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IMMUNOFLUORESCENCE MICROSCOPY OF A MYOPATHY

α-Actinin is a Major Constituent of Nemaline Rods

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SUMMARY

A biopsy of skeletal muscle taken from a child with the clinical symptoms of congenital nemaline myopathy was studied. Light and electron microscopy revealed rod-like structures within the muscle fibres, and thus confirmed the clinical diagnosis. Indirect immunofluorescence, using specific antibodies against actin and desmin (both derived from chicken gizzard) as well as against α-actinin and tropomyosin (both from porcine skeletal muscle) revealed that the rods consist of massive accumulations of α-actinin. Desmin seems to be peripherally associated with the rods. Anti-actin and anti-tropomyosin did not stain the rods; however, a masking effect could not be ruled out. These findings support previous hypotheses that nemaline rods can be taken to be lateral polymers of normal Z-disks.

Nemaline myopathy was first described in 1963 by Shy et al. [1] as a congenital non-progressive disease of skeletal muscle in a child. Light microscopic and electron microscopic analyses of muscle biopsies taken from this patient as well as subsequent studies on similar afflictions (for references, see [2]) have shown large numbers of rod-like structures accumulated within the muscle fibres. These rods are usually between 1 and 5 μm in length and 1 μm in diameter. They appear to be extremely electron-dense and distort the regular myofibrillar arrays. The close proximity of smaller rods to the Z-line, as well as their fine structure suggested that these rods originated from the Z-line [3–6]. Electron micrographs revealed a characteristic periodicity of the fine structure of nemaline rods: in longitudinal sections, thin filaments were seen to run parallel to the long axis of the rod [6, 7]. These filaments were connected with each other through periodically spaced lines perpendicular to the long axis of the rod. In cross sections, the rods showed square arrays of filaments arranged in a 'basket-weaving' pattern, quite similar to the Z-line lattices [4, 6, 8, 9, 10].

Since, on the basis of the evidence described above, the nemaline rods were considered to be abnormally enlarged Z-disks, and since the chemical composition of normal Z-disks is as yet unknown, a number of studies have been undertaken to determine the protein composition of nemaline rods, with the aim of learning more about Z-disk constitution. As purified tropomyosin displays a basket-weaving type pattern in sectioned crystals which superficially resembles Z-disk structures, earlier reports suggested that nemaline rods, as well as Z-disks, may be mainly composed

of tropomyosin [4, 5]. Later studies have suggested that longitudinal thin filaments comprising the backbone of the rods consist of actin [6, 7], or that the nemaline rods are deposits of breakdown products of an abnormal myosin [11].

The structural resemblance of the rods to the Z-disks led other investigators to the assumption that they might contain α-actinin [7, 8]. This protein makes up at least 50% of the normal Z-disk proteins [12]. Prolonged extraction of normal Z-disks with low ionic strength buffers or incubation with a Ca-activated neutral protease releases α-actinin into the supernatant and results in the structural disintegration of this organelle. The same was found for nemaline rods [7, 10]. Immunofluorescence with antibodies against α-actinin, however, yielded contradictory results. Whereas Sugita and co-workers reported that the rods do not stain [13, 14], Schollmeyer and coworkers obtained a positive result [15].

In this paper, we report that affinity purified antibodies against porcine skeletal muscle α-actinin intensely stained nemaline rods in a muscle biopsy of a young boy, while antibodies against another Z-disk protein, desmin [16], did not bind to the rods themselves but indicated the presence of desmin around or between individual rods. Anti-actin and anti-tropomyosin did not react with the rods. These findings imply that this genetic disorder results in an abnormal accumulation of α-actinin in skeletal muscle, and support the previous hypotheses that nemaline rods can be interpreted as being laterally replicated Z-disks.

MATERIALS AND METHODS

Sample preparation

The biopsy was taken from the left deltoid muscle of a 2-year-old boy via an open biopsy procedure. The clinical features of this child were characteristic of congenital nemaline myopathy. Sucking and swallowing in the first months of life had been impaired and motor development was delayed. Examination revealed hypoplasia and hypotonia of the skeletal muscles, a myopathic face and scaphocephaly. Tendon reflexes could not be elicited. One part of the material was divided into pieces of approx. 4×3×3 mm. The pieces were frozen in isopentane which had been cooled in liquid nitrogen and stored at −85°C. Similarly treated material from a muscle biopsy of a child of comparable age were used as control tissue. Following histological and histochemical examination, this control muscle had been judged to be not pathologically changed.

Longitudinal and transverse sections some 2–3 μm thick were cut on a Slee cryostat. 0.5 μm thick sections were cut on a Reichert OMU 3 Ultramicrotome with FC-2 cryo-attachment, at −30 to −40°C. They were mounted on gelatin-coated glass slides, and fixed in 3.5% formaldehyde in phosphate-buffered saline, pH 7.3 (PBS). Sections were stored in PBS and 0.02% NaN_3 at 4°C and used for indirect immunofluorescence up to 10 weeks after cutting. During that period, structural integrity as well as antibody-binding capacity remained unchanged.

Antibody characterization

Anti-actin was raised in rabbits against chicken gizzard actin and purified by affinity chromatography on Sepharose–actin as previously described [17]. Anti α-actinin was raised in rabbits against porcine skeletal muscle α-actinin and purified by affinity chromatography on Sepharose–α-actinin in the same way. Purification, specificity and cross-reactivity of both antibodies have been extensively described [18–22]. Anti-tropomyosin was raised in rabbits against porcine skeletal muscle tropomyosin. The antiserum stained I-bands of isolated fibrils. Eight percent of the protein in a crude IgG preparation bound to an affinity column made with tropomyosin–Sepharose and could be eluted with 4 M $MgCl_2$. In this study, non-purified serum was used in a dilution 1 : 30. Anti-desmin was generously donated by Dr Franke, German Cancer Research Center, Heidelberg. It had been raised in guinea pigs against chicken gizzard desmin. Specificity and cross-reactivity of the non-purified serum have been described elsewhere [23]. In this study, a dilution of 1 : 20 was used. FITC-conjugated goat anti-rabbit IgG and sheep anti-guinea pig IgG (second antibodies) were purchased from Miles Laboratories and diluted 1 : 10 and 1 : 20, respectively.

Controls included staining of sections with preimmune sera or without the specific (first) antibody. Fluorescence in these cases was negligible.

Indirect immunofluorescence procedure

The fixed sections were extracted with Triton X-100 and acetone before antibody application as previously described for tissue culture cells [17]. Incubation

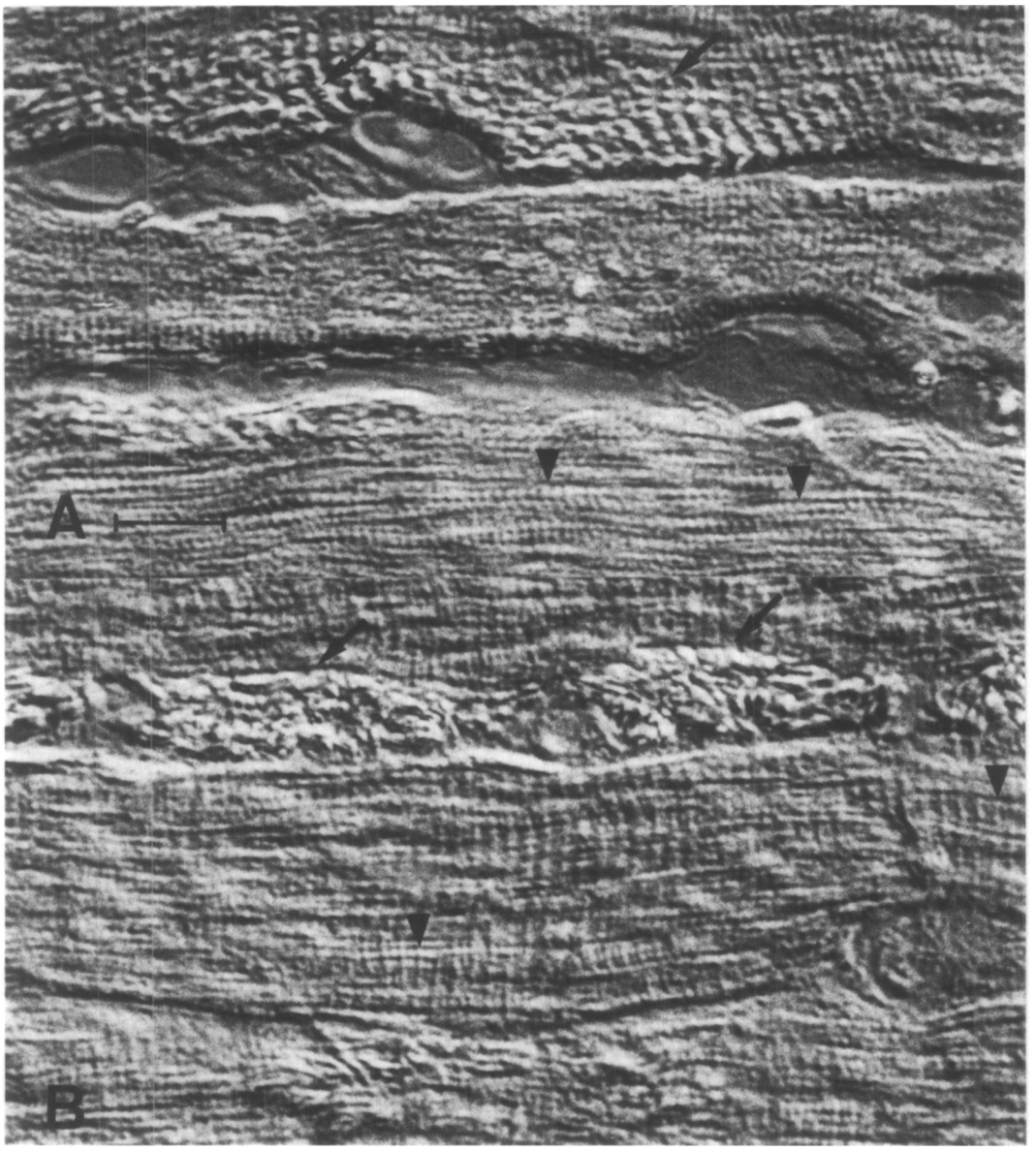


Fig. 1. Longitudinal sections (2–3 μm) of a frozen muscle biopsy from a myopathy patient, as seen in phase interference microscopy. With this technique, normal Z-lines stand out as prominent structures (*arrowheads*). The diseased muscle fibre in the top region of (*A*) demonstrates a thickening and fragmentation of the Z-line structure and the accumulation of rods, mostly with their long axis perpendicular to the Z-line (*A*). In another diseased fibre, islands of rods are seen in a row alongside the long axis of the fiber (*B*, *arrows*). Bar, 10 μm.

with the first and the second antibody (20 μl each slide carrying 4 sections) was carried out for 45 min at 37°C. Samples were embedded in Elvanol (Serva, Heidelberg) and examined with a Zeiss photomicroscope equipped with phase contrast, phase interference and epifluorescence optics. Photographs were taken on Kodak Tri X Pan film with the camera being set to automatic exposure. Prints were made with varying exposure times, to bring out optimal contrasts.

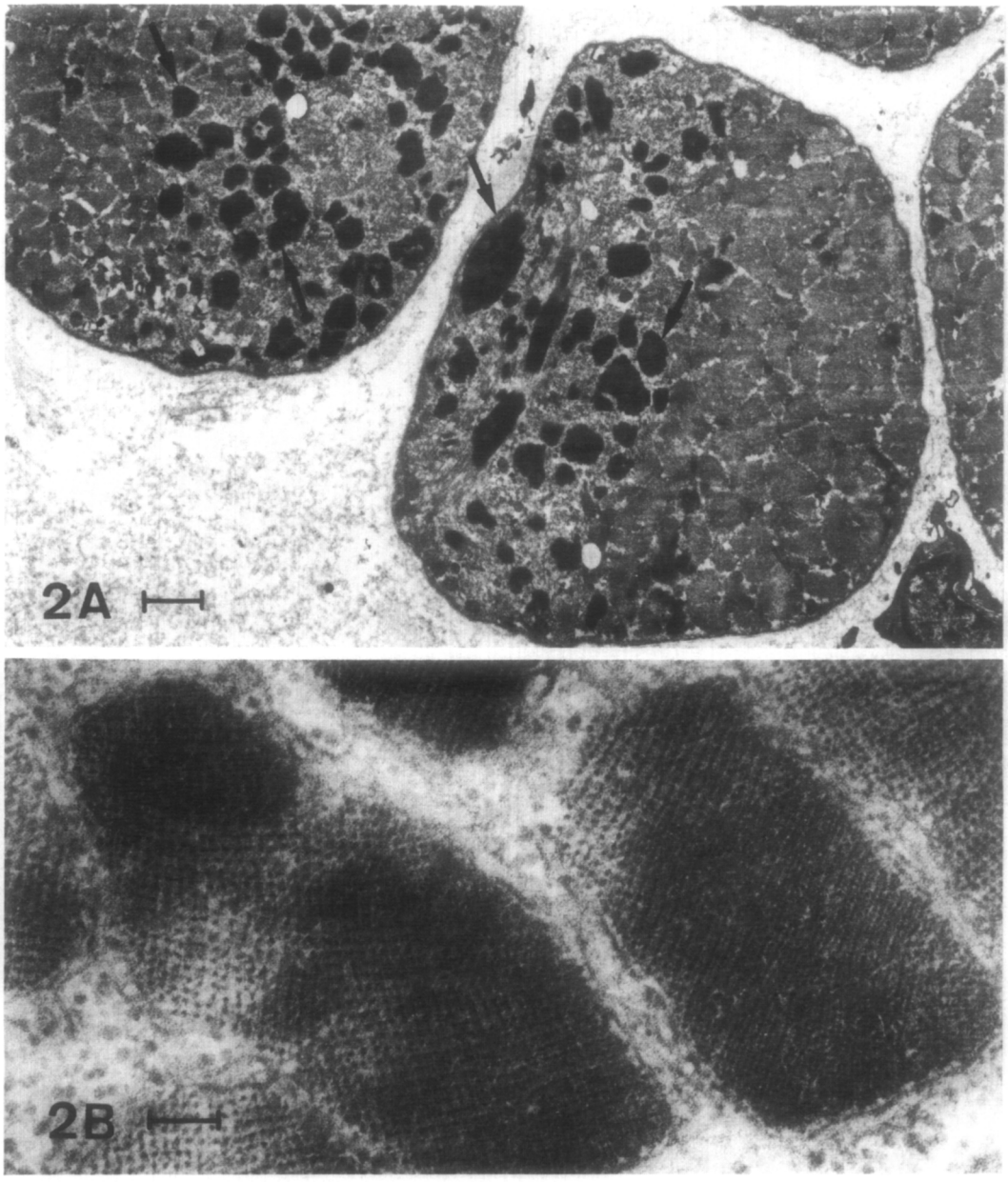
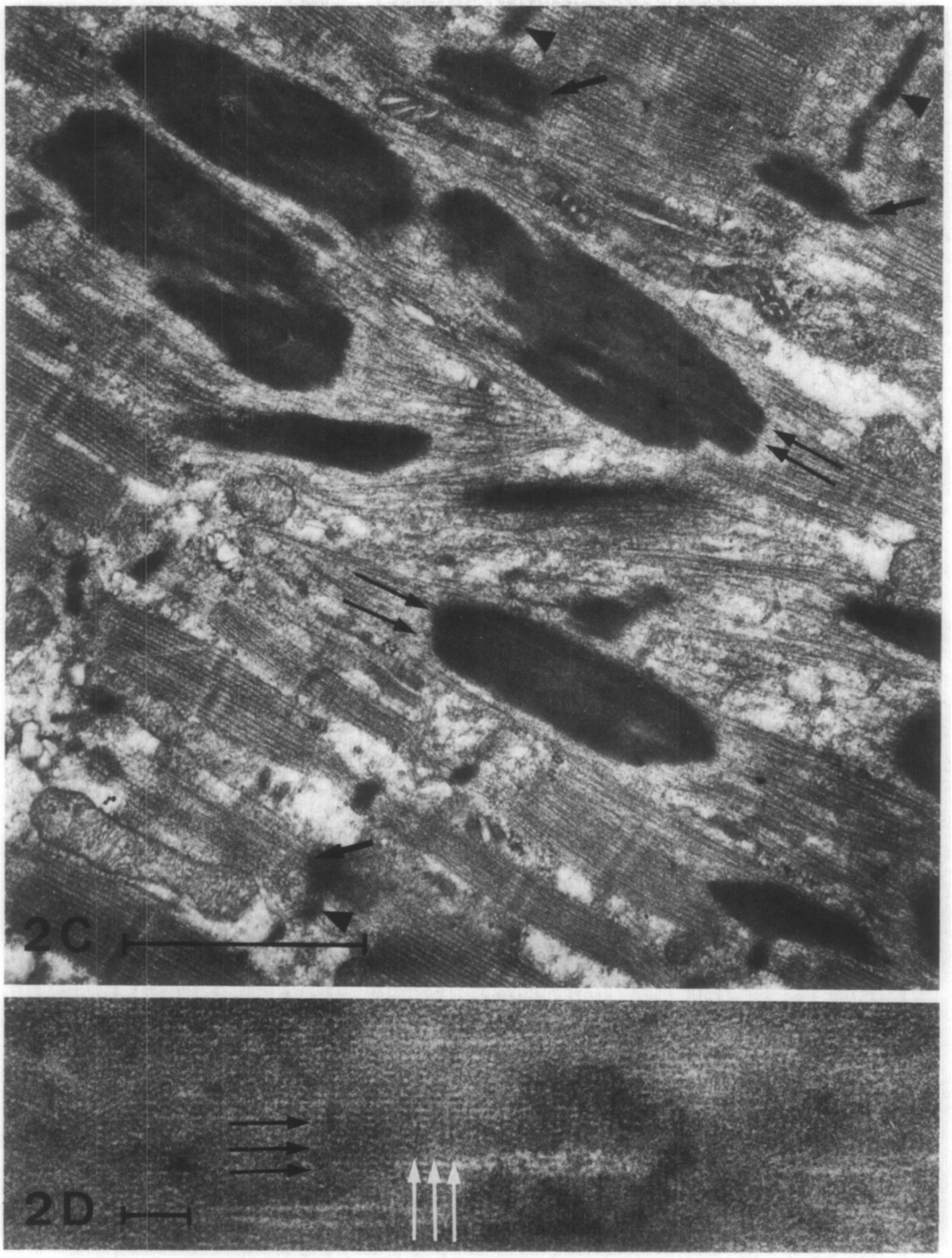


Fig. 2. Electron micrographs of the same muscle biopsy described in fig. 1. (*A, B*) Transverse; (*C, D*) longitudinal sections. Nemaline bodies appear as electron-dense particles excentrically located in one region of the muscle fibre (*A, arrows*). At higher magnification, the square lattice of those rods is obvious. In longitudinal sections, small rods (*C, short arrows*) are seen in the proximity of abnormally thick Z-lines (*arrowheads*). Larger rods grossly distort the myofibrils, but still appear connected to thin filaments (*C, long arrows*). At higher magnification, two periodicities are seen in longitudinally sectioned rods: filaments run parallel to the long axis of the rod (*D, black arrows*), cross striations are located perpendicular to them (*D, white arrows*). Bar, (*A, C*) 1 μm; (*B, D*) 0.1 μm.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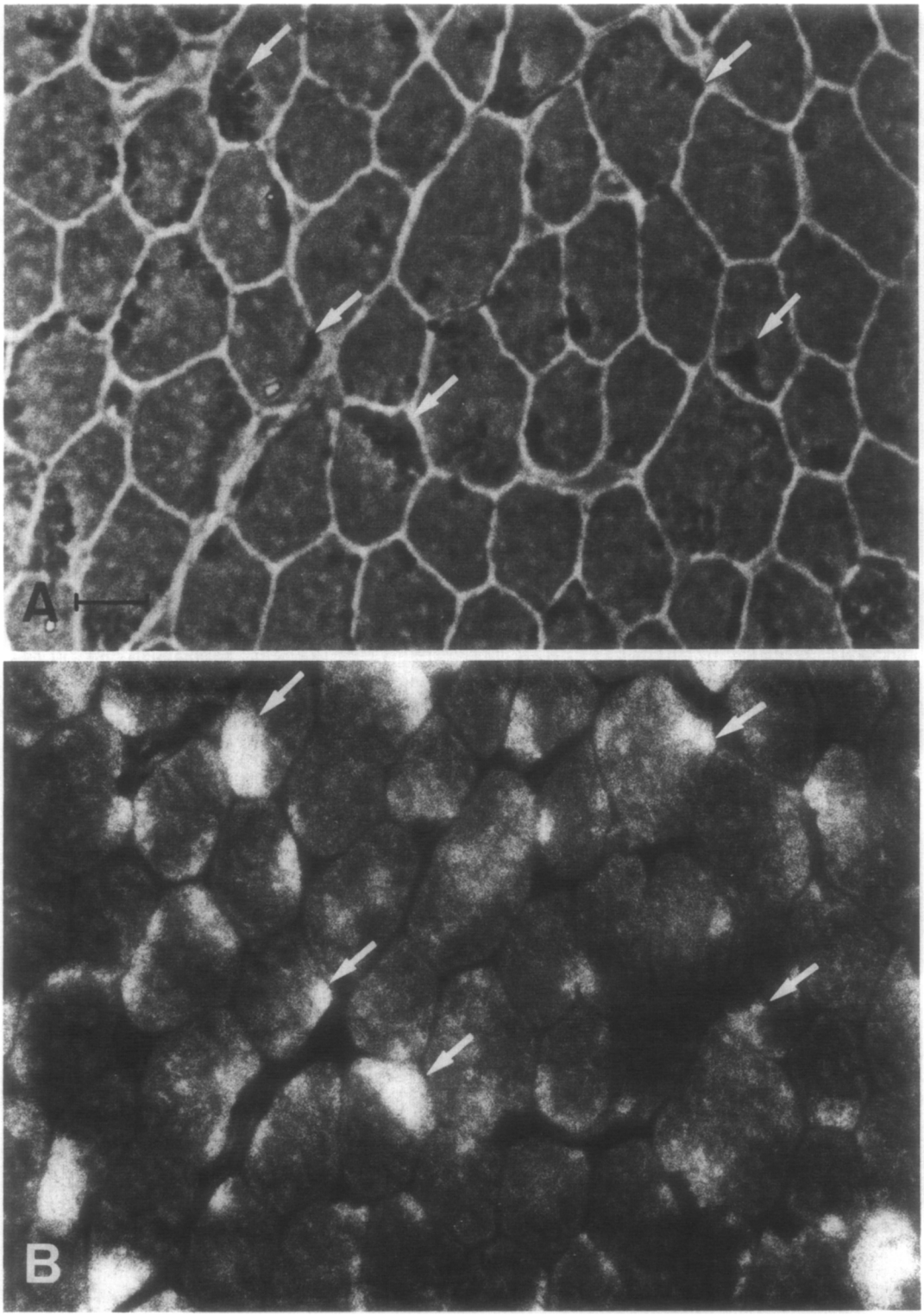
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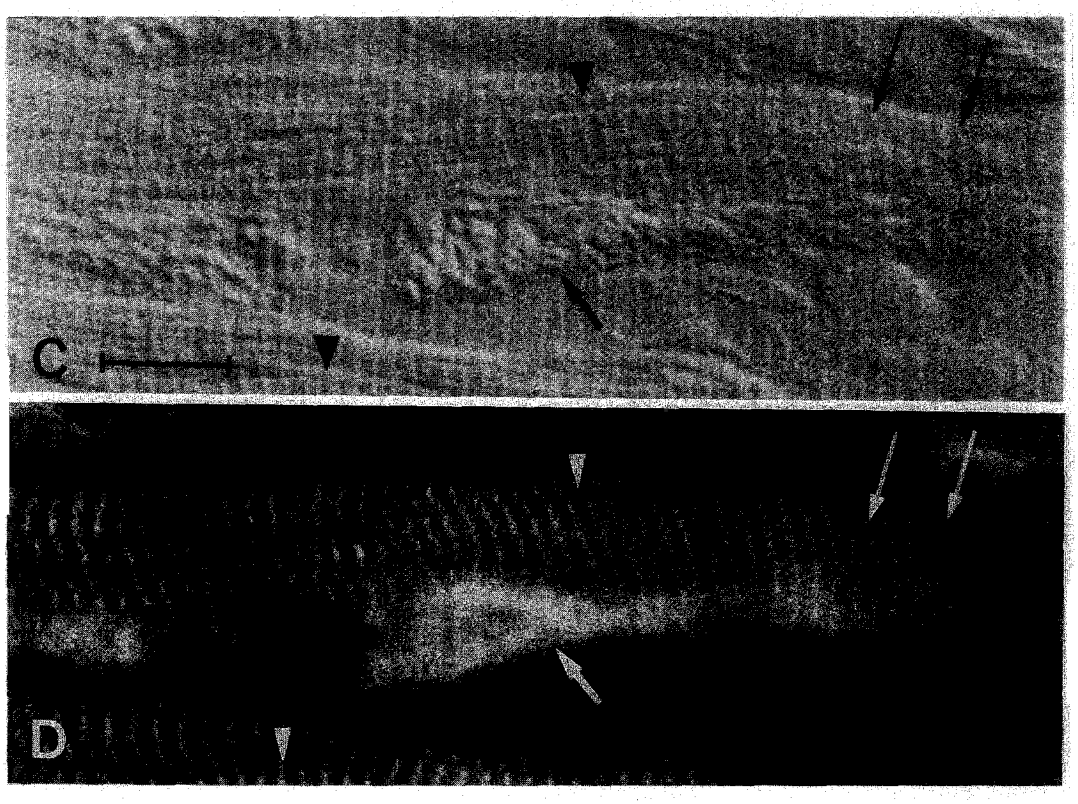


Fig. 3. Transverse (*A*, *B*) and longitudinal (*C*, *D*) sections (2–3 μm) of the nemaline myopathy biopsy, stained with anti-α-actinin. (*A*) Phase contrast; (*C*) phase interference; (*B*, *D*) epifluorescence microscopy. A one-to-one correspondence of nemaline rods (*A*) and brightly fluorescent areas within diseased muscle fibres (*B*) is obvious (*arrows*). Normal Z-lines (*C*, *thin arrows*) show binding of the antibody in narrow, brightly fluorescent lines (*D*, *thin arrows*). Areas of Z-line thickening (*C*, *D*, *arrow heads*) and rod formation (*C*, *D*, *fat arrows*) display a homogeneous, diffuse, but bright fluorescence. Bars, 10 μm.

Electron microscopy

A small part of the biopsy material was fixed for 2.5 h in a 2% glutaraldehyde solution buffered with cacodylate (pH 7.3), containing 25 mM CaCl. It was post-fixed for 2 h in 1% osmium tetroxide, dehydrated and embedded in Epon. Transverse and longitudinal sections 1 μm thick were stained with toluidine blue. Thin sections were stained with uranyl acetate followed by lead citrate and were examined in a Philips EM 201 electron microscope.

RESULTS

In the phase interference microscope, the abnormalities in frozen sections of the biopsy were quite prominent. Longitudinal sections revealed the association of these rods with the Z-lines. While in some regions the Z-lines appeared normal, they were remarkably thickened in others, and in still other regions, they gave rise to rods (fig. 1*A*). Considering an individual Z-line, the longest rods, with their long axis parallel to the myofibrillar filaments, were closest to the sarcolemma. Large conglomerates of such rods were piled up at the margins of muscle fibres whose centres appeared still intact. Frequently, such accumulations of rods were seen in rows along one side of an individual muscle fibre (fig. 1*B*, arrows). In cross sections, such rows appeared as excentrically located piles of rods in one,

but rarely in two corners of transversely cut muscle fibres (cf fig. 2*A*).

In the electron microscope, the rods appeared as extremely dense particles. In transverse sections, affected muscle fibres contained rods of variable diameter which formed excentrically located groups (fig. 2*A*, arrows). The square lattice of interwoven filaments, similar to the Z-disk lattice, was revealed at high magnification (fig. 2*B*). The distance between the dots marking the corners of the squares was approx. 100–150 Å, which is in agreement with previous measurements (cf [6, 7]). Longitudinal sections through an affected area showed widened and somewhat 'fluffy' Z-lines (fig. 2*C*, arrowheads) with smaller rods located nearby (fig. 2*C*, short arrows). Large rods had completely distorted the regular myofibrillar arrangement, but their tapered ends still maintained structural continuity with the microfilaments of the previous I-bands (fig. 2*C*, long arrows). At higher magnification, the characteristic pattern of longitudinal filaments, spaced approx. 150 Å apart, with perpendicular striations showing a periodicity of about 150–200 Å, could be seen in large rods (fig. 2*D*, arrows). Thus, clinical symptoms (see Material and Methods) as well as light and electron microscopic findings were in agreement with previous descriptions of congenital nemaline myopathy.

Indirect immunofluorescence with specific antibody against skeletal muscle α-actinin demonstrated a brilliant staining of the regions where nemaline rods were accumulated in cross sections through diseased muscle fibres. There was a one-to-one correspondence between the areas occupied by those rods, as seen in phase contrast, and the fluorescence image (fig. 3*A*, *B*). Transverse sections through normal control muscle showed only a weak, spotty fluorescence after incubation with anti-α-actinin, probably indicating areas of Z-disks at or close to the surface of the section (not shown). In longitudinal sections, areas with thickened Z-lines as well as with marginal accumulations of rods showed bright fluorescence (fig. 3*C*, *D*), while in other regions normal Z-line staining was observed.

Staining of transverse sections with specific anti-actin, however, revealed no correlation between the location of nemaline rods and fluorescence (fig. 4*A*, *B*). The staining was distributed in a patchy manner, similar to the one seen in the control sections (not shown). The light regions probably reflect areas where the plane of the section runs through the I region of myofibrils. The exclusion of anti-actin binding at the site of nemaline rods could be most clearly seen in 0.5 μm longitudinal sections, where individual non-fluorescent rods could be distinguished, surrounded by strongly fluorescent I-bands (fig. 4*C*, *D*).

Incubation of sections with serum against skeletal muscle tropomyosin also gave a negative result; the fluorescence pattern showed no correlation with the pattern of rod accumulation (fig. 5*A*, *B*). Staining with anti-tropomyosin gave essentially the same pictures as staining with anti-actin, also in that there was no difference between the staining patterns of nemaline myopathy sections and sections through control muscle (not shown).

Guinea pig serum directed against chicken gizzard desmin cross-reacted with human muscle. In transverse sections, spotty staining was seen which appeared to be denser and more intense at the sites of nemaline rod accumulations (fig. 6*A*, *B*). However, in contrast to the case of anti-α-actinin staining, there was no one to one correspondence between the location of the

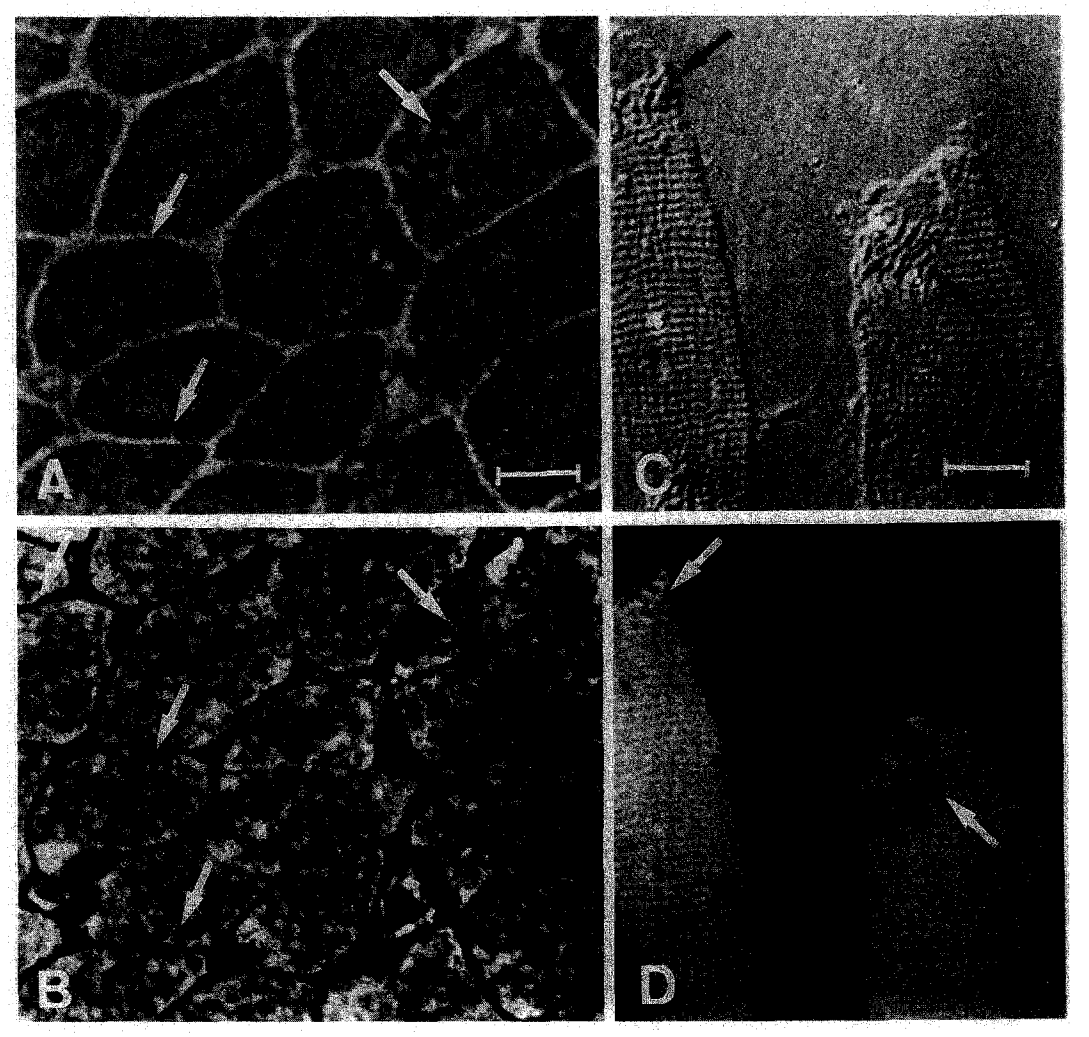
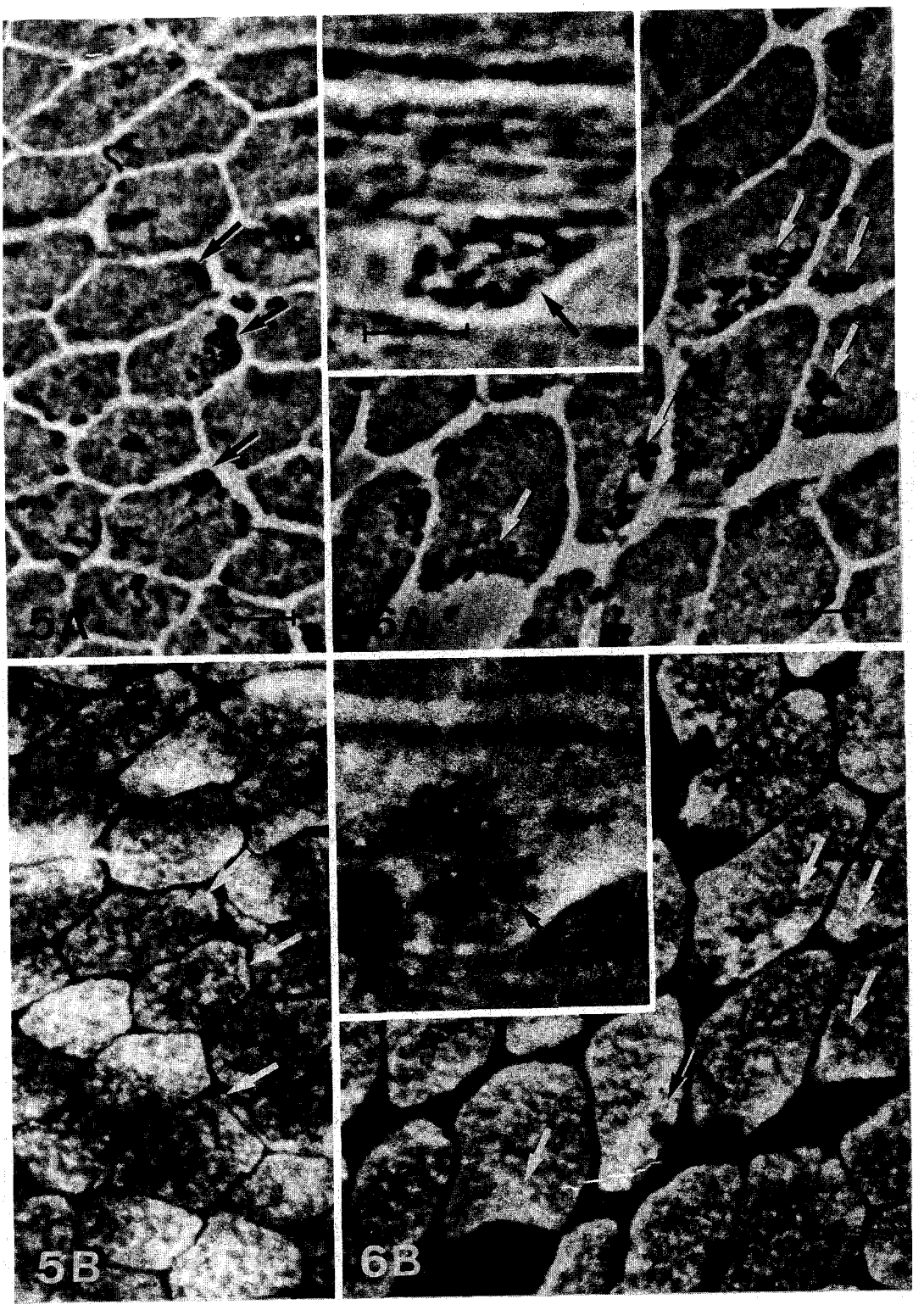


Fig. 4. Transverse and longitudinal sections of nemaline myopathy sample, stained with anti-actin. (*A*) Phase contrast; (*B*) epifluorescence microscopy of 2–3 μm thick transverse sections. No correspondence of nemaline rods (*arrows*) to fluorescence is seen. (*C*) Phase interference; (*D*) epifluorescence microscopy of 0.5 μm frozen longitudinal sections. Rods are surrounding a nucleus. The antibody against actin stains the I-bands, but does not stain nemaline rods (*arrows*). Bars, 10 μm.

rods and the fluorescence. At higher magnification, longitudinal sections revealed that not the rods themselves but their surroundings were stained with anti-desmin (fig. 6*A*, *B*, insets). The fluorescent striations seen in longitudinal sections (fig. 6*B*, inset) corresponded presumably to Z-lines or to the adjacent I-regions, since desmin was reported to be a component of normal Z-lines (cf [16, 24]) and since this serum stained the regions of I–Z–I junctions in isolated rat myofibrils and frozen sections of rat cardiac muscle (Schmid, Jockusch & Franke, unpublished).

DISCUSSION

From the four antibodies against structural proteins of skeletal muscle which we used in this study (anti-actin, anti-tropomyosin,


5A
6A
5B
6B

anti-α-actinin, anti-desmin), only anti-α-actinin reacted strongly with the nemaline rods. Thus, our results confirm previous suggestions and preliminary evidence for the presence of this protein in such abnormal developments of the Z-line [26]. Yamaguchi et al. demonstrated that the thin filaments running parallel to the long axis through the nemaline rod are actin-like in their binding capacity to heavy meromyosin but do not stain with anti-actin [7]. These authors suggested that the antibody does not sufficiently penetrate the electron-dense material (presumably α-actinin). In our experiments, neither anti-actin nor anti-tropomyosin stained the rods significantly. These results do not exclude the possibility that the actin filaments within the nemaline rods may be associated with tropomyosin, as in the I-band, or, that tropomyosin may be present in a local distribution typical for Z-disks. There is still some controversy as to whether or not tropomyosin is a constituent of normal Z-lines (cf [15, 24, 25, 26]). In the case of desmin, however, it seems unlikely that the lack of staining of rod bodies with anti-desmin may be due to a masking effect. It has been shown on isolated Z-disk sheets that desmin is located preferentially at the margin of individual Z-disks occupying the space between such disks in an isolated sheet ([16, 24]). If nemaline rods represent lateral polymers of normal Z-disk components, one would expect desmin to be present around their margins not within the rods proper. Our results confirmed this expectation.

Since actin (and possibly tropomyosin) is present within nemaline rods [7] but masked, while α-actinin is easily accessible to the antibody, α-actinin must be a major component of nemaline rods. As in the Z-disk, the presence of α-actinin in the rods is associated with the presence of amorphous, electron-dense material and a lattice-type arrangement of filaments. The arrangement of α-actinin molecules within the Z-disk and their possible relationship to either the amorphous material or the Z-disk filaments is unknown. Prolonged low ionic strength extraction or treatment with Ca-activated protease removes the amorphous material and releases α-actinin into the supernatant, but leaves the lattice largely intact [7, 10]. However, since no biochemical studies have been made on the composition of extracted Z-disks or nemaline rods, one cannot exclude the possibility that α-actinin is participating in some lattice structures. In addition, the released molecules which were termed 'α-actinin' by Ebashi et al. [27], may themselves be a proteolytic cleavage product of a larger, so far unknown Z-disk protein.

Our present knowledge on α-actinin is very limited. This protein, as purified from skeletal muscle, binds to F-actin in vitro, apparently cross-linking individual filaments [28]. When incubated with Z-line extracted fibrils, α-actinin binds to the thin filaments in the I-band in a temperature-dependent fashion; at 37°C, α-actinin binds predominantly to the Z-line region, while at 0°C, it cross-links thin filaments form-

Fig. 5. Transverse sections (2–3 μm) through nemaline myopathy sample, stained with anti-tropomyosin. (*A*) Phase contrast; (*B*) epifluorescence microscopy. The antibody binds to some regions of the cross sectioned muscle fibres (presumably in the I-band), but no correspondence to nemaline rods (*arrows*) is seen. Bar, 10 μm.

Fig. 6. Transverse and longitudinal sections (2–3 μm) through the nemaline myopathy sample, stained with anti-desmin. (*A*) Phase contrast; (*B*) epifluorescence microscopy is increased in areas of rod accumulations (*arrows*). Higher magnifications of longitudinal sections, however, demonstrate that not the nemaline rods themselves, but their surroundings are stained brightly with antidesmin (insets in *A*, *B*). Bar, 10 μm.

ing an I-band network, probably displacing tropomyosin [29].

The aberration of the muscle structure in patients with congenital nemaline myopathy might reflect the loss of the normal restriction of α-actinin to the ends of actin filaments in the Z-disk region. Alternatively, the dislocation of α-actinin could be ascribed to a defect in the regulation of muscle protein turnover leading to accumulation of α-actinin outside the Z-disk region. Since, however, the disease is non-progressive, the aberrant α-actinin in this disorder must at least participate in the general turnover of muscle proteins after birth.

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