Molecular Forms of N-CAM and Its RNA in Developing and Denervated Skeletal Muscle

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Abstract. The neural cell adhesion molecule (N-CAM) is present in both embryonic and perinatal muscle, but its distribution changes as myoblasts form myotubes and axons establish synapses (Covault, J., and J. R. Sanes, 1986, *J. Cell Biol.*, 102:716–730). Levels of N-CAM decline postnatally but increase when adult muscle is denervated or paralyzed (Covault, J., and J. R. Sanes, 1985, *Proc. Natl. Acad. Sci. USA.*, 82:4544–4548). To determine the molecular forms of N-CAM and N-CAM-related RNA during these different periods we used immunoblotting and nucleic acid hybridization techniques to analyze N-CAM and its RNA in developing, cultured, adult, and denervated adult muscle.

As muscles develop, the extent of sialylation of muscle N-CAM decreases, and a 140-kD desialo form of N-CAM (generated by neuraminidase treatment) is replaced by a 125-kD form. This change in the apparent molecular weight of desialo N-CAM is paralleled by a change in N-CAM RNA: early embryonic muscles express a 6.7-kb RNA species which hybridizes with N-CAM cDNA, whereas in neonatal muscle this form is largely replaced by 5.2- and 2.9-kb species. Similar transitions in the desialo form of N-CAM, but not in extent of sialylation, accompany differentiation in primary cultures of embryonic muscle and in cultures of the clonal muscle cell lines C2 and BC3H-1. Both in vivo and in vitro, a 140-kD desialo form of N-CAM and a 6.7-kb N-CAM RNA are apparently associated with myoblasts, whereas a 125-kD desialo form and 5.2- and 2.9-kb RNAs are associated with myotubes and myofibers.

After denervation of adult muscle, a ~12–15-fold increase in the levels of N-CAM is accompanied by a ~30–50-fold increase in N-CAM RNA, suggesting that N-CAM expression is regulated at a pretranslational level. Forms of N-CAM and its RNA in denervated muscle are similar to those seen in perinatal myofibers.

THE neural cell adhesion molecule (N-CAM)[1] occurs in a variety of molecular forms that are selectively expressed at various stages of development and in various regions of the nervous system. Highly sialylated forms, prevalent in embryonic and neonatal brain, are gradually replaced by less sialylated forms as development proceeds (3, 7, 28). In adults, N-CAM exists as a family of related glycoproteins (~180, ~140, and ~120 kD in rodents) which appear to differ mainly in the length of their cytoplasmic and/or transmembrane domains (8, 10). The relative abundance of these three forms varies during development and among brain regions (3, 29). Finally, within individual molecular weight classes, additional heterogeneity has been detected using monoclonal antibodies (3, 14, 34). Some of the variation in N-CAM form may arise at the RNA level, in that N-CAM RNA exists in multiple species, which vary in their relative and absolute abundances during development (9, 11). To understand how N-CAM regulates and is regulated by developmental interactions, it will be important to discover the factors that determine which forms of N-CAM and its RNA are expressed by particular cells and at particular stages.

Although N-CAM has been analyzed in greatest detail in brain, we believe that muscle provides several advantages for studies of the cell and molecular biology of this molecule. The neuromuscular junction is perhaps the most accessible and best characterized of all synapses, and a wealth of information is available about its structure and function. Furthermore, the development of the neuromuscular junction and of muscle per se have been studied intensively, and several mechanisms by which nerve and muscle influence each other's development have been discovered (26, 31). Finally, we have provided a detailed description of the distribution of N-CAM in developing and adult muscle (6) and have shown that N-CAM levels increase when muscle is denervated or paralyzed and thus are regulated trans-synaptically, through an activity-dependent mechanism (5). Here, to begin to study the molecular biology of N-CAM in muscle, we have asked (*a*) which forms of N-CAM and its RNA are present in

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; E, embryonic day; N-CAM, neural cell adhesion molecule; P, postnatal day.

developing muscle; (*b*) whether transitions among forms observed in vivo also occur in vitro; and (*c*) whether the denervation-induced increase in N-CAM levels is regulated at the RNA level.

Materials and Methods

Animals

Timed pregnant Sprague-Dawley rats were obtained from Chappel Breeders (St. Louis, MO). The first day of pregnancy was designated embryonic day 0 (E0), and the day of birth, E21–E22, is also referred to as postnatal day 0 (P0). Diaphragms of adult male rats (150–200 g) were denervated by cutting the phrenic nerve intrathoracically (19). The hindlimbs of adult female Swiss mice (30–40 g) were denervated by cutting the sciatic nerve in the thigh.

Cell Cultures

For primary cultures, forelimbs of E19 rats were dissociated to prepare muscle cultures as described by Sanes and Lawrence (32). The clonal cell lines BC3H-1 (24) and C2 (35) were maintained in log phase by trypsination and replating in Dulbecco's modified Eagle's medium containing 10% fetal calf plus 10% newborn calf serum. BC3H-1 cells were replated at 2×10^4 cells/ml and C2 cells were replated at 5×10^3 cells/ml in 15-cm gelatin-coated culture dishes containing 25 ml medium. To induce fusion and differentiation of C2 cells, cultures were refed with medium containing 10% horse serum on day 4; BC3H-1 cells were not re-fed.

Immunoblot Analysis

Detergent extracts were prepared by homogenizing tissues in 4 vol phosphate-buffered saline (PBS) containing 1% Nonidet P-40, 100 μg/ml leupeptin (Sigma Chemical Co., St. Louis, MO), 0.6 trypsin inhibitor units/ml Aprotinin (Sigma Chemical Co.), 1 mM phenylmethylsulfonyl chloride, and 1 mM EDTA and then centrifuging at 25,000 *g* for 15 min. The protein concentration of supernatants was determined using the Pierce BCA reagent (Pierce Chemical Co., Rockford, IL) and bovine serum albumin (BSA) as standard. Aliquots of supernatants were mixed with sample buffer (final concentrations: 2% SDS, 10 mM dithiothreitol, 5% glycerol, 20 mM Tris-HCl, pH 6.8), heated to 56°C for 5 min, and electrophoresed in 0.5-mm-thick SDS/7% polyacrylamide gels (16). Molecular mass markers used to calibrate the gels were: myosin, 200 kD; *Escherichia coli* RNA polymerase β-subunit, 160 kD; β-galactosidase, 116 kD; *E. coli* RNA polymerase δ-subunit, 90 kD; pyruvate kinase, 57 kD. Proteins were transferred from the gels to nitrocellulose filters (BA 80, Schleicher & Schuell, Keene, NH) in 96 mM glycine/12.5 mM Tris/20% MeOH for 60–90 min at a field strength of 5 V/cm (33). N-CAM was detected by the sequential incubation of filters with rabbit anti-rat-N-CAM (6), peroxidase-conjugated second antibody (Cappel Laboratories, Cochranville, PA), and 0.01% diaminobenzidine/0.005% H_2O_2/25 mm sodium citrate, pH 6. The peroxidase reaction product was intensified as describd by Newman et al. (23). Molecular weight markers were detected using Auro Dye (Janssen Pharmaceutica, Inc., Piscataway, NJ).

