig. 1, *C* and *D*). This difference was maintained until St 40, when N-cadherin was also down regulated in the dTC-treated muscles. Therefore, the down regulation of N-cadherin expression appeared to be delayed, but not abolished in the absence of motoneuron activation of developing myotubes. dTC treatment produced no visible alterations in N-cadherin immunostaining in the sciatic or intramuscular nerves, which as described for control hindlimbs by Hahn and Covault (1993), was extremely low in the intramuscular nerves, but high on bundles of axons in the sciatic nerve which resulted in the patchy appearance seen in Fig. 1 (*C* and *D*).

Myotubes were characterized with electron microscopy by the presence of myofibril bundles, and during both primary and secondary myogenesis, a nonuniform distribution of N-cadherin was observed on myotube surfaces. As shown for St 31 (Fig. 2, *A* and *B*) and 38 (Fig. 2 *C*), N-cadherin immunoreaction product was most abundant on the regions of plasma membranes (asterisks) that form close appositions between primary myotubes (identified by their large diameter and arrangement of myofibrils at the cell periphery) and the smaller and electron-dense myoblasts or newly developing myotubes. However, it was reduced or absent from the appositions between more mature myotubes (Fig. 2, *arrows*). During primary myogenesis (St 31) and at the onset

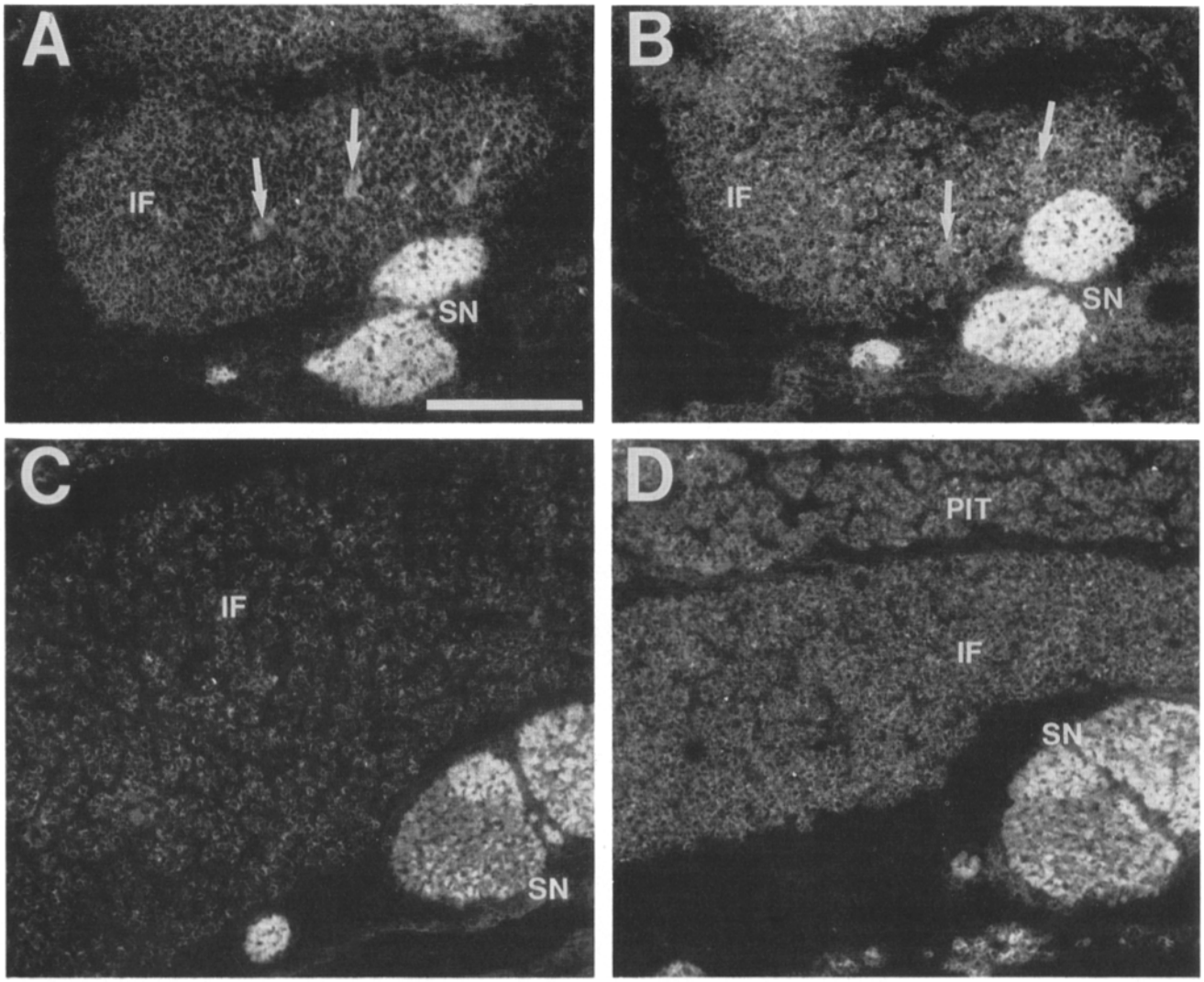


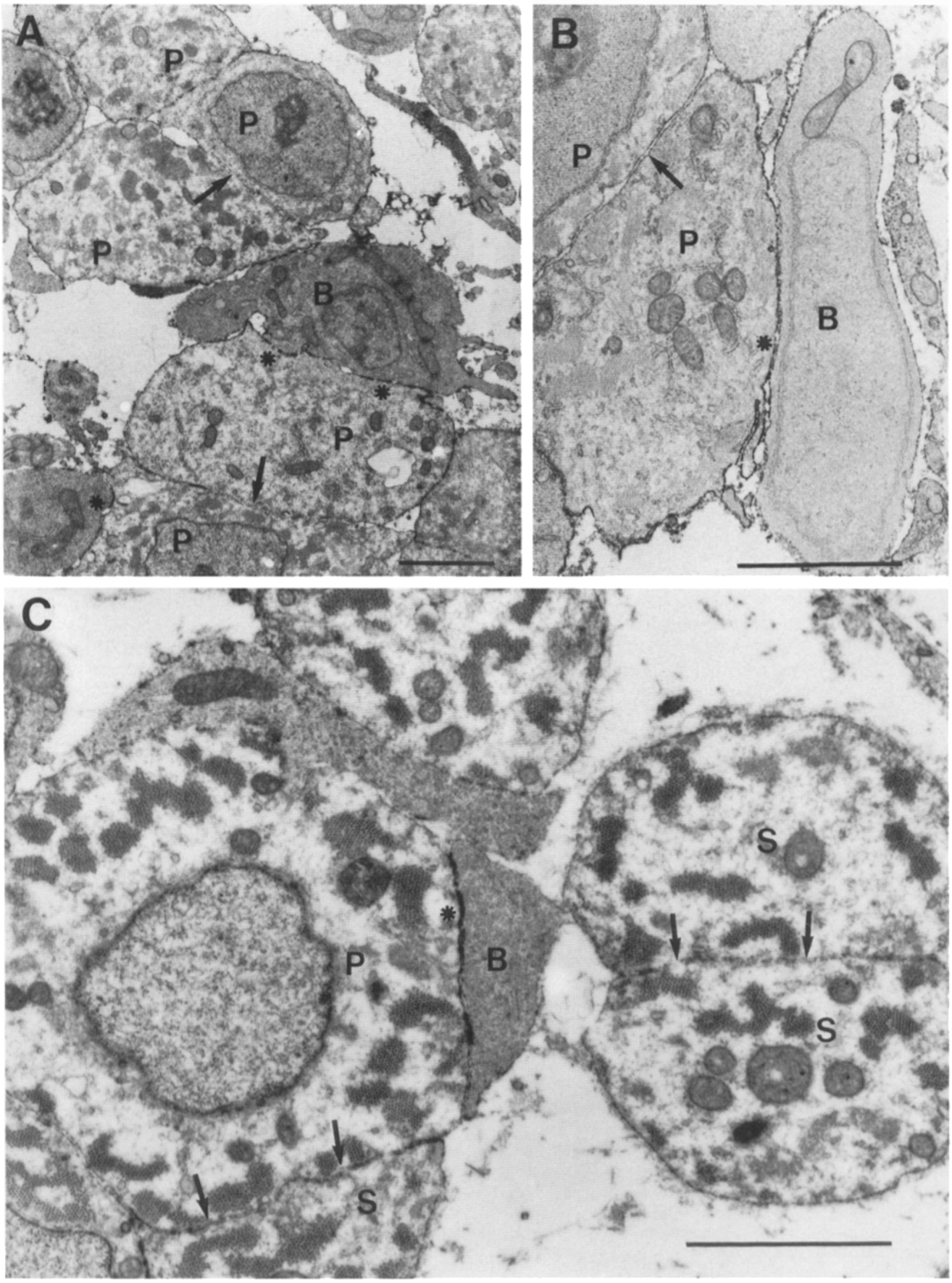
Figure 1. Comparison of N-cadherin immunofluorescence in developing hindlimb muscles of control (*A* and *C*) and in dTC-treated (*B* and *D*) chick embryos. (*A* and *B*) Iliofibularis (*IF*) muscle and sciatic nerve trunks (*SN*) at St 31 during primary myogenesis. (*C* and *D*) View of the iliofibularis muscle, and sciatic nerve at St 35 during the onset of secondary myogenesis. N-cadherin immunostaining is more intense in the dTC-treated muscle at both stages, and the intensity difference is greater at St 35, while no intensity differences are apparent in the sciatic nerve. Intramuscular nerves in the iliofibularis muscle (*arrows*) are weakly N-cadherin positive in both cases. *PIT*, posterior iliotibialis muscle. Bar, 200 μm.

of secondary myogenesis (St 35, Fig. 3 *B*), mature and forming myotubes and myoblasts were observed with N-cadherin on their free surfaces, but by St 38 (Fig. 2 *C*) this was greatly reduced, reflecting what had been observed with immunofluorescence (Fig. 1 *C*). In addition to the staining observed on muscle cell profiles, N-cadherin reaction product was also observed on the extracellular surfaces of fibroblasts (which were large, only loosely associated with the myotube clusters, and which contained abundant polyribosomes and rough endoplasmic reticulum), and on extracellular matrix material containing collagen fibrils.

EM localization of N-cadherin in activity-blocked muscle showed that in addition to the overall increase in N-cadherin expression observed by immunofluorescence, there was an obvious alteration in its distribution. In st 35 control muscles N-cadherin was low or absent on the apposed surfaces of more mature myotubes (Fig. 3 *B*), whereas in dTC-treated muscle intense N-cadherin staining was present at these appositions (Fig. 3 *A*, *asterisks*).

