TISSUE & CELL 1984 16 (1) 17–29

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FILAMENT-DIRECTED INTERCELLULAR CONTACTS DURING DIFFERENTIATION OF CULTURED CHICK MYOBLASTS

Key words: cytoskeleton, muscle, cell interaction, microfilaments

ABSTRACT. Detergent-extracted, critical point dried chicken myoblasts at early stages of development in tissue culture were observed by electron microscopy. It was found that the organization of filaments within these cells changes significantly during development. A particular specialization of the cellular filament framework is the formation of microprocesses; long extensions of the cellular filament system. These microprocesses appear to be involved in cell-to-cell contact. The filaments of these processes appear to integrate with the filament system of a contacted cell, and possibly transmit tension from one cell to another. The role of these structures in effecting muscle differentiation by forming cytoplasmic connections and the implications for muscle gene expression are discussed.

Introduction

During the differentiation of muscle, characteristic morphological changes occur. As early as 1917, Lewis and Lewis noted that single cells growing out from explants of embryonic muscle tissue were bipolar or fusiform in shape. These morphotypic cells (myoblasts) were then seen to fuse together to form the multinuclear syncytium known as the myotube. With the adaptation of these cells to clonal tissue culture, Konigsberg (1963) was able to show that these bipolar cells are indeed the precursors to the myofiber.

The study of muscle differentiation has been facilitated by the degree of homogeneity in the population of myoblasts which can be obtained (Konigsberg, 1963) as well as in the degree of synchrony during the fusion process (Shainberg *et al.*, 1969; Paterson and Strohman, 1972). The synchronous fusion of cells in the population, easily viewed in the phase contrast microscope, has allowed the temporal correlation of this morphological event with the production of the myofibrillar proteins (Paterson and Strohman, 1972; Buckley and Konigsberg, 1974; Emerson and Beckner, 1975), the production of muscle specific enzymes (Coleman and Coleman, 1968), and the identification of muscle-specific gene transcripts (Paterson *et al.*, 1974; Kessler-Icekson *et al.*, 1978; Paterson and Bishop, 1977; Strohman *et al.*, 1977; Devlin and Emerson, 1978).

Despite the high degree of resolution obtained with current molecular and biochemical technology, there exists only an imprecise correlation of macromolecular synthesis with gross changes in morphology as seen by phase contrast microscopy. This is due to the heterogeneous nature of the cell culture system and the low resolution of the light microscope for describing changes in cellular structure. In an effort to improve the resolution used to define myoblast differentiation, a scanning electron microscope study of this process was carried out (Huang *et al.*, 1978). This work indicated that prior to the formation of the myotube, long actin-rich processes ('microprocesses') were extended from myoblasts, sometimes over a distance of 200 μm. When processes contacted each other, they fused prior to the union of the

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Received 15 November 1982.
Revised 27 June 1983.

respective cell bodies as seen in the light microscope. The internal structure of myoblasts undergoing differentiation was then investigated further by detergent extraction of the cytosol in order to reveal the filamentous framework of these cells, which is composed principally of actin, myosin and intermediate filament proteins (Pudney and Singer, 1979). During differentiation this structural aspect of the cell becomes reorganized from a diffuse network of filaments in pre-myoblast cells into linear, axially orientated filament bundles characteristic of myoblasts fusing together (Pudney and Singer, 1980). More recently, Fulton *et al.* (1981) have observed changes in the filament framework of myoblasts fusing into myotubes and have related these events to changes in the surface lamina of these cell types. High voltage microscopy (Peng *et al.*, 1981) has elucidated a possible cytoskeletal foundation of sarcomere formation.

In the present study we propose that this reorganization of filament systems is an important stage in the process of muscle differentiation. In particular, the microprocesses, formed by projections of these filaments, may be involved in important intercellular contacts prior to myoblast fusion.

Materials and Methods

Whole mounts of Triton X-100 cells were prepared according to the method of Pudney and Singer (1979). In brief, myoblasts were isolated and cultured from 11-day chicken embryo breast (pectoralis) muscle. These myoblasts were plated on to carbon/Formvar-coated gold electron microscope grids attached to glass coverslips and cultured for a period of 48 hr. At various times during the differentiation of the myoblasts (24 and 48 hr post-plating) the coverslips bearing the grids were removed from the culture medium, briefly washed, extracted in a buffer containing 0·1 M NaCl, 10 mM Tris, pH 7·4, 3 mM $MgCl_2$ and 1% Triton X-100 (2 min), fixed in 0·2 M S-collidine buffered 5% gluteraldehyde, and dehydrated through a graded series of alcohol. Finally the grids were removed from the coverslips for critical point drying through CO_2. Following critical point drying, they were lightly coated with carbon and observed in a JEOL 100S electron microscope. Cells not extracted with Triton X-100 were treated exactly as above but observed in the HVEM (EM7 Mk 11) located in the Electron Optics Laboratory, Division of Laboratories and Research, New York State Department of Health, Albany, New York, under the direction of Dr. D. Parsons.

Electrophoresis of newly synthesized proteins remaining after extraction with Triton X-100 was done by labelling the cells in the presence of ^{35}S-methionine (NEN) for 1 hr before extraction. After extraction in detergent, cytoskeletal remnants were washed thoroughly with buffer minus the Triton X-100 and the protein remaining on the dish was dissolved in sample buffer for isoelectric focusing. The samples were then analyzed by the two-dimensional gel electrophoresis technique of O'Farrell (1975).

Results

Chicken presumptive myoblasts, cultured on Formvar-covered and carbon-coated gold grids, undergo the typical process of differentiation seen using the traditional collagen substrate. Over 80% of these cells fuse into myotubes. When these cells are subjected to Triton X-100 extraction after the method of Pudney and Singer (1979), an elaborate, anastomosing network of filaments is revealed. Drying through the critical point of CO_2 prevents the disruption of this filament system due to surface tension. The removal of soluble proteins reveals the insoluble filament system in great clarity. This system has been shown previously to consist mainly of actin, myosin and intermediate filament protein (Pudney and Singer, 1979). The spatial organization of the filamentous structures that remain after Triton extraction can then be examined by stereoscopic microscopy (see Fig. 1). The filamentous organization of presumptive myoblasts consists of a dense ramifying network of filaments (Fig. 1). Previous investigation has demonstrated that this filamentous reticulum is composed mainly of actin (Pudney and Singer, 1979).

As the presumptive myoblasts proceed in their developmental program to form myoblasts, they become elongated and assume a bipolar morphology, with an elongate nucleus (Fig. 2). These morphological characteristics allow them to be easily distinguished

Fig. 1. Stereoscopic reconstruction of a portion of a myoblast taken at the edge of the remnant filamentous structure remaining after extraction with Triton X-100 illustrating the three-dimensional organization of filaments. Tilt 5° from original axis. ×62,000.

from fibroblasts. The network of filaments also becomes reorganized and realigned with the longitudinal axis of the myoblast. During this stage the myoblasts develop long cytoplasmic microprocesses. This is a characteristic of cultured, differentiating chick muscle cells. Immunofluorescent studies have shown them to be rich in actin (Huang *et al.*, 1978). These microprocesses establish contact either with each other (Fig. 3) or with myoblast cell bodies (Fig. 4). The microprocesses generally have a uniform diameter of 0·1 μm but appear to be able to increase in thickness. Scanning electron microscopy has revealed that the microprocess stage during myoblast development into myotubes has a duration of approximately 20 hr and over 80% of the cells in the population elaborate these structures in this time period (Huang *et al.*, 1978). Microprocesses have not been reported in other cell types subjected to detergent extraction, nor have we seen them in chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells or the human line


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WI 38. The extending microprocesses exhibit splayed ends resulting from the unraveling of the microfilament, reminiscent of the 'growth cone' of axons (Yamada *et al.*, 1971). Following detergent extraction, the fine structure of the microprocesses could be examined and were seen to be composed principally of microfilaments. At higher magnificatlons in Figs. 5 and 6, these microfilaments may be seen at the junction of two microprocesses from contacting cells (as shown in Fig. 3). When individual microprocesses contact each other at their tips, the filaments forming the frayed ends comingle with each other (Fig. 5). Microprocesses may also establish contact at an oblique angle as in Fig. 6. In this micrograph, the fraying of the microprocess tip is more noticeable. The interactions of the microprocesses become so intimate that individual cell boundaries are lost. Thus, as in Fig. 6, the microprocesses rather than simply intersecting undergo a complex realignment which seemingly reorientates both microprocesses into a single filament bundle.

Contact between the filament systems of two cells by means of a microprocess can also be seen without detergent extraction using high voltage electron microscopy (Fig. 7). In this preparation, a myoblast is contacting a presumptive myoblast through a microprocess. The filaments within the process can be seen to integrate with the filamentous reticulum of the contacted cell. Fine filamentous projections from the process appear to attach the microprocess to the substrate. When microprocesses contact myotubes there is a distinct realignment of the filaments within the microprocess (Figs. 8, 9). Thus the filaments of the microprocesses accommodate themselves to the filament organization present in the myotube, making an abrupt turn to run parallel with these filaments.

Occasionally, dense material can be seen at the point of contact between a microprocess and the filaments of a contacted cell (Fig. 10). In order to investigate the relationship between the filaments of the microprocesses with those present in a contacted cell more precisely, stereoscopic micrographs were prepared. Stereoscopic microscopy illustrates that direct contact between filament systems occurs within the matrix of the cellular filament system, towards the substrate surface of these cells (Fig. 11). By comparison, Fig. 12 shows two cells with the morphology of fibroblasts which are in contact with each other but not fusing. The filament systems of each cell overlap but remain distinctly independent.

Muscle cultures were also examined 48 hr after plating on to grids. At this stage, many cells had produced microprocesses which were in contact with each other. Figs. 13 and 14 illustrate a case where fusion has been established between microprocesses and it is apparent that considerable tension can be transmitted through these structures. This is implied by the distortion of the filamentous network of these cells. The shape and position of the nucleus appear to be affected by this distortion of the network. Often the

Fig. 2. Bipolar myoblast. ×2000.

Fig. 3. Fusing myoblasts. In this example the microprocesses developed by each cell appear to be fused together. ×2000.

Fig. 4. In this micrograph the extraordinary length (approximately 100 μm) and thinness (approximately 100 nm) of the microprocess can be appreciated. In this example the microprocess is fusing with a cell body (arrowhead). ×4000.

Fig. 5. At higher magnification, the microprocess can be seen to be composed of microfilaments and in this case the fusion of the tips of two microprocesses result in a comingling of these microfilaments. ×7800.

Fig. 6. When two microprocesses fuse at an angle with each other, the interaction of the microfilaments is clearly demonstrated. ×6000.

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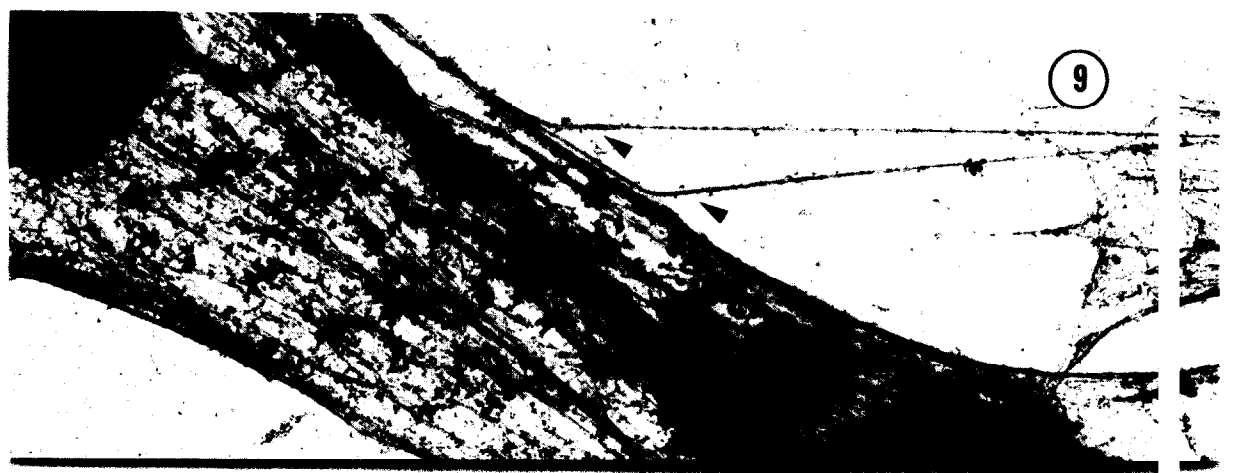

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nucleus appears to be pulled towards the microprocess so that it lies eccentrically within the cytoplasm. Cells which are apparently in the process of fusing can be seen to be connected by conduits of cytoplasm considerably thicker than the microprocesses (Figs. 15–18). These fusion bridges involve the elaborate integration of the filamentous elements of the two cells (Figs. 16, 18). Tension is also generated by these bridges which is again demonstrated by the distortion of the contacted cell and thus may pull these cells together (Figs. 15, 16). The nucleus can become considerably distorted by forces appearing to pull it into the intercellular connection (Fig. 17).

Two-dimensional gel electrophoresis of the Triton X-100 insoluble filament network of presumptive myoblasts (Fig. 19a) compared to a population of cells a day later (when the myoblast processes are the most abundant) indicate a dramatic change in the complexity of the filamentous proteins (Fig. 19b). The synthesis of abundant proteins which remain after detergent extraction increases from three, present in cells prior to cell contact, to at least five additional abundant proteins when cells are in contact. Most of these newly synthesized proteins have similar electrophoretic identities to proteins involved in muscular contraction and possibly the organization of the sarcomere.

Discussion

This work attempts to describe the process of muscle cell differentiation in tissue culture by focusing on the reorganization of the filament structure of these cells after Triton extraction. The notable observation from this work is the formation of tissue-specific highly attenuated projections of this filament system (microprocesses) which could act as to mediate intercellular contact. The transformation of single-celled myoblasts into a syncytium (myotube) may require the participation of these microprocesses. The interaction of these microprocesses with each other, or with cell bodies, establishes a connection between the cells resulting in the union of their respective filamentous systems. The establishment of this connection appears to generate considerable tension indicating that possibly these cells are actively drawn together. Presumably the microconnection between two cells develops into a cytoplasmic bridge which facilitates the integration of the filament network of both cells into a syncytium. Unpublished data using time lapse cinematography with Nomarski optics supports this viewpoint.

Thin-section ultrastructural studies on myoblast fusion have concentrated on the stage of syncytium formation when the cells are in close proximity (Lipton *et al.*, 1972; Shimada, 1971). Microprocesses were not seen in these studies, possibly due to the thinness of the plane of section and the low probability of encompassing a microprocess wholly within this plane. Similarly, freeze-fracture studies on these cells were also performed when the cells were in close enough proximity to observe gap junctions (Kalderon *et al.*, 1977; Rash and Staehelin, 1974). Another study observed 'filopodia' connecting fusing myoblasts (Chiquet *et al.*, 1975). These could correspond to our fusion processes. The technique of detergent extraction applied in this work allows the visualiza-

Fig. 7. High voltage micrograph of unextracted myoblasts. The long microprocess developed by one of these cells is fusing with the body of pre-myoblast. Note the thin filamentous extensions which possibly anchor the microprocess to the substratum (arrowhead). ×2700.

Fig. 8. When a microprocess fuses with another cell the filaments of the microprocess react by organizing themselves along the axis of orientation of the recipient cells, in this case a myotube. ×6900.

Fig. 9. Another example of filaments from one cell (arrowhead) orientating themselves to accommodate with the filaments present in the recipient cell, which again is a myotube. ×12,000.

tion of the entire microprocess in high resolution. This approach reveals that the microprocess is comprised of a microfilament bundle which can be viewed as a specialization of the filament framework of the cell.

The microprocesses extend the reach of the cell by as much as 20 nuclear diameters, thereby increasing the probability of intercellular contact. Since the differentiation of these cells requires their fusion, any morphological mechanism which promotes cellular association can also promote differentiation and explain the long-observed directed nature of myoblast and pre-myoblast interactions (see, for instance, Backmann, 1980). This represents a different viewpoint from previous literature (Konigsberg, 1971) which has focused on soluble factors which may effect cellular associations during muscle differentiation, presumably by their interaction with the cell membrane. These views may not be mutually exclusive since morphological changes may arise in direct response to the secretion of diffusible substances.

The concept of fusion at a distance through microprocesses may suggest a mechanism for the activation of gene expression in these cells. As described in the Introduction, the definition of differentiation has relied on the formation of syncytia (myotubes) as a result of myoblast fusion. This morphological event is most often observed with the phase contrast microscope. If this morphological process is observed at higher resolution, cytoplasmic intercommunication occurs through microprocess connections not visible in the phase contrast microscope. Models requiring syncytia formation for the activation of the expression of muscle specific genes must, therefore, consider these initial interconnections. Contrarily, myoblasts prevented from fusing in calcium-deficient medium can be shown to produce muscle-specific proteins (Emerson and Beckner, 1975; Vertel and Fischman, 1976; Moss and Strohman, 1976). Hence it has been concluded that specific gene activation does not require the formation of syncytia (Delain and

Fig. 10. High-power micrograph of fusing region between a microprocess (arrowhead) and a myoblast cell body. A dense area (arrow) marks the point of fusion. The microprocess passes beneath the nucleus of the myoblast.

Fig. 11. Stereoscopic reconstruction of an area of fusion between two myoblasts. Note the reaction of the filament bundles (arrowhead) which pass beneath the cell before fusing with the filaments. Tilt 10° from original axis. ×24,000.

Fig. 12. This micrograph demonstrates two myoblasts in physical contact but not fusing. There is no interaction between the apposing filamentous structures (arrowheads). ×5200.

Fig. 13. Two myoblasts fusing by means of their microprocesses which illustrates the asymmetric positioning of the nuclei which can occur as a result of tension. ×4100.

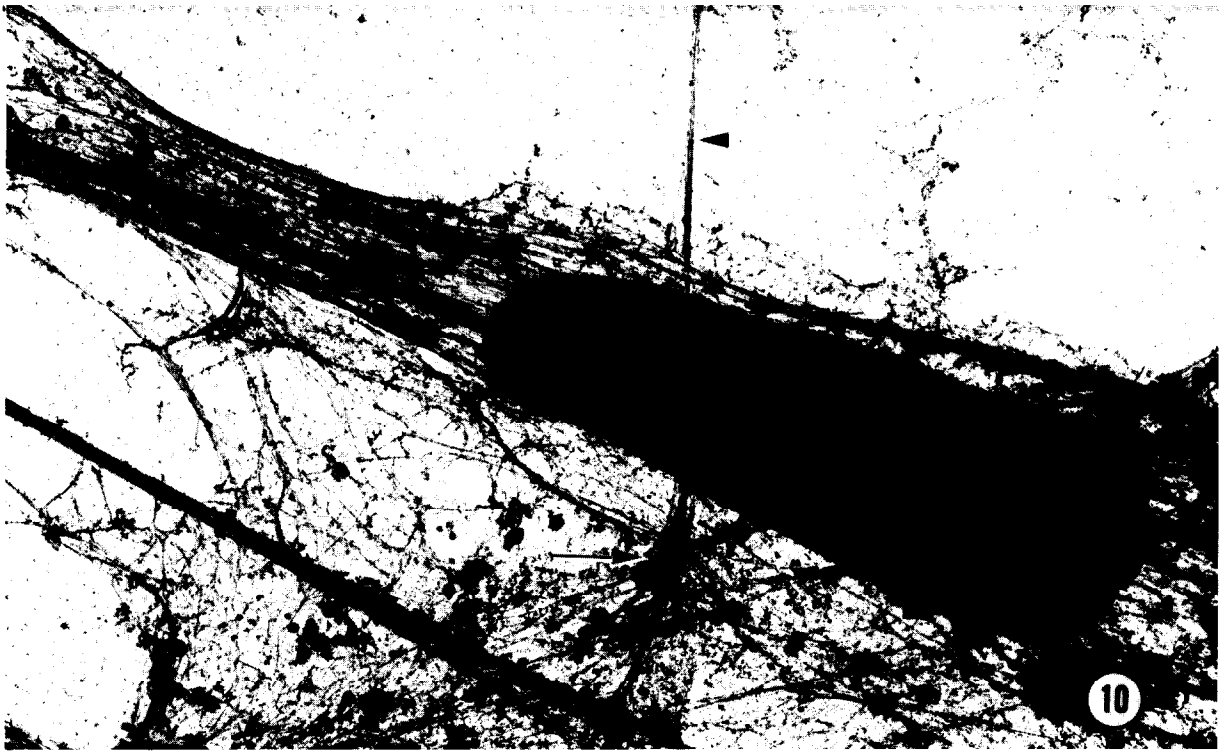
Fig. 14. This micrograph illustrates a long microprocess developed by a myoblast fusing with the cell body of a recipient myoblast. Note the acentric position of the nucleus of the myoblast developing the microprocess. ×4000.

Fig. 15. The cytoplasmic bridge (arrowhead) established through a fusion process (possibly a more developed stage of the microprocess) illustrating the tension that can be developed which subsequently deforms the recipient myoblast. ×7800.

Fig. 16. High power micrograph of the fusing area seen in Fig. 15 demonstrating the comingling of the filamentous structures. ×16,200.

Fig. 17. Following the initial event of fusion, the cells become closely apposed with a shortening and thickening of the original microprocess. ×7000.

Fig. 18. High-power micrograph of the area of fusion present in Fig. 17 illustrating the interaction between the filamentous organizations. ×20,000.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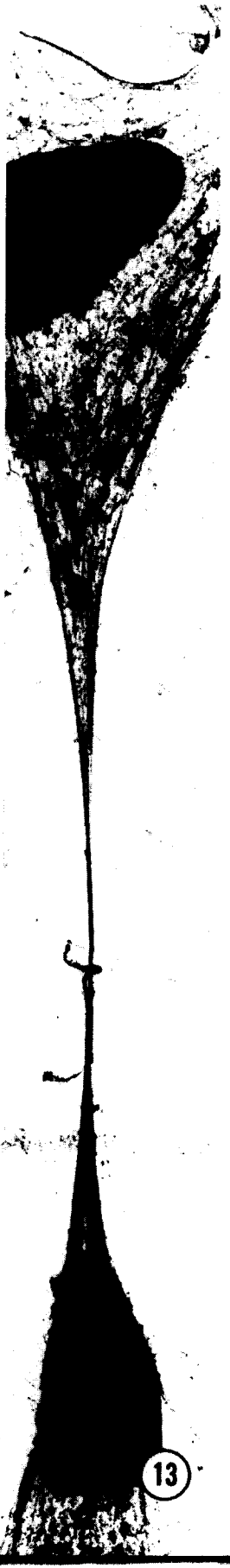

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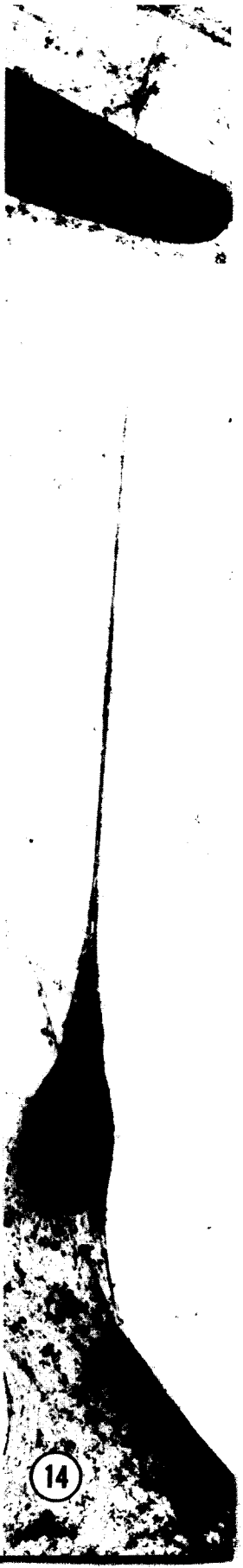

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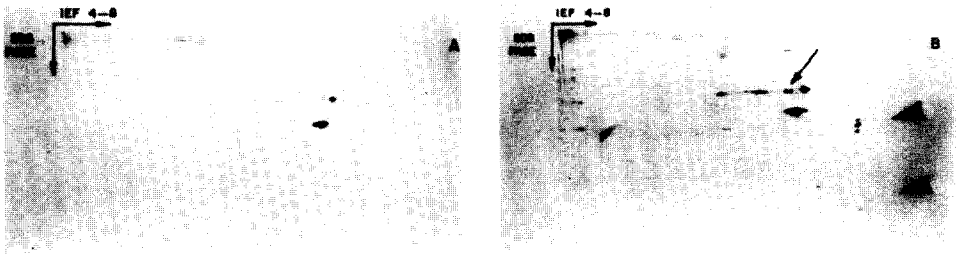

Fig. 19. Two-dimensional gel electrophoresis of labelled Triton X-100 insoluble proteins from pre-myoblast cultures (a) and cultures of myoblasts following microprocess development (b). The large arrows point to the tropomyosins (top) and light chains of myosin (bottom). The small arrow points to desmin, a muscle-specific intermediate filament protein.

Wahrmann, 1975). It is possible, however, that myoblasts can be interconnected through microprocesses but not be able to effect complete fusion into a myotube in calcium deficient culture media. Hence they would appear to be in a non-fused state when observed by conventional light microscopy.

The process of myoblast differentiation defined here considers only the differentiation of the cellular filament system. In this respect, the bipolar myoblast can be considered a differentiated cell (Trotter and Nameroff, 1976). The morphological reorganization of this system in presumptive myoblasts to a bipolar myoblast with microprocesses presumably requires gene activation. Possibly, proteins resulting from this activation continually modify the cell framework during differentiation. Consistent with this view is the appearance of additional proteins associated with the detergent insoluble framework. These additional proteins have the same identity as some contractile proteins present in muscle cells and thus may interact with the filament network to initiate the morphological reorganization. They may also be involved in the tension generated on the microprocesses connecting the cells, or effect the orientation of the sarcomere. The axis established between two conjoined cells may form a linear scaffold on which to build a contractile apparatus. Recent evidence from high voltage microscopy (Peng *et al.*, 1981) suggests that the cellular filament system induces sarcomere formation. Muscle cells developing in suspension, without a fixed substrate with which to establish an axis of orientation, are able to organize sarcomeres but not parallel arrays of myofibrils (Puri *et al.*, 1980).

The distortion of the nucleus by filaments was originally noted in the electron microscopic study of muscle tissue by Franke in 1969 (Franke and Schinko, 1969; Franke, 1970). We find a similar distortion of this organelle in cultured cells. Tension transmitted by filaments connecting the cells may be transferred to the nucleus and result in its elongation. While little is known about the effects of nuclear shape on gene function, the characteristic elongated nuclei of muscle cells may belie some specialization in molecular function. Thus, from the perspective of both morphological and molecular investigation the filament network of developing muscle cells appears to be a valuable unifying principle for future approaches to the analysis of myogenesis.

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