

Localization of C-Protein Isoforms in Chicken Skeletal Muscle: Ultrastructural Detection Using Monoclonal Antibodies

J. E. DENNIS, T. SHIMIZU, F. C. REINACH, and D. A. FISCHMAN

Department of Cell Biology and Anatomy, Cornell University Medical College, New York 10021; and Department of Neurology, Brain Research Institute, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan

ABSTRACT Monoclonal antibodies (McAbs) specific for the fast (MF-1) and slow (ALD-66) isoforms of C-protein from chicken skeletal muscle have been produced and characterized. Using these antibodies it was possible to demonstrate that skeletal muscles of varying fiber type express different isoforms of this protein and that in the posterior latissimus dorsi muscle both isoforms are co-expressed in the same myofiber (17, 18). Since we had shown that both isoforms were present in all sarcomeres, it was feasible to test whether the two isoforms co-distributed in the same 43-nm repeat within the A-band, thereby establishing a minimum number of C-proteins per repeat in the thick filaments.

Here we describe the ultrastructural localization of C-protein in myofibers from three muscle types of the chicken using these same McAbs. We observed that although C-protein was present in a 43-nm repeat along the filaments in all three muscles, there were marked differences in the absolute number and position occupied by the different isoforms. Since McAbs MF-1 and ALD-66 decorated the same 43-nm repeats in the A-bands of the posterior latissimus dorsal muscle, we suggest that at least two C-proteins can co-localize at binding sites 43 nm apart along thick filaments of this muscle.

Purified C-protein is a single polypeptide chain of ~140,000 daltons, present in thick filaments of vertebrate skeletal and cardiac muscle (14, 23). It binds to myosin rod, light meromyosin (9), myosin subfragment 2 (19), and F-actin (10). It inhibits actin-activated ATPase (10), and alters myosin assembly in vitro (8). C-protein has been localized with the aid of polyclonal antibodies to seven stripes in each half of the A-bands of rabbit psoas (3, 13) and chicken pectoralis (16) muscle. The physiological role of C-protein is unknown, but various functions have been suggested, such as thick filament length determination (6), regulation of crossbridge movement (14), thick filament structural support (13, 16), and thick filament conformational change during muscle activation (4). Since C-protein is the only thick filament protein that co-distributes with myosin outside the bare zone, a determination of its organization in the thick filament is necessary to understand the packing structure of this filament.

Recently, isoforms of C-protein have been described in different muscle types in both rabbit (1, 23) and chicken (17). We have generated monoclonal antibodies (McAbs)¹ specific

¹ *Abbreviations used in this paper:* ALD, anterior latissimus dorsi;

for fast and slow C-proteins of the adult chicken, and have studied their distribution in different muscle types (17). We have demonstrated that C-protein isoforms co-exist within single sarcomeres of the posterior latissimus dorsi (PLD) muscle (18). In this report we describe ultrastructural localization of these monoclonal antibodies in chicken pectoralis major (PM), anterior latissimus dorsi (ALD), and PLD muscles. Our results not only confirm those of Craig and Offer (3) and Pepe and Drucker (16) showing that C-protein is located at 43-nm repeats in the A-bands of different muscles, but also demonstrate that different C-protein isoforms have characteristic distributions, that similar isoforms can exhibit different distributions in different muscles, and that more than one isoform of C-protein can co-exist within the same 43-nm repeat of a single A-band.

MATERIALS AND METHODS

Antibodies: Monoclonal antibodies to C-protein isoforms of fast-twitch (MF-1) and slow-tonic (ALD-66) muscle were previously described (17). For

McAbs, monoclonal antibodies; PLD, posterior latissimus dorsi; PM, pectoralis major; RAM, rabbit anti-mouse.

the present experiments, the McAbs secreted into tissue culture medium (200 ml volume) were partially purified by ammonium sulfate precipitation. Protein precipitated between 20–50% saturation was collected, resuspended in 10 ml of solution A (0.1 M KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mM dithiothreitol, 1% glucose, 0.1 mM phenylmethylsulfonyl fluoride, 0.05% NaN₃, 10 mM phosphate buffer, pH 7.0), and dialyzed against 1 liter of solution A overnight, at 4°C. Some batches of MF-1 were further purified on a Sepharose-4B (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) goat-anti-mouse-IgG affinity column. The ammonium sulfate precipitate was first dialyzed against PBS (0.15 M NaCl, 10 mM sodium phosphate buffer, pH 7.0), adsorbed to the affinity column, and washed extensively with PBS. Bound protein was eluted with 0.2 M glycine-HCl (pH 2.3), neutralized with 0.2 M Tris (tris[hydroxymethyl]aminoethane)-HCl, pH 8.6, and dialyzed overnight against solution A. All antibody solutions were cleared by centrifugation at 12,000 g for 30 min before use. Rabbit anti-mouse (RAM) lyophilized whole antiserum (Cappel Laboratories) and purified nonimmune mouse IgG (Cappel Laboratories West Chester, PA) were reconstituted as indicated by the manufacturer.

Tissue Preparation: All procedures were conducted at 4°C. Pectoralis major, ALD, and PLD muscles were obtained from adult white leghorn chickens. The animals were etherized and sacrificed by intravenous injection of 10 ml of 20 mM NiCl₂, 0.1 M NaCl, 0.1 M MgCl₂ (to inhibit muscle contraction). Exposed muscles were bathed in 20 mM Tris-maleate buffer pH 7.0, 1 mM NiCl₂, 0.1 M NaCl, 10 mM MgCl₂, 2 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01% NaN₃, and 2–3-mm thick strips of muscle were dissected parallel to the long axis, tied to wooden sticks, immersed into solution B (20 mM Tris-maleate buffer pH 7.0, 0.1 M NaCl, 10 mM MgCl₂, 2 mM EGTA, 1% glucose, 0.5 mM dithiothreitol, 0.1 M phenylmethylsulfonyl fluoride, 0.05% NaN₃) plus 0.5% Triton X-100 for 30 min, transferred to fresh solution A (with 0.5% Triton X-100) for 6 h (2 changes), placed in solution A (without Triton X-100) overnight, and used the next morning for antibody labeling.