NCAM

Although N-cadherin immunofluorescence decreased during secondary myogenesis, immunofluorescence for total NCAM remained intense until after St 39 (Fig. 4, *A*, *C*, and *E*), when it begins to decrease (Tosney et al., 1986; Covault and Sanes, 1986). In contrast to the effect of dTC treatment on N-cadherin expression, immunofluorescence did not reveal any obvious differences in total NCAM expression during most of myogenesis. As in control muscle, immunofluorescence remained bright until St 39 when NCAM expression is down regulated (Fig. 4, *B*, *D*, and *F*). However, by St 38 (Fig. 4, *E* and *F*), a slightly lower intensity of immunostaining was observed in the activity-blocked muscles. As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with N-cadherin, the intense NCAM staining observed in the sciatic and intramuscular nerves of control muscle was unaltered.

Despite the apparent lack of change in NCAM expression that was observed at the light microscopic level during normal development, EM analysis revealed a change in its distribution. Throughout development NCAM immunoreaction product was observed on both the free and apposed surfaces of primary myotubes, myoblasts, and fibroblasts (Fig. 5, *A* and *B*). However, at early stages (st 31), some portions of muscle cell membranes that shared appositions with other cells within clusters were not immunopositive (see for example Fig. 9 *B*, *arrows*), but these unstained portions decreased, so that by St 38 uniform NCAM staining was observed on all surfaces of all cell types within the cluster (Fig. 6, *A* and *B*). In addition to NCAM immunostaining on the myotube surface, immunoreaction product was observed on the internal surfaces of small intracellular vesicles (Fig. 6 *B*). Some NCAM-positive vesicles were also observed in contact with the cytoplasmic surface of the cell membrane, while others had membranes continuous with the cell membrane. Both coated and non-coated NCAM-positive vesicles were observed, and some of the non-coated vesicles formed complexes, resembling the caveolae described by Yamada (1955) in epithelial cells and by Franzini-Armstrong (1991) in developing skeletal muscle. Such vesicles could be involved in NCAM's delivery to or removal from the cell surface, or they could be parts of forming T-tubules, which are known to express NCAM (Covault and Sanes, 1986). Like N-cadherin, NCAM was also observed on the surfaces of non-muscle cells such as fibroblasts, intramuscular axons and Schwann cells, as well as on what appeared to be ECM-containing collagen fibrils. Some of this extracellular NCAM may have been due to interactions between heparin sulphate proteoglycogan contained in the extracellular material and the heparin sulphate-binding site on NCAM (see Cole et al., 1986).

As with immunofluorescence, EM analysis (Fig. 7) of dTC-treated muscles did not reveal any perturbation in the distribution of NCAM, since NCAM was observed on both the free and apposed surfaces of myotubes and myoblasts, as was the case with control muscle.

Polysialic Acid

Unlike the relatively uniform expression of total NCAM, the amount of PSA associated with NCAM exhibited a striking temporal regulation during development (Fig. 8). During the period in which total NCAM immunostaining remained intense and invariant (St 28–39), PSA immunofluorescence was initially visible only on some myotube surfaces at low intensity levels, (St 28, not shown). Staining then increased at a slow rate throughout primary and early secondary myogenesis, until beginning at St 37 (Fig. 8 *A*) it increased sharply to peak at St 38 (Fig. 8 *C*), and then rapidly declined at St 39 (Fig. 8 *E*). While the overall expression of total NCAM was only slightly affected by activity blockade, PSA expression was dramatically altered. Immunofluorescence with mAb 5A5 revealed that activity blockade virtually abolished the presence of PSA on myotube surfaces (Fig. 8, *B*, *D*, and *F*), even at St 38 when PSA was dramatically up-regulated in the control muscle. This effect was not particular to the iliofibularis muscle, as it occurred in other hindlimb muscles as well. However, the effect was restricted to myotubes, since immunostaining for PSA in the sciatic nerves of the dTC-treated embryos did not differ from that in control muscle (Fig. 8 *C*). Although activity blockade produces an up-regulation of polysialylated NCAM in nerve trunks at early embryonic stages (St 27–32) (Landmesser et al., 1991), by St 35 PSA expression on intramuscular nerves had decreased to very low levels in both control and dTC-treated embryos, and no difference in staining intensity was observed.

Electron microscopy revealed that polysialylated NCAM displayed an unusual cellular distribution: while total NCAM was expressed on all surfaces of muscle cells, polysialylated NCAM was always restricted to their free surfaces. At St 31, when PSA levels were observed to be low by immunofluorescence, only portions of the free surfaces of muscle cells were immunopositive (Fig. 9 *A*), PSA was notably absent from regions between closely apposed cells (arrows Fig. 9 *A*, *arrows*). Although NCAM was also not yet uniformally distributed, it was present over more of the cell surface than PSA (compare Fig. 9, *A* and *B*). Between St 31 and 38, PSA immunostaining became more extensive over the free surfaces of cells, so that by St 38, when PSA expression peaks, entire clusters appeared to be encapsulated by PSA (Fig. 9 *C*). As at St 31, PSA was virtually excluded from the apposed surfaces of cells within clusters; however staining often extended from the free surfaces a short distance, especially between cells at the edges of the cluster (Figs. 9, *C* and 10 *A*, *asterisks*). In contrast, total NCAM was by now abundant on all cell surfaces, including those between closely apposed myotubes (Fig. 10 *B*).

The absence of PSA from cell appositions within clusters did not appear to be an artifact due to a failure of the immunoreagents to penetrate to these surfaces, because although the 5A5 antibody used to detect PSA was of IgM iso-

Figure 2. Electron microscopic localization of N-cadherin on developing myotubes. (*A*) Myotube clusters in St 31 iliofibularis muscle. N-cadherin is present on the free surfaces of primary myotubes (*P*) and electron-dense blast cells (*B*) and where primary myotubes appose blast cells (*asterisk*). N-cadherin is low or excluded from regions where primary myotubes closely appose each other (*arrows*). (*B*) Higher magnification view of St 31 muscle to show an N-cadherin-positive region where the cell membranes of a blast cell (*B*) and a primary myotube (*P*) appose each other (*asterisk*), and an N-cadherin negative region where two primary myotubes appose each other (*arrow*). (*C*) St 38 iliofibularis muscle during secondary myogenesis, when N-cadherin expression is down regulated. N-cadherin immunostaining persists on portions of the free surfaces of muscle cells, but is most dense at appositions between blast cells and myotubes (*asterisk*). Arrows indicate expanses of membrane that form appositions between a primary and secondary myotube in a cluster (*left*), and between two secondary myotubes shown on the right (*S*) that have separated from a cluster. In these regions N-cadherin reaction product is present, but sparser than at appositions with blast cells (*asterisk*). Bars, 2 μm.

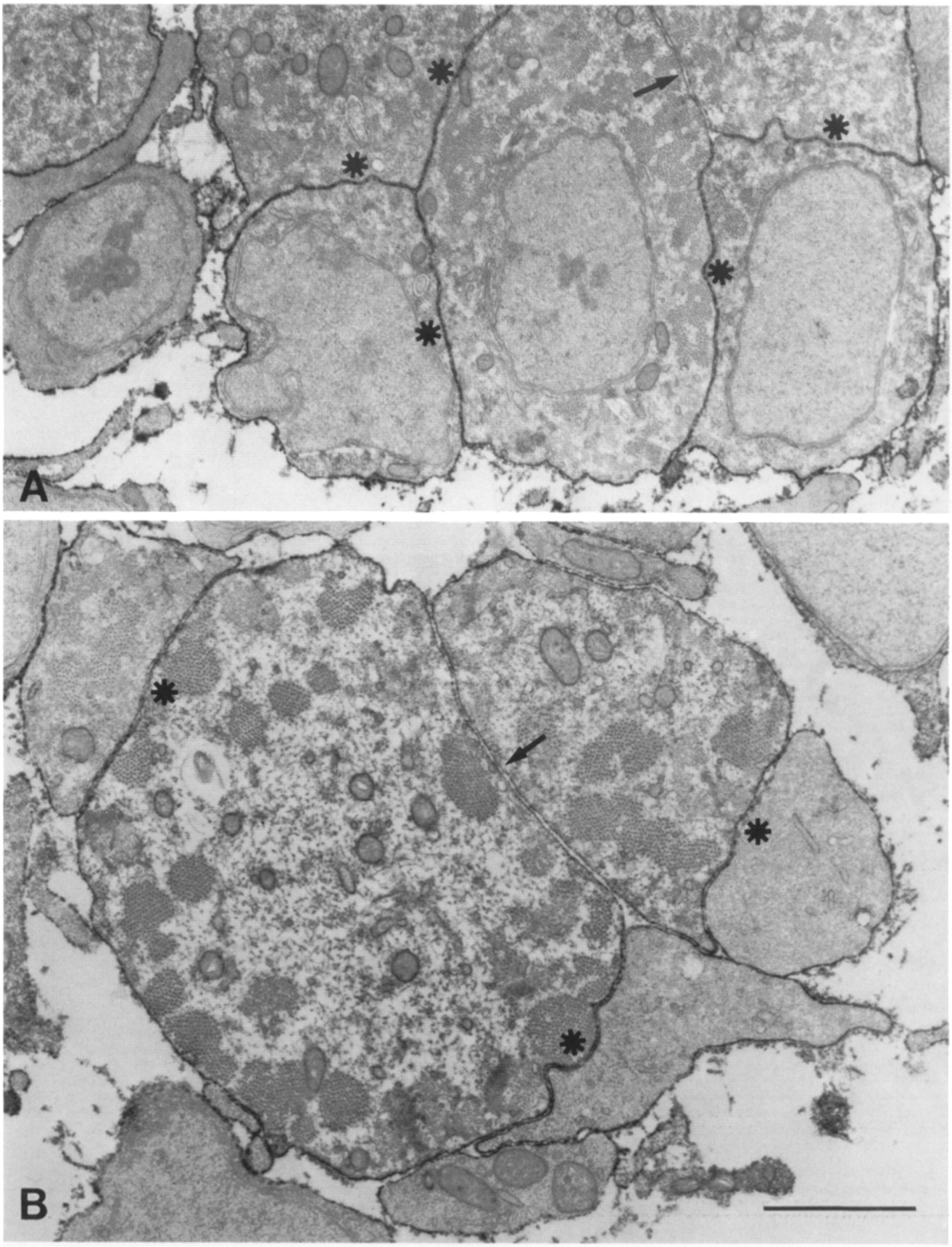

Figure 3. Evaluation of N-cadherin distribution in dTC-treated iliofibularis muscle at St 35, when N-cadherin immunofluorescence is increased over control levels. (*A*) Portion of an extremely large myotube cluster, characteristic of those in activity blocked muscle, showing five myotubes that are N-cadherin positive not only on their free surfaces, as observed in control muscles, but also on most of their apposed surfaces (*asterisks*). One region between two myotubes (*arrow*) is N-cadherin negative, but these regions were rarely found in the dTC-