To remove sialic acid, Nonidet P-40 extracts were treated with neuraminidase from *Clostridium perferigens* (1 U/ml, Sigma Type X) in 25 mM Na acetate, pH 5, for 30 min at 37°C. These conditions were deemed adequate for complete desialylation of muscle N-CAM in that quadrupling the concentration of neuraminidase and doubling the time of incubation did not affect the molecular weight or amount of digestion products detected by immunoblotting. As a control, duplicate samples were incubated with neuraminidase in the presence of 20 mM 2-deoxy-2,3-dehydro-N-acetyl neuraminic acid (Boehringer Mannheim Biochemicals, Indianapolis, IN), a specific inhibitor of neuraminidase activity (17). Samples were then mixed with sample buffer and analyzed as described above.

Peptide Mapping

N-CAM was affinity purified (6) from neuraminidase-treated Nonidet P-40 extracts of BC3H-1 3-d cultures, P0 rat intercostals, or adult rat brain and electrophoresed on 7% polyacrylamide gels. Coomassie Blue–stained bands containing 1–2 μg protein were cut out and digested with 75 ng *Staphylococcus aureus* V8 protease (Boehringer Mannheim Biochemicals) as described by Cleveland et al. (4). Digestion products were separated by electrophoresis on 15% polyacrylamide gels and detected by silver staining. Concanavalin A (26 kD) and avidin (17 kD) were used as molecular mass markers.

Preparation and Hybridization of RNA

After dissection, tissue samples were immediately frozen in liquid N_2. Total RNA was extracted from pooled samples by homogenization in guanidine hydrochloride and then fractionated by electrophoresis, transferred to filters, and hybridized with nick-translated total plasmid DNA, as described previously (18). Hybrids were detected by autoradiography and quantitated by densitometry of appropriately exposed autoradiographs. cDNA-containing plasmids used were pM1.3 for N-CAM (11) and p6H for the AChR δ-subunit (15). Sizes were assigned to RNAs hybridizing with pM1.3 as described in reference 9.

Enzyme-linked Immunosorbent Assay

Nonidet P-40 extracts were diluted in PBS, and 400-μl aliquots were filtered through nitrocellulose sheets using a 96-well filtration apparatus (Schleicher & Schuell). The sheets were incubated sequentially with 10 mg/ml BSA in PBS, rabbit anti-rat-N-CAM antibodies in PBS-BSA, and peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) in PBS containing 50% normal goat serum. Filters were washed in PBS, 0.05% Tween-20 for 30 min and cut into 96 individual squares, which were then incubated separately in tubes containing 1.2 ml of 0.04% o-phenylenediamine, 0.01% H_2O_2, 0.1 M Na citrate, pH 5 for 40 min. The reaction was stopped by the addition of 0.6 ml of 4 N H_2SO_4, and the optical density at 492 nm was measured. For each sample, a series of dilutions containing 0–10 μg protein was filtered in triplicate. Optical densities were corrected by subtracting values obtained from identically prepared filters incubated with nonimmune rabbit serum. Affinity-purified adult rat brain N-CAM (6) was used to prepare a standard curve for each experiment.

Results

Molecular Forms of N-CAM in Developing Muscle

Immunohistochemical studies have demonstrated that N-CAM is present in both embryonic and perinatal muscle but that its distribution changes as myoblasts fuse to form myotubes, axons establish synapses, and myotubes and synapses mature (see Figs. 8–11, in reference 6). To learn whether changes in the distribution of N-CAM are accompanied by alterations in its molecular form, we prepared detergent extracts from E15, E17, and P0 muscles and analyzed them by immunoblotting.

On immunoblots of developing muscle, antibodies to N-CAM stained a band of M_r 140,000, which sometimes appeared as a doublet, and a smear extending from ~140 to ~220 kD (Fig. 1*A*, lanes *1–3*). Similar results have been reported for chicken (30) and mouse (27) muscle N-CAM, whereas human muscle N-CAM has been reported to have an apparent molecular mass of ~180 kD (20). The average apparent molecular weight of the smear decreased during development, whereas the fraction of the immunoreactive material migrating at 140 kD increased. This developmental transition is similar to that previously observed in brain (3, 12, 28), in which a high molecular weight smear is replaced by sharper, lower molecular weight bands (Fig. 1*B*, lanes *1* and *2*); differences are that the diffuse material is of higher apparent molecular weight in brain (M_r ~180,000–250,000) than in muscle, and that adult brain contains bands of M_r 120,000 and 180,000 as well as 140,000. Thus, in muscle as in brain, N-CAM changes in form as development proceeds.

In embryonic brain, the dispersity and high molecular weight of N-CAM is known to be due to a high degree of sialylation (7, 28). To determine whether embryonic muscle N-CAM is also sialylated, extracts were incubated with neuraminidase to remove sialic acid before being submitted to immunoblotting. Neuraminidase treatment resulted in the loss of the high molecular weight heterodisperse material and the appearance of discrete bands at 140 and 125 kD (Fig. 1*A*, lanes *5–7*); these changes were not seen when 2-deoxy-2,3-