Antibody Labeling: The muscles were removed from sticks and cut or teased into 1 × 10-mm bundles while immersed in solution A, bathed in solution A plus 0.5% Triton X-100 for 30 min, and rinsed in solution A. After incubating the fiber bundles in solution A plus 1% BSA for 10 min, they were placed in a primary antibody solution. Specimens were placed on a rotary mixer in the cold room and incubated for 14 h followed by a 2-h (five changes) wash in solution A. Some of the specimens were then incubated for 2 h with RAM IgG diluted 1:10 in solution A plus 1% BSA and washed as described above. Subsequently, all specimens were fixed and prepared for electron microscopy.

The following labeling protocols were used for each of the three muscles studied: (a) MF-1 only; (b) ALD-66 only; (c) MF-1 followed by RAM IgG; (d) ALD-66 followed by RAM-IgG; (e) RAM IgG only. ALD-66 was used as a control antibody for PM muscle and MF-1 was the control antibody for ALD muscle. Since PLD muscle bound both antibodies, nonimmune mouse IgG followed by RAM IgG was used as its control. The results with or without second antibody labeling were equivalent; the second antibody only enhanced the staining pattern produced by the primary antibody.

Electron Microscopy: The fiber bundles were fixed with 2.5% glutaraldehyde in PBS overnight at 4°C, washed three times with 0.1 M sodium cacodylate buffer (pH 7.0), postfixed 1 h in 1% OsO₄ in 0.05 M sodium cacodylate buffer (pH 7.0), dehydrated in a graded series of ethanol, transferred to propylene oxide, and embedded in Epon 812. Sections 50–80 nm thick were stained with uranyl acetate and lead citrate, and observed in a JEOL 100 CX II electron microscope at 80 kV. Magnifications were calibrated using a negatively stained catalase standard.

Image Enhancement: Photographic enhancement of lateral repeats was achieved by multiple exposures of electron microscope negatives on photographic paper (5). The shift of the photographic paper was perpendicular to the myofibril axis and four exposures were superimposed to enhance laterally spaced densities.

Data Analysis: Electron microscope negatives of well aligned A-bands were selected for optical diffraction. Scattering diagrams were generated on an optical diffractometer in the laboratory of Dr. P. Ross Smith (New York University School of Medicine) and recorded on Kodak Plus-X film. Absolute optical diffraction measurements of calibrated electron microscope negatives revealed a reflection at 13.6 ± 0.3 nm ($n = 8$)² corresponding to the true 14.3-nm myosin repeat. The difference in these two values is accounted for by ~5% tissue shrinkage during specimen preparation. The 14.3-nm reflection was used as an internal standard for measuring the C-protein repeats. Direct measurements of the A-bands of negatives calibrated with catalase crystals showed that ALD muscle A-bands measured 1.59 ± 0.042 μm ($n = 8$), whereas in PM and PLD muscles the values were 1.63 ± 0.038 μm ($n = 8$) and 1.53 ± 0.017 μm

² All numerical values are presented as the mean ± one standard deviation.

($n = 5$), respectively. Based on these results we assumed an A-band length of 1.60 μm and used this value as an internal standard for all labeling measurements made directly from prints (15).

RESULTS

The protocol used in this study for isolating fiber bundles produced relaxed areas throughout the specimen which exhibited excellent ultrastructural preservation when compared with traditional glycerinated preparations. This permitted the observation of broad areas with all the sarcomeres uniformly labeled (Fig. 1). Good alignment of thick filaments of both PM and PLD muscle were obtained, thus permitting the measurement of C-protein repeats by optical diffraction. In the ALD muscle, it was not possible to obtain perfectly aligned thick filaments throughout an A-band and the measurements had to be done directly on the micrographs, using regions of the A-bands in which parallel thick filaments exhibited transverse registration of their M-lines.

Three aspects of the antibody labeling pattern were determined for each muscle-McAb combination: (a) The lateral spacing of the labeled stripes. (b) The number and relative position of the labeled stripes. (c) The position of the labeled stripes relative to the lateral edges of the pseudo-H or bare zone.

The lateral spacing of labeled C-protein stripes was 42.6 ± 1.0 nm ($n = 21$), as determined by optical diffraction of A-bands from PM and PLD muscles. No significant difference in the C-protein repeat was detected between different muscle type (Table I). The labeling repeat of McAb ALD-66 in PLD and ALD muscles was similar when measured with micrographs internally standardized to A-band lengths of 1.60 μm (15) (Table I).

Measurements from micrographs were used for mapping of C-protein labeling patterns in the three muscle types (Fig. 2). Each transverse stripe was mapped along the A-band between positions 1 and 11, as defined by Craig (2). Similar banding patterns have also been observed by Wilson (22) and Sjoström (20) but we have chosen to follow Craig's numeration.

Pectoralis Major (Figs. 3 and 4)

When incubated with MF-1, PM muscle exhibited eight labeled stripes in each half of the A-band. Seven stripes were always detected at positions 5–11, whereas an eighth stripe at position 3 showed variations in label intensity. The distance between the end of the bare zone and the label at position 3 was 93 ± 6 nm ($n = 18$), that between the label at positions 3 and 5 was 86 ± 4 nm ($n = 8$), and that between the bare zone and position 11 was 451 ± 8 nm ($n = 8$). At times we observed some density at position 2, but as can be seen in Fig. 4, this electron density was also present in the control sections, and did not correspond to a site of antibody deposition. No labeling of PM muscle was observed with ALD-66, excluding the red stripe region deep in the pectoralis muscle, which contains mixed fiber types (17).