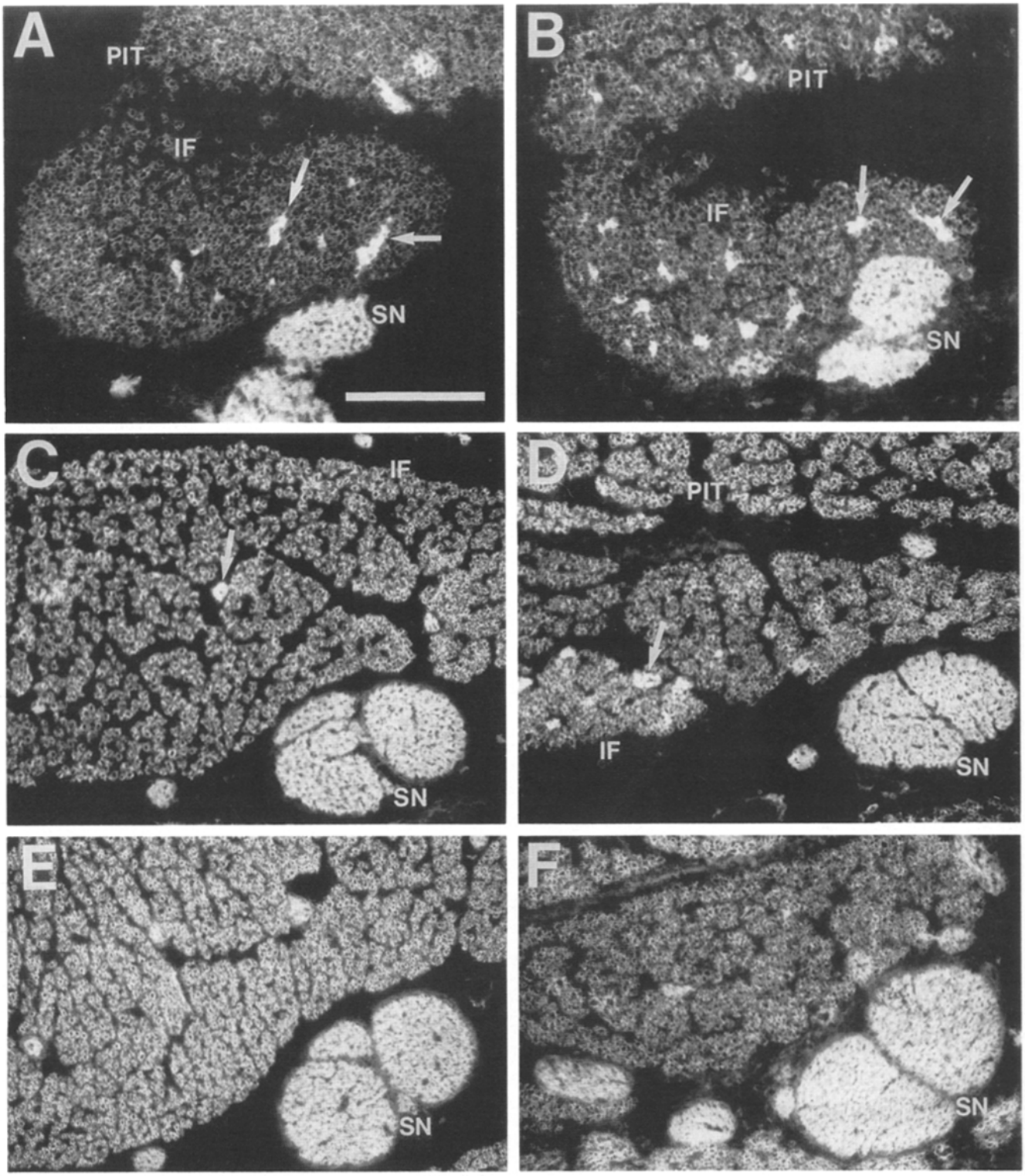

Figure 4. NCAM immunofluorescence in control (*A*, *C*, and *E*) and dTC-treated (*B*, *D*, and *F*) developing muscle with 5E antibody which recognizes all protein isoforms of NCAM. (*A* and *B*) Staining at St 31 during primary myogenesis; (*C* and *D*) at St 35 during the onset of secondary myogenesis; (*E* and *F*) at St 38 during secondary myogenesis. The myotubes, intramuscular nerves (*arrows*) and sciatic nerve trunks (*SN*) of the control embryos are intensely immunopositive through St 38. Staining is similar to control in the dTC-treated embryos until St 35, but by St 38, immunostaining in the dTC-treated muscle (*F*) is somewhat less intense than in the control embryo (*E*). *IF*, iliofibularis muscle; *PIT*, posterior iliotibialis muscle. Bars: (*A* and *B*) 100 μm; (*C* and *D*) 150 μm; (*E* and *F*) 200 μm.

treated muscles. (*B*) Plasma membranes of myotubes and blast cells in a control iliofibularis muscles are also N-cadherin positive on their free surfaces, and at regions where primary myotubes appose newly formed secondary myotubes or myoblasts (*asterisks*), but are not stained where more mature primary myotubes appose each other (*arrow*). Bars: (*A*) 2 μm; (*B*) 1.4 μm.

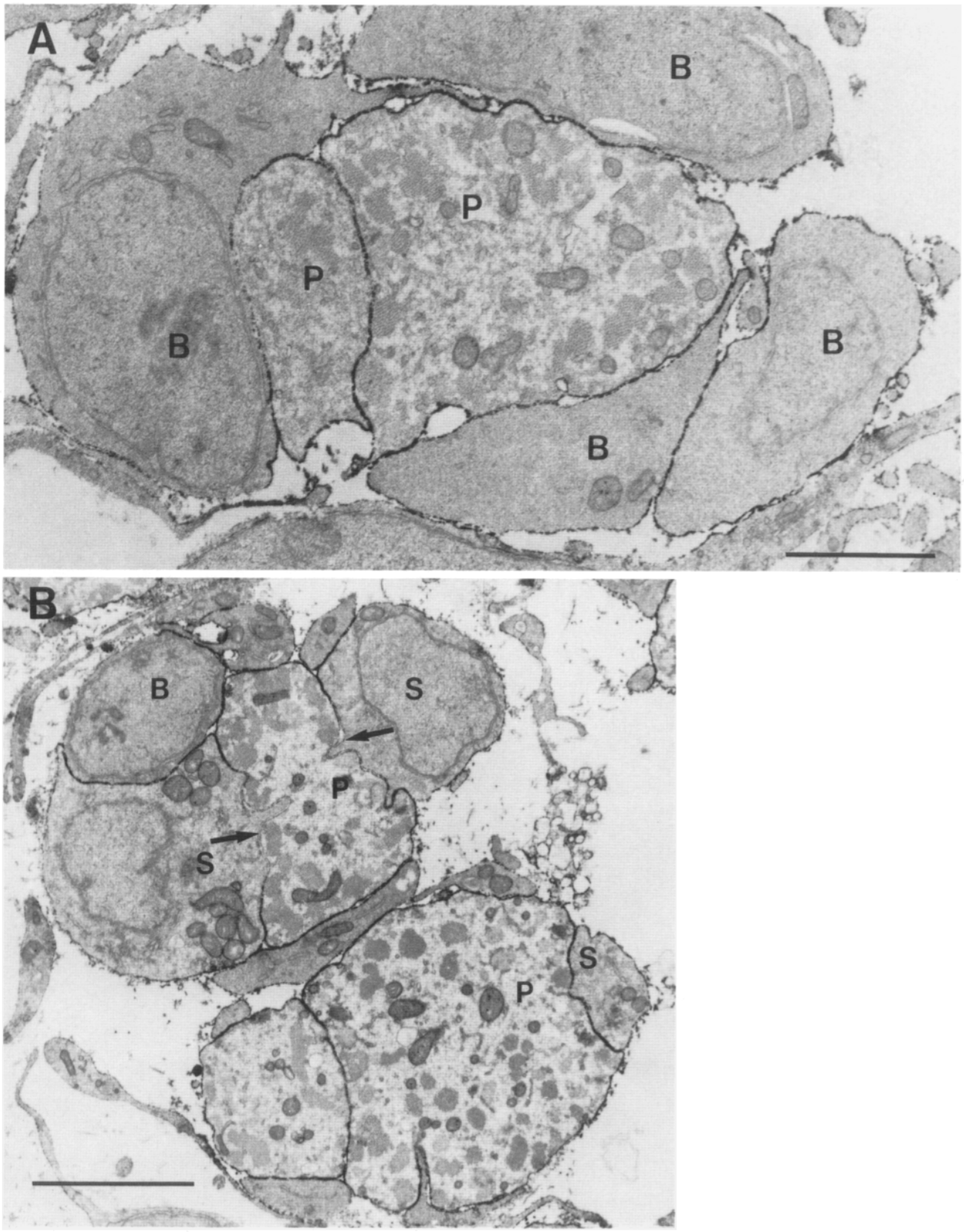

Figure 5. Distribution of total NCAM in the iliofibularis muscle. (*A*) During primary myogenesis (st 31), 5E immunoreaction product for total NCAM is present on free and apposed plasma membrane surfaces of two primary myotubes (*P*) and several blast cells (*B*). (*B*) Low-magnification electron micrograph of NCAM localization in a myotube cluster at the onset of secondary myogenesis (st 35). The surfaces of primary myotubes (*P*), forming secondary myotubes (*S*), and blast cells (*B*) of two clusters are nearly entirely NCAM positive, although portions of apposed surfaces within the cluster contain very little NCAM (*arrows*). Bars: (*A*) 2 μm; (*B*) 3 μm.