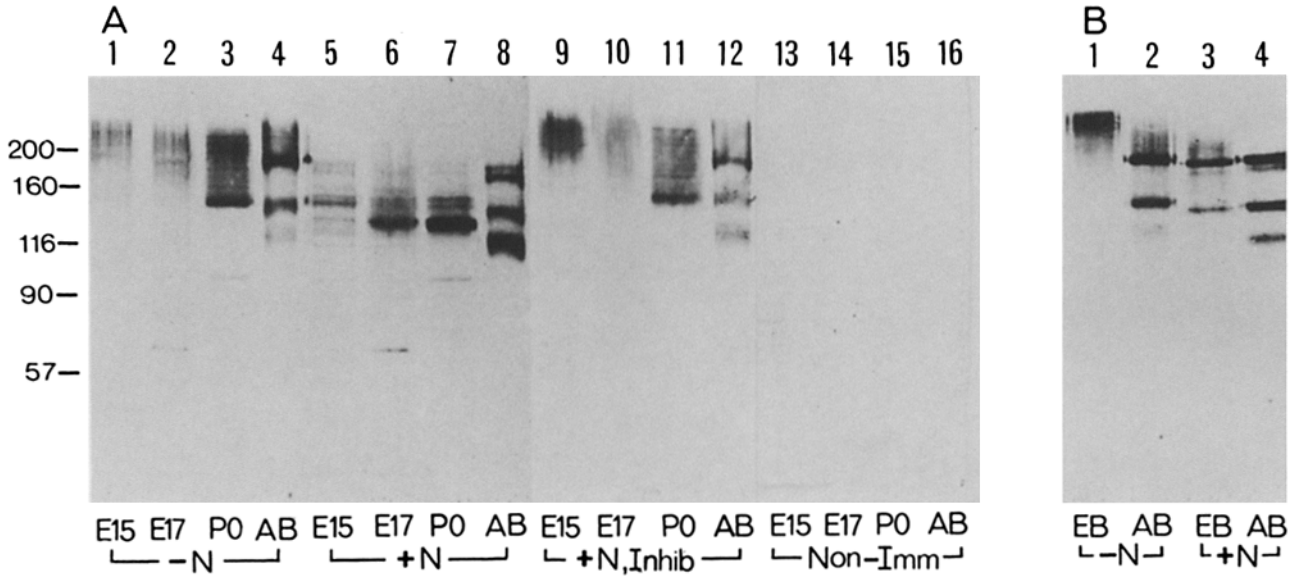

Figure 1. (*A*) Immunoblot analysis of native and neuraminidase-treated N-CAM in developing muscle. Nonidet P-40 extracts of E15 (lanes *1*, *5*, and *9*), E17 (lanes *2*, *6*, and *10*), or P0 (lanes *3*, *7*, and *11*) rat muscle or of adult rat brain (lanes *4*, *8*, and *12*) were probed with anti-N-CAM serum and peroxidase-conjugated second antibody. Samples in lanes *5–8* were incubated with neuraminidase to remove sialic acid, and samples in lanes *9–12* were incubated with both neuraminidase and 2-deoxy-2,3-dehydro-N-acetylneuraminic acid, a specific inhibitor of neuraminidase activity. Lanes *13–16* are identical to lanes *9–12* but were probed with nonimmune serum. The amount of sialic acid associated with muscle N-CAM decreases during development. Neuraminidase digestion produces two major desialo N-CAMs, of 140 and 125 kD; E15 muscle contains predominately the 140-kD form and P0 muscle, the 125-kD form. (*B*) Nonidet P-40 extracts of E16 (lanes *1* and *3*) or adult (lanes *2* and *4*) rat brain probed with anti-N-CAM and peroxidase second antibody. Samples in lanes *3* and *4* were treated with neuraminidase. Embryonic brain N-CAM contains large amounts of sialic acid. Adult brain contains a 120-kD native form and a ~115-kD desialo form of N-CAM not found in embryonic brain. Muscle samples contained 30 μg protein and brain samples 8 μg protein per lane.

dehydro-N-acetyl neuraminic acid, a specific inhibitor of neuraminidase (17), was included in the incubation mixture (Fig. 1*A*, lanes *9–11*). Thus, muscle N-CAM, is highly sialylated.

Both the 140- and 125-kD forms of muscle N-CAM generated by neuraminidase are electrophoretically distinguishable from the desialo forms generated from brain N-CAM (cf., Fig. 1*A*, lanes *7* and *8*). It was therefore important to confirm that both muscle forms were, in fact, authentic N-CAM. To this end, we isolated both desialo muscle forms as well as 135- and 115-kD desialo brain N-CAM by affinity chromatography and preparative gel electrophoresis, subjected them to limited proteolysis, and used gel electrophoresis and silver staining to compare the peptides generated. Fig. 2 shows that the 140- and 125-kD desialo forms from muscle were both highly related to desialo brain N-CAM. A second concern was that the smaller (125 kD) form might have been created artifactually (e.g., by proteolysis) from the larger (140 kD) form during the digestion with neuraminidase. However, several observations demonstrate that this is probably not the case. First, generation of the 125-kD band was blocked by a specific inhibitor of neuraminidase (Fig. 1*A*, lanes *9–11*). Second, this form was observed in both rat intercostal and diaphragm muscle extracts as well as in chicken muscle (not shown) and in cultured mammalian muscle (see below). Third, neuraminidase treatment generated 140- and 125-kD material from N-CAM that had been depleted of degradative enzymes which might be present in the crude extracts by affinity purification on anti-N-CAM-agarose before neuraminidase treatment. Finally, the formation of the 125-kD band was not prevented by boiling the muscle extracts to inactivate proteases before treating them with neuraminidase, or by including a variety of protease inhibitors in the incubation. Thus, muscle contains two distinct "core" (desialo) forms of N-CAM. We do not know whether the desialo forms differ from each other and from brain forms in primary sequence and/or in post-translational modifications.

Comparison of desialylated N-CAM from muscles of different ages (Fig. 1, lanes *5–7*) revealed that the proportions of 140- and 125-kD forms varied during development: the 140-kD form predominated at E15, whereas the 125-kD form predominated at P0. Thus, the proportions of desialo forms of N-CAM as well as the extent of their sialylation are developmentally regulated in muscle.

Molecular Forms of N-CAM in Cultured Muscle

On E15, when muscle N-CAM is highly sialylated and predominantly of the 140-kD desialo form, myoblasts are abundant, myotubes have just begun to form, and synaptic transmission is newly established. By P0, when muscle N-CAM is less sialylated and predominantly of the 125-kD desialo form, muscles are depleted of myoblasts, myotubes are abundant, and synapses have matured. Thus, the observed molecular alterations in N-CAM might reflect changes in the cellular composition of the muscle, formation of myotubes from myoblasts, maturation of myotubes, an influence of innervation on the developing muscle, and/or changes in the amount of the small but unavoidable contamination of our muscle extracts with nerve N-CAM. To test these ideas, we determined the forms of N-CAM synthesized by muscle cells in culture.