Anterior Latissimus Dorsi (Figs. 5 and 6)

When incubated with McAb ALD-66, the ALD muscle demonstrated nine labeled stripes in each half of the A-band. The nine stripes correspond to positions 3 through 11. Positions 3 and 11 were located respectively 106 ± 5 nm ($n = 8$) and 467 ± 9 nm ($n = 8$) lateral to the margin of the pseudo-H zone. Nine stripes were observed in all preparations. Since thick filaments in A-bands of the ALD muscle are not in

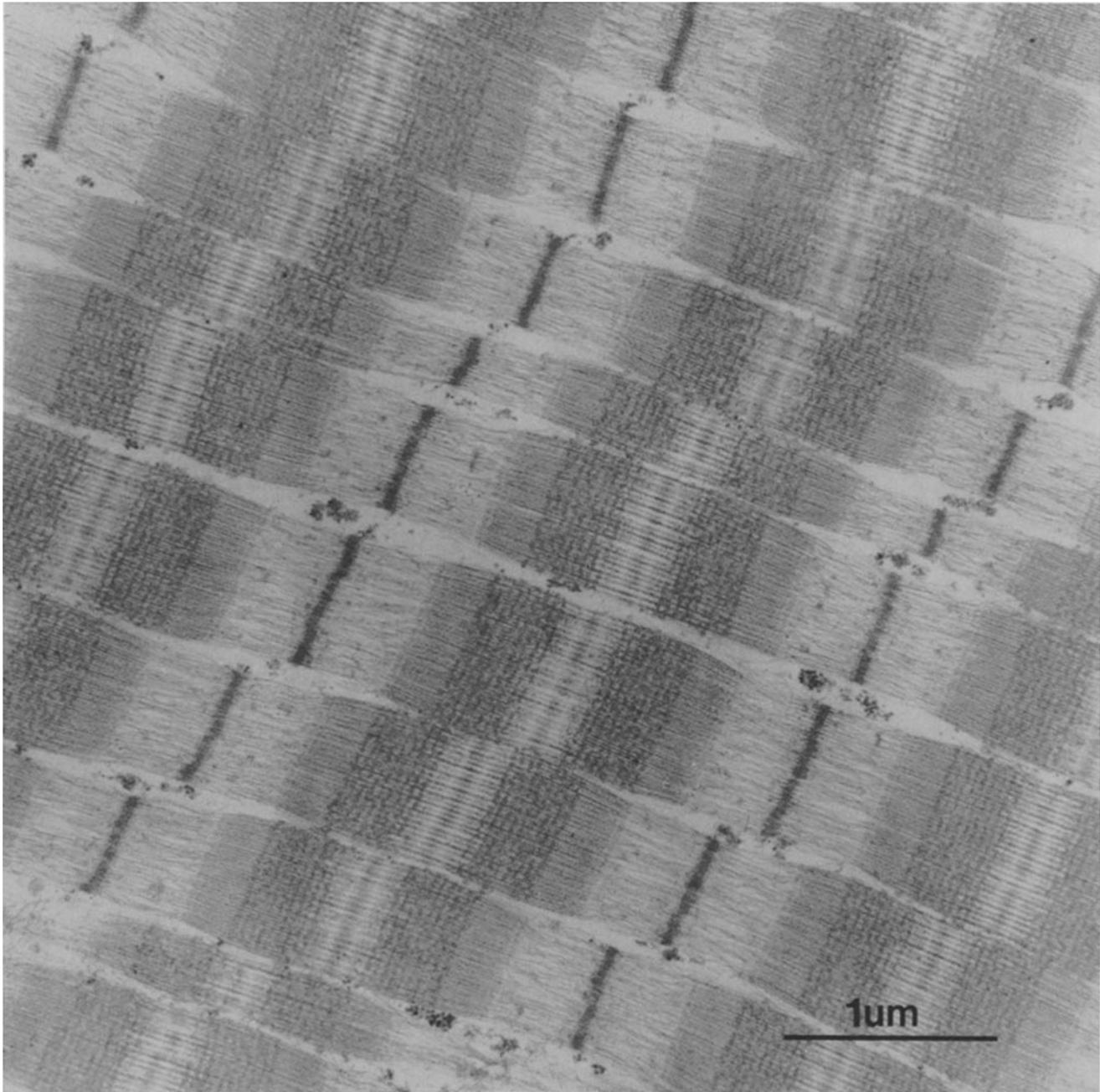


FIGURE 1 Electron micrograph of PLD muscle incubated with McAb ALD-66 followed by rabbit anti-mouse (RAM) IgG. A consistent staining pattern of 18 stripes is seen within each A-band. Intensity of labeling diminished at a distance of 10–15 sarcomeres below the surface of each fiber, presumably from diffusion limitation of the antibody. No alteration of the labeling pattern was observed in sarcomeres having diminished labeling intensity. $\times 34,500$.

TABLE I
Labeled C-Protein Repeat Measurements

Muscle	McAb	C-protein repeat nm	Method
PM	MF-1	42.6 ± 1.1 (11)	A
PLD	MF-1	42.7 ± 0.8 (10)	A
PLD	ALD-66	42.1 ± 1.5 (8)	A
PLD	ALD-66	44.6 ± 2.0 (20)	B
ALD	ALD-66	45.0 ± 1.9 (18)	B

A, measured from diffraction patterns of micrographs; B, measured from printed micrographs. Values in parentheses equal the number of sarcomeres analyzed.

perfect side-to-side register, the C-protein stripes may not line up transversely across the whole A-band. As a result, artificial stripes can be created in the image translation experiments (Figs. 5A and 6A). Thus, we suggest that caution must be exercised in interpreting the image translations of ALD sarcomeres. No labeling was observed in the ALD muscle incubated with MF-1 with or without secondary antibody.