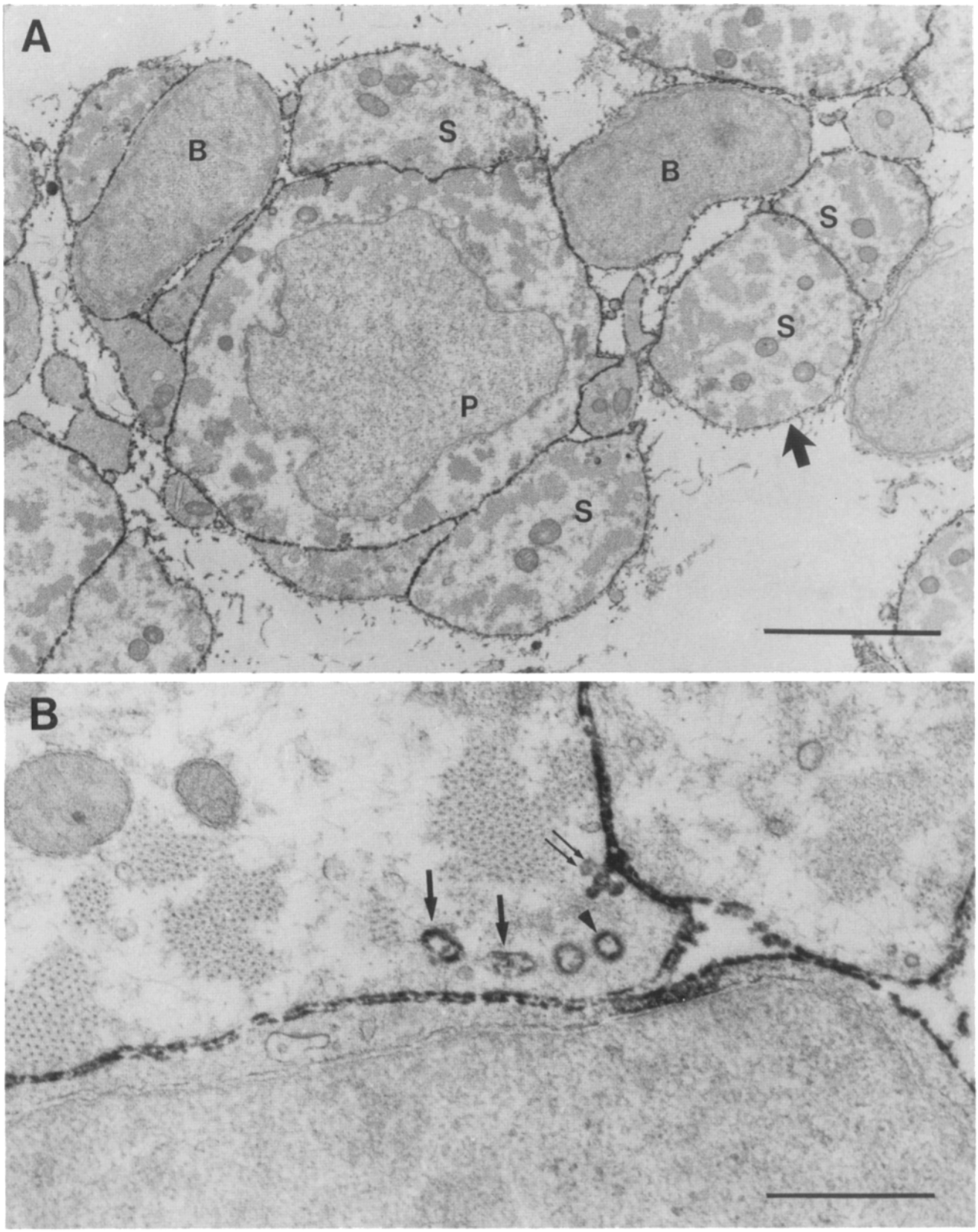

Figure 6. The distribution of total NCAM becomes uniform on cell surfaces during secondary myogenesis. (*A*) NCAM immunostaining on primary (*P*), secondary (*S*), and blast (*B*) muscle cells within a cluster, and on two nearby secondary myotubes that are no longer associated with a cluster (*arrow*). By St 38, all muscle cell types are NCAM positive along all of their surfaces, and NCAM-negative regions observed at earlier stages are no longer visible. (*B*) NCAM is present not only extracellularly on free and apposed plasma membranes, but also intracellularly on the internal surfaces of large and small non-coated vesicles or calveolae (*arrows*). A coated vesicle indicated by the arrowhead also contains NCAM immunoreaction product. Double arrow points to a caveolae complex that is continuous with the plasma membrane. Bars, (*A*) 2.0 μm; (*B*) 0.64 μm.

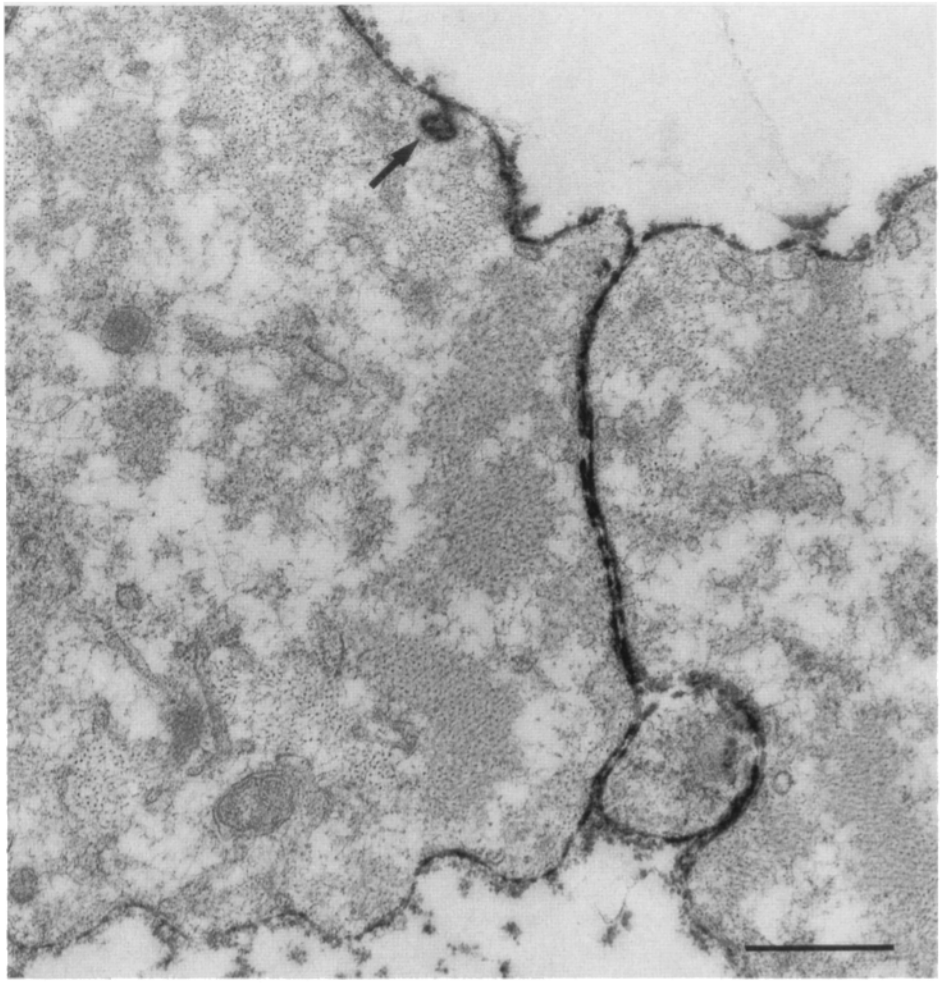

Figure 7. The distribution of NCAM is not perturbed by dTC treatment. Electron micrograph of the iliofibularis muscle of a St 38 dTC-treated embryo containing myotubes that are NCAM positive on their free and apposed surfaces. The myotubes also contain NCAM-positive vesicles (*arrow*). Bar, 0.5 μm.

type and therefore large, the same restricted distribution was found with another anti-PSA antibody of IgG isotype (Fig. 10 *C*). Since the 5E antibody used to visualize the NCAM polypeptide is also an IgG, and was detected on all cell surfaces, we concluded that the absence of 5A5 immunoreaction product at cell–cell appositions was not an artifact of poor antibody penetration.

Since no PSA immunostaining was observed in dTC-treated muscle at the light microscope level, we did not study PSA in these muscles at the EM level.

In summary then, chronic paralysis of developing muscles produced three alterations in the normal expression of adhesion molecules as detected by immunofluorescence and immunoelectron microscopy: (*a*) N-cadherin expression was enhanced and its down regulation delayed; (*b*) a dTC-induced decrease of NCAM levels was detected after St 38; and (*c*) polysialylated NCAM, which was restricted to the free surfaces of myotubes, was not expressed in the dTC-treated muscles, despite the continued expression of total NCAM.

Immunoblot Analysis of NCAM Isoforms in Developing Control and dTC-treated Muscle

Since, mAb 5E does not distinguish among the different isoforms of NCAM, it is possible that although overall NCAM expression was only slightly affected by dTC treatment, larger changes in the expression of individual isoforms may have occurred. While all three major NCAM isoforms have been shown to be capable of mediating neurite adhesion and outgrowth when transfected into 3T3 cells (Doherty et al., 1990), the possibility exists that the different isoforms could be playing different roles during more complex developmental events in intact tissue. For example, the switch from transmembrane isoforms to a GPI-linked isoform has been suggested to play a role in myoblast fusion (Dickson et al., 1990). It is also possible that the different isoforms could be differentially sialylated, providing the possibility of targeting PSA to specific cell surfaces. It is known from other systems that such targeting is associated with GPI linkages (Dotti et al., 1991; Powell et al., 1991; Dotti and Simons, 1990; Lisanti et al., 1988; Brown et al., 1989). For these reasons we examined the molecular weight forms of NCAM expressed during muscle development in the presence and absence of nerve activity and determined which of these NCAM isoforms contained PSA.

The different isoforms of NCAM expressed during primary and secondary myotube formation were examined with SDS-PAGE combined with immunoblot analysis (Fig. 11). Thigh muscle homogenates from embryos at St 28 and St 32 (during different stages of primary myogenesis), St 35 (at the

