Proc. Nat. Acad. Sci. USA
Vol. 70, No. 6, pp. 1688–1692, June 1973

Actin in Dividing Cells: Contractile Ring Filaments Bind Heavy Meromyosin

(cell division/cytokinesis/cleavage/HeLa cells/microfilaments)

THOMAS E. SCHROEDER

Friday Harbor Laboratories, University of Washington, Friday Harbor, Wash. 98250

Communicated by Keith R. Porter, March 26, 1973

ABSTRACT **Many microfilaments and microtubules are well preserved after glycerol-extraction of HeLa cells at room temperature (22°). Incubation in heavy meromyosin from rabbit skeletal muscle results in conspicuous and characteristic "decoration" of microfilaments of the contractile ring. Decoration is completely prevented by 10 mM ATP or 2 mM pyrophosphate, and fails to occur if heavy meromyosin is either omitted or replaced by egg albumin, a nonspecific protein. Decorated microfilaments have a substructure consisting of polarized, repeating arrowheads 27–35 nm apart. The specificity of these results strongly suggests that microfilaments of the contractile ring in HeLa cells are closely related to muscle actin. Very thin undecorated strands among the microfilaments of the contractile ring possibly represent a myosin component. These findings are discussed in terms of: the actomyosin-like properties of the contractile ring as a mechanochemical organelle that causes cell cleavage; the probable universal occurrence of actin-like protein in all dividing animal cells; and the contractile ring's combined sensitivity to cytochalasin B and its affinity for heavy meromyosin, a combination unique among microfilamentous organelles.**

The contractile machinery in cells is often, if not universally, fibrillar in nature. Certain fibrillar structures, those called microfilaments, are thought to be the causative agents of cellular contractions such as shortening, constriction, and some changes in cell shape. According to a current working hypothesis, such microfilaments are closely akin to muscle actin, the chief contractile protein of thin myofilaments. Direct biochemical characterization of microfilaments is usually impossible. However, in certain favorable cases where protein extraction and purification have been feasible, definite actomyosin-like proteins have been described, as in amoebae, slime molds, blood platelets, and sea urchin eggs (see Pollard's review, ref. 1). Actin has also been detected in many other cell types by applying exogenous heavy meromyosin (HMM) to glycerol-extracted cells (2). HMM, the soluble tryptic fragment of muscle myosin, interacts with actin under defined conditions to produce a morphologically distinctive complex. Localized HMM-binding permits the identification of actin or an actin-like component. The latter designation reflects the convention that actin can only be confirmed by rigorous biochemical tests.

Cell cleavage is an equatorial constriction that essentially completes the division process in animal cells. It typically lasts about 10 min. Independent contractility of the equatorial cell cortex has been demonstrated and the contractile tension has even been measured (see Rappaport's reviews, ref. 3). The notable fibrillar structure associated with the cleavage process is the contractile ring (CR), whose short-lived presence as a band or ring-like array of microfilaments corresponds very closely with the geometry and progress of the cleavage constriction (4, 5).

CR microfilaments measure 4–6 nm in diameter in most intact cells, are of indefinite length, are closely associated with the plasmalemma, are circumferentially oriented, and are restricted to the region of the cleavage furrow. Their resemblance to actin was noted when originally described (6) in jellyfish eggs. In studies involving newt eggs Perry and coworkers (7) have recently demonstrated that the CR complexes with HMM, and they therefore concluded that it contains an actin-like component. Unfortunately their conclusion is not automatically applicable to contractile rings in other cells because CR microfilaments in amphibian eggs seem to be unique on two grounds: (*i*) they are thicker (8–10 nm) (8, 9) than CR microfilaments elsewhere (4–6 nm) and (*ii*) whereas cytochalasin B brings about disruption of CR microfilaments in HeLa cells and sea urchin eggs (4, 5), the drug elicits very little response in cleaving amphibian eggs, either in terms of inhibiting contraction or disrupting the CR (8, 10, 11). In this last connection, it might be reasoned that since cytochalasin B has little or no effect on native actomyosin from muscle (12–14), *only* CR microfilaments of amphibian eggs are akin to actin, and that the thinner, cytochalasin-sensitive CR microfilaments of other cells are composed of something quite different.