Cells dissociated from embryonic muscle divide and fuse

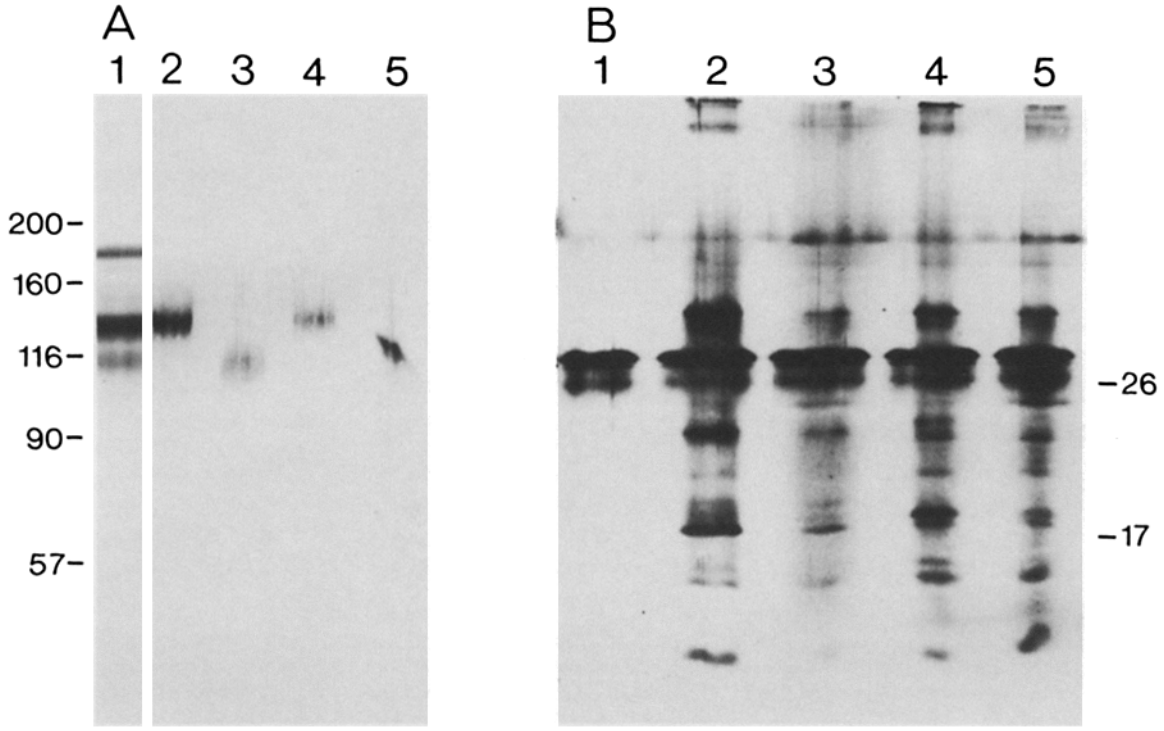

Figure 2. V8 protease digests of brain and muscle N-CAMs. (*A*) Desialo N-CAMs before digestion, visualized by silver staining. Lane *1* contains total desialo adult brain N-CAM, whereas subsequent lanes contain individual desialo forms isolated by preparative gel electrophoresis: 135-kD brain N-CAM (lane *2*); 115-kD brain N-CAM (lane *3*); 140-kD muscle N-CAM (from 3-d BC3H-1 cultures [see below], lane *4*); and 125-kD muscle N-CAM (from P0 intercostals, lane *5*). (*B*) Peptides produced by V8 protease digestion (reference 4) of individual desialo forms. Lane *1* contains enzyme only; lanes *2–5*, as in *A*.

in culture to form striated, contractile myotubes; myoblasts in the cultures begin to fuse 2 d after they are plated, and fusion is essentially complete by day 5. N-CAM is present on the surface of myoblasts in 2-d cultures, and on all myotubes and some mononucleated cells in postfusion cultures (see Fig. 12 in reference 6). Immunoblotting showed that in both 2- and 7-d cultures, N-CAM was present in both ~140-kD and higher molecular mass heterodisperse forms (Fig. 3, lanes *1* and *2*). Treatment with neuraminidase revealed that the predominant desialo forms in 2- and 7-d cultures were 140 and 125 kD, respectively (Fig. 3, lanes *3* and *4*). Thus, the 140-kD desialylated form appears to be associated with myoblasts, whereas the 125-kD desialylated form appears to be associated with myotubes in vitro. Furthermore, these results indicate that the transition from a 140- to a 125-kD desialo form can occur in the absence of innervation.

Although primary cultures offer a simpler system than fresh muscle for study, they still contain a heterogeneous population of cells. To ask whether transitions in N-CAM forms can occur in a single cells, we used two clonal muscle cell lines, C2 and BC3H-1. C2 cells fuse to form spontaneously contracting myotubes in culture and acquire patches rich in AChRs and acetylcholinesterase upon fusion (13). BC3H-1 cells do not fuse but acquire many other aspects of a differentiated muscle phenotype with time in culture (24). In both cell lines, 3-d (nominally undifferentiated) cultures contained predominantly the 140-kD desialo form of N-CAM, and 7-day (differentiated) cultures contained predominantly the 125-kD desialo form (Fig. 3, lanes *7, 8, 11*, and *12*). Thus, the transition from 140- to 125-kD forms accompanies differentiation of clonal cell populations and probably occurs within single cells.

Whereas the developmental transition from 140- to 125-kD desialo form in vitro resembled that seen in vivo, the transition from more to less sialylation did not occur in culture. In fact, in both primary and clonal cell cultures, N-CAM was more highly sialylated in differentiated (7 d) cultures than in undifferentiated (2 or 3 d) cultures (Fig. 3, lanes *1, 2, 5, 6, 9*, and *10*). This result demonstrates that both 125- and 140-kD forms can be heavily sialylated and that transitions in extent of sialylation and between core forms can be independently regulated.