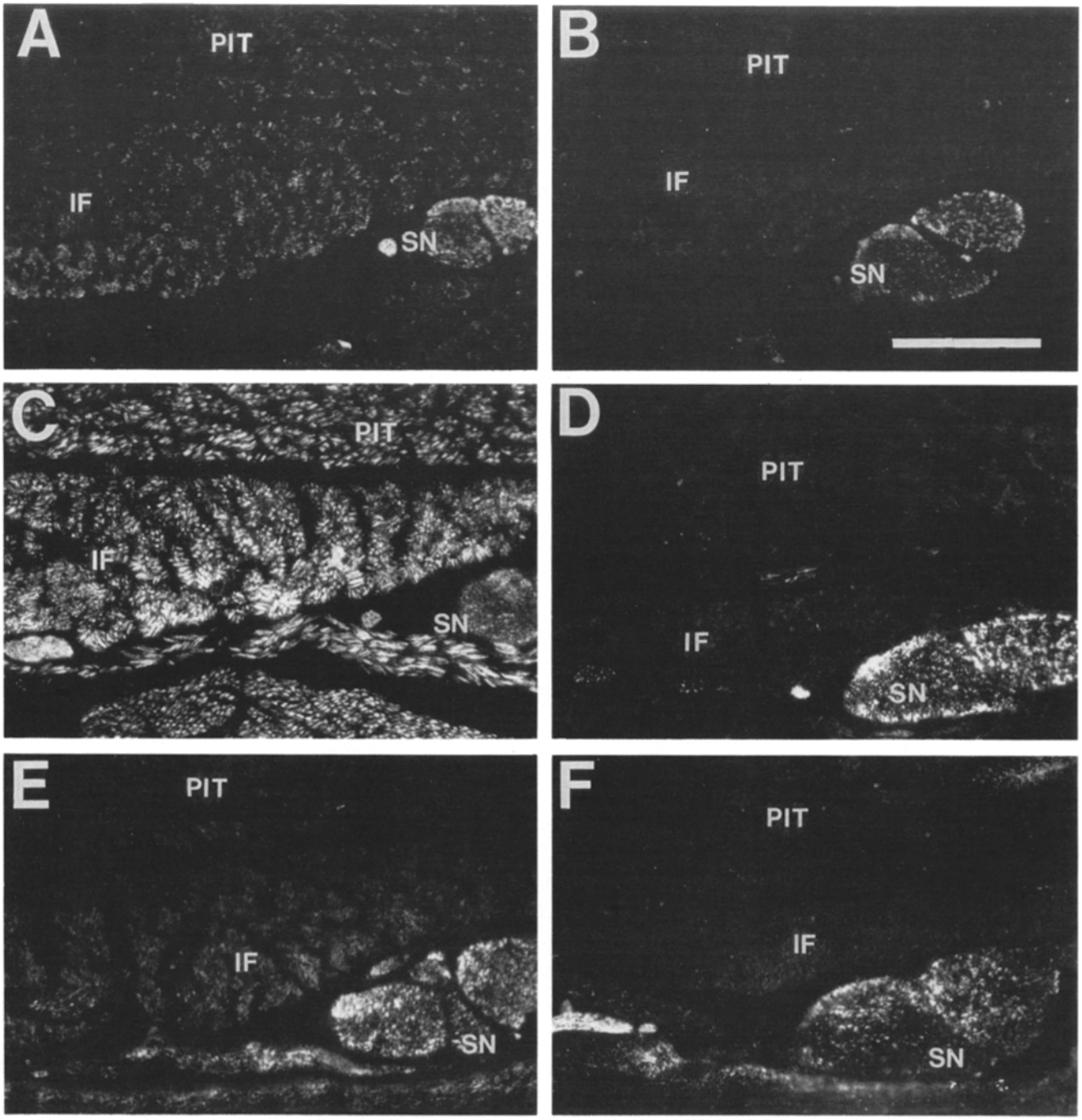

Figure 8. Immunofluorescence for polysialylated NCAM with PSA-specific mAb 5A5 in control (*A*, *C*, and *E*) and dTC-treated (*B*, *D*, and *F*) developing muscle. (*A*, and *B*) St 37. (*C* and *D*) St 38. (*E* and *F*) St 39. A rapid and transient upregulation of polysialylated NCAM occurs between St 37 and 39 in control muscle, but in dTC-treated muscle, virtually no polysialylated NCAM is expressed on myotubes, although immunostaining of the sciatic nerve remains identical to that in control embryos. *IF*, iliofibularis muscle; *PIT*, posterior iliotibialis; *SN*, sciatic nerve. Bar, 300 μm.

onset of secondary myogenesis), and St 38 and 40 (when secondary myogenesis is well underway) contained NCAM of 5 different molecular weights when desialylated with neuraminidase: 180, 155, 145, and 130 kD, and in many but all of the samples, 120 kD (Fig. 11 *A*, lanes 2–6). In order to determine which of these may have been due to the presence of intramuscular nerve in the muscle samples, sciatic nerve homogenates were examined, and found to contain approximately equal amounts of 180-, 145-, and 120-kD NCAM. Since the muscle samples contained only a minor amount of 180-kD NCAM, which is considered to be nerve specific, (but see Tassin et al., 1991), we concluded that only a very minor portion of the 145- and 120-kD isoforms could be due to contamination by nerve.

The predominance of the different molecular weight isoforms of NCAM were observed to vary according to de-

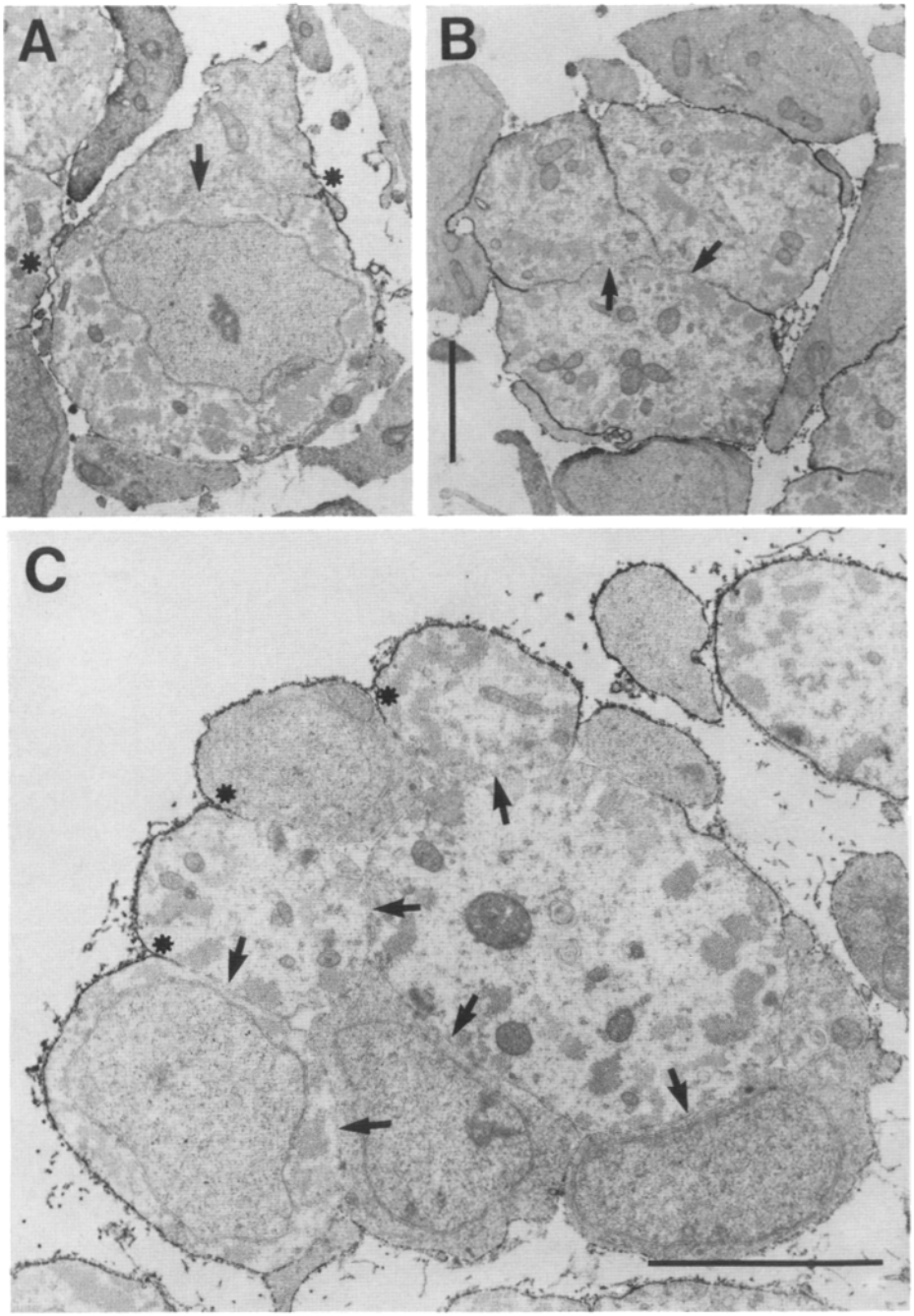

Figure 9. Immuno-electron microscopy for polysialylated NCAM with mAb 5A5. (*A*) Low-magnification view of the patchy distribution of polysialylated NCAM on the free surfaces of myotube clusters at St 31. Patches of PSA were usually located on regions of cell membranes that were close to, but not congruent with other cells (*asterisk*). Nearly all apposed surfaces are PSA negative (*arrows*). (*B*) A comparison with the distribution of total NCAM at St 31 shows that although total NCAM is also expressed non-uniformly, its distribution is more extensive than that of PSA. Arrows show NCAM-negative patches of apposed membranes. (*C*) At St 38, when immunofluorescence for PSA peaks, 5A5 reaction product remains restricted to the cluster periphery, forming a glycocalyx-like coat that encloses the entire myotube cluster. However, as at earlier stages, reaction product is not present on most apposed surfaces within the cluster (*arrows*), except at some regions near the periphery of the cluster (*asterisks*). PSA reaction product is also present scattered throughout the extracellular matrix. Bars: (*A*) 3 μm; (*B*) 2.4 μm; (*C*) 2.7 μm.

velopmental stage (in Fig. 11 *A*, compare lanes *2–6*). At St 28, most of the NCAM present in muscle had an apparent molecular weight of 145 kD, and this remained the predominant isoform through St 35. However, by St 38 expression of the 130-kD NCAM had increased with respect to the other forms, and by ST 40 it had become the major isoform. At the same time, the proportions of NCAM running at 145 and 155 kD also decreased. In addition, the amount of the 145-kD isoform relative to the 155-kD isoform decreased after St 28, with both isoforms being equally represented by St 35.

Following activity blockade (Fig. 11 *B*), the initial pattern of NCAM expression was unaltered. Thus at St 28, most of the NCAM was of the 145-kD isoform, and over the next several stages the other isoforms (155 and 130 kD) increased as in control muscle. However, the subsequent shift after St 35, in which the 130-kD isoform increases and the 145- and 155-kD isoforms decrease, did not occur in the dTC-treated muscle (compare the control Fig. 11 *A*, lane 6 with the dTC-treated muscle Fig. 11 *B*, lane *3*); instead the relative levels of expression remained similar to those observed at St 32.

When the intensities of the different NCAM bands were compared within each immunoblot and expressed as a percentage of the total NCAM (Fig. 11 *C*), in both control and dTC-treated muscle at St 28, the 145-kD NCAM was found to comprise about 60% of the total muscle NCAM while the 130-kD form comprised less than 10%. However, while the proportion of the 145-kD form had decreased in control muscle by St 39 to 25% and that of the 130-kD form had in-

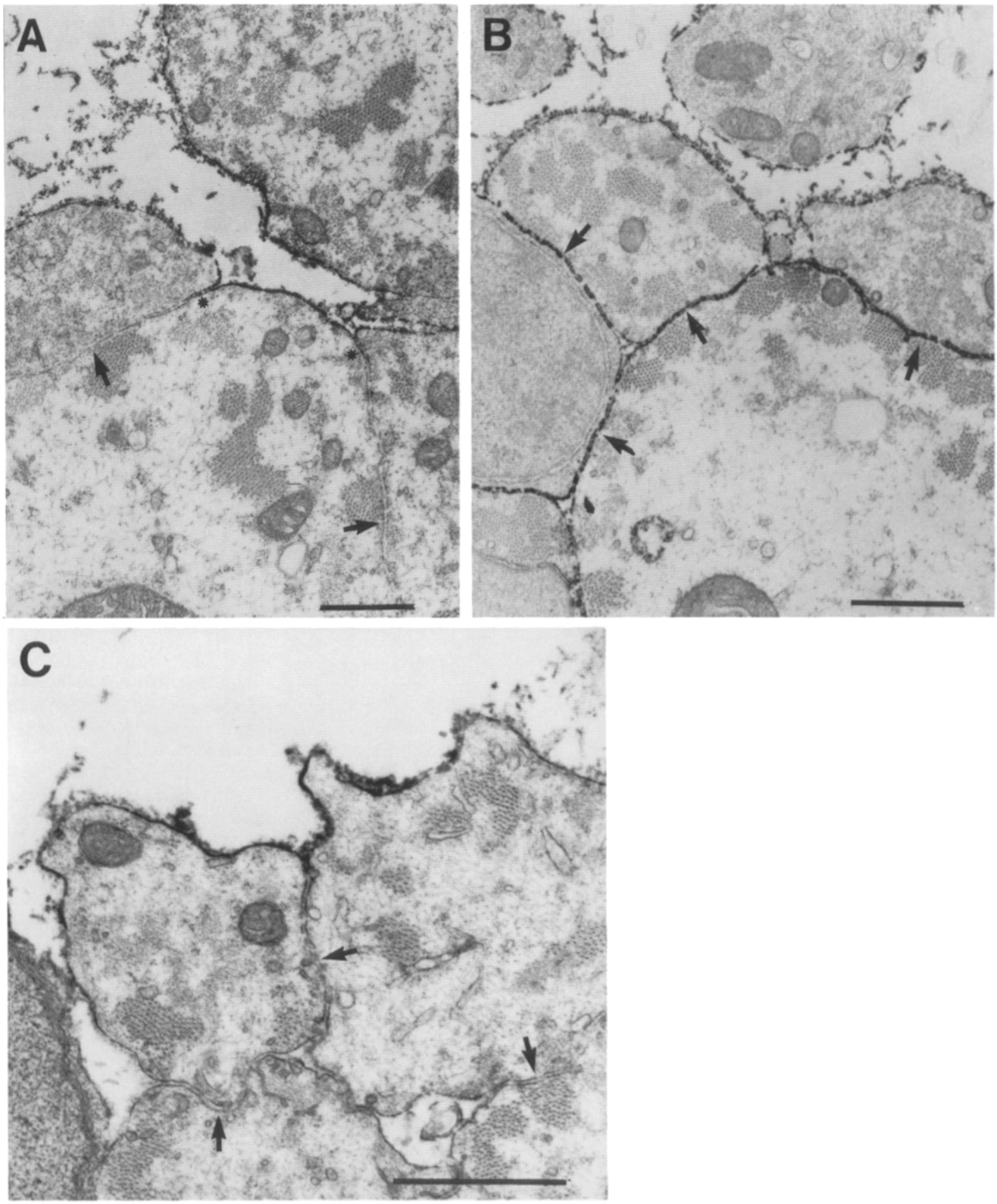

Figure 10. Comparison of total and polysialylated NCAM immunostaining in St 38 iliofibularis muscle. (*A*) Portion of a myotube cluster with 5A5 reaction product for PSA predominantly located at the free surfaces. PSA is also seen penetrating for short distances between closely apposed cells near the periphery of the cluster (*asterisks*). The shapes of the PSA-positive cell profiles suggest that the cleft between them has formed by the separation of the upper cell from the other cells. No PSA is present on most of the apposed myotube surfaces (*arrows*). (*B*) Total NCAM, on the other hand, is present not only on the free surfaces of myotubes, but also on all of the apposed surfaces within the cluster (*arrows*). (*C*) Immunostaining with an IgG isotype antibody against PSA produces the same distribution of reaction product (at the free surfaces of cells, but not at cell-cell appositions) as 5A5, which is an IgM class antibody. Therefore, the distribution pattern of PSA observed with 5A5 is not an artifact due to a failure of the larger IgM antibody to penetrate to cell surfaces within clusters. Bars: (*A*) 0.5 μm; (*B*) 1 μm; (*C*) 0.8 μm.

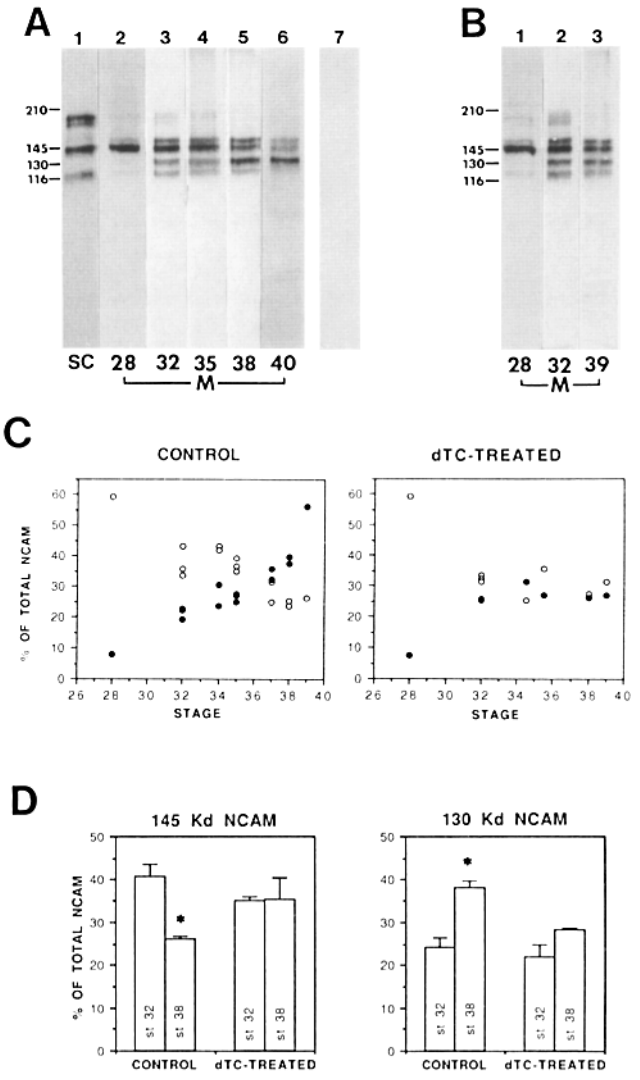

Figure 11. Activity blockade prevents developmental changes in the molecular weight isoforms of NCAM expressed in hindlimb muscles. Neuraminidase-desialylated proteins extracted with NP-40 from muscle or sciatic nerve homogenates were separated by SDS-PAGE and immunoblotted with 5E antibody and alkaline phosphatase-conjugated secondary antibody. (*A*) Western blots of control embryo NCAM isoforms in St 38 sciatic nerve (*SC*, lane *1*) and in muscle at St 28, 32, 35, 38, and 40 (*M*, lanes *2–6*, respectively) stained with 5E. In lane *7*, St 38 muscle proteins were processed only with the second antibody as a control for nonspecific staining. Calculated (145 and 130 kD) and protein standard M_r (210 and 116 kD) are shown on the left. (*B*) 5E Western blots of St 28, 32 and 39 muscle NCAM (lanes *1–3*, respectively) from dTC-treated embryos. (*C*) Quantitative representation of the developmental change in predominant NCAM forms. Densitometric scans were made of NCAM immunoblots and the quantity of the 145- (○) and the 130-kD (●) bands were expressed as a percentage of the total density of all muscle NCAM forms (155, 145, 130, and 120 kD). Control muscle expresses predominantly 145-kD NCAM at St 28, but after St 36 the 130-kD form becomes predominant. In dTC-treated muscle, the 145-kD form is also initially predominant, but by St 32 the 145- and 130-kD forms are equally represented. Each point is the value obtained from a representative gel. (*D*) Comparisons of the relative quantities of NCAM isoforms at st 32 and st 38. Asterisks indicate significant changes (a decrease for 145-kD NCAM and an increase for 130-kD NCAM) that occurred in the control muscle between st 32 and 38 ($P < 0.01$, unpaired *t*-test). No differences were detected between St 32 and 38 in the dTC-treated muscle. Values are means ± S.E. from three separate experiments. * $P \leq 0.01$, unpaired *t*-test.

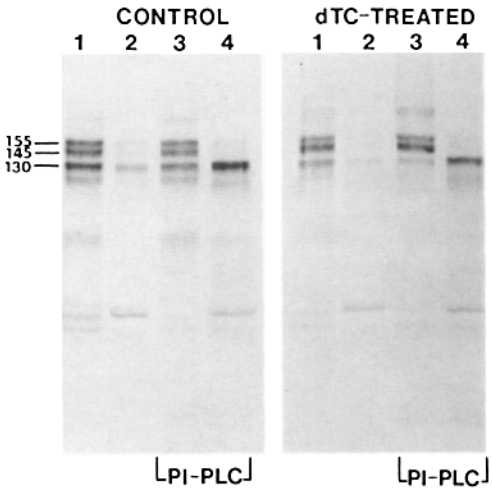

Figure 12. GPI-linkage of the 130-kD NCAM form in control and dTC-treated muscle. St 38 muscle membranes were incubated in buffer in the absence (lanes *1* and *2*) or the presence (lanes *3* and *4*) of phosphatidyl inositol-specific phospholipase C (*PI-PLC*). The resulting soluble (lanes *2* and *4*) and insoluble (lanes *1* and *3*) fractions were separated and processed for NCAM Western blot analysis with 5E antibody. Incubation of both control and dTC-treated muscle with PI-PLC resulted in an increase in the amount of 130 kD (and some 120 kD) NCAM in the soluble fraction (lane *4*), and an accompanying decrease in the amount of 130-kD NCAM in the insoluble membrane fraction (lane *3*), as compared to the non-enzyme-treated membranes (lanes *1* and *2*). Calculated M_r (kD) are indicated on the left.

creased to over 55%, in dTC-treated muscles, the 145- and 130-kD isoforms remained equally represented after St 32, each comprising about 30% of the total NCAM. A statistical analysis showed that a significant decrease in 145-kD NCAM and an increase in 130-kD NCAM occurred in control muscle between st 32 and 38, but that these changes did not occur in the dTC-treated muscle (Fig. 11 *D*).

To determine which of the isoforms of muscle NCAM corresponded to extracellular lipid-linked NCAM, crude muscle membrane preparations were incubated in GPI-specific PLC (Knudsen et al., 1989). GPI-PLC solubilized the 130-kD NCAM (and a minor quantity of the 120-kD NCAM) at all stages when this isoform was expressed (St 32, 35, 38, and 39), as shown in Fig. 12 for a St 38 muscle sample. However, GPI-PLC treatment never removed all of the 130-kD NCAM, and at St 38 when the abundance of this form was very high, only about half was solubilized by the enzyme. In studies of other GPI-linked proteins a portion of these proteins are also often resistant to PLC, apparently because they have alternative or additional linkages to the membrane (Ferguson et al., 1985; Almquist and Carlsson, 1988). While we did not determine if such alternative linkages occurred for the 130-kD NCAM, the release of NCAM by means other than via an effect on the GPI anchor (such as phosphatidyl choline hydrolysis or proteolysis) were ruled

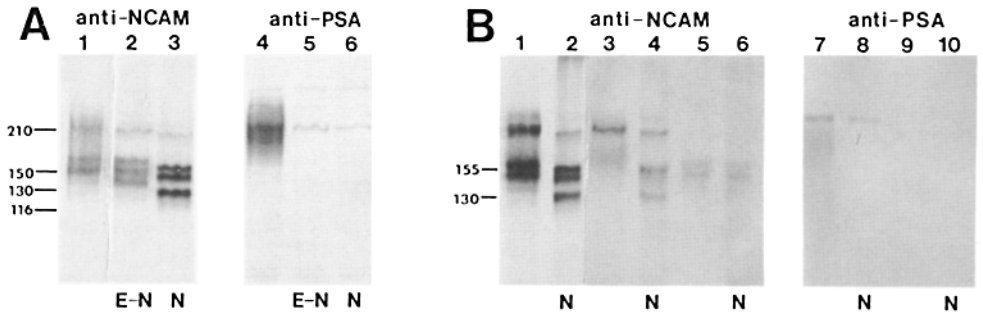

Figure 13. Polysialic acid is differentially represented among the molecular weight forms of NCAM. (*A*) Western blot analysis of St 38 hindlimb muscle extracts that were left fully glycosylated (lanes *1* and *4*), or treated with either endo-N (*E-N*) to remove PSA (lanes *2* and *5*) or neuraminidase (*N*) to further remove sialic acid residues (lanes *3* and *6*). Lanes *1–3*, immunostaining for NCAM with 5E. Lanes *4–6*, immunostaining for PSA-NCAM with 5A5. See text for description. (*B*) Demonstration of polysialic acid on nearly all of the 130-kD NCAM and on very little of the 145-kD NCAM. NCAM extracted from St 38 muscle was separated by SDS-PAGE, and the gel was cut into two parts at the 165-kD region. Proteins eluted from the two portions were either left glycosylated, or were desialylated with neuraminidase, and analyzed with 5E (lanes *1–6*) and 5A5 (lanes *7–10*) Western blotting. Non-treated (lane *1*) and enzyme-treated (lane *2*) total muscle NCAM; non-treated (lanes *3* and *7*) and enzyme-treated (lanes *4* and *8*) NCAM eluted from the >165-kD portion of the gel. Non-treated (lanes *5* and *9*), and enzyme-treated (lanes *6* and *10*) NCAM from the <165-kD portion of the gel. Desialylation of the upper portion of the gel which contains PSA (lane *7*) results in 155- and 130-kD NCAM, but very little 145-kD NCAM (lane *4*), while the PSA-negative lower portion (lane *9*) is only somewhat neuraminidase sensitive, producing 155 and 145 kD but no 130-kD NCAM.

out by the ability of zinc chloride to completely inhibit the effect of the enzyme (see Table I). Furthermore the molecular weight of the enzyme-solubilized NCAM (130 kD) was not smaller than that remaining in the membrane, as would have been expected had solubilization occurred by proteolysis. Although the amount of 130-kD NCAM was reduced following dTC treatment, this too was removed from membrane preparations by PI-PLC as in controls (Fig. 12, compare lanes *3* and *4* in both control and dTC treated), indicating that it was also lipid linked.

Polysialylation of NCAM Isoforms

Since the 130-kD NCAM isoform was considerably reduced in the dTC-treated muscles, the possibility that this form carried most of the PSA (and thus contributed to the absence of PSA in activity-blocked muscle) was explored in St 38 muscle when PSA levels are maximal. Muscle extracts treated with an endoneuraminidase that is specific for the α-2,8 sialyl linkages in PSA, which we term endo-N (Finne et al., 1983; Acheson, 1991), and non-enzyme-treated muscle extracts were analyzed by SDS-PAGE and immunoblotting for NCAM or PSA. NCAM from non-enzyme-treated muscle ran as a polydispersed band between 220 and 140 kD (Fig. 13 *A*, lane *1*), and PSA immunostaining occurred only above 155 kD (Fig. 13 *A*, lane *4*). Following complete desialylation of NCAM with neuraminidase, St 38 muscle produced three distinct bands at 155, 145, and 130 kD (Fig. 13 *A*, lane *3*). Treatment with Endo-N, which leaves five sialic acid residues, resulted in three bands running at slightly higher molecular weights, as would be expected (Fig. 13 *A*, lane *2*). Since the bands running at the position of the two higher molecular weight bands could also be detected in the untreated sample (Fig. 13 *A*, lane *1*), and since this portion of the gel did not stain for PSA (Fig. 13 *A*, lane *4*) we concluded that at least a portion of the desialo 145- and 155-kD NCAM isoforms did not contain PSA. In contrast, all of the 130-kD isoform appeared to be polysialylated, since no band corresponding to that isoform was present in the native sample (Fig. 13 *A*, lane *1*). Following endo-N treatment, a distinct band appeared at 140 kD (Fig. 13 *A*, lane *2*) and following complete removal of sialic acid with neuraminidase, this material ran at 130 kD (Fig. 13 *A*, lane *3*).

In confirmation of this conclusion, when native NCAM was eluted from the PSA-immunopositive portions of SDS-PAGE gels (i.e., that running above 165 kD) and then desialylated, mostly 155- and 130-kD isoforms, but only a minor portion of the 145-kD isoform, were obtained (Fig 13 *B*, lane *4*). Conversely, the lower molecular weight, PSA-negative region of the gel contained only 155- and 145-kD NCAM, but no 130-kD NCAM (Fig. 13 *B*, lane *6*). Together, these observations demonstrate that of the four desialo-molecular weight forms of NCAM expressed by embryonic muscle, the vast majority of the 130-kD form is polysialylated, while most of the 145-kD form lacks PSA. The 155-kD NCAM appears to be composed of both polysialylated and non-polysialylated forms expressed in nearly equal proportion.

Further evidence that PSA is present on the 130 kD, PLC-extractable form of NCAM was provided by ELISA (Table I). Anti-NCAM absorbed onto ELISA wells was used to remove NCAM from crude membrane and soluble muscle fractions of St 38, PLC-treated muscle. The resulting quantities of NCAM and associated PSA present in the two fractions were measured in parallel sets of wells with either a second antibody against NCAM or with anti-PSA. PLC solubilized more than 38% of the PSA present in the muscle samples, along with over 25% of the muscle NCAM, while in control (non-enzyme-treated) samples only about 3% of the PSA was solubilized. The higher proportion of PSA than NCAM released by PLC is indicative of the PLC-sensitive (and therefore, lipid-linked) form of NCAM carrying more PSA than the other NCAM forms remaining in the membrane fraction.

Discussion

N-Cadherin Expression Is Correlated with Specific Stages of Myogenesis

Confirming the previously published results of Hahn and

Covault (1992), we have found N-cadherin to be prevalent during primary myogenesis and to be down regulated during secondary myogenesis. However, by immunocytochemical analysis at the EM level, we have also been able to detail the pattern of N-cadherin expression on primary and secondary myotubes and on the myoblasts which give rise to them. Although N-cadherin expression was highest during primary myogenesis, its expression was not confined to primary myotubes since N-cadherin reaction product was observed on both primary and secondary myotubes, and particularly on the myoblasts giving rise to both of these. For both primary and secondary myotubes, N-cadherin expression was initially high in myoblasts and young myotubes, and then decreased as the myotubes matured. Myotubes formed in vitro have also been observed to undergo a progressive loss of N-cadherin as they mature (Mege et al., 1992). The presence of N-cadherin on the free surfaces of primary myotubes suggests that it may provide a permissive substrate for myoblast fusion. Thus N-cadherin adhesion might not only mediate fusion between myoblasts, as demonstrated in vitro by Knudsen et al. (1990*b*) and Mege et al. (1992), but might also facilitate interaction between myoblasts and myotubes, including fusion and/or alignment.

Whereas the free surfaces of maturing myotubes were N-cadherin positive, the surfaces that apposed other maturing myotubes lacked N-cadherin. This apparent compartmentalization of the membrane surfaces may play a role in allowing maturing myotubes to separate from each other. Such a role is strongly suggested by perturbations observed in the myotube clusters of dTC-treated embryos in which intense N-cadherin immunostaining was present between nearly all myotubes within these clusters, and these maturing myotubes failed to separate from the clusters.

N-cadherin could become absent from membrane surfaces that appose other myotubes by a selective removal of the glycoprotein from these surfaces by lateral movement in the membrane. However, it is probable that anchoring of N-cadherin not only to the cytoskeleton, but also to the opposite cell, might compromise its ability to move. Another possibility is that N-cadherin at these sites would undergo proteolysis or endocytosis. However, no N-cadherin–positive vesicles were observed to suggest the latter mechanism. Alternatively, the N-cadherin observed may be a cleavage product which is synthesized by and released from myoblasts and which becomes bound heterophilically to the free surfaces of the maturing myotubes which have already ceased to synthesize the glycoprotein. Active proteolytic fragments of N-cadherin have been found (Roark et al., 1992), and heterophilic interaction of N-cadherin with another CAM, R-cadherin, has been hypothesized by Redies et al. (1992). Another possibility is that in maturing myotubes, newly synthesized N-cadherin is not inserted into portions of the membranes that form appositions with other cells. However, this would differ from the known localization of other cadherins such as E-cadherin at cell–cell contacts between epithelial cells (Shore and Nelson, 1991; Volk and Geiger, 1984) and desmogleins and desmocollins at desmosomal junctions (Koch et al., 1990; Collins et al., 1991). In any case, N-cadherin is clearly absent from these appositions in control muscles but present following activity blockade. Thus, whatever mechanism is responsible for the distribution of N-cadherin, it is regulated by muscle activity. In addition, the persistence of N-cadherin in these regions following activity blockade may contribute to the failure of myotubes to separate from the clusters.

Regulation of NCAM Isoforms during Primary and Secondary Myogenesis

In denervated adult chicken muscle, which has been reported to express only 125-kD NCAM (Covault et al., 1986), PI-PLC treatment solubilized NCAM that migrated as a single band corresponding to 130 kD (data not shown). Thus the 130-kD NCAM in the present study apparently corresponds to lipid-linked NCAM referred to as 120- or 125-kD NCAM by other investigators (He et al., 1986; Covault et al., 1986; Dickson et al., 1987; Moore et al., 1987; Barton et al., 1988; Hemperly et al., 1986; Covault and Sanes, 1986; Cunningham et al., 1987; Knudsen et al., 1989, 1990*b*). The 145-kD isoform which was not PLC-extractable corresponds to the transmembrane glycoprotein (Owens et al., 1987; Knudsen et al., 1989, 1990b). We also detected a second band at 155 kD that was not removed by PI-PLC and was thus presumably not lipid-linked. The 145- and 155-kD isoforms have recently been detected in chicken thigh muscle by another group (Yoshimi et al., 1993), where both have been judged to be transmembrane based on their reactivity with cytoplasmic domain-specific antibodies. Some molecular weight variations in these reported forms could also be due to variations in glycosylation other than sialylation or to differing phosphorylation states (Sorkin et al., 1984; Rutishauser et al., 1988; Hoffman et al., 1982). However Yoshimi et al. (1993) have reported that in chick thigh muscle from the same embryonic stages that we have studied, the 155-kD isoform has the muscle-specific domain (MSD) insert (Dickson et al., 1987), whereas the 145-kD isoform does not. They also reported that the MSD domain is present in the 130-kD isoform.

In contrast to N-cadherin, myotubes continue to express NCAM until very late in development. However, while total NCAM expression did not vary during most of myogenesis, expression of specific isoforms was differentially regulated during primary and secondary myogenesis. Because antibodies that would distinguish among the different muscle NCAM isoforms were not available, we were unable to determine if primary or secondary myotubes differentially expressed the different isoforms on their surfaces. However, immunoblot analysis revealed that expression of the 130-kD lipid linked isoform increased markedly during secondary myogenesis. These findings are similar to those made by Covault and Sanes (1986), Tassin et al. (1991), and Moore et al. (1987), in developing mouse and chick muscle (see also Roubin and Goridis, 1992).

Although some of these studies, especially those in culture, have been interpreted to indicate that the 145-kD transmembrane isoform is expressed in myoblasts whereas the 130-kD lipid-linked form predominates in myotubes, our observations are more compatible with the 145-kD isoform being expressed in primary myotubes, and the 130-kD isoform being associated with secondary myoblasts and the myotubes they give rise to (see also Yoshimi et al., 1993). Consistent with this possibility, transfecting C2 myoblasts with lipid-linked NCAM enhanced fusion (Dickson et al., 1990), and PLC extraction or immunological blocking of PI-linked pro-

teins on myoblast surfaces inhibited fusion (Knudsen et al., 1989).

As reported by Yoshimi et al. (1993) we found the 155-kD isoform to be transiently expressed, being high between St 32-38 and then being down regulated along with the 145-kD isoform later in development. The tightly and differentially regulated expression of all three isoforms during in vivo myogenesis suggests that they may be playing distinct roles. Histological localization of each of these isoforms at different stages of myogenesis at the light and electron microscopy level would be helpful in suggesting some of the potential roles they might be playing.

Since no switch in expression of the 145- to the 130-kD form of NCAM occurs in activity blocked muscles, this regulation, due to alternative splicing, appears to be dependent on functional innervation. Secondary myogenesis does occur in dTC-treated muscles, but the normal exponential increase in secondary myotube formation is severely reduced (Fredette and Landmesser, 1991*b*). Therefore this reduction in the number of differentiating secondary myoblasts may be responsible for the decreased quantity of lipid-linked NCAM observed in activity-blocked muscle.

Polysialylation of Particular NCAM Isoforms

Of the major isoforms of NCAM expressed by developing muscle, only the 155-kD transmembrane and the 130-kD lipid-linked molecules were polysialylated. Since our electron microscopy analysis revealed that individual cells express both non-polysialylated and polysialylated NCAM, the heterogeneity of polysialylation would have to be explained either by a sialytransferase differentially recognizing specific isoforms or by a temporal regulation of the PSA biosynthetic pathway. In the latter case, the earliest NCAM isoforms synthesized (145 and a portion of the 155 kD) would escape polysialylation, whereas the isoforms that are synthesized later (the 130- and a portion of the 155-kD form) would be polysialylated, as synthetic activity is increased. In developing chicken brain, Breen and Regan (Breen et al., 1987; Breen and Regan, 1988) have also observed that 140-kD NCAM is not polysialylated while other NCAM isoforms carry PSA. This was also attributed to developmental upregulation of PSA synthesis after expression of the 140-kD NCAM. Although the simplest explanation compatible with our data is a developmentally regulated biosynthetic pathway for PSA that can use all forms of NCAM, other possibilities can not be rigorously excluded until metabolic labeling studies are performed. In developing retina (Bartsch et al., 1990) the 140-kD form of NCAM also escapes polysialylation. However, in other systems, such as the developing spinal cord and sciatic nerve (Hoffman et al., 1982; Rutishauser et al., 1983, 1988; Finne et al., 1983; Tang et al., 1992) and in Wilms tumor cells (Zuber and Roth, 1990), the 140-kD NCAM is polysialylated. Although little is known about the synthetic pathway for PSA in muscle, our study clearly indicates that PSA synthesis is regulated either directly or indirectly by nerve activity, since the usual marked upregulation of PSA did not occur following activity blockade, even though some of the 130-kD isoform was still synthesized.

The pattern of PSA regulation in muscle suggests that polysialylation is actively involved in the breaking of cell–cell bonds during myotube separation. This proposal is supported by the fact that in other systems PSA appears to regulate cell–cell interactions by acting as a physical impediment to molecular and/or membrane interactions (Acheson et al., 1991; Rutishauser et al., 1988; Landmesser et al., 1988, 1990; Tang et al., 1992; Zhang et al., 1992). In the present case, while down regulation of N-cadherin could prevent future adhesion between myotubes, the introduction of large, bulky PSA groups into the intracellular space could promote the initial separation of the cells. The absence of polysialic acid on dTC-treated myotubes which fail to separate from clusters is consistent with this interpretation. Furthermore, myotube clusters increase in size between St 28 and 32, and then decrease to remain at a constant size through St 39, even though new myotubes continue to form within the clusters (unpublished observations). Some mechanism must therefore exist to maintain the cluster size, and to prevent the clusters from becoming increasingly large, as occurs in the dTC-treated embryos. We have observed that virtually all of the 130-kD lipid-linked NCAM is polysialylated at St 38 (after which 130-kD NCAM but not PSA immunostaining persists) and that the expression of 130-kD NCAM and patches of PSA immunoreactivity can be observed at St 32. Therefore expression of polysialylated NCAM, along with a down regulation of N-cadherin, may mediate the separation of myotubes from clusters as they form and mature.

Several groups have observed that PSA can be restricted to specific regions of NCAM-positive cells (Bartsch et al., 1990; Rougan et al., 1993). In cultured hippocampal neurons NCAM is expressed on both axons and dendrites but polysialic acid is restricted to axons and is concentrated near the peripheral regions of the axon and growth cone (Kim, W. T., W. F. Collins, and A. N. Van Den Pol, 1992, *Soc. Neurosci. Abst.* 18:944). In Wilms tumor cells PSA is sparse or absent at cell–cell appositions where NCAM is present (Zuber and Roth, 1990). In our studies, we observed that virtually all of the 130-kD NCAM isoform contains PSA. It follows, therefore, that this isoform must be restricted to the PSA-immunostained locations on cell surfaces. This raises the intriguing possibility that the 130-kD NCAM may be targeted to the free surfaces of developing muscle cells by virtue of the molecule's GPI anchor, which is known to serve as a targeting signal for glycoproteins in epithelial cells and in hippocampal neurons (Dotti et al., 1991; Dotti and Simons, 1990; Lisanti et al., 1988; Brown et al., 1989), and also for lipid-linked NCAM transfected into epithelial cells (Powell et al., 1991). However, preliminary observations of the distribution of another GPI-linked molecule, T-cadherin, failed to show any such compartmentalization on developing myotubes (Fredette, B., unpublished observations). Since no PSA was observed at any stage on surfaces internal to the cluster, diffusion of the molecule to the outer surfaces of the cluster is unlikely. It is not possible to draw any conclusions about localization of the other NCAM isoforms, since they are either not polysialylated (145-kD NCAM) or only a portion is polysialylated (155-kD NCAM). Thus, these forms may be distributed on any of the NCAM-positive muscle surfaces.

After St 38, polysialic acid immunostaining of muscle cells rapidly decreases whereas the 130-kD NCAM remains predominant. Therefore either PSA synthetic activity is rapidly down regulated while 130-kD NCAM continues to be

synthesized, or some mechanism exists at the muscle surface either to remove PSA from NCAM or to remove the entire polysialylated NCAM molecule from the myotube surface. In either case, the PSA present on the myotube surface at St 38 must be replaced with non-polysialylated NCAM within 24 h, which makes simple replacement by protein turnover unlikely. Although no evidence for endogenous endoneuraminidase has been reported in developing muscle, this possibility remains to be explored further. Another possibility is that the GPI-linked, PSA-containing NCAM could be shed into the extracellular space. Indeed some of our EM observations and others from Wilms tumor cells (Zuber and Roth, 1990) where the PSA immunoreaction product appears as a thick fuzzy coat is at least suggestive of this possibility. In Aplysia sensory neurons, apCAM, another member of the Ig superfamily with some homology to NCAM, can be removed by endocytotic removal of the molecule from the surface (Dailey et al., 1992). As in their study, we have also observed coated vesicles that are NCAM immunopositive and which could be involved in removing NCAM from the cell surface. However, these were not PSA immunopositive.

In summary, this study has provided a detailed description of N-cadherin and NCAM isoform expression in relation to the cell interactions which occur during primary and secondary myogenesis. We had previously observed that although both primary and secondary myogenesis occurs in the hindlimbs of dTC-treated embryos, the quantity of both types of myotubes was drastically reduced (Fredette and Landmesser, 1991b). The present study has shown that the expression of NCAM, PSA, and N-cadherin are also perturbed in muscles that develop in the absence of functional innervation, leading us to suggest that although both N-cadherin and NCAM adhesion are essential for the formation of myotubes (Knudsen et al., 1989, 1990*a*,*b*), their continued expression and regulation is also essential to later stages of myogenesis. One of these later events, namely separation of myotubes from clusters, is clearly dependent on nerve activity. Neuromuscular blockade also perturbs CAM expression by abolishing PSA expression, by preventing an NCAM isoform switch that normally occurs after St 32, and by delaying the down regulation of N-cadherin at myotube–myotube appositions. Whether or not these perturbations in CAM expression are directly responsible for preventing the separation of myotubes from the clusters, and perhaps ultimately in reducing the number of myotubes that are able to form in a muscle, remains to be directly tested. Although PSA has been shown to attenuate the cell–cell interactions mediated by several cell adhesion molecules including NCAM, it is not known if N-cadherin function can be similarly affected. Experiments are currently in progress to enzymatically remove PSA from NCAM and to block N-cadherin function with antibodies during in ovo development. In preliminary experiments, removal of PSA alone, by injection of endo-N, did not result in the greatly enlarged clusters caused by activity blockade. Thus the normal down regulation of N-cadherin, together with the upregulation of PSA may both be required to allow myotubes to separate from clusters. In this case it may be necessary to interfere with several adhesion systems simultaneously to mimic the effect of activity blockade.

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