

The M_r 165,000 M-protein Myomesin: A Specific Protein of Cross-striated Muscle Cells

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ABSTRACT The tissue specificity of chicken 165,000 M-protein, tentatively named "myomesin," a tightly bound component of the M-line region of adult skeletal and heart myofibrils, was investigated by immunological techniques. Besides skeletal and heart muscle, only thymus (known to contain myogenic cells) was found to contain myomesin. No myomesin could, however, be detected in smooth muscle or any other tissue tested. This result was confirmed *in vitro* on several cultured embryonic cell types. Only skeletal and heart muscle cells, but not smooth muscle or fibroblast cells, showed the presence of myomesin. When the occurrence and the distribution of myomesin during differentiation of breast muscle cells in culture were studied by the indirect immunofluorescence technique, this protein was first detected in postmitotic, nonproliferating myoblasts in a regular pattern of fluorescent cross-striations. In electron micrographs of sections through young myotubes, it could be shown to be present within the forming H-zones of nascent myofibrils. In large myotubes the typical striation pattern in the M-line region of the myofibrils was observed. Synthesis of myomesin measured by incorporation of [³⁵S]methionine into immunoprecipitable protein of differentiating cells increased sharply after ~48 h in culture, *i.e.*, at the time when the major myofibrillar proteins are accumulated. No significant amounts of myomesin were, however, found in cells prevented from undergoing normal myogenesis by 5'-bromodeoxyuridine.

The results indicate that myomesin (a) is a myofibrillar protein specific for cross-striated muscle, (b) represents a highly specific marker for cross-striated muscle cell differentiation, and (c) might play an important role in myofibril assembly and/or maintenance.

The temporal appearance of myofibrillar proteins as well as the composition and organization of myofibrils in differentiating muscle cells have been subjects of intense studies in recent years (for review, see references 1, 5, 11, 13, 17, 19, 24, and 30). Investigations of the properties of a number of newly detected, so-called minor components of the myofibrillar compartment (12, 14, 25, 28, 31, 48, 53) have provided a better insight into the molecular organization of the unit of contractility, the sarcomere. There is, however, still considerable lack of knowledge regarding the sequence of appearance and organization of the myofibrillar structures during development (1, 13, 19, 37, 49). One of the major transverse structures in a mature cross-striated muscle fiber, the Z-band, has been described as the initial site of myofibrillogenesis (19), whereas the second one, the so-called M-line, has only recently been found to play a role in the organization of the assembly of the myofibrillar structure (10, 12, 16, 43).

Our own investigations on the structure and function of the M-line region of chicken skeletal and heart muscle indicate the presence of two major protein components: the muscle type isoprotein of creatine kinase (MM-CK) (48, 50, 52) and the 165,000 M-protein (42, 43).

The M-protein was first isolated from cross-striated skeletal muscle by Masaki and Takaiti (26) and more recently by Trinick and Lowey (44) and Strehler et al. (42). It is also present in myofibrils lacking a visible M-line, *e.g.*, those from chicken heart muscle (41-43, 51), while the second M-line protein MM-CK most certainly is responsible for the electron-dense material forming the visible M-line (43, 48, 50, 52). We propose that the 165,000 M-protein be called "myomesin".¹ The new terminology will, firstly, help to distinguish this muscle protein from other components with similar molecular

¹ Myomesin: derived from Greek *μυών μέση*.

weights and, secondly, avoid confusion with "M-protein" of other types. Myomesin is a tightly bound component of the M-line region. It can only be extracted quantitatively under conditions where the myofibrils are virtually destroyed (43); in vitro experiments also indicate a high affinity of this protein for myosin (23). Thus, a key role of myomesin in the molecular organization of the thick filaments and their spatial organization can be assumed. In this communication we report on three interdependent approaches to better understand the characteristics of myomesin. Immunological techniques employing specific antibodies against chicken myomesin were used (a) to determine whether myomesin is restricted to cross-striated skeletal and heart muscle cells or whether it can be found in smooth muscle and in nonmuscle cells as well; (b) to study the time of appearance and the location of myomesin in differentiating and in differentiation-blocked muscle cells; and (c) to obtain data on the rate of synthesis of myomesin in differentiating cells.

MATERIALS AND METHODS

Cell Cultures

Cells from the heart muscle (3) of 8- to 10-d chicken embryos and from the breast muscle and the gizzard (47) of 10- to 12-d chicken embryos were obtained as described. 1×10^6 cells in 4 ml of standard medium (85 parts minimal essential medium [MEM], 10 parts horse serum, 5 parts chicken embryo extract, supplemented with antibiotics [47]) were usually plated into 60-mm gelatinized tissue culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) containing four to six sterilized and gelatinized glass coverslips. Breast muscle cells in suspension were grown according to Puri et al. (36). Incubation conditions were as those already described (47). Fibroblast cells were obtained either by dissociating the connective tissue surrounding the breast muscle of 11-d chicken embryos or by subculturing 72-h-old primary breast muscle cultures after trypsinization and filtration of the cell suspension through 10- μ m nylon mesh (47). To prevent overgrowth of primary skeletal muscle cell cultures by fibroblasts, 1- β -D-arabinofuranosyl cytosine (ara-C; Sigma Chemical Co., St. Louis, Mo.) was added to the cultures after 48 h to a final concentration of 5×10^{-6} M. Normal differentiation was prevented by culture in medium containing 5'-bromodeoxyuridine (2.5 μ g/ml; BrdUrd, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) or in standard medium with 1.7 mM EGTA.

Antibody

The characterization of myomesin and of the affinity column-purified rabbit anti-chicken myomesin antibody has been described in recent publications from this laboratory (42, 43). Preimmune serum or IgG fraction prepared as described by Sober and Peterson (40) from the same rabbits was always used in control experiments.

Indirect Immunofluorescence Technique

Cells grown on coverslips were washed for 5 min in phosphate-buffered saline (PBS), fixed for 30 min in 3% (wt/vol) paraformaldehyde in PBS, 0.1 mM CaCl₂, 1 mM MgCl₂, washed again in PBS, incubated for 30 min in 0.1 M glycine in PBS (quenching), and permeabilized for 3-5 min in 0.2% Triton X-100 in PBS according to Ash et al. (2). After washing in PBS, the coverslips were incubated for 15-30 min on a drop of PBS containing antimyomesin IgG (20 μ g/ml) or IgG from preimmune serum (40 μ g/ml), washed with PBS (15 min, three changes of PBS), and then incubated for 15-30 min in fluorescein-conjugated goat-anti-rabbit IgG (Grand Island Biological Co., Grand Island, N. Y.) diluted 1:50 in PBS. The coverslips were then extensively washed with PBS and mounted on slides with 90% glycerol. Cells were observed under a Zeiss Standard Model 18 microscope equipped with epifluorescence optics.

Electron Microscopy

Cells were prefixed for 30 min with 3% (wt/vol) paraformaldehyde in PBS, 0.1 mM CaCl₂, 1 mM MgCl₂, washed with PBS, permeabilized for 30 min with 0.2% Triton X-100 in PBS containing 5 mg/ml bovine serum albumin, and

incubated for 60 min in the same solution containing 40 μ g/ml antimyomesin IgG or 100 μ g/ml preimmune IgG. After washing with PBS, cells were fixed with 3% glutaraldehyde in PBS (15 min), postfixed in 1.5% OsO₄ in PBS (30 min), dehydrated in ethanol, and embedded in Epon 812. Thin sections were cut on an LKB-Ultratome 4801 A (LKB Produkter AB, Stockholm, Sweden), poststained with 2% uranyl acetate in H₂O (45 min) followed by lead citrate (0.13 M Pb(NO₃)₂, 0.19 M Na-citrate in H₂O, 15 min), and observed under a Siemens Elmiskop 102 electron microscope at 100 kV.

Determination of the Rate of Myomesin Synthesis in Cultured Cells

Primary breast muscle cell cultures (2×10^6 cells/100-mm gelatinized Falcon tissue culture dish) were labeled at different intervals after plating for 2 h with 250 μ Ci of [³⁵S]methionine (New England Nuclear, Boston, Mass., sp act 500 Ci/mM) in 2.5 ml of culture medium (86.5% MEM, 10% horse serum, 3.5% embryo extract). Labeled cultures were washed three times with PBS and stored frozen at -20°C. Cells were scraped from plates in 500 μ l of PBS, 0.1% sodium dodecyl sulfate, 0.5% Triton X-100, and then sonicated (three times 15 s, maximal output). An aliquot of the cell homogenate was used for the determination of DNA (4).

The rest of the homogenate was digested for 15 min at 37°C with 0.12 vol of an RNAase/DNAase solution (10 mg of pancreatic DNase I (Sigma Chemical Co.) and 5 mg of pancreatic RNAse (Sigma Chemical Co.) in 10 ml of 0.5 M Tris-HCl, 0.05 M MgCl₂, pH 7) and the total acid-precipitable radioactivity was measured as described by Caravatti et al. (6). Myomesin was isolated from the radioactive cell extract by immunoprecipitation as described for MM-CK (6, 35). 1-2 μ g of carrier myomesin was added to the radioactive cell extract containing 2×10^6 acid-insoluble dpm and the specific immunoprecipitation was performed with 10 μ l of antimyomesin serum. Immunoprecipitates were separated by electrophoresis on 8% polyacrylamide slab gels in the presence of 0.1% SDS (21), and the Coomassie Brilliant Blue-stained myomesin bands were cut out from the gels and counted in a Packard Tri-Carb 2650 scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) as described (6).

Preparation of Tissue Extracts

Tissues were excised on ice from chicken embryos or freshly killed adult chicken, cut into small pieces, and either used directly or stored in liquid nitrogen until use. About 0.5 g of tissue was homogenized with 1-2 ml of extraction buffer (0.6 M KCl, 1 mM EDTA, 1 mM MgCl₂, 10 mM Na₂P₂O₇, 0.3 mM dithiothreitol, 0.1 M K-phosphate buffer, pH 6.4) in a Dounce homogenizer (Kontes Co., Vineland, N. J.) and extracted at 4°C for 6-12 h. After centrifugation in a Beckman microfuge B (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 5 min, the supernates were used for double immunodiffusion tests against affinity-purified antimyomesin IgG.

RESULTS

Tissue Specificity of Myomesin

Using the antibody against myomesin, we investigated the cross-reactivity in a number of chicken tissue extracts as well as in different cell types in culture, because the question of whether this protein can be found only in muscle tissues or in nonmuscle tissues as well has not yet been conclusively answered. The results of the double immunodiffusion cross-reactivity tests in tissue extracts are summarized in Table I. Out of 15 adult tissues, only breast and leg skeletal muscle, ventricular and atrial heart muscle, and thymus show a cross-reaction with antimyomesin antibody. A positive reaction could also be found in the same tissues from 11- and 19-d-old chicken embryos (embryonic thymus was not tested). Thus, myomesin seems to be present uniquely in myofibril-containing cross-striated skeletal and heart muscle. The reaction with thymus extract is not surprising because it is well known that mammalian thymus contains a population of myogenic cells capable of forming myotubes showing well-developed myofibrils (18, 54). The same was found recently for chicken thymus (J. C. Perriard, personal communication). The data in Table I ap-

TABLE 1
Detection of Myomesin in Tissue Extracts from Chicken

Tissue	11-d Em- bryos	19-d Em- bryos	Adult
Breast muscle	(+)	+	+
Leg muscle	ND	+	+
Heart	(+)	+	Ventricle: + Atrium: +
Gizzard	-	-	-
Crop	-	-	-
Gut	ND	-	-
Lung	ND	ND	-
Liver	-	-	-
Kidney	ND	ND	-
Spleen	ND	ND	-
Thymus	ND	ND	+
Oviduct	ND	ND	-
Ovary	ND	ND	-
Pancreas	ND	ND	-
Brain	-	-	-

(+), Very weak reaction; ND, not determined.

parently contradict the results obtained by Schollmeyer et al. (38) who are, to our knowledge, the only authors who describe the presence of an M-protein in cells different from the skeletal or heart muscle type, namely in chicken gizzard, human platelets, human uterus, and mouse 3T3 fibroblast cells.

Fig. 1*a-d* show primary cultures of embryonic chicken skeletal and heart muscle cells stained for myomesin by the indirect immunofluorescence technique and compared to identically treated primary cultures of chicken gizzard smooth muscle cells (Fig. 1*e* and *f*) and chicken fibroblasts (Fig. 1*g* and *h*). No indication for the presence of myomesin can be found either in fibroblasts or in gizzard cells. Control experiments done with IgG from preimmune serum show no fluorescence in all cell types studied. Cryosections from adult tissues incubated with antimyomesin IgG and subsequently stained with fluorescence-labeled second antibody confirmed these results: There is strong M-line fluorescence in skeletal muscle tissue but no fluorescence in gizzard tissue (not shown).

Myomesin in Developing Primary Muscle Cell Cultures

In isolated chick myofibrils, myomesin is associated exclusively with sarcomere M-lines (42-44). Thus, the myofibrillar M-line localization of myomesin as revealed by the indirect immunofluorescence technique can be used as a very sensitive assay for the accumulation of this protein during differentiation. Staining with antimyomesin antibody demonstrates that still proliferating presumptive myoblasts (6 h in culture) do not accumulate detectable amounts of antigen (Fig. 2*a* and *b*) while 18 h later nascent myotubes already show some distinct fluorescence in a cross-striated arrangement (Fig. 2*c* and *d*). However, later the myotubes become stuffed with aligned myofibrils (Fig. 2*e* and *f*), and myomesin can be easily localized within the H-region of the single sarcomeres (Fig. 2*i* and *k*).

It is fair to assume that the accumulation of myomesin is coordinately regulated with the accumulation of the major myofibrillar proteins characteristic of differentiated muscle cells. Accumulation of a number of muscle-specific isoproteins

is thought to begin after withdrawal of myogenic cells from the mitotic cycle and can occur independent of fusion into myotubes (7, 9, 27, 29, 33, 46, 49). Myogenic cells were cultured in suspension and later allowed to attach and spread for 24 h according to Puri et al. (36). As can be seen in Fig. 3*a* and *b*, most of the cells show bright, regular cross-striations extending into the processes of the still mostly unfused myoblasts after incubation with antimyomesin antibody. A similar result could be obtained when myogenic cells were cultured in EGTA-containing medium (Fig. 3*c* and *d*) which is known to prevent fusion but not differentiation of myogenic cells (45, 46). Addition of the thymidine analogue BrdUrd to the medium not only prevents presumptive myoblasts from becoming postmitotic but also prevents accumulation of muscle-specific proteins or isoenzymes (6, 32, 34, 45-47). This is also true for the accumulation of myomesin as is shown in Fig. 3. No significant immunofluorescence can be detected in proliferating cultures which have been subcultured in BrdUrd-containing medium (Fig. 3*e* and *f*); a switch from BrdUrd medium to standard medium, however, allows the formation of myotubes, and the concomitant appearance of myomesin-specific fluorescence within myofibrils can be observed (Fig. 3*g* and *h*).

In addition, myomesin synthesis in developing skeletal muscle cell cultures was measured by immunoprecipitation techniques (6, 35). Significant synthesis was not detectable during the first 2 d of culture; the low background synthesis could be attributed to the presence of a few contaminating early-fusing myotubes in the culture. During the next 2 d, synthesis rose sharply to much higher values at 96 h (Fig. 4). Culture in BrdUrd-containing medium resulted in suppression of significant myomesin synthesis, while synthesis was resumed if cells were transferred into standard medium.

Thus, these results corroborate the results from the immunofluorescence studies and demonstrate that accumulation of myomesin into M-line structures parallels its synthesis. A similar behavior has been described for, e.g., the MM-type isoenzyme of creatine kinase (6, 34). The decrease in myomesin synthesis after day 4 may be a culture artifact or reflects a net decrease in the rate of myomesin synthesis as has been observed also for myosin heavy chain (8).

Ultrastructural Localization of Myomesin in Myogenic Cells

We have shown in previous reports that myomesin is an integral component of myofibrils in adult cross-striated muscle even if no visible M-line can be detected (42, 43). This assertion apparently documents that the array of the thick filaments of myofibrils does not necessarily need to be accompanied by what is a visible M-line which, as we assume, is made up, at least partially, of MM-CK (50, 52). In addition, it has also been reported that during myofibril assembly in developing muscle a visible M-line (i.e., electron-dense material) appears only relatively late as compared to the formation of the Z-line structure (11, 13, 19). Judging, however, from the immunofluorescence observations and from the synthesis rate studies described above, we expected myomesin to be present very early during myofibrillar assembly. Thus, we had been interested in corroborating the idea of an analogy between the situation in chicken heart myofibrils and the one in newly assembled myofibrils in early myotubes or postmitotic myoblasts. Cells were fixed after 48 h in culture, subsequently