Molecular Forms of N-CAM-related RNA in Developing and Cultured Muscle

We used a clonal cDNA probe specific for N-CAM mRNA (pM 1.3; 9) and techniques of blot hybridization to determine the species of N-CAM RNA present in developing and cultured muscle. As previously described (9, 12), pM 1.3 detected RNA species of 7.4, 6.7, 5.2, and 2.9 kb in extracts of adult brain (Fig. 4, lane *2*); the two larger species were abundant but the two smaller species barely detectable in embryonic brain (Fig. 4, lane *1*). Unlike Goridis et al. (11) and Gennarini et al. (9), we did not detect a 4.3-kb species in either developing or adult brain. Because we do not know the coding potential of any of these species, we refer to them as N-CAM-related RNA or N-CAM RNA, rather than as mRNA.

Developing and cultured muscle contained RNAs of 6.7, 5.2, and 2.9 kb that were recognized by pM 1.3 (Fig. 4, lanes *3–6*). We presume that these species are N-CAM RNAs because their recognition by pM 1.3 is probe specific, they were absent from N-CAM poor tissues such as liver, and they

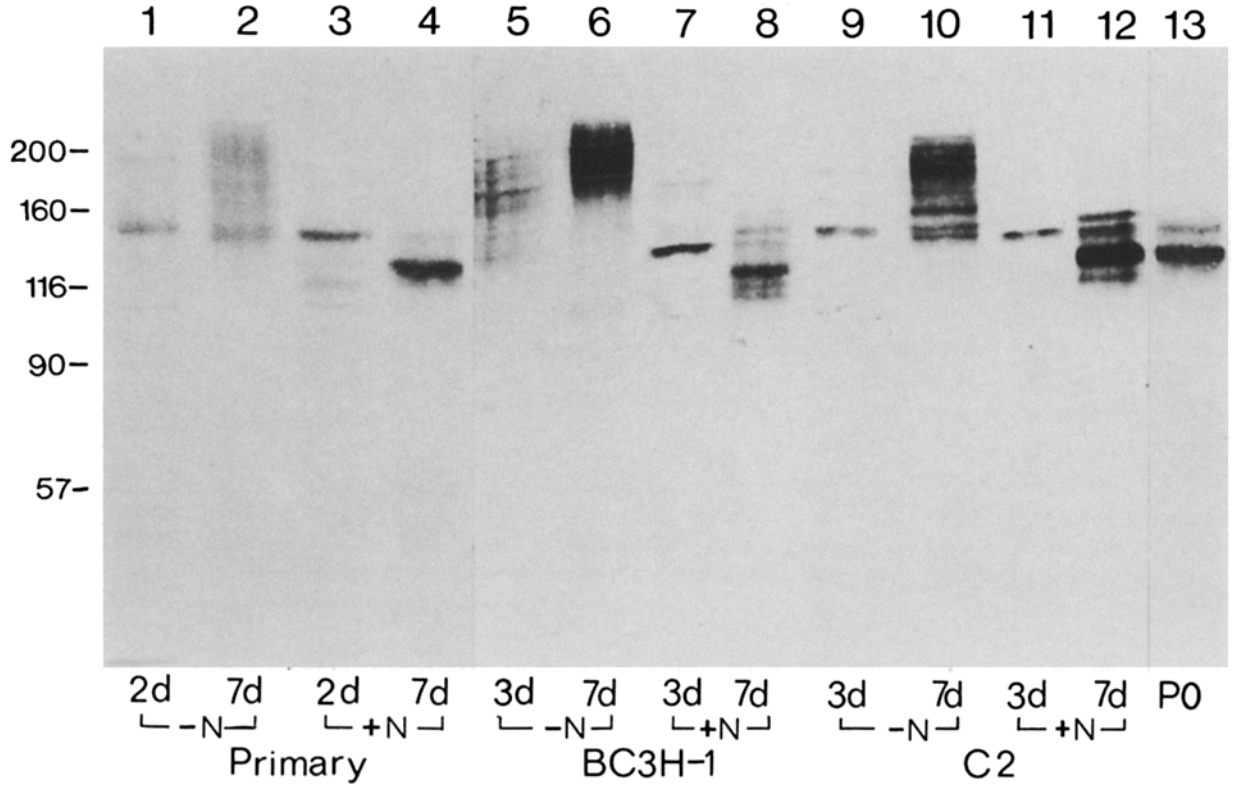

Figure 3. Immunoblot analysis of native and neuraminidase-treated N-CAM from muscle cultures. Nonidet P-40 extracts of primary muscle cultures containing myoblasts (lanes *1* and *3*) or mature myotubes (lanes *2* and *4*), 3-d (lanes *5* and *7*) or 7-d (lanes *6* and *8*) cultures of BC3H-1 cells, or 3-d (lanes *9* and *11*) or 7-d (lanes *10* and *12*) cultures of C2 cells were probed with anti-N-CAM serum and peroxidase second antibody. Samples in lanes *3, 4, 7, 8, 11,* and *12* were treated with neuraminidase. Lane *13* contains neuraminidase treated N-CAM from P0 diaphragm, for comparison. Differentiation of muscle in both primary cultures and in the clonal cell lines BC3H-1 and C2 is accompanied by a change in the apparent molecular mass of desialo N-CAM from 140 to 125 kD. Samples contained 30 μg (lanes *1–4*), 50 μg (lanes *5–12*), or 25 μg (lane *13*) protein.

migrated with N-CAM-specific RNAs from brain on agarose gels. No RNA of 7.4 kb was detected in muscle with pM 1.3. The relative proportions of the three N-CAM RNA species detected changed during development, both in vivo and in vitro. Thus, the 6.7-kb species was far more abundant than the 5.2- and 2.9-kb species in E15 muscle, and 3-d BC3H-1 cells. In contrast, the 5.2- and 2.9-kb RNAs were more abundant than the 6.7-kb form in perinatal (E21-P0) muscle, 7-d primary cultures, and 7-d BC3H-1 (Fig. 4, lanes *3–6*, and Table I). Thus, for N-CAM-related RNA as for N-CAM, multiple molecular forms are present in muscle, and the relative proportions of the forms change during development.

N-CAM and Its RNA in Denervated Adult Muscle

Levels of N-CAM, which are low in normal adult muscle, increase after denervation (2, 5, 27). Immunoblotting revealed that N-CAM in denervated muscle is similar in form to the N-CAM in perinatal muscle: it is predominantly a 140-kD form, with some heterodisperse material of higher molecular mass, and is converted to a major band of 125 kD by neuraminidase (Fig. 5*A*, lanes *1–6*). There is too little N-CAM in normally innervated adult muscle to detect reliably on immunoblots; however, concentration of adult N-CAM using anti-N-CAM agarose reveals that it contains both 140- and 125-kD desialo forms (Fig. 5*A*, lane *8*). Analysis by enzyme-linked immunosorbent assay demonstrated that levels of N-CAM increase 12–15-fold (12-, 13-, 13-, and 15-fold in four assays) during the first 2 wk after denervation (Fig. 5*B*): detergent extracts of innervated and 7–10-d-denervated rat diaphragm contained 0.036 ± 0.04 and 0.48 ± 0.04 ng N-CAM/μg protein, respectively. In comparison, we found ~8.7 ng/μg protein in extracts of adult brain.

To determine whether the induction of N-CAM by denervation is regulated at a pretranslational step, we used pM 1.3 to detect N-CAM-related RNA in innervated and denervated rat muscle. N-CAM RNA was barely detectable in normal muscle but increased markedly in abundance after denervation (Fig. 6*A*). As was the case for N-CAM protein, N-CAM RNA in denervated muscle resembled N-CAM RNA from P0 muscle (i.e., the 5.2- and 2.9-kb species were more abundant than the 6.7-kb species). Densitometry of appropriately exposed autoradiographs showed that the abundance of the 6.7-kb form increased only twofold, whereas the 5.2- and 2.9-kb species increased ~30–50-fold by 10–14 d after denervation. Thus, an increase in N-CAM RNA accompanies and presumably underlies the induction of N-CAM after denervation.

We have previously shown that levels of acetylcholine receptor (AChR) α-subunit mRNA increase after denervation of mouse leg muscle; this increase is sufficient in speed and magnitude to account for the development of denervation supersensitivity in skeletal muscle (18). To determine whether levels of N-CAM RNA and AChR mRNA are similarly regulated after denervation, we compared the levels of N-

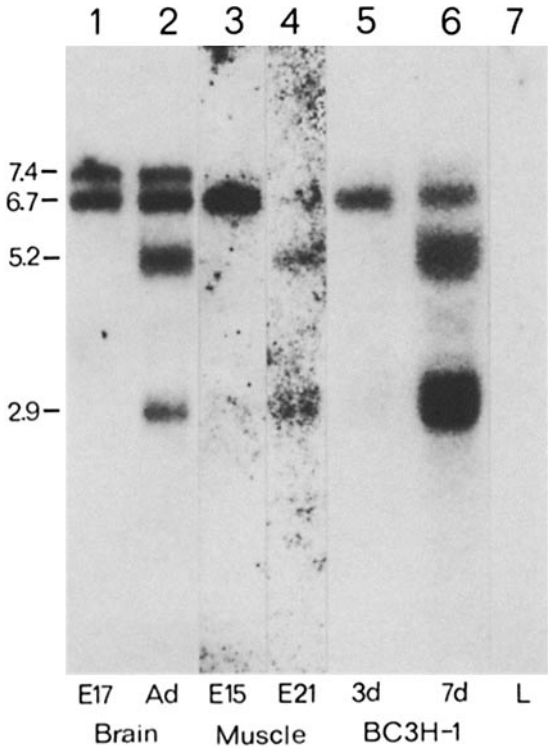

Figure 4. Blot hybridization of nick-translated N-CAM cDNA to muscle RNA. RNA isolated from E17 (lane *1*) or adult rat brain (lane *2*), from E15 (lane *3*), or E21 (lane *4*) rat intercostals, from undifferentiated (lane *5*) or differentiated (lane *6*) cultures of BC3H-1 cells, and from rat liver (lane *7*) was fractionated by gel electrophoresis, transferred to filters, incubated with ^{32}P-pM1.3-DNA specific for N-CAM, and exposed to x-ray film. All lanes contain 5 μg total RNA. E15 intercostals and undifferentiated muscle cultures contain predominately a 6.7-kb N-CAM RNA species, whereas P0 intercostals and differentiated cultures contain largely 5.2- and 2.9-kb species. Adult brain contains RNAs of 7.4, 6.7, 5.2, and 2.9 kb, which hybridize to the N-CAM cDNA probe; embryonic brain contains only the 7.4- and 6.7-kb forms. Liver RNA (which contained readily detectably β-actin mRNA in parallel experiments) contained no detectable N-CAM RNA.

CAM RNA in mouse leg muscles denervated for 1, 4, or 8 d, and on duplicate gels we determined the abundance of AChR δ-subunit mRNA, using a cloned cDNA probe specific for this subunit (15). Fig. 6, *B* and *C* show that N-CAM and AChR δ-subunit RNAs increased in parallel after denervation and that both followed a time course similar to that previously determined for AChR α-subunit mRNA. Whereas the kinetics of RNA increase depend on rates of degradation as well as on rates of synthesis, this result is consistent with the notion that expression of genes for N-CAM and AChR is regulated in parallel.

Discussion

Immunohistochemical studies have shown that N-CAM in rat skeletal muscle changes in distribution during embryogenesis, declines in level and becomes concentrated at synapses postnatally, and reappears after denervation (5, 6; see also references 20 and 27). Because both N-CAM (3, 12) and its RNA (9, 11, 21) are known to exist in multiple forms in brain, it seemed possible that changes in form might accompany the transitions we had documented histologically. If they

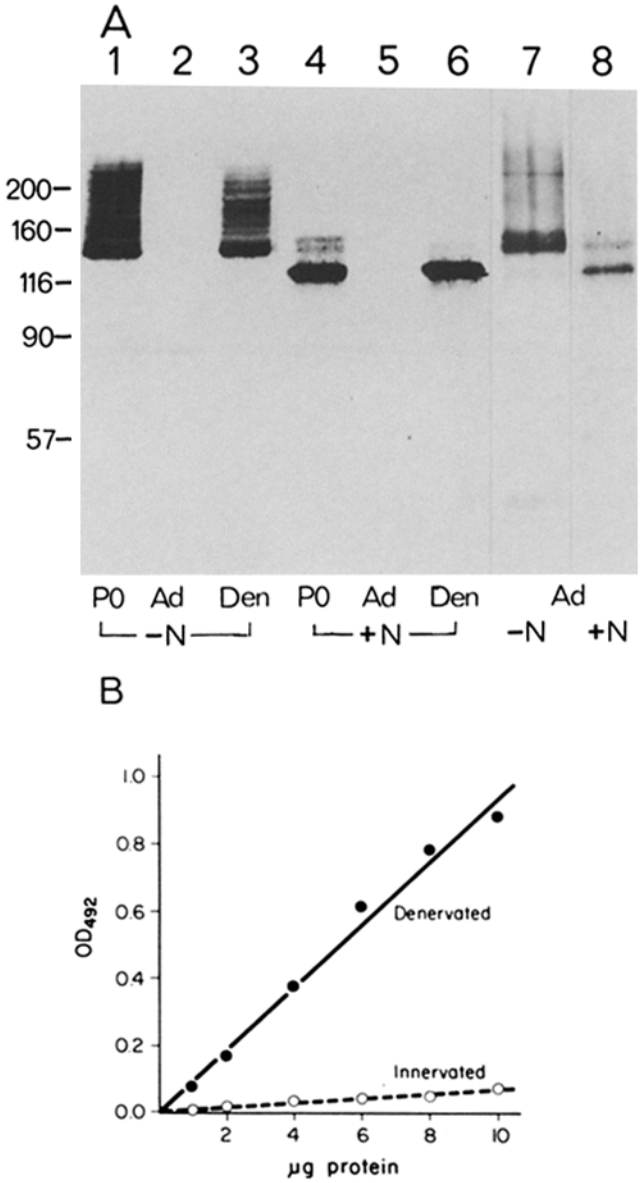

Figure 5. N-CAM accumulates in denervated muscle. (*A*) Immunoblot analysis of normal and denervated adult muscle N-CAM. Lanes *1–6*, Nonidet P-40 extracts of P0 (lanes *1* and *4*), normal adult (lanes *2* and *5*) or 1-wk-denervated adult (lanes *3* and *6*) rat diaphragm were probed with anti-N-CAM serum and peroxidase second antibody. Samples in lanes *4–6* were treated with neuraminidase. Denervation induces the accumulation of the 125-kD desialo form of N-CAM characteristic of postnatal muscle. Lanes *7* and *8*, N-CAM was concentrated from an extract of normal adult diaphragm by affinity chromatography to render detectable the small amount of N-CAM present in innervated muscle; the sample in lane *8* was treated with neuraminidase. Normal adult diaphragm contains both the 140- and 125-kD forms of N-CAM. Samples in lanes *1–6* each contained 50 μg protein. (*B*) Enzyme-linked immunosorbent assay quantitation of N-CAM in Nonidet P-40 extracts of normal (○) and 10-d-denervated (●) adult rat diaphragm. Denervation results in a 12–15-fold increase in muscle N-CAM.

did, muscle would be an ideal system in which to study the relationships between the molecular and cell biologies of N-CAM. We therefore used immunoblotting to determine the molecular forms of N-CAM and blot hybridization to determine the species of N-CAM RNA in developing, denervated,

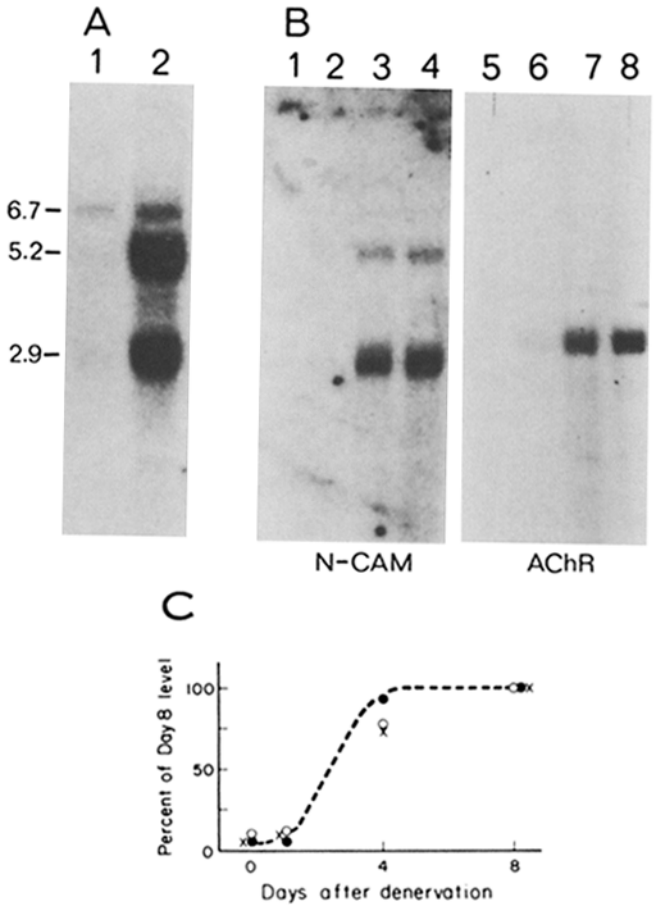

Figure 6. Denervation induces the accumulation of N-CAM RNA. (*A*) RNA from normal adult (lane *1*) or 10-d-denervated adult (lane *2*) rat diaphragm was fractionated by gel electrophoresis, transferred to filters, and hybridized with ^{32}P-pM1.3-DNA specific for N-CAM. Denervation induces the accumulation of N-CAM RNAs. (*B*) RNA from normal (lanes *1* and *5*), 1-d-denervated (lanes *2* and *6*), 4-d-denervated (lanes *3* and *7*) or 8-d-denervated (lanes *4* and *8*) adult mouse leg muscle, probed with N-CAM cDNA (lanes *1–4*) or with a cDNA-specific for the δ-subunit of AChR (lanes *5–8*). (*C*) Time course of the increase in N-CAM and AChR-specific RNAs after denervation of mouse leg muscle. Levels of N-CAM 5.2-kb (○), 2.9-kb (●), and AChR δ-subunit (X) RNAs obtained by densitometric scanning of films such as those shown in *B* are expressed as a percentage of the value obtained on day 8 for each species. Values for AChR α-subunit (– – –) mRNA were redrawn from reference 18. The time courses of increase for both 5.2- and 2.9-kb N-CAM RNAs and for both AChR-subunit mRNAs are similar.

and cultured muscle. Our main results are the following: (*a*) As muscles develop in vivo, the degree of sialylation of their N-CAM decreases and a 140-kD desialo form is replaced by a 125-kD desialo form. (*b*) The transition in desialo forms but not in degree of sialylation occurs as embryonic and clonal muscle cells differentiate in vitro in the absence of nerves. (*c*) Multiple species of muscle RNA hybridize to N-CAM cDNA, and the relative abundance of these species changes during development, both in vivo and in vitro. (*d*) The denervation-induced increase in N-CAM protein is accompanied by an increase in N-CAM RNA and is therefore probably regulated pretranslationally.