This issue is taken up here by investigation of the ability of CR microfilaments of the 4- to 6-nm variety to bind HMM under defined conditions. HeLa cells are used since their contractile ring has been previously characterized (4). In addition, since CR microfilaments of HeLa cells have been shown to be sensitive to cytochasalin B, these experiments also test the hypothesis that microfilaments that bind HMM are insensitive to cytochalasin B, and vice versa (15).

MATERIALS AND METHODS

Monolayer cultures of HeLa cells were generously provided by Dr. George Kenny and Mr. Frank Cartwright, University of Washington. These cells were grown on 60-mm plastic petri dishes in Minimum Essential Medium containing 10% fetal-calf serum. After two days of growth, the medium was decanted and gently replaced with 50% glycerol–50% standard salt solution (SSS) after Ishikawa (2). SSS consisted of 0.1 M potassium chloride, 5 mM magnesium chloride, and 6 mM sodium phosphate buffer (pH 7.0). Extractions were conducted at

Abbreviations: CR, contractile ring; HMM, heavy meromyosin; SSS, standard salt solution.

room temperature (22°), like all subsequent steps. After 24 hr the glycerol solutions were gently removed and the preparations were rinsed twice in SSS for 30 min. Extracted monolayers were incubated for 2 hr in the following media and then rinsed for 5 min in SSS, except where indicated otherwise. (*1*) Standard salt solution. (*2*) 0.5 mg/ml HMM* in SSS. (*3*) 0.5 mg/ml HMM in SSS with 10 mM disodium ATP (pH adjusted to 7.0), and rinsed in SSS with 10 mM ATP. (*4*) 0.5 mg/ml HMM in SSS with 2 mM sodium pyrophosphate, and rinsed in SSS with pyrophosphate. (*5*) 0.5 mg/ml of egg albumin in SSS.

All preparations were fixed in 2.5% glutaraldehyde–0.1 M sodium phosphate buffer (pH 7.0) for 30 min and then post-fixed in 1.0% osmium tetroxide in the same buffer for 20 min. A brief water rinse was followed by 0.5% aqueous uranyl acetate for 1 hr. Subsequent dehydration, infiltration, embedding, cell selection, and ultramicrotomy were as described (4). Where perpendicular sections of cleaving cells were taken, the presence of microtubules of stem-bodies verified the precise equatorial plane (Figs. 3, 5, and 6).

RESULTS

Cell morphology after glycerol-extraction

After gentle extraction at room temperature, HeLa cells in monolayer culture retain much of their normal shape. Since cleavage is the event under present investigation, the dumbbell-shaped cell is of special concern here (Figs. 1, 2, and 3). Inner cell contents are approximately normal in distribution. The plasmalemma is frequently perforated and irregularly elevated above the cytoplasm, which is demarcated by many small granules, probably glycogen. Membranous organelles are always deformed, although remnants of mitochondria and rough endoplasmic reticulum are not difficult to recognize. The nuclear envelope seems particularly resistant to glycerol-extraction. The mitotic apparatus is intact; typical microtubules are present in large numbers (Fig. 5)—a fact that can be attributed to the temperature of extraction and incubation.

The contractile ring and other microfilaments

Extraction in glycerol followed by incubation in low ionic strength solution (SSS) preserves the contractile ring (CR) as a thin, discontinuous band of 4- to 6-nm microfilaments beneath the cleavage furrow (Figs. 5 and 8). The undecorated CR is rarely more than about 0.1 μm in thickness. Its microfilaments appear to be somewhat disarrayed. However, the predominantly circumferential alignment is still very discernible. With regard to microfilaments in the CR, preparations treated with HMM plus ATP or pyrophosphate cannot be distinguished from the SSS controls (Fig. 7). In addition, the CR is unchanged when the nonspecific protein, egg albumin, is substituted for HMM in the incubation medium (Fig. 7). This evidence establishes that CR microfilaments do not have a nonspecific affinity for proteins in general.

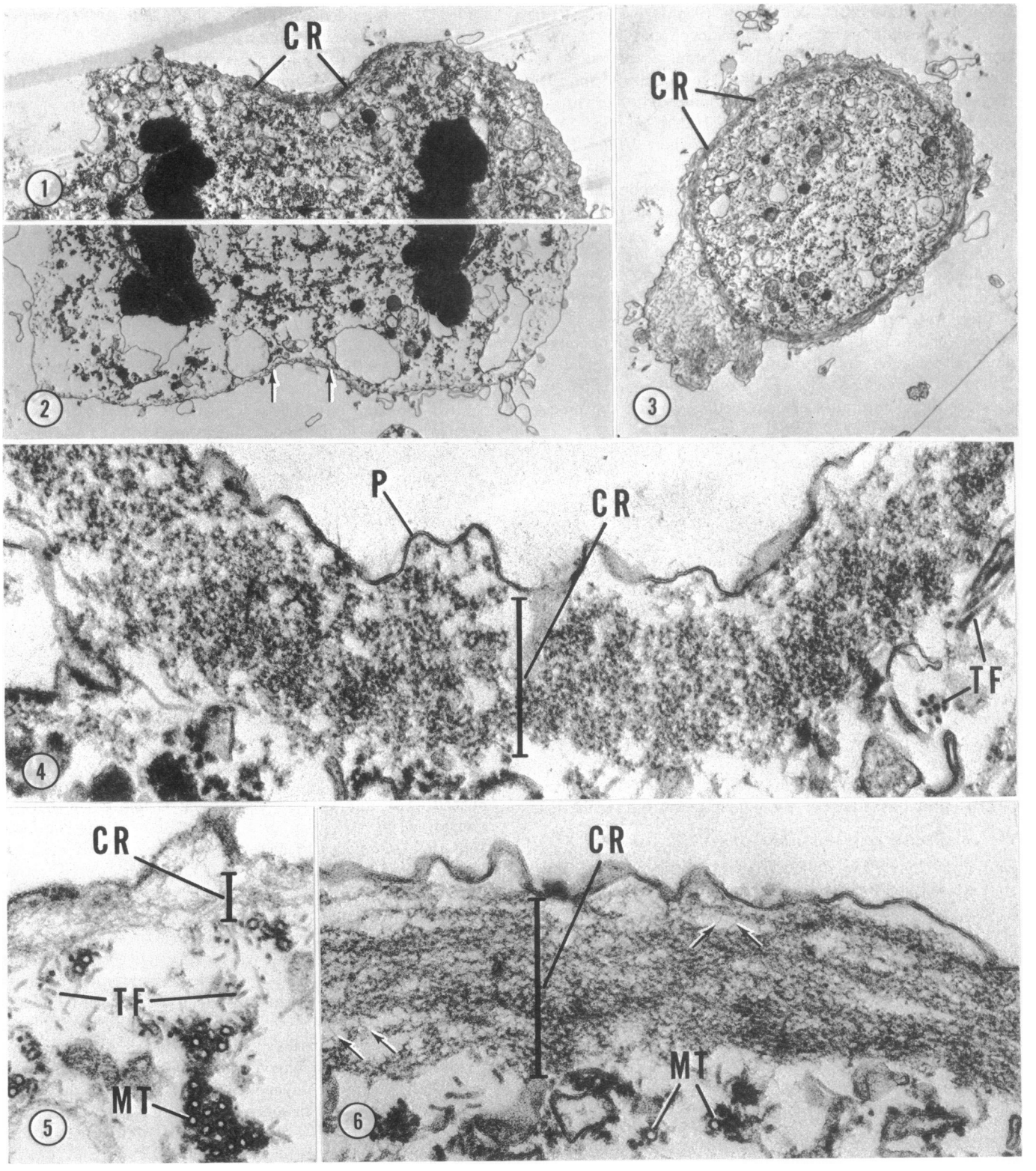
Cleaving cells that have been incubated in HMM *without* polyphosphates consistently display so-called "decorated" microfilaments, almost all of which reside within the normal domain of the CR. The extent and nature of decoration is the same in each of eighteen carefully sectioned, dumbbell-shaped cleaving cells. The decorated CR appears as a particularly thickened band, often 0.25 to 0.5 μm thick. It is rarely discontinuous, and is distinctly confined to the cleavage furrow proper (Fig. 1). It encircles the cell equator as a complete band (Fig. 3). In favorable longitudinal sections (in which CR microfilaments are normally seen end-on) the decorated CR presents a lattice-like appearance of loosely clustered electron-dense dots adorned and interconnected by material of lesser electron density (Fig. 4). The dense dots measure 4–6 nm across and are thought to be individual CR microfilaments—corresponding to undecorated microfilaments in any of the control preparations. The decorating material radiates irregularly from the dense dots and is interpreted to be HMM bound to the microfilamentous cores. The minimum distance separating decorated microfilaments appears to be about 20 nm.

In equatorial sections, such as Figs. 3 and 6, decorated CR microfilaments are clearly aligned circumferentially, but individual filaments are usually difficult to distinguish separately from their neighbors. Amid broad arrays of decorated microfilaments, as in Fig. 6, a quasi herring-bone pattern is seen. Occasionally, where filaments are especially well separated, the fuzzy decoration of individual microfilaments may be resolved as repeating arrowheads, at least for short distances. This pattern is strikingly similar to the characteristic substructure of the actin–HMM complex which in planar, negatively stained preparations has about a 36-nm periodicity of polarized arrowheads (18, 19). In Figs. 9 and 10 the arrowheads on CR microfilaments are likewise polarized and are spaced 27–35 nm apart. This spacing is felt to be in close enough accord with that of decorated actin if one considers that the decorated CR microfilaments are variably oriented within sections of finite thickness, causing some fore-shortening.

Upon close examination, there is another interesting component of the HMM-decorated contractile ring, besides microfilaments. There are many, very thin, undecorated strands that link together clusters of decorated microfilaments (Figs. 6 and 10). These whisker-like strands measure about 2 nm across and emerge periodically from arrowhead complexes (Fig. 10). These structures, here designated M-fibers, may possibly represent an oligomeric form of myosin. M-fibers may be an intrinsic component of the CR, but as they have not yet been detected in control preparations, they may have been introduced with exogenous HMM during incubation. This latter possibility is considered unlikely.

Decorated microfilaments are not exclusively restricted to the CR. Small discontinuous patches occur in the cortical cytoplasm of most cells, and a few decorated microfilaments are scattered deeper in the cytoplasm, regardless of the stage in the cell cycle. But nowhere are decorated microfilaments as numerous, as well aligned, or as consistently seen as in the CR of cleaving cells. A few decorated microfilaments lie within the domain of the mitotic apparatus and, to be sure, some of these are oriented parallel to its microtubules. However, in

* Over the past 3 years several batches of HMM have been used. Early experiments (16) were marred by inconsistent results probably caused by deterioration and inactivation of HMM. For present experiments, HMM from rabbit skeletal muscle was obtained as a lyophilized powder (17) from Dr. Ralph Yount, Washington State University, for which I am grateful. On the basis of its enzymatic assay, 31% of the powder, by weight, was HMM; 46% was sucrose; and 13% was potassium chloride. Fresh solutions containing 1.61 mg/ml of this powder yielded 0.5 mg/ml solutions of active HMM.



FIGS. 1 and 2. Low-magnification electron micrographs of cleaving cells sectioned parallel to long axes. Contractile ring (*CR*) is pronounced after HMM treatment (Fig. 1) and hardly visible (*arrows*) after SSS incubation alone (Fig. 2). ×6000.

FIG. 3. Perpendicular section at plane of equatorial cleavage furrow. Belt-like contractile ring (*CR*) is decorated by incubation in HMM. ×8000.

FIG. 4. Contractile ring (*CR*) viewed in same plane as Fig. 1 appears as a broad array of dense dots (microfilaments) surrounded by less dense decoration (HMM). *P*, plasmalemma. *TF*, thick filaments. ×82,000.

FIGS. 5 and 6. Contractile ring (*CR*) in SSS (Fig. 5) is diminutive and very pronounced after HMM treatment (Fig. 6). Thick filaments (*TF*) sometimes appear hollow. *MT*, microtubules of stem-bodies. *Arrows*, M-fibers thought to be oligomeric myosin. Both ×82,000.

general, these findings do not lend much support to the recent claims (20–22) that microfilaments occur in the mitotic apparatus in functionally significant numbers.

Thick filaments, microtubules, and membranes

Decoration of structures other than microfilaments by HMM has not been detected in these experiments. Membranes of all kinds appear comparable in all preparations, whether or not HMM was present. Microtubules are present in large numbers after extraction and incubation in all media except HMM plus ATP. Wall structure of microtubules is well defined, and occasionally one sees globular interconnections between nearby tubules. It is interesting to record, however, that cells incubated in HMM plus 10 mM ATP almost totally lack their microtubules. The significance of this finding is not presently known.

Thick filaments 10–13 nm across are plentiful and easily distinguished from other structures in glycerol-extracted cells. They do not display an affinity for HMM. In cleaving cells, thick filaments often appear in fascicles of 5–25 filaments that trace sinