Chick Myotendinous Antigen. I. A Monoclonal Antibody as a Marker for Tendon and Muscle Morphogenesis

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ABSTRACT Extracellular matrix components are likely to be involved in the interaction of muscle with nonmuscle cells during morphogenesis and in adult skeletal muscle. With the aim of identifying relevant molecules, we generated monoclonal antibodies that react with the endomysium, i.e., the extracellular matrix on the surface of single muscle fibers. Antibody M1, which is described here, specifically labeled the endomysium of chick anterior latissimus dorsi muscle (but neither the perimysium nor, with the exception of blood vessels and perineurium, the epimysium). Endomysium labeling was restricted to proximal and distal portions of muscle fibers near their insertion points to tendon, but absent from medial regions of the muscle. Myotendinous junctions and tendon fascicles were intensely labeled by M1 antibody. In chick embryos, "myotendinous antigen" (as we tentatively call the epitope recognized by M1 antibody) appeared first in the perichondrium of vertebrae and limb cartilage elements, from where it gradually extended to the premuscle masses. Around day 6, tendon primordia were clearly labeled. The other structures labeled by M1 antibody in chick embryos were developing smooth muscle tissues, especially aorta, gizzard, and lung buds. In general, tissues labeled with M1 antibody appeared to be a subset of the ones accumulating fibronectin. In cell cultures, M1 antibody binds to fuzzy, fibrillar material on the substrate and cell surfaces of living fibroblast and myogenic cells, which confirms an extracellular location of the antigenic site. The appearance of myotendinous antigen during limb morphogenesis and its distribution in adult muscle and tendon are compatible with the idea that it might be involved in attaching muscle fibers to tendon fascicles. Its biochemical characterization is described in the accompanying paper (Chiquet, M., and D. Fambrough, 1984, *J. Cell Biol.* 98:1937–1946).

Interactions between muscle fibers and nonmuscle cells (e.g., tendon fibroblasts and neurons) are essential for both the development and the proper functioning of skeletal muscles. It is known that during muscle morphogenesis in the chick limb nonmuscle mesenchyme cells organize the spatial arrangement of the developing myotubes (4, 5). Nonmuscle cells are also thought to be involved in the splitting of premuscle masses into individual muscles (6, 28); they eventually penetrate the muscle to form its connective tissue layers, i.e., epimysium, perimysium, and (at least in part) the endomysium that surrounds single muscle fibers (4). Tendons are able to develop autonomously, i.e., in the absence of muscle, but degenerate if no proper junctions to muscle fibers are established (18). The mechanical coupling between skeletal muscle fibers and their tendons at the myotendinous junction (31) must involve components of the extracellular matrix contributed by each cell type. In these morphogenetic events involving both muscle and connective tissue cells, extracellular matrix components might mediate the cellular interactions while functioning as an organizing scaffold (6, 29, 33). To understand these processes, the molecular composition of the extracellular matrix of skeletal muscle must be determined. Components identified so far on the surface of muscle fibers include fibronectin and several types of collagen (20, 27), laminin (27), acetylcholinesterase (25), and a heparan sulfate proteoglycan[1] (1). However, this list probably still lacks many other developmentally and functionally important molecules.

[1] Bayne, E. K., M. J. Anderson, and D. M. Fambrough, manuscript in preparation.

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Our laboratory has been generating monoclonal antibodies against endomysium components with the aim of studying the interaction of muscle fibers with connective tissue and nerve cells during development[1] (1, 7, 11, 12). In two instances we used chick type V collagen preparations (35) as an immunogen. Type V collagen once was believed to be enriched in the endomysium compared to other collagen types (3), a finding which was recently questioned by a study using monoclonal antibodies (24). From our hybridoma libraries prepared from spleen cells of mice immunized with type V collagen we obtained several antibodies which, although not directed against type V collagen (8), reacted with the endomysium of chick skeletal muscle. The most interesting of these, called M1 antibody, is characterized in this and the accompanying paper (8). M1 antibody recognizes an extracellular matrix component that appears early during embryogenesis in perichondrium and in developing ligaments and tendons, is later prominent in myotendinous junctions, and extends into the endomysium at the tips of muscle fibers. Based on its developmental appearance and distribution in vivo, we speculate that the "myotendinous antigen" recognized by M1 antibody might be involved in establishing and maintaining connections between muscle fibers and tendon fascicles.

MATERIALS AND METHODS

Isolation of Monoclonal Antibody M1: Chick type V collagen was isolated from pepsin-digested homogenates of 16-d-old chick embryos according to von der Mark and von der Mark (35). Upon SDS PAGE, the preparations yielded the typical pattern of α_1 (V) and α_2 (V) chains (in a ratio of 2:1) migrating above of the α_1 (I) chain of a type I collagen standard (not shown). A small amount of contaminants with faster migration rates was visible on the gels. Two BALB/cJ mice were immunized intraperitoneally with type V collagen solution (300 μg/mouse in 100 μl) suspended in 100 μl complete Freund's adjuvant. Material for the injections was kindly provided by Dr. K. von der Mark (Max Planck Institut, Munich). After 1 mo, the mice were boosted with 150 μg of the same preparation in 300 μl phosphate-buffered saline injected into the tail vein. This was repeated after 2 d, and 2 d later the mice were killed and their spleens removed. Lymphocytes were isolated from each spleen separately, fused with 10^7 SP 2/0 myeloma cells (19) each, and resulting hybridoma lines grown as described by Kennett et al. (17). Hybridoma culture supernates were screened in a solid phase radioimmune assay in which microtiter wells coated with the type V collagen preparation (100 μg/ml, 2 h at 20°C) were incubated with the supernates for 1 h before reaction with ^{125}I-labeled rabbit anti-mouse Fab for another hour. After each incubation step, the microtiter wells were washed three times for 10 min with borate-buffered saline containing 0.5% bovine serum albumin. About 70 culture supernates giving values 5–40-fold above background in this assay were rescreened by immunofluorescence on cryosections of chick anterior latissimus dorsi muscle. Supernate of hybridoma line 2BD3, from which monoclonal antibody M1 was derived, was moderately active in the radioimmune assay and specifically labeled the endomysium around single muscle fibers. Selected hybridoma lines were cloned in soft agar. IgG secreting clones were identified by the formation of precipitation rings after overlaying the colonies with goat anti-mouse IgG antiserum (11). Clones were picked up with pasteur pipettes, grown in flasks, rescreened, and injected into pristane primed BALB/cJ mice to generate ascites tumors. Immunoglobulins were precipitated from ascites fluid with 50% saturated ammonium sulfate. After dialysis against 10 mM potassium phosphate, pH 8.0, the preparation was loaded on a DE-52 (Whatman Laboratory Products Inc., Clifton, NJ) column. IgG was eluted with 40 mM potassium phosphate, pH 6.8, and stored in 1-ml aliquots at −70°C. On SDS gels under reducing conditions, monoclonal antibody M1 derived from hybridoma clone 2BD3-1 revealed a single heavy chain of M_r γ50,000 and a single light chain (not shown). Judging from its electrophoretic and chromatographic behavior and from its reaction with anti-mouse IgG antibodies, M1 antibody is an IgG. To confirm its monoclonal origin, we subcloned the original clone 2BD3-1 in soft agar. Culture supernates of all six secreting subclones tested produced an identical immunofluorescence staining pattern typical for M1 antibody. All the experiments described in this and the accompanying paper (8) were performed with a single batch of purified M1 antibody (1.95 mg/ml) originating from the original clone 2BD3-1.

TABLE I

*Radioimmunoassay with M1 Antibody**

Coating of microtiter wells‡	Antibodies‖		
	P3 × 63	B3	M1
Conditioned medium	37	113	515¶
Gelatin;§ cond. med.	11	172	146
Chick serum	5	32	22
Gelatin;§ chick serum	10	285	20
Fibronectin	14	433	12
Myotendinous antigen	38	41	664
Gelatin§	0	0	14

* The radioimmunoassay was performed as described in Materials and Methods.

‡ Microtiter wells were incubated for 1 h at 20°C with 100 μl of fibroblast conditioned medium, chick serum (1:10 diluted), chick serum fibronectin (6) (40 μg/ml), and affinity-purified myotendinous antigen (8) (~20 μg/ml), respectively.

§ Where indicated, the microtiter wells were precoated with 1 mg/ml gelatin for 30 min at 20°C. This increased the signal obtained with antibody B3 (since gelatin selectively binds fibronectin), but *decreased* binding of M1 antibody.

‖ The antigen-coated microtiter wells were incubated with 50 μl of a solution containing 10 μg/ml of one of the following three monoclonal antibodies: P3 × 63 (nonspecific antibody), B3 (antifibronectin), and M1 (antimyotendinous antigen). ^{125}I-labeled rabbit anti-mouse Fab was used as a second antibody. Numbers represent the average of duplicate wells and are expressed as cpm above the gamma counter background (40 cpm).

¶ The binding of M1 antibody was blocked by mixing it with polyclonal antiserum against myotendinous antigen. Addition of 5% preimmune serum to the M1 antibody yielded 362 cpm, of antifibronectin antiserum, 356 cpm, and of anti-myotendinous antigen-antiserum, 31 cpm.

Other Immunoreagents: Monoclonal antibodies 31-2 (an anti-chick laminin)[1] and B3 (which reacts with chick cellular and plasma fibronectin [12]) were kind gifts of Dr. E. K. Bayne (Merck, Sharp, and Dohme Laboratories, Rahway, NJ) and Dr. J. M. Gardner (Massachusetts Institute of Technology, Cambridge, MA), respectively. Rhodamin-conjugated sheep anti-mouse IgG and fluorescein-conjugated sheep anti-rabbit IgG antisera were obtained from Cappel Laboratories (Cochranville, PA). Rabbit anti-mouse Fab, a generous gift of Dr. P. Gearhart (Johns Hopkins Medical School), was iodinated by the chloramine-T method (14) for radioimmune assays. Antiserum against electrophoretically pure human plasma fibronectin (which cross-reacts with chick cellular fibronectin) was prepared and characterized as described (10). A specific antiserum against myotendinous antigen was generated as follows. The antigen was purified from fibroblast conditioned medium by affinity chromatography on M1 antibody-Sepharose as described in the accompanying paper (8). Immunoblotting (8) confirmed that the antigen preparation was not contaminated with fibronectin. 100 μg of the antigen were injected into a rabbit in complete Freund's adjuvant. The rabbit was boosted after a month with the same material (in incomplete adjuvant) and antiserum obtained a week later.

Specify Controls: Several experiments were performed to ascertain the target specificity of both the monoclonal antibody M1 and the polyclonal antiserum against myotendinous antigen. To control for nonspecific antibody binding, we used the unrelated monoclonal mouse IgG, P3 × 63, and rabbit preimmune serum, respectively. At the specified antibody concentrations, these two reagents produced very low backgrounds in all assays described in the two papers (see, e.g., Table I of this paper; see Fig. 8 in reference 8). M1 antibody is avian-specific, whereas the polyclonal antiserum reacts with a related mammalian antigen (not shown). M1 antibody does not bind to purified chick serum fibronectin (8) and, within the sensitivity limits of our assay, does not recognize any material in chick serum (Table I). M1 antibody and anti-myotendinous antigen antiserum yielded identical immunofluorescence staining patterns which differed from the fibronectin distribution (see Fig. 3). The antiserum against myotendinous antigen precipitated the same metabolically labeled material as M1 antibody (not shown). Anti-myotendinous antigen-antiserum blocked binding of M1 antibody, but not of monoclonal antifibronectin B3, to the respective target (Table I). Polyclonal antifibronectin antiserum, on the other hand, did not interfere with M1 antibody binding, as evidenced by the double-labeling experiments shown in this paper.

Immunofluorescence of Cryostat Sections and Cell Cultures: Anterior longissimus dorsi (ALD)[2] muscle of adult chickens was frozen in liquid isopentane. Unfixed pieces of chick embryos staged according

[2] *Abbreviations used in this paper*: ALD, anterior longissimus dorsi.

to Hamburger and Hamilton (15) were mounted in Tissue Tek (Miles Laboratories, Elkhart, IN) and frozen on dry ice. Sections of 4 μm (adult muscle) and 14 μm (embryos) were made on a cryostat microtome (Minotome; Damon/IEC) at −20°C and processed for indirect double immunofluorescence as described in Wakshull et al. (36). Monoclonal antibodies were used at 10 μg/ml, and antisera and second antibodies were routinely diluted one hundred fold in borate-buffered saline containing 0.5% bovine serum albumin.

Primary skin fibroblast and breast muscle cells were obtained from 11-d-old chick embryos as described (32). 2×10^5 fibroblasts were plated per nongelatinized, and 5×10^5 myogenic cells per gelatinized (32) 60-mm Falcon tissue culture dish. Cultures were grown in 4 ml Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% horse serum (Gibco Laboratories) and 2% chick embryo extract. For immunofluorescence staining of living cells, culture dishes were washed with Hanks' balanced salt solution and incubated with 10 μg/ml monoclonal antibody in balanced salt solution for 1 h at 37°C. After washing with balanced salt solution cell cultures were fixed with 2% paraformaldehyde, 100 mM lysine, 60 mM sucrose, 20 mM sodium phosphate, pH 7.2. Dishes were washed again and then incubated with antifibronectin antiserum followed by second antibodies as described for cryostat sections (36). (Living cultures could be incubated with monoclonal antibodies, but not with antifibronectin antiserum which interferes with cell attachment [10]). Cell cultures as well as cryostat sections were mounted in 90% glycerol buffered with 20 mM sodium phosphate (pH 7.3) and examined with a Zeiss microscope equipped with epifluorescence optics. Photographs were taken on Kodak Tri-X film and push-developed in Diafine.

RESULTS

M1 Antigen Is Localized in the Endomysium of Chick Muscle

The monoclonal antibody characterized in this paper originates from a hybridoma library where Type V collagen purified from pepsin digests of 16-d-old chick embryos was used as an immunogen (see Materials and Methods). 45 hybridoma culture supernates reacted strongly with the immunogen in a solid phase radioimmunoassay, seven of which preferentially stained the endomysium in cryosections of adult chick muscle. Of these seven, three exhibited a staining pattern very similar to the monoclonal antilaminin antibody 31,[1] whereas the other four labeled fuzzy material apparently located between the basement membranes of adjacent muscle fibers. Experiments of the kind described in the accompanying paper (8) demonstrated that the antibodies secreted by these four hybridoma lines were not directed against Type V collagen, but against a minor contaminant present in the immunogen (not shown). Because of our interest in the composition of the endomysium, i.e., the extracellular matrix surrounding individual muscle fibers, we nevertheless cloned these hybridomas and injected the clones into mice. The monoclonal IgG purified from ascites fluid which is described in this and the following (8) paper was designated M1 antibody.

When M1 antibody was used for immunofluorescent staining of adult chick ALD muscle in cryostat sections (Fig. 1*A*), the endomysium was stained, whereas in the perimysium (around muscle fiber bundles) and in the epimysium (around muscles) only capillaries and perineurium were labeled. As mentioned, the fuzzy staining differed from a typical basement membrane staining found with antilaminin antibody 31 (Fig. 1*C*). Interestingly, only the space between single muscle fibers, but not muscle fiber surfaces facing the perimysium, were labeled (Fig. 1*A*). This was not the only nonuniformity of the M1 antibody staining pattern found on the surface of muscle fibers: regional differences were also found along the longitudinal axis within ALD muscle. Fig. 1, *A–C*, depict cross sections through the proximal part of the muscle near its insertion on the vertebral column; a similar staining pattern was found distally (not shown). In medial regions of the muscle (Fig. 1, *D–F*), by contrast, M1 antibody labeling was completely absent from the endomysium and only occasional capillaries and perineurium were fluorescent (Fig. 1*D*). Such regional specializations in the staining pattern were not found with antilaminin antibody 31, which stained the basement membrane of muscle fibers over their entire length (Fig. 1, *C* and *F*). Similarly, antifibronectin antibody B3 labeled fuzzy material in the endomysium (but also in peri- and epimysium) along the whole length in ALD muscle (Fig. 1, *B* and *E*).

M1 Antigen Is a Component of Myotendinous Junctions and Tendons

The question remained whether the M1 antibody staining pattern observed in ALD muscle truly reflects the distribution of the antigen or merely differential accessibility of antigenic sites. Although there is as yet no definitive answer to this problem, several observations indicate that M1 antigen is not masked in places where no antibody staining is found. First, no other monoclonal antibody we tested so far exhibited a similar differential labeling pattern (see above); it is therefore not a generalized phenomenon. Second, M1 antibody labeled tendon fascicles (see Fig. 2) which are very dense structures, but (in contrast to B3 antifibronectin antibody) did not stain loose connective tissue around tendons and muscles. This is the opposite of what one would expect if tissue penetration by the antibody were a problem. Third, treatment of cryosections with hyaluronidase or acetic acid (which is known to unmask antigenic sites of collagenous peptides [24]) did not change M1 antibody staining patterns (not shown). We therefore believe that the labeling patterns represent a differential distribution of myotendinous antigen in the endomysium of ALD muscle, which might be related to a function exhibited at the tips of muscle fibers. This idea was supported by the finding that myotendinous junctions were very heavily labeled by M1 antibody (Fig. 2). In the case of ALD muscle, where the muscle fibers directly insert on the bones, M1 antibody staining was found throughout the periosteum (Fig. 2*D*). In cases in which muscle fibers are attached to a tendon, the labeling extended from the endomysium to the myotendinous junctions and the tendon fascicles (Fig. 2*B*). As best as can be judged at the level of the light microscope, M1 antigen appears to be continuous between a muscle and the skeletal elements it moves; it is therefore tempting to hypothesize that M1 antigen actually is involved in anchoring muscle fibers to tendons and bones.

Fibronectin (revealed by antibody labeling) is present in myotendinous junctions and tendons as well (Fig. 2, *A*, *C*, and *E*). However, high power micrographs of embryonic tendon demonstrate that M1 antibody binds to a class of coarse extracellular matrix fibrils (Fig. 2*F*) which differ morphologically from, and only partially overlap with, the more delicate fibronectin fibrils (Fig. 2*E*).

M1 Antibody Is a Marker for Embryonic Tendon Development

During chick limb development, tendons are able to form autonomously, i.e., in the absence of muscle (18). Together with the mesenchyme surrounding the muscle primordia, they are thought to organize the formation and arrangement of

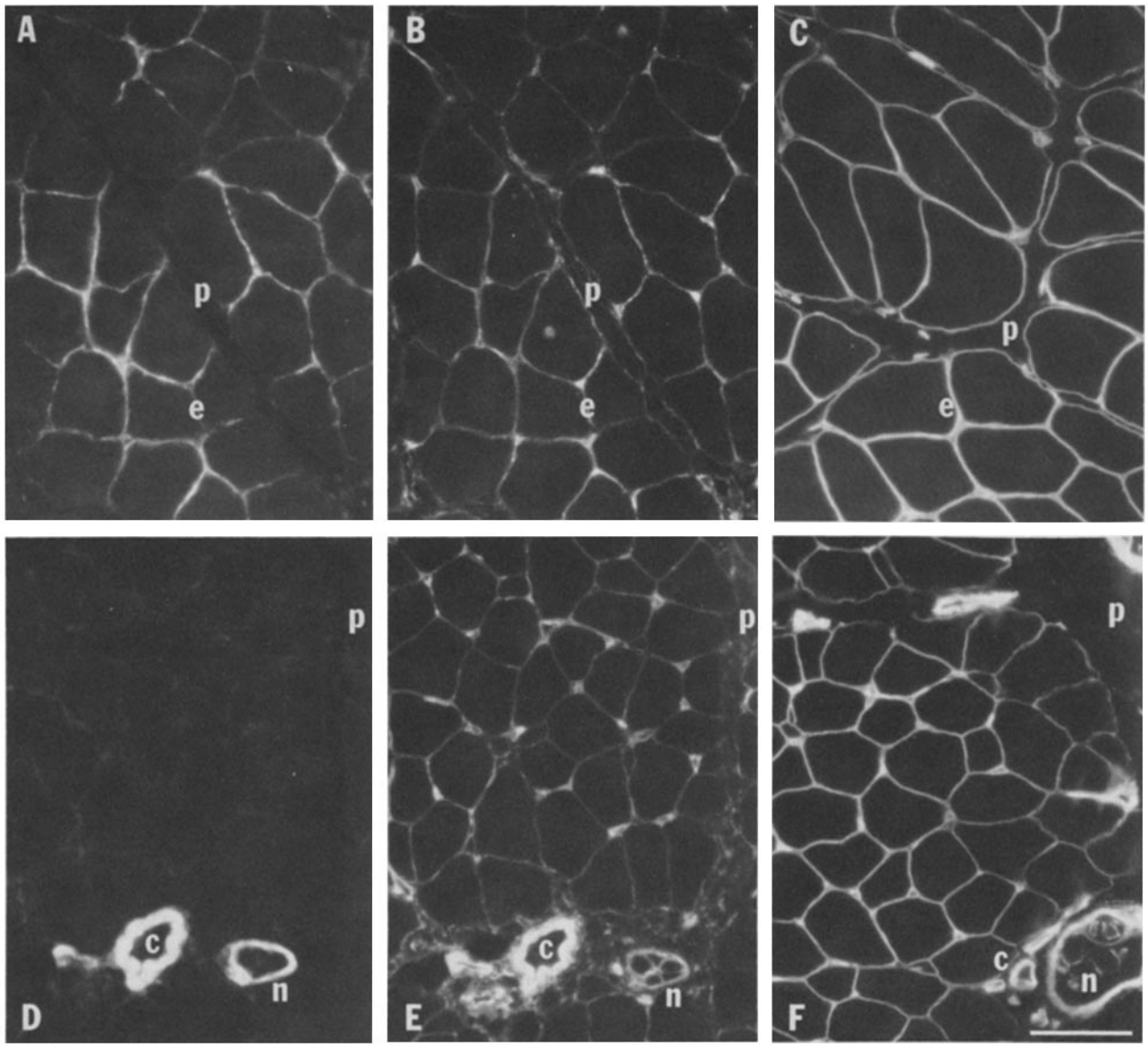

FIGURE 1 Comparison of M1 antibody staining with fibronectin and laminin distribution in chick ALD muscle. Cryostat sections of the proximal (*A–C*) and the medial (*D–F*) part of the same ALD muscle were stained by indirect immunofluorescence with M1 antibody (*A* and *D*), antifibronectin antiserum (*B* and *E*), and antilaminin antibody 31-2 (*C* and *F*), respectively. Picture pairs (*A* and *B*) as well as (*D* and *E*) each represent a single section stained simultaneously with monoclonal antibody M1 and rabbit antifibronectin antiserum, incubated with the two respective second antibodies, and photographed with rhodamine (*A* and *D*) or fluorescein (*B* and *E*) optics. *C* and *F* represent different sections of corresponding muscle regions. M1 antibody stains fuzzy material in the endomysium in proximal, but not medial, regions of the muscle. *e*, endomysium; *p*, perimysium; *c*, capillary; *n*, nerve. Bar, 30 μm.

individual muscles (28). Since the myotendinous antigen seems to form a topographical bridge between adult tendons and muscle fibers; we wondered whether it was already present during the important morphogenetic processes in the embryo limb bud when the first contacts between tendon primordia and developing myotubes are established.

The first structures in the developing chick wing bud labeled by M1 antibody are the perichondria of the developing humerus and (somewhat later) of the radius and ulna around day 4. By stage 27/28 (day 5), additional staining was found in mesenchymal areas extending from the perichondria of radius and ulna towards the developing premuscle masses (Fig. 3*B*). At the time the muscle anlagen are split into individual muscles (stage 29 and later), M1 antibody labeling was detected in the tendon primordia located between individual muscles (Fig. 3*D*). In longitudinal sections, the stained tendon primordia could be observed to attach to the muscles and bones (Fig. 4). It is noteworthy that M1 antibody staining is found in tendon anlagen at a time when Type I collagen (which makes up the bulk material of adult tendons) is confined to the perichondrium and the peripheral dermis in the developing wing (29). It is therefore possible that the epitope recognized by M1 antibody is part of preliminary structures involved in early contacts between presumptive tendon cells and developing myotubes, and that these structures are only later transformed into mature tendons. In any case, M1 antibody seems to be a very useful marker for following early tendon development and hence for studying the morphogenetic events involved in the mechanical coupling of motor muscles to the bones they move. Fig. 3, *E* and

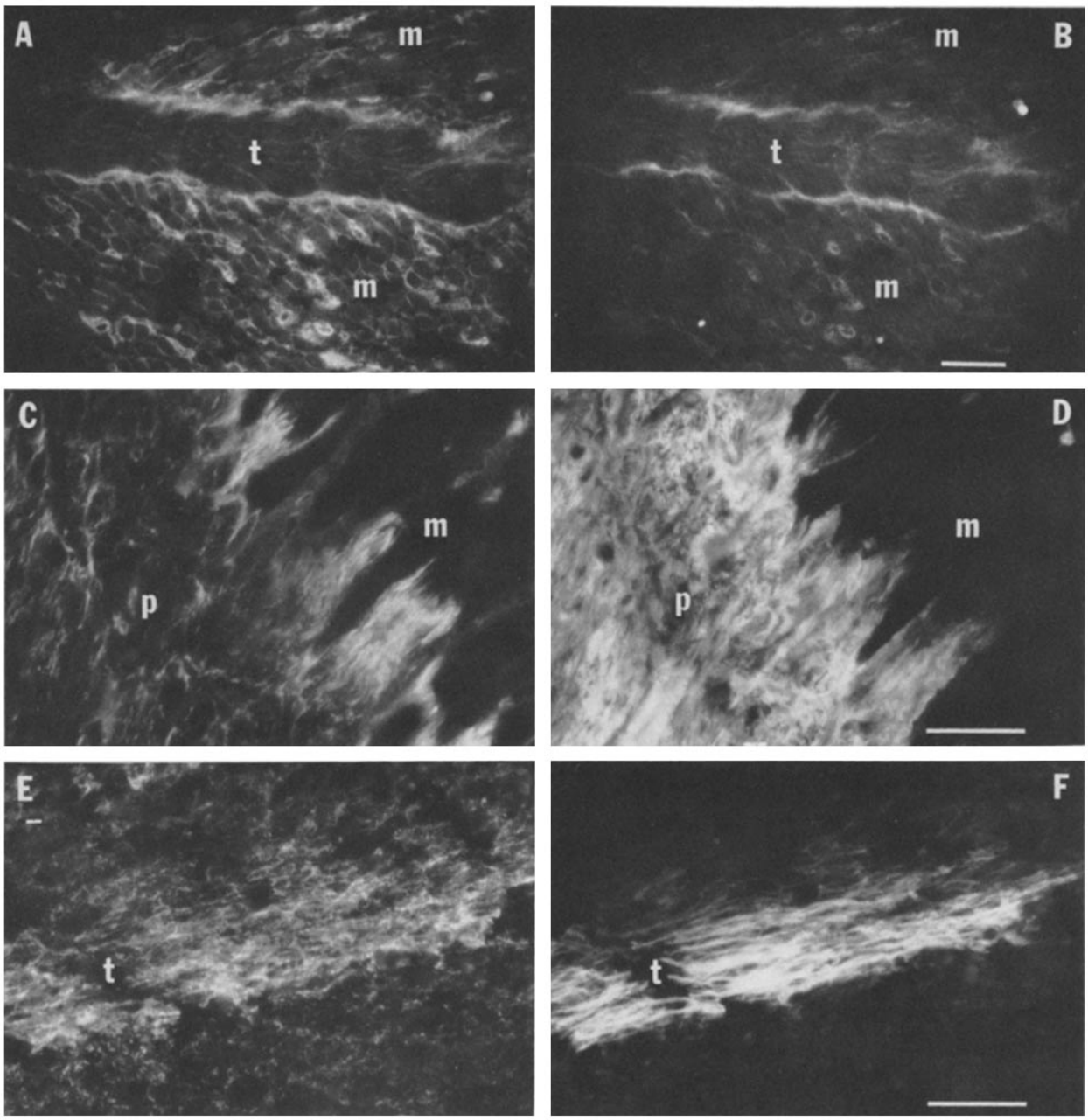

FIGURE 2 Labeling of myotendinous junctions and tendons with M1 antibody. Longitudinal cryostat sections through a 19-d-old chick embryo leg muscle (*A* and *B*) the proximal insertion of an adult ALD muscle (*C* and *D*), and a tendon primordium in a 7-d-old chick embryo wing bud (*E* and *F*) were stained simultaneously with antifibronectin antiserum (*A*, *C* and *E*) and M1 antibody (*B*, *D*, and *F*) by indirect immunofluorescence. *m*, muscle fibers; *t*, tendon; *p*, periosteum. Bar, 30 μm.

F, shows a cryostat section that was double-labeled in a reciprocal manner compared to all other figures in this paper, i.e., with monoclonal antifibronectin B3 (Fig. 3*E*) and polyclonal anti-myotendinous antigen-antiserum (Fig. 3*F*). This antiserum produces a staining pattern strikingly similar to M1 antibody (Fig. 3*D*) but different from both antifibronectin antibodies (Fig. 3, *C* and *E*). Hence, we have an independent probe for the antigen that is very likely to recognize several epitopes on a unique molecule.

M1 Antibody Labels Developing Smooth Muscle Tissue

We made cryostat cross-sections through the trunk of chick embryos to determine what other structures besides perichondrium, ligaments, and tendons are labeled by M1 antibody. In 3-d-old embryos (stage 20), myotendinous antigen was present in the basement membrane surrounding the notochord as well as the aorta; in the head region, the lens capsule also contained the antigen (not shown). At day 4 (stage 24), M1 antigen seemed to accumulate in the region of the developing vertebrae (not shown), and at 5.5 d (stage 28) it was found in the processes of the vertebrae and the ligaments connecting them, whereas the chondrogenic areas apparently excluded M1 antigen (Fig. 5). At the same time, the multilayered aortic wall was heavily labeled by M1 antibody, and myotendinous antigen began to appear in the developing lung buds, esophagus, and gizzard. However, no myotendinous antigen appeared in dermis, epidermis, heart muscle, and liver, all of which were heavily labeled by antifibronectin

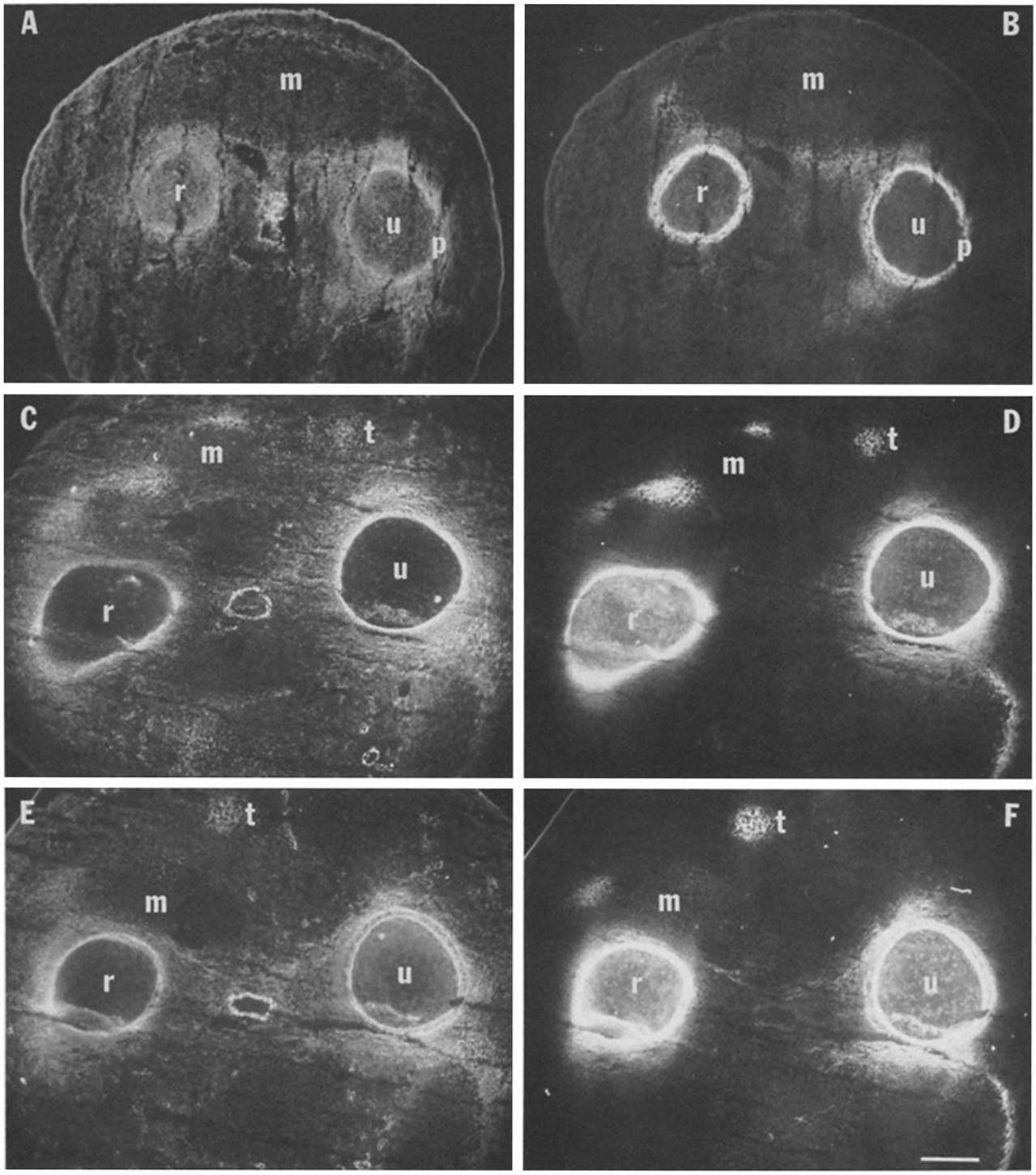

FIGURE 3 Labeling of perichondrium and tendon primordia by M1 antibody in the developing chick wing. Cryostat sections through day 5 (*A* and *B*) and day 6½ (*C* and *D*) chick embryo wing buds were stained by double immunofluorescence labeling with antifibronectin antiserum (*A* and *C*) and M1 antibody (*B* and *D*). Section (*E* and *F*), which was slightly more proximal on the same limb as *C* and *D*, was double-stained with monoclonal antifibronectin B3 (*E*) and polyclonal antimyotendinous antigen-antiserum (*F*). *r*, radius; *u*, ulna; *p*, perichondrium; *t*, tendon; *m*, premuscle mass. Bar, 100 μm.

antibody. In the dermis of 11-d-old embryos, myotendinous antigen was found exclusively at the base of developing feather papillae. Heart muscle and liver tissue remained negative for the antigen, whereas gizzard (especially its tendinous sheets) was very brightly stained by M1 antibody (not shown). In general, the tissues labeled by M1 antibody (but not the class of matrix fibrils; see Fig. 2, *E* and *F*) appeared to be a subset of fibronectin-accumulating structures. Neural tissue was completely negative for both antifibronectin and M1 antibody labeling at all stages investigated (Fig. 5); we do not yet know when during development myotendinous antigen appears in the perineurium (where it is present in adult muscle, Fig. 1 *D*).

In conclusion, a second class of tissues (besides perichondrium, tendons, and ligaments) labeled by M1 antibody during embryogenesis are structures containing smooth muscle, especially the major blood vessels, feather papillae, and the

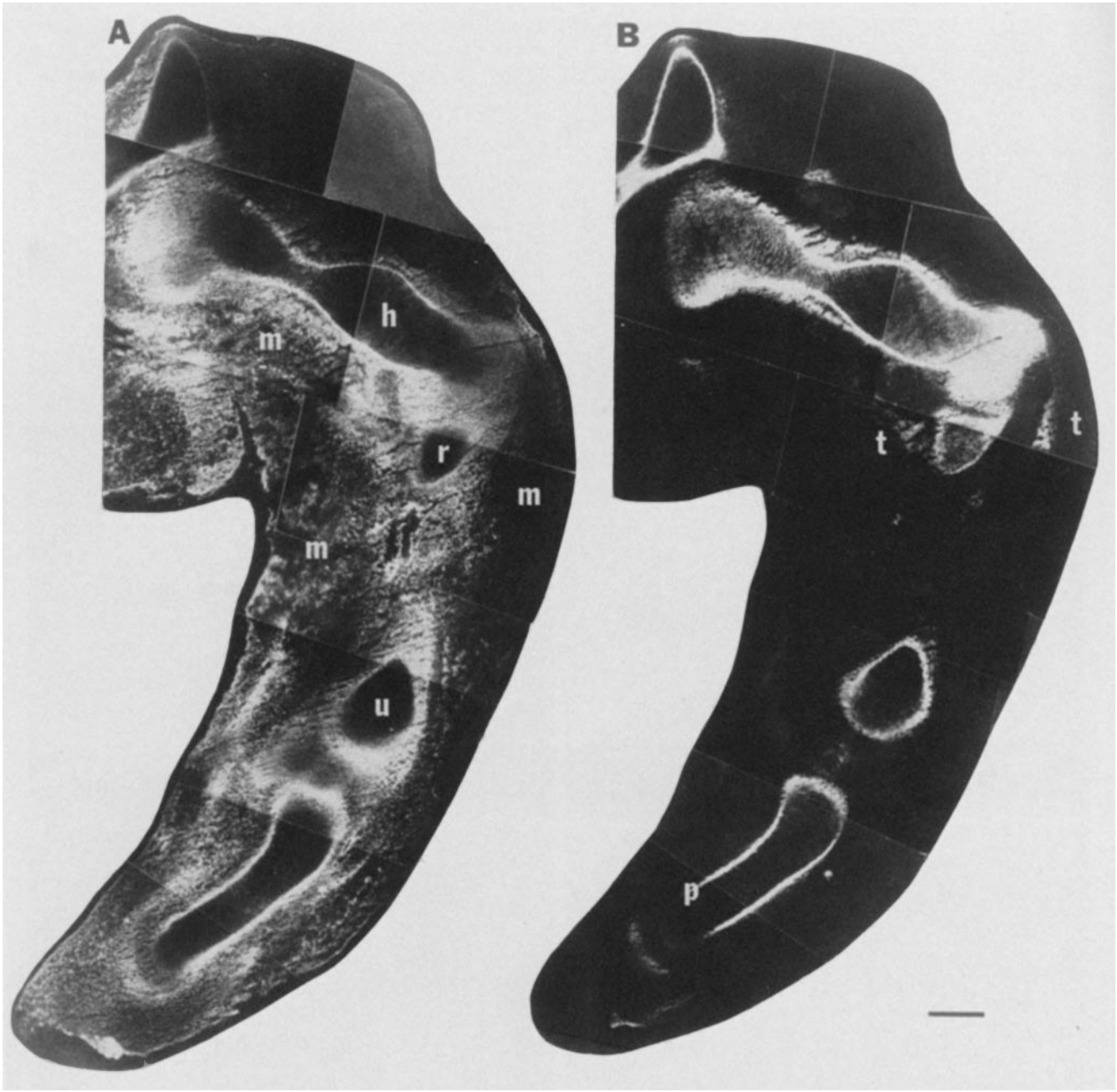

FIGURE 4 Labeling of developing perichondrium and tendons with M1 antibody in a 7-d-old chick embryo wing bud. A longitudinal section was stained by double immunofluorescence with antifibronectin antiserum (*A*) and M1 antibody (*B*). *h*, humerus; *r*, radius; *u*, ulna; *p*, perichondrium; *m*, premuscle mass; *t*, tendon. Note that the tendon primordia connect bones with premuscle masses, which appear as areas weakly stained by antifibronectin (6). Bar, 200 μm.

gizzard. Together with the lung and certain ligaments, these are also major structures that accumulate elastin during late embryonic development and after hatching (26); this fact might turn out to be important in searching for the function of myotendinous antigen.

Myotendinous Antigen Is an Extracellular Matrix Component Accumulated in Cell Cultures

The peculiar tissue staining pattern of M1 antibody in vivo does not reveal from which cell types the antigen originates. As a first step in addressing this question, we looked at the accumulation of myotendinous antigen in cell cultures. To establish, at the same time, the extracellular location of the antigen, we incubated living cultures of myogenic cells and fibroblasts with M1 antibody. Cells were then fixed and incubated with antifibronectin antiserum followed by second antibodies (see Materials and Methods).

In a 72-h old myogenic culture stained with polyclonal antifibronectin antibodies, the gelatinized substrate exhibits diffuse fluorescence due to absorbed serum fibronectin; the myotubes appear as dark shadows with fluorescent margins (Fig. 6*A*; see reference 6). In these cultures, material reacting with M1 antibody was found deposited in fuzzy patches and streaks on the substrate between and underneath cells (Fig. 6*B*). This material was enriched on the surfaces of myotubes and especially abundant around fibroblasts that also accumulated fibronectin fibrils (Fig. 6, *A* and *B*). Compared to the relative sparsity of fibronectin on the surface of myotubes, myotendinous antigen was relatively abundant. It was obvious

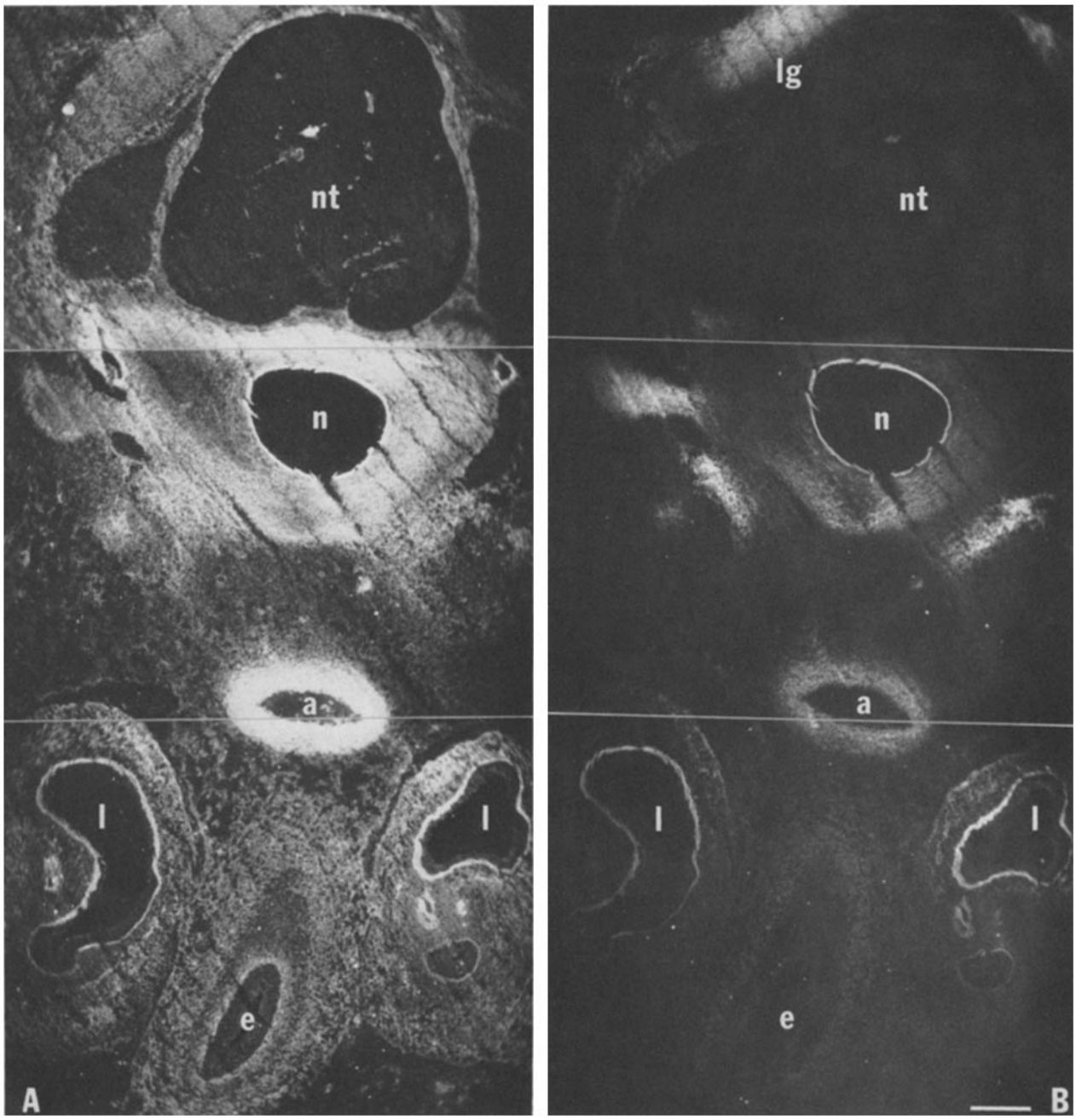

FIGURE 5 Cryostat cross-section through the trunk region of a 5½-d-old chick embryo simultaneously stained with antifibronectin antiserum (*A*) and M1 antibody (*B*). *lg*, intervertebral ligament; *nt*, neural tube; *n*, notochord; *a*, aorta; *l*, lung buds; e, esophagus. Bar, 100 μm.

that fibronectin and myotendinous antibody were not co-distributed. This was even more pronounced in subconfluent fibroblast cultures (Fig. 6, *C* and *D*), where, in fact, different types of cellular staining patterns could be distinguished: some cells were intensely labeled by antifibronectin, others by M1 antibody, others by both. Often, trails of fibronectin fibrils or myotendinous antigen patches were not associated with cells (not shown). In the many cases where these trails did not overlap, they might have been deposited by different types of fibroblasts. In confluent fibroblast cultures where a dense fibronectin network had developed (Fig. 6*E*), myotendinous antigen partially co-distributed with broad fibronectin fibrils, but never completely overlapped with the fibronectin pattern (Fig. 6*F*).

In general, accumulation of material reacting with M1 antibody was higher in fibroblast than in myogenic cultures, which agrees with the data on biosynthesis described in the accompanying paper (8). In myogenic cultures, myotendinous antigen was associated not only with the cell-free substrate and with fibroblast-like cells, but also with myotube surfaces. Since fibroblasts release much more newly synthesized myotendinous antigen than myotube cultures (8), it is possible that some of the myotube-associated material is not produced by the myotubes themselves, but exported by neighboring myoblasts and fibroblasts. This possibility is further addressed in the accompanying paper (8) and might suggest a possible function of the antigen as a link between nonmuscle and muscle cells in myotendinous junctions.