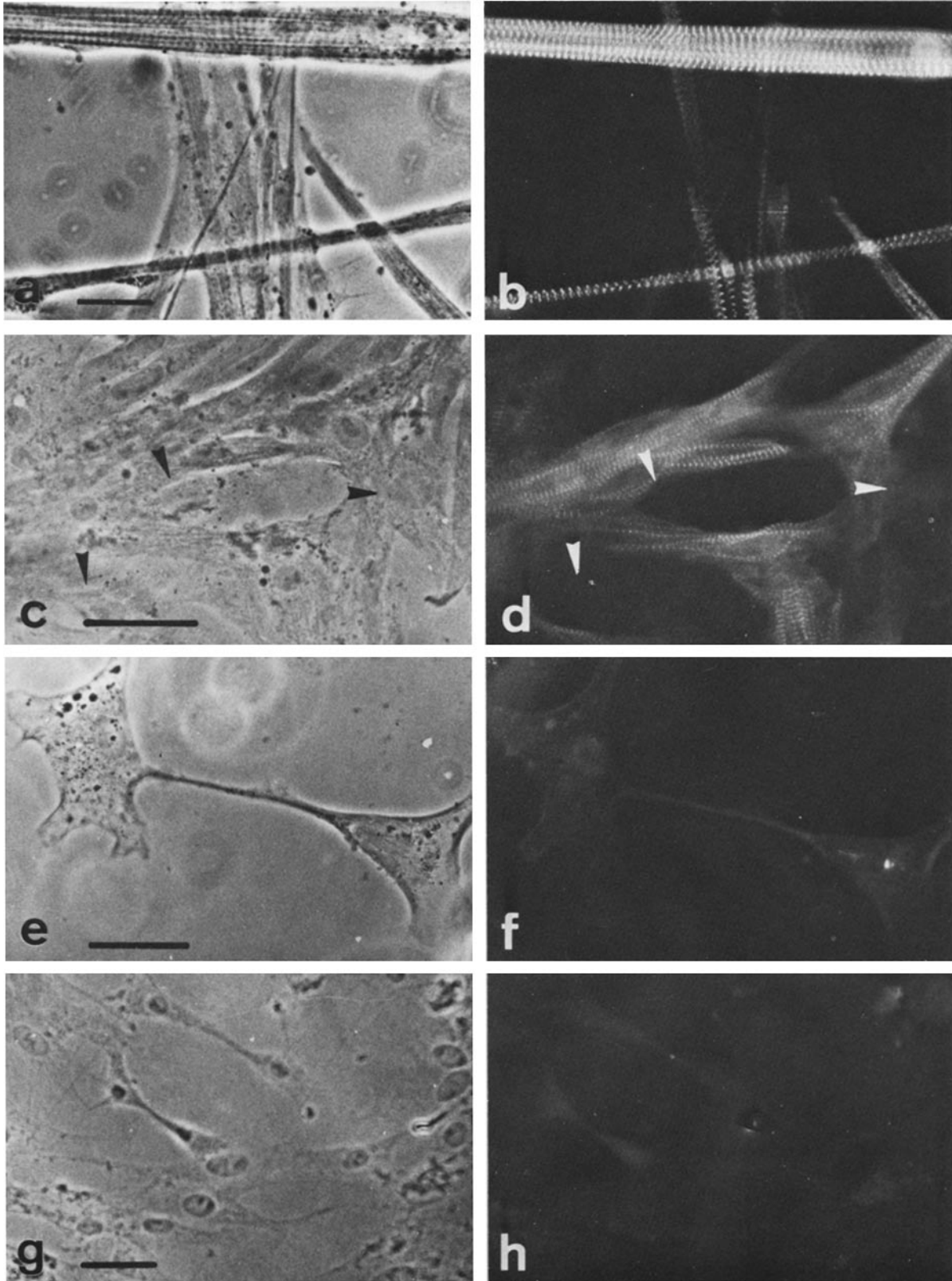


FIGURE 1 Indirect immunofluorescence on 96-h chicken cell cultures using affinity-purified antibody against myomesin. (a and b) breast muscle; (c and d) heart muscle; (e and f) smooth muscle (gizzard); (g and h) fibroblast cultures. Left side: phase contrast; right side: epifluorescence optics. Arrows (c and d): Note the absence of myomesin-staining in fibroblasts contaminating the heart cell culture. Bars, 40 μ m.

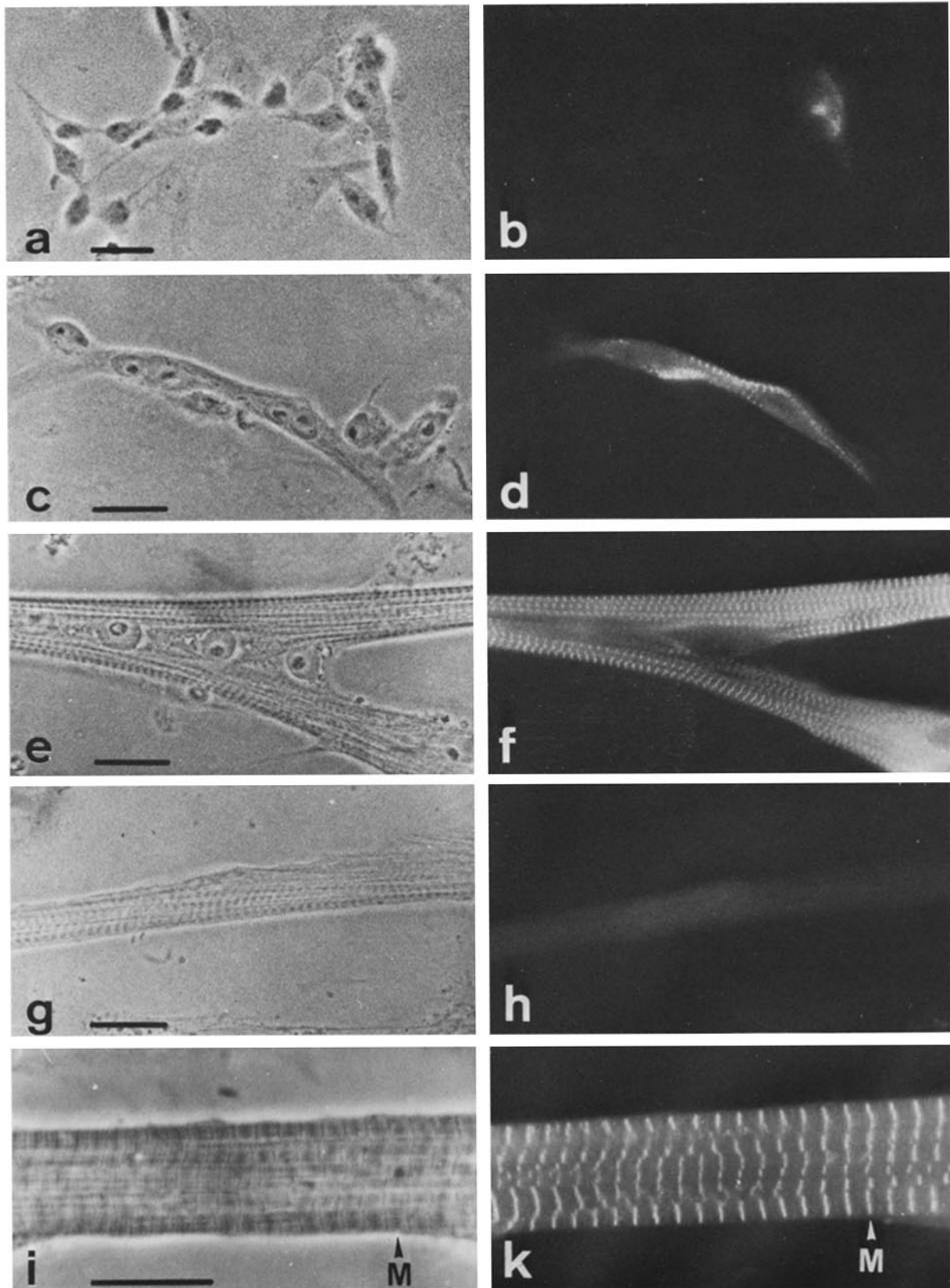


FIGURE 2 Indirect immunofluorescence using affinity-purified antibodies against myomesin. Chicken breast muscle cell cultures were 6 h (a and b) (representing proliferating presumptive myoblasts), 24 h (c and d), and 6 d (e and f) after plating. (g and h) 6-d culture incubated with preimmune IgG. (i and k) close-up of myofibrils in a 15-d-old myotube. Left side: phase contrast; right side: epifluorescence optics. M, M-line region. Bars, 20 μ m.

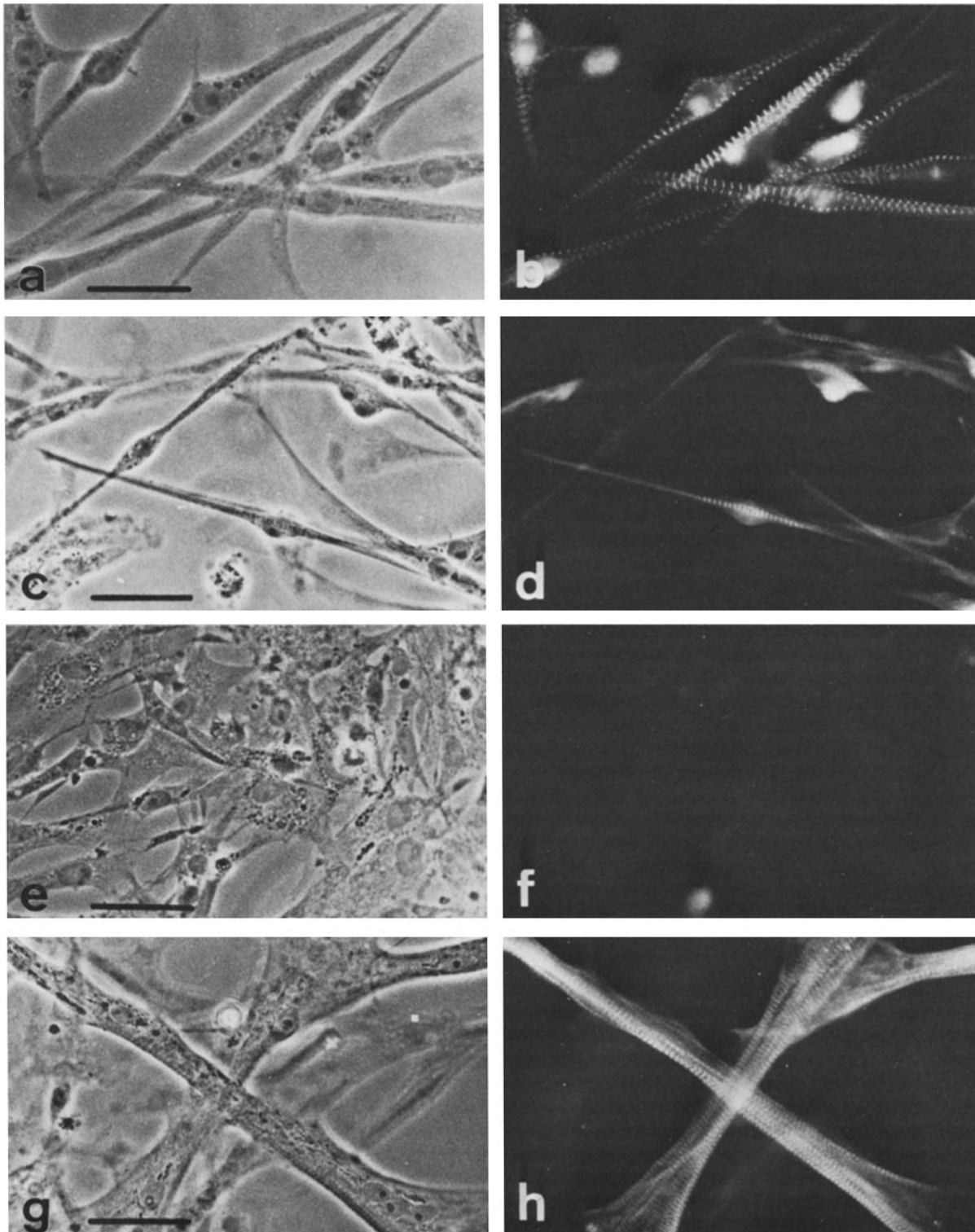


FIGURE 3 Indirect immunofluorescence using myomesin-specific antibody on chicken breast muscle cell cultures grown in suspension for 48 h and allowed to attach to substrate for 24 h (a and b), grown for 72 h in 1.7 mM EGTA-containing medium (c and d), grown for 96 h in BrdUrd-containing medium (e and f), or grown for 48 h in BrdUrd-containing medium and then switched to standard medium for 48 h (g and h). Left side: phase contrast; right side: epifluorescence optics. The diffuse additional fluorescence occasionally observed stems from myofibrils out of focus or from background staining varying with cell thickness. Bars, 40 μ m.

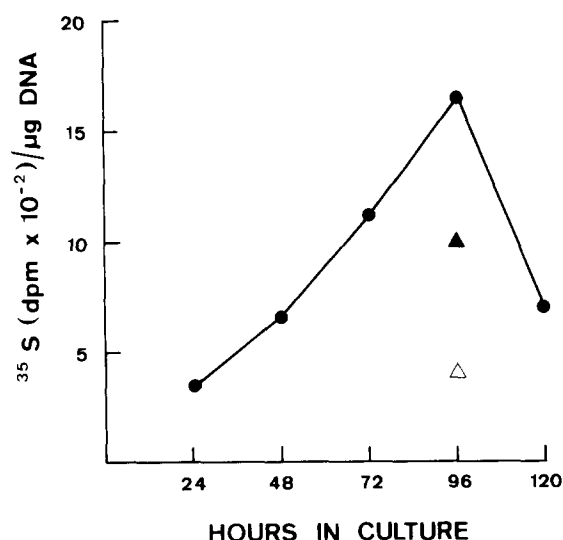


FIGURE 4 Synthesis of myomesin during myogenesis in vitro. Incorporation of [^{35}S]methionine into myomesin synthesized in chicken breast muscle cells was measured. Cells were pulsed for 2 h with [^{35}S]methionine at the times after plating indicated. Each value represents the average of two measurements. Δ , Value found in confluent cultures grown in the presence of BrdUrd. \blacktriangle , Value found in cultures grown for 48 h in BrdUrd-containing medium and then switched to standard medium for 48 h.

incubated with antimyomesin IgG, sectioned, and prepared for electron microscopy. There is indeed no visible M-line detectable in most of the sarcomeres of untreated or preimmune IgG-incubated myofibrils of young myotubes as shown in Fig. 5a. After incubation with specific antimyomesin antibody, a strong IgG deposit in the M-line region can be observed (Fig. 5b), indicating the presence of the antigen at this stage of differentiation. The data suggest that myomesin itself does not contribute to the electron-dense M-line (very probably made up by primary M-bridges [20, 22, 39, 43]) which seems to be formed at a later stage and which consists predominantly of MM-creatine kinase (28, 43, 48, 50, 52). Traces of electron-dense material within the M-line region in individual sarcomeres very probably represent the beginning integration of MM-CK into the maturing sarcomeres. A similar sequence of events has been observed in immunofluorescence studies where MM-CK hardly can be detected within the M-line of myofibrils in very young myotubes (unpublished observation).

DISCUSSION

The results presented here demonstrate that the 165,000 dalton M-protein myomesin, a component of the M-line region of skeletal and heart myofibrils, represents an excellent marker of cross-striated muscle cells. In contrast to published data (38), no myomesin could be found in any other cell type, neither in smooth muscle cells nor in other motile systems, e.g., the stress fibers of fibroblasts. It has to be stated, however, that Schollmeyer and co-workers (38) purified the putative M-protein from chicken gizzard, whereas we refer to myomesin isolated from skeletal muscle tissue; thus the observed differences could originate from the different tissue sources used, and the possible existence of isoproteic forms of myomesin should therefore be kept in mind. A number of molecular weights have been reported for putative M-proteins ranging from M_r 140,000 to

190,000 (11, 12, 23, 26, 42–44); therefore the possibility that different myofibrillar proteins have been discussed can also not be excluded.

Myomesin from chicken skeletal muscle is a tissue-specific protein but seems not to display a distinct species specificity, e.g., shows cross-reaction of antibody with hamster myofibrils (M. Eppenberger, personal communication) and with quail myofibrils (D. Turner, personal communication). The antibody against the putative M-protein from chicken gizzard has been reported to react with human tissues and mouse 3T3 cells (38). The high antigenicity of myomesin combined with a remarkable organelle specificity make this protein well suited for the study of the progress of muscle cell development, particularly of myofibrillogenesis. The data presented here indicate that myomesin can be found in myogenic cells as soon as the very first myofibrils are observed. It is remarkable that the small amounts of myomesin synthesized (constituting only 0.036% of the total protein synthesized at the time of maximal synthesis) and accumulated are so easily stained with the immunofluorescence technique. Obviously, the antigen is highly concentrated in the M-line region, thus little or no myomesin is available anywhere else within the cell. This is also true for cells differentiating in suspension cultures where M-line-containing myofibrils wound about the nucleus can be detected as long as the cells are not allowed to attach to a substrate (36). Furthermore, myomesin can be used as a sensitive marker for the localization of muscle cells in somites and in myotomes during early embryonic development in vivo as has been shown on cryostat sections of as early as stage 24 (15) chick embryos (M. Chiquet, personal communication).