Posterior Latissimus Dorsi (Figs. 7 and 8)

Labeling of the PLD muscle was observed after incubation with either McAb ALD-66 or MF-1. The labeling patterns of

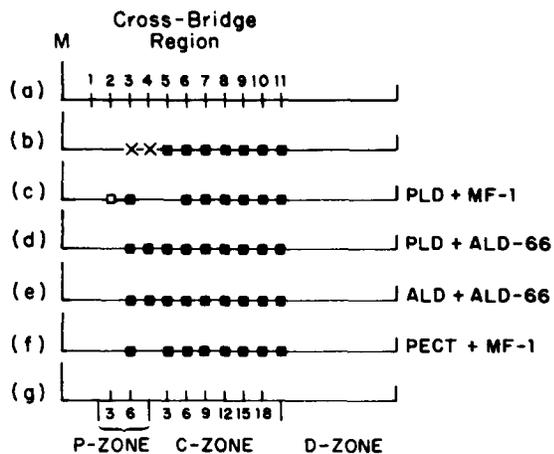


FIGURE 2 Schematic representation of skeletal muscle half A-bands showing labeling patterns of monoclonal antibodies in different muscles. ALD-66 and MF-1 are two monoclonal antibodies. *PLD*, posterior latissimus dorsi; *ALD*, anterior latissimus dorsi; *Pect*, pectoralis major. (a) Numbering of the 43.0-nm repeats observed in negatively stained A segments as described by Craig (2), and as used in the text. (b) Labeling pattern for C-protein in chicken muscle as described by Pepe and Drucker (16). X, stripes not consistently observed in all preparations; ●, stripes definitely ascribed to C-protein antibody labeling. (c) Labeling pattern of McAb MF-1 in chicken PLD muscle. □, stripes consistently observed in all specimens; □, stripe rarely observed. (d) Labeling pattern of McAb ALD-66 in chicken PLD muscle. Nine stripes were consistently observed

these McAbs differed in the number of decorated stripes, their A-band position and their intensity. PLD muscle incubated with ALD-66 exhibited a pattern identical to ALD muscle after labeling with this same antibody: nine stripes were observed in each half of the A-band corresponding to positions 3 through 11. Position 3 was located 111 ± 10 nm ($n = 20$) distal to the bare zone whereas position 11 was 455 ± 8 nm ($n = 20$) away from the bare zone. The stripes did not exhibit any variation in labeling intensity. PLD muscle incubated with MF-1 exhibited eight stripes in each half of the A-band corresponding to positions 2, 3, and 6 through 11. The stripe at position 2 was sometimes unlabeled or poorly labeled, whereas positions 3, and 6 through 11 were always labeled. A gap in labeling was always observed at positions 4 and 5. The

at positions 3–11. Coincident binding of two C-protein isoforms occurs in PLD muscle at positions 3 and 6–11. (e) Labeling pattern of McAb ALD-66 in chicken ALD muscle. The labeling pattern was identical to that of ALD-66-labeled PLD muscle. (f) Labeling pattern of McAb MF-1 in chicken pectoralis muscle. Eight stripes were consistently observed at positions 3 and 5–11, with a gap at position 4. (g) Schematic presentation of skeletal muscle A-bands, as described by Sjostrom and Squire (20), from negatively stained cryosections of human tibialis anterior muscle. Prominent lines in the C-zone at positions C1, C3, ..., C21 were interpreted to be due to C-protein molecules. Other prominent lines were observed at positions P3 and P6 in the P-zone and position M9 in the M-region (this corresponds to position 1 in a above). The spacing between these presumptive nonmyosin proteins is ~ 43.0 nm.

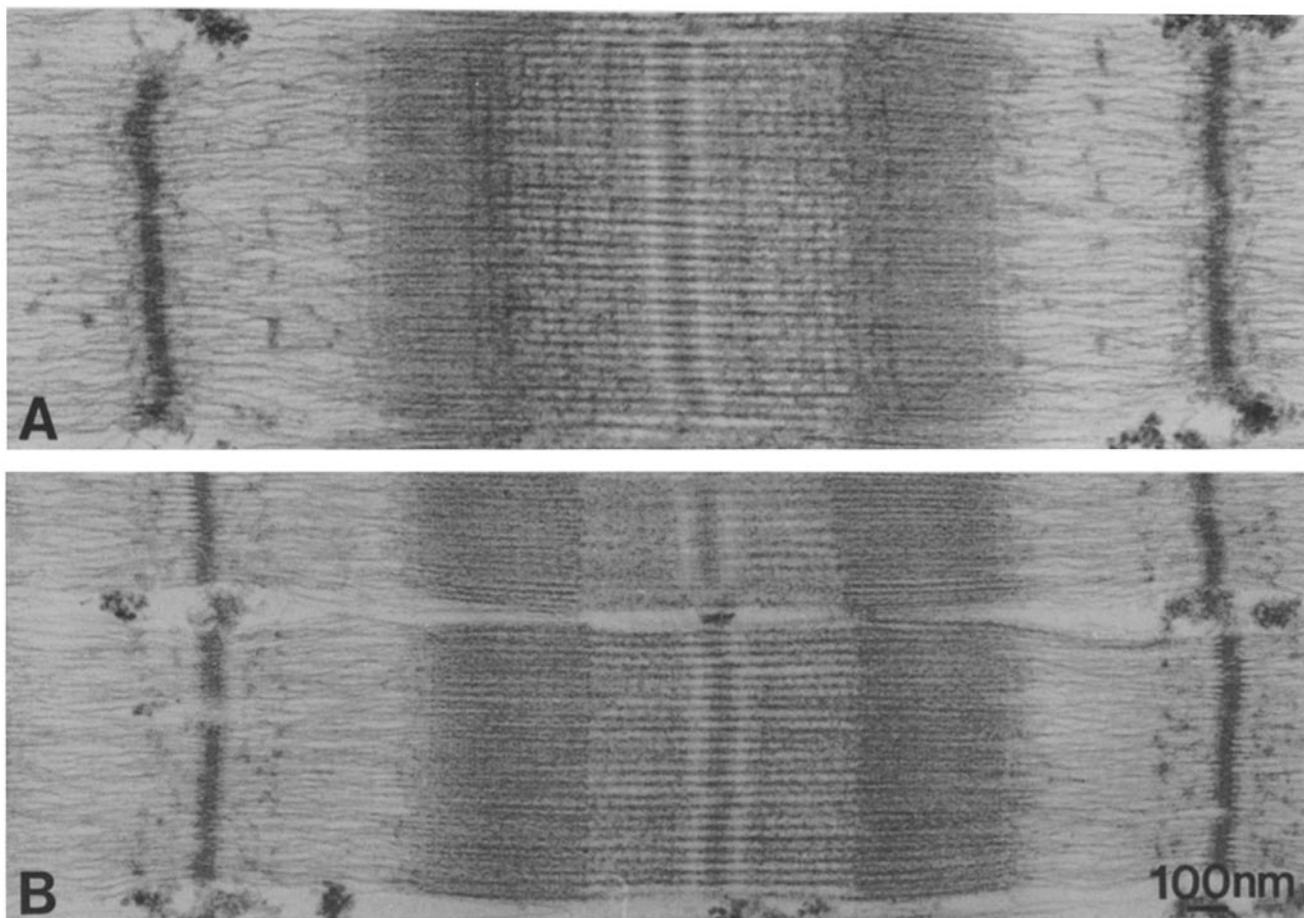


FIGURE 3 Electron micrographs of McAb-labeled PM muscle. (A) MF-1 followed by RAM, showing eight stripes in each half of the A-band; (B) control specimen incubated with ALD-66 followed by RAM IgG. $\times 54,500$ (a); $\times 52,100$ (b).

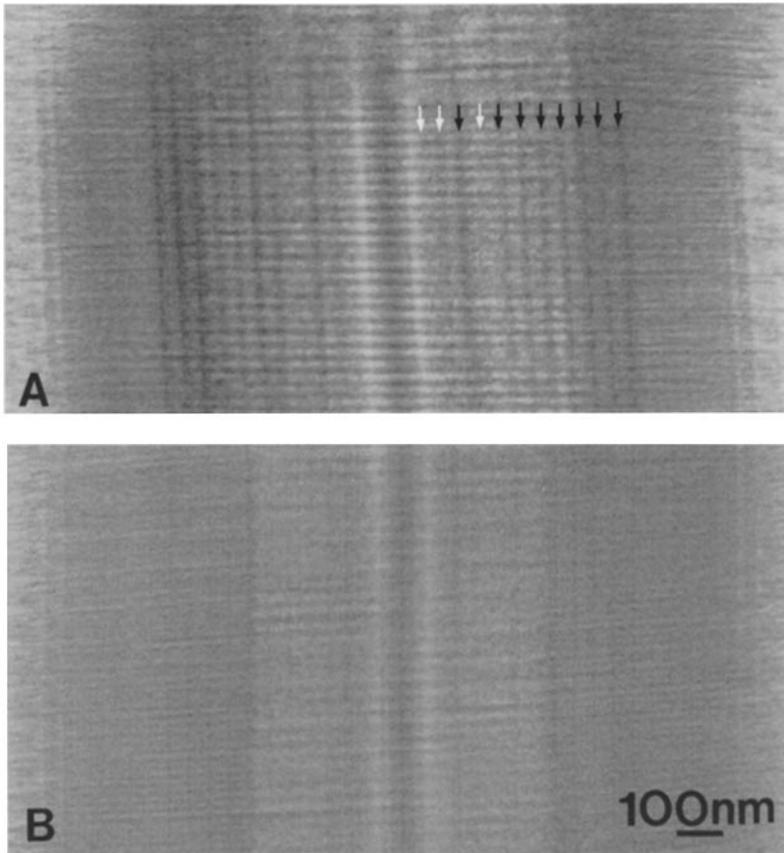


FIGURE 4 Linear photographic translations of electron microscope negatives from PM muscle incubated with (A) MF-1 and (B) ALD-66 McAbs. The arrows show the eleven 43-nm repeats in the A-band. Black arrows indicate the position of the repeats labeled by the monoclonal antibodies; white arrows show the position of the unlabeled repeats.

distance from the end of the bare zone to the labeling at position 2 was 54 ± 4 nm ($n = 20$), position 3: 95 ± 6 nm ($n = 20$), position 6: 233 ± 9 nm ($n = 20$), and position 11: 456 ± 9 nm ($n = 20$). No labeling was observed in PLD muscle incubated with nonimmune mouse IgG with or without secondary antibody.

When the MF-1 labeling pattern in PLD muscle was compared with the pattern observed in pectoralis muscle, it became apparent that these differed in three respects: (a) PLD muscle was never labeled at position 5, whereas PM muscle was consistently labeled at this site; (b) position number 3 was consistently labeled in PLD muscle, whereas labeling of this stripe was inconsistent in PM muscle; and (c) although labeling of position 2 in PLD muscle was sometimes detected, it was never seen in PM muscle. In PLD muscle, coincident labeling of positions 3 and 6 through 11 was always observed with McAbs MF-1 and ALD-66.

DISCUSSION

From the analysis of the McAb staining patterns in ALD, PLD, and PM muscle we can conclude that (a) different isoforms of C-protein in these three muscles are all located within a series of 43 nm repeats in the A-bands, presumably on thick myofilaments; (b) the number and positions of the 43-nm repeats occupied by the different isoforms is different in muscles that contain only one isoform, e.g., ALD and PM muscles; (c) in the PLD muscle, which contains two isoforms within all sarcomeres in all myofibers, it was observed that some 43-nm repeats contained both isoforms whereas other repeats contained only one isoform (see Fig. 2). We conclude that, not only do variant muscle fiber types contain different

isoforms of thick filament proteins, but these are distributed along the thick filaments in a manner characteristic of the specific fiber type.

We have shown the localization of C-protein isoforms at three positions proximal to the seven C-protein stripes previously described (3, 16). The positions of these stripes are consistent with the observations on cryosections of human tibialis anterior muscle, which suggest that nonmyosin proteins are located along three 43.0-nm repeats in the "P-zone" of the A-band (20). Pepe and Drucker (16) have observed that a 9th inner stripe (corresponding to position 3) is consistently labeled with polyclonal C-protein antibody, whereas an 8th stripe (corresponding to position 4) is either faint or missing. Craig and Offer (3) used antiserum absorbed with C-protein to show that the 9th stripe contained a protein contaminant of their original C-protein antigen preparation. However, antiserum purified by passage through a C-protein affinity column still labeled the 8th and 9th stripes at the edge of the fiber (3). Since their work was done with polyclonal serum it was not possible to exclude the presence of other antibodies to minor components of the thick filament (including variant C-protein isoforms) which labeled the proximal (8th and 9th) stripes.

In our experiments, using McAbs, we can be certain of the homogeneity of the antibody preparation. We cannot rigorously exclude the possibility of an unknown antigen present in the inner stripes that can bind the McAb. However, in previous experiments we have shown that in whole muscle homogenates, and during C-protein purification, only the major C-protein polypeptide binds the antibodies (17, 18).

We have also shown coincident binding of both McAbs at positions 3 and 6 through 11 of PLD muscle. Since we have

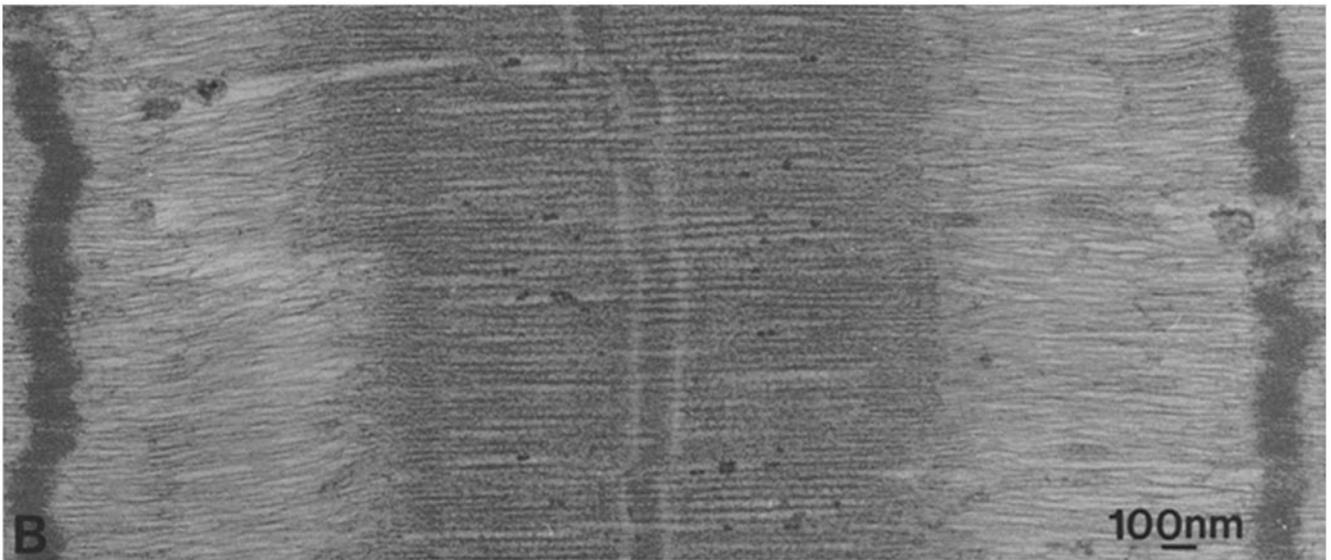
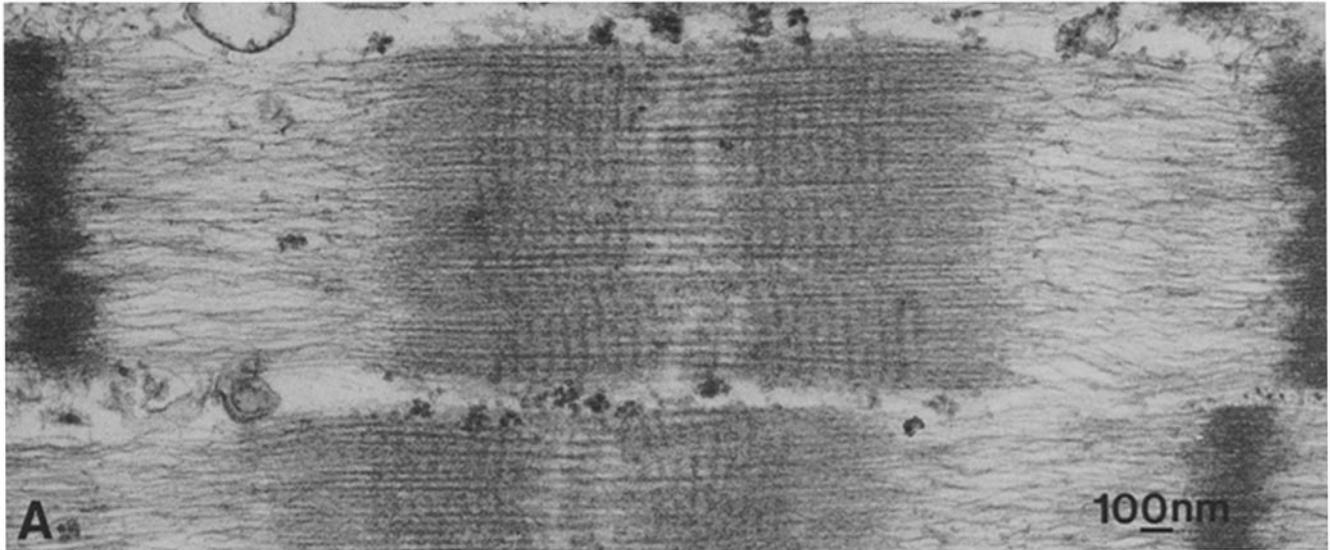


FIGURE 5 Electron micrographs of McAb-labeled ALD muscle. (A) ALD-66 followed by RAM-labeled muscle showing nine stripes in each half of the A-band; (B) control specimen incubated with MF-1 followed by RAM. $\times 47,400$ (A); $\times 53,100$ (B).

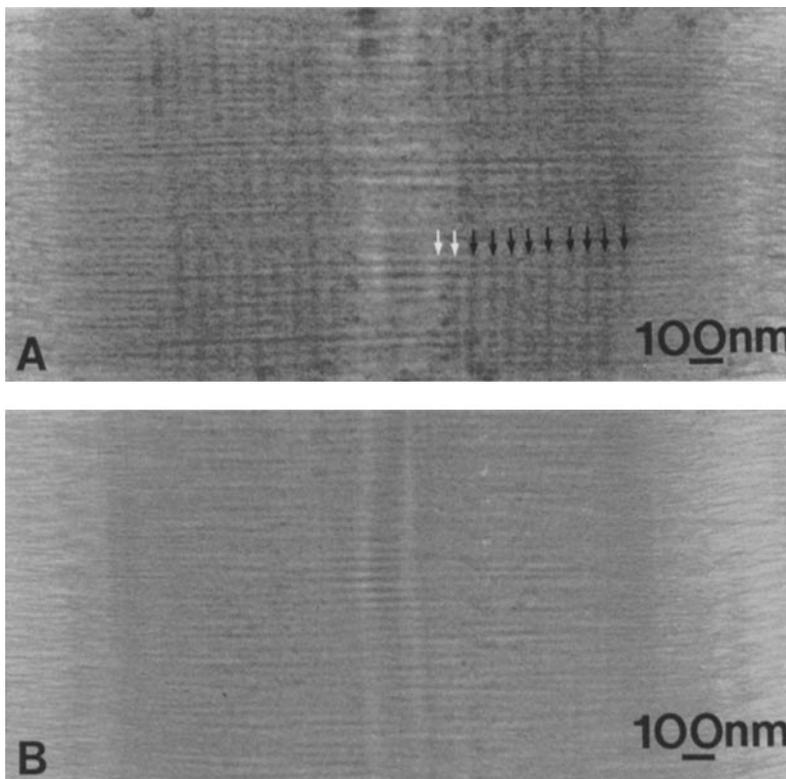


FIGURE 6 Translation of negatives from specimens shown in Fig. 5. (A) ALD-66-labeled ALD muscle; (B) MF-1-labeled ALD muscle. The arrows show the eleven 43-nm repeats in the A-band. Black arrows indicate the position of the repeats labeled by the monoclonal antibodies; white arrows show the position of the unlabeled repeats.

demonstrated that both antibodies bind to different C-protein isoforms in this muscle, we conclude that at least two dissimilar C-protein molecules are localized on each thick filament along positions 3 and 6 through 11. Offer (14) estimated, from the ratio of C-protein to actin in myofibrils, that there were $\sim 37 \pm 7$ C-protein molecules per thick filament. In ALD and PLD muscle labeled with ALD-66 antibody we consistently observed nine stripes in each half of the A-band; this would correspond to 2.05 C-protein molecules per stripe. For PM muscle we observed a maximum of eight stripes, and for PLD labeled with ALD-66 and MF-1, nine and eight stripes, respectively. This would correspond to 2.31, 2.05, and 2.31 C-protein molecules per stripe respectively. However, Morimoto and Harrington (11) estimated 52 ± 5 C-proteins per filament, from the molar ratios of myosin to C-protein. These data would indicate there are 2.9 C-protein molecules per stripe if we assume there are 18 stripes per filament. Clearly, more data are needed to accurately establish the number of C-proteins per stripe.

The simplest model would predict the presence of 2–3 C-protein molecules at each of the 18 stripes along the thick filament, 9 in each half A-band. These could be occupied by a homogeneous population of C-protein molecules, as in the outer stripes of PM and ALD muscle, or by a heterogeneous mixture of two different C-protein isoforms as in the case of the PLD muscle. This model could be extended to the inner stripes where, depending on the muscle type, the sites could be occupied partially by C-protein or with other minor protein components of the thick filament yet to be isolated. Evidence that these components might exist is provided by the labeling obtained by Craig and Offer (3) using absorbed polyclonal antiserum.

The major implication of the present study is that not only do thick filaments of “fast” and “slow” muscle fibers differ in isoform composition, but they also differ in the distribution of these isoforms along the myofilaments. Ignoring other components of the thick filament, C-protein distribution could be affected both by the structure of the particular C-protein isoform and the composition and organization of the underlying myosin molecules. The complexity of the problem is significant during myogenesis where different myosin isoforms appear sequentially during the course of embryonic and postnatal development (21). We have recently analyzed the expression of C-protein isoforms in the PM during chicken development using the same panel of McAbs (12) and have observed a period in the growth of PM in which “fast” and “slow” isoforms of C-protein co-exist in all myofibers. This phenomenon represents a developmental analogue of that seen in the adult PLD muscle. Thus, during the course of myogenesis, thick filaments must change in their composition of both myosin and C-protein, suggesting a complex interplay of these two sets of proteins. The physiological implications of these observations are uncertain but they reemphasize the need for inclusion of cross-bridge heterogeneity in models of thick filament construction and function.

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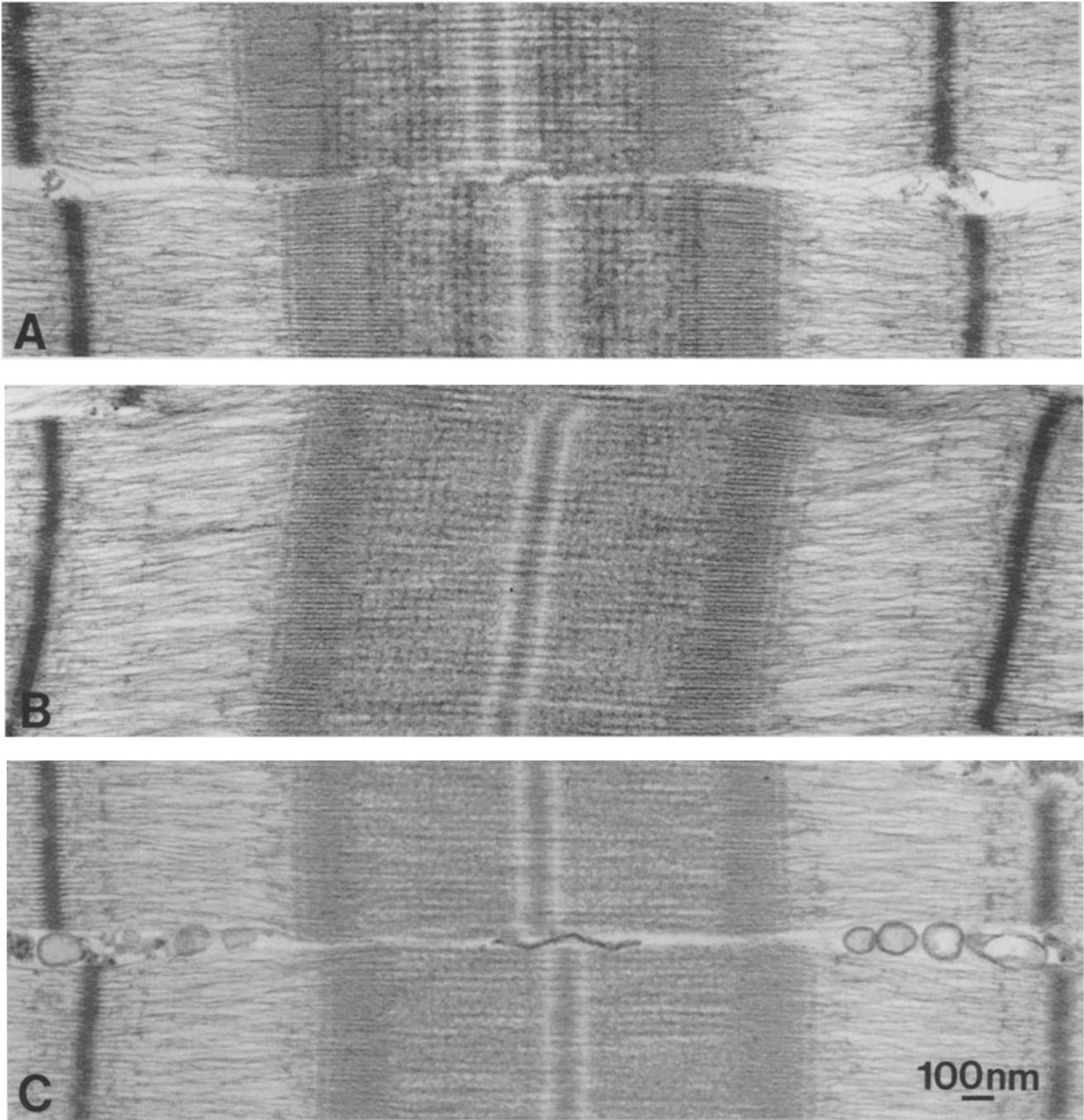


FIGURE 7 Electron micrographs of McAb-labeled PLD muscle. A shows a pattern of nine consecutive stripes in PLD incubated with ALD-66 followed by RAM. B shows the six-plus-two labeling pattern obtained with McAb MF-1 followed by RAM. C shows a control specimen of PLD muscle incubated with nonimmune mouse IgG followed by RAM $\times 52,500$ (A); $\times 53,800$ (B); $\times 53,900$ (C).

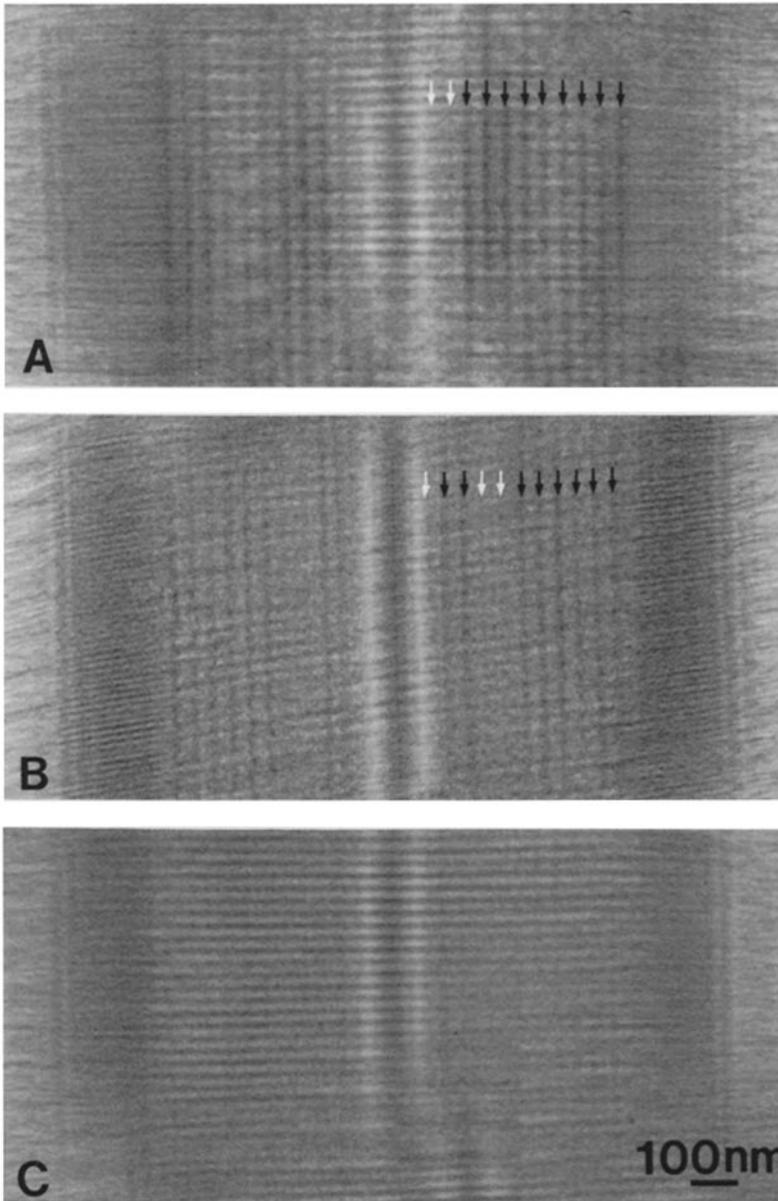


FIGURE 8 Photographic translation of negatives from PLD specimens shown in Fig. 7. (A) ALD-66 decoration of PLD muscle; (B) MF-1 decoration of PLD muscle; (C) control specimen incubated with nonimmune mouse IgG followed by RAM IgG. The arrows show the eleven 43-nm repeats in the A-band. Black arrows indicate the position of the repeats labeled by the monoclonal antibodies; white arrows show the position of the unlabeled repeats.

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