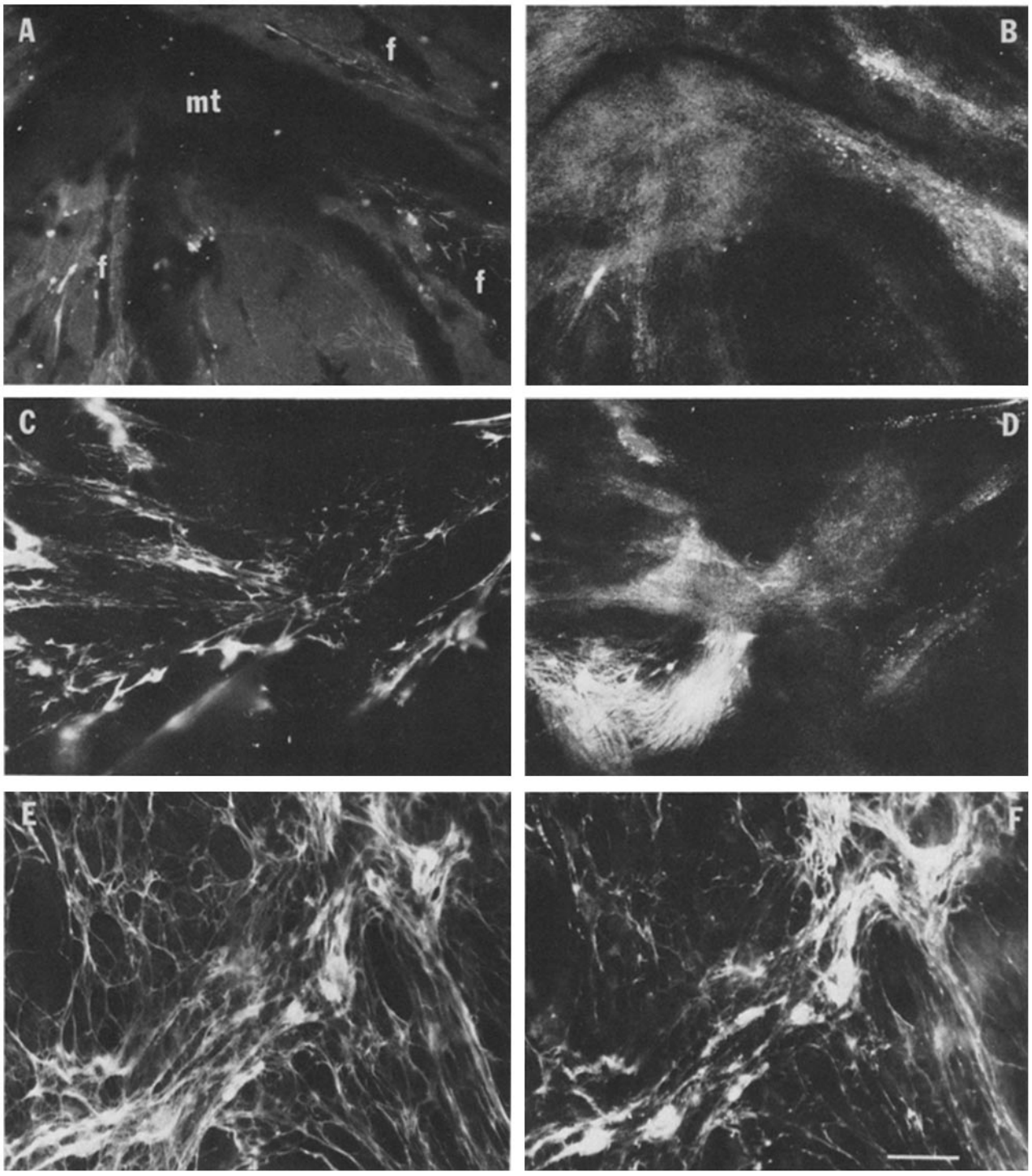

FIGURE 6 M1 antibody staining of extracellular material in chick muscle and fibroblast cultures. 72-h-old muscle cultures (*A* and *B*), 72-h-old subconfluent (*C* and *D*), and 6-d-old confluent (*E* and *F*) primary fibroblast cultures were incubated with M1 antibody while living, then fixed, then incubated with antifibronectin followed by second antibodies as described in Materials and Methods. Note that the antifibronectin staining (*A*, *C*, and *E*) and the M1 antibody staining (*B*, *D*, and *F*) overlap only partially. Bar, 20 μm.

DISCUSSION

Experiments using chimeric avian embryos have established that nonmuscle mesenchyme cells guide skeletal muscle morphogenesis in the developing limb (18, 34). We and others have suggested that extracellular matrix components might be involved in the transfer of morphogenetic information from nonmuscle to muscle cells (6, 29). Fibronectin (for a review, see reference 16) might be a good candidate, since connective tissue cells synthesize much more of it than myogenic cells (6), whereas myogenic cells strongly react to exogenous fibronectin by attachment, elongation, and contact guidance (33). However, it is extremely difficult to establish a directionality of fibronectin action, since it is so widespread in vivo (23) and its origin within a given extracellular matrix is not normally known. Using monoclonal antibodies, we were therefore searching for components that might developmentally and functionally connect muscle with nonmuscle

cells, but exhibit a simpler, more specialized distribution in the developing limb than fibronectin.

M1 antibody was chosen for detailed study because the myotendinous antigen recognized by it seemed to meet these criteria. Its distribution was clearly different from fibronectin and laminin. In vivo, it seemed (in contrast to fibronectin) to be accumulated only by a subclass of connective tissue cells, mainly those of perichondrium/periosteum, ligaments, and tendons. The antigen could be detected early during limb development at places where tendon primorodia make contact with the muscle anlagen. Moreover, myotendinous antigen was found on the surface of muscle fibers at specialized sites. The uneven M1 antibody staining pattern on muscle fibers was puzzling, but seemed to reflect the actual antigen distribution (see Results). This nonhomogeneous pattern rises questions about the developmental origin of myotendinous antigen on muscle fiber surfaces. If it is produced by the muscle fibers themselves, they must have been able to restrict deposition to specific areas of the endomysium near and at the myotendinous junctions. Alternatively, the antigen could be deposited by tendon fibroblasts that invade the endomysium for some distance. In the accompanying paper (8), we show that cultured fibroblasts produce at least seven times more myotendinous antigen per nucleus than myogenic cells. It is therefore possible that a small number of fibroblasts, which are known to be present in the endomysium (4), synthesize the antigen and deposit it at specialized sites on the surface of muscle fibers. In principle, this argument holds true for other endomysium components including fibronectin (6) and even the basal lamina (21). While it is clear, for example, that type I collagen bundles are deposited by fibroblasts present between tendon fascicles (30), the identity and cellular origin of the material linking tendon collagen fibrils to muscle fibers are not known (31). The results presented in this paper show that the myotendinous antigen recognized by M1 antibody is continuous between tendons and muscles. Our hypothesis, that myotendinous antigen might be one of the components by which limb nonmuscle cells attach muscle fibers to tendon fascicles, remains to be proven, however.

While the focus of this paper is on the possible involvement of myotendinous antigen in tendon fibroblast–muscle fiber interactions, other features of it are worth considering. For example, myotendinous antigen seems to appear in tendon primordia before they accumulate considerable amounts of type I collagen (29), which is the major component of adult tendons (30). Also, major elastic tissues like blood vessel walls, lung buds, and the gizzard stain with M1 antibody early during embryonic development, while elastin is synthesized and accumulated mainly during late embryogenesis and after hatching (26). It is therefore possible that in both cases myotendinous antigen is part of an embryonic extracellular matrix scaffold, which might be important for the coordinated deposition of the definitive structures, i.e., collagenous and elastic fibers, during tissue morphogenesis (13). Another interesting aspect of myotendinous antigen is its apparent abundance in a narrow zone around chondrogenic areas of the developing wing. Archer et al. (2) have stressed the importance of this zone, i.e., the perichondrium, for the morphogenesis of the long bones. It is thought that mechanical pressure generated by the matrix build-up within chondrogenic areas causes the peripheral nonchondrogenic cells to flatten and align circumferentially, thereby defining the perichondrium (2, 37). The aligned perichondrial fibroblasts might counteract mechanical stress by the deposition of extracellular matrix (22); myotendinous antigen could be deposited (together with type I collagen and fibronectin [9, 34]) during the formation of a tough, cylindrical, perichondrial sheet. This sheet might constrain the chondrogenic area and thereby allow only longitudinal expansion of the prospective diaphysis of the long bones (2).

In conclusion, we hypothesize that myotendinous antigen, which is characterized as being an apparently novel glycoprotein complex in the accompanying paper (8), is accumulated by a subset of connective tissue cells in places where mechanical stresses are known to occur during tissue morphogenesis (2, 28). It remains an open question if the antigen is deposited in response to environmental factors (such as mechanical stress), or if there are distinct lineages of connective tissue cells producing it. Likewise, we do not know as yet whether myotendinous antigen is indeed (as we suspect) a mechanical building block within a given extracellular matrix. However, its early appearance during morphogenetically important processes in the chick embryo make it worth studying.

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