Analysis of muscle N-CAM by immunoblotting reveals that the extent of its sialylation decreases during development in vivo, a transition analogous to the embryonic to adult (E→A) conversion that occurs in brain (3, 12, 28). Analysis of neuraminidase-treated N-CAM reveals an additional level of heterogeneity: a 140-kD desialo form of N-CAM is replaced by a 125-kD form as development proceeds (Table I). Such heterogeneity has not been noted in previous studies of desialo N-CAM from chick (30) or mouse muscle (27): in chick this failure was presumably due to analysis of only early embryonic tissue. The transition between desialo forms occurs in primary cultures of muscle, indicating that it can occur in the absence of nerves, and in clonal cell lines, suggesting that it can occur within single cells. In primary cultures, C2 cells, and presumably in vivo, the 140-kD form is associated with mononucleated myoblasts and the 125-kD form is associated with myotubes. Similarly, whereas BC3H-1 cells do not fuse, a 140–125-kD switch occurs as the cells acquire a number of characteristics of the myotube phenotype. We do not know whether, in cell populations that do fuse, the transition in forms is simultaneous with and/or obligatorily linked to fusion. However, a change in the molecular form of N-CAM appears to be part of the program of muscle development.

A clue to the mechanism of this transition comes from comparing the relative abundance of desialo forms and of N-CAM-related RNAs in various situations. A 6.7-kb RNA species is correlated with the 140-kD glycoprotein core, whereas 5.2- and 2.9-kb forms are correlated with the 125-kD core (Table I). The coding potential of the N-CAM RNAs is unknown, and the 2.9-kb species may not be large enough to encode N-CAM (2.9 kb = 967 codons = a 120-kD protein, based on the amino acid composition in reference 7). However, it is tempting to speculate that the 6.7-kb species encodes the 140-kD desialo form and that the 5.2- and/or 2.9-kb species encode the 125-kD form. By the same line of reasoning, and consistent with other studies (9), the presence of a 180-kD protein and a 7.4-kb RNA in brain and their absence from muscle (Table I) suggest that the 7.4-kb RNA may encode the 180-kD form of N-CAM. If these speculations are correct, the developmental transition in N-CAM forms in muscle would resemble transitions in forms of myosin and other contractile proteins, which also have developmentally regulated embryonic and adult isoforms encoded by different RNAs (25). Unlike embryonic and adult myosin heavy chains, which are clearly products of different genes (25), different forms of N-CAM appear to be the product of a single gene (11, 21). The synthesis of different N-CAM RNAs is thus presumably analogous to the synthesis of multiple troponin T and myosin light chain RNAs from single genes by use of alternative transcription initiation/termination sites and alternative RNA splicing (1, 22).

In adult muscle, N-CAM expression is regulated by innervation. The effect of innervation is mediated in large part by electrical and/or contractile activity, since N-CAM accumulates not only in denervated muscles but also in muscles chronically paralyzed by the pharmacologic blockade of nerve conduction (5). In many respects the regulation of N-CAM expression in muscle is similar to that of AChRs (discussed in reference 6). We previously showed that the increase in AChRs after denervation is preceded by an increase in AChR mRNA (18). In this study, we present similar results for N-CAM. In rat diaphragm, denervation induces a 12–15-fold increase in N-CAM and a 30–50-fold increase in N-CAM RNA. In the mouse leg, the time course of increase in N-

Table I. Molecular Forms of N-CAM and Its RNA in Developing, Adult, and Cultured Muscle and in Brain

Source	Anti-N-CAM-stained muscle cells*	N-CAM (desialo forms)	N-CAM RNA
		kD	*kb*
E15 muscle	Myoblasts, myotubes	140 ≫ 125	6.7 ≫ 5.2, 2.9
E17 muscle	Myoblasts, myotubes	140, 125	6.7, 5.2, 2.9
Perinatal (E21-P0) muscle	Myofibers	125 ≫ 140	5.2, 2.9 ≫ 6.7
Adult muscle	Satellite cells, myofibers	140, 125	6.7, 5.2, 2.9
Denervated adult muscle	Myofibers > satellite cells	125 ≫ 140	5.2, 2.9 ≫ 6.7
2-d primary muscle cultures	Myoblasts	140	6.7, 5.2, 2.9
7-d primary muscle cultures	Myotubes	125 ≫ 140	5.2, 2.9 ≫ 6.7
3-d C2 cells	Myoblasts	140	ND
7-d C2 cells	Myotubes	125 ≫ 140	5.2, 2.9 ≫ 6.7
3-d BC3H-1 cells	Myoblasts	140	6.7
7-d BC3H-1 cells	Differentiated myocytes	125 ≫ 140	5.2, 2.9 ≫ 6.7
E17 brain	—	180, 135	7.4, 6.7
Adult brain	—	180, 135, 115	7.4, 6.7, 5.2, 2.9

ND, not detected.

* Immunohistochemical data summarized from references 5 and 6.

CAM RNA after denervation paralleled that for the AChR α- and δ-subunits. Thus, the synthesis and/or turnover of N-CAM and AChR mRNAs are similarly regulated by innervation.

Our immunohistochemical studies show that N-CAM is appropriately positioned to participate in a number of different intercellular interactions during muscle development (6). Changes in the chemical nature of N-CAM could be important in at least two of these interactions. First, N-CAM is present on myoblast surfaces and might participate in the intercellular recognition that precedes fusion. If the 140-kD form of N-CAM were more effective than the 125-kD form in promoting fusion, a switch by myotubes to the 125-kD form could limit further fusion of myoblasts with them. Second, N-CAM may mediate early interactions between motor axons and myotubes. Accumulation of the 125-kD form of N-CAM by myotubes could provide a means for embryonic axons to distinguish them from 140-kD N-CAM rich myoblasts. By employing antibodies that recognize individual forms of N-CAM, it should be possible to gain a better understanding of the subcellular locations and the functions of the various forms of N-CAM in muscle. Such studies may also help us to understand the significance of the variety of N-CAM forms expressed in central nervous tissue.

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