It is becoming clear that the lack of a visible M-line either early in muscle differentiation or in a particular muscle type such as chicken cardiac muscle does not mean that there is no myomesin; on the contrary, in such muscles myomesin has been shown to interact strongly with other structural components in the M-region from which it can hardly be released (42, 43). Even if the relationship between M-line material and myofibrillar assembly and myofibril maintenance is still uncertain, a key role of myomesin in myofibrillogenesis has to be assumed. Several facts support such an assumption: (a) the synchronous appearance of myomesin and other myofibrillar proteins in differentiating myogenic cells; (b) antibodies against myomesin stain a transverse structure within the nascent H-zone of small myofibrils consisting of only few aligned myofilaments (while it is impossible to detect any myomesin when only single myofilaments in a still random fashion are present), and (c) in vitro experiments show a strong binding of the 165,000 M-protein (= myomesin) to myosin and myosin fragments (23).

All the results discussed demonstrate clearly that myomesin represents a differentiation marker for myofibrillogenesis unsurpassed so far. One certainly must strive for a better understanding of its function in myofibril assembly and myofibril maintenance. Experiments directed toward this goal are underway in our laboratory.

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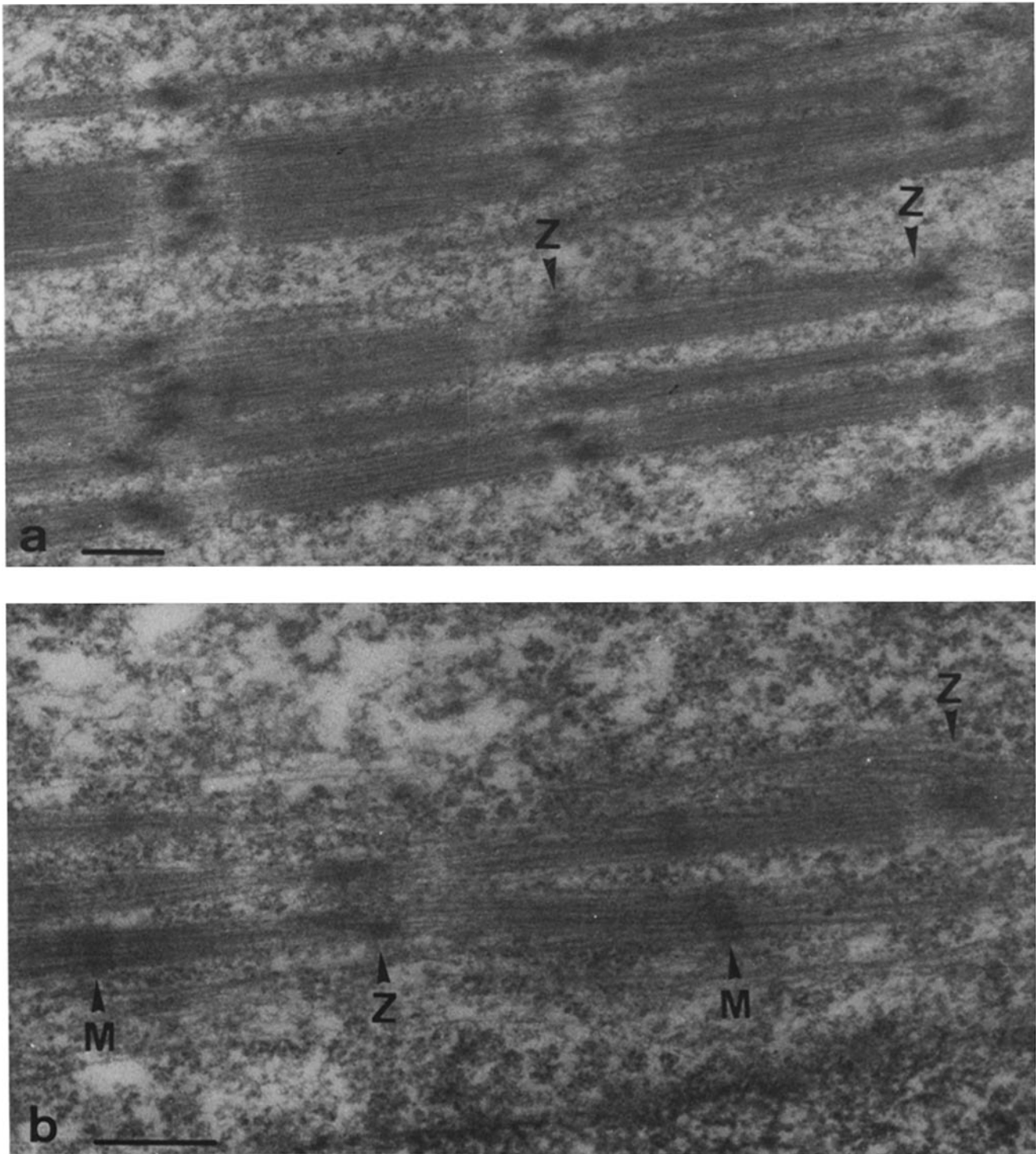


FIGURE 5 Localization of myomesin in nascent myofibrils in small myotubes of chicken breast muscle cells 48 h after plating. (a) Incubation with preimmune IgG; (b) incubation with antibody against myomesin. Z, Z-line; M, M-line region. Bars, 0.4 μ m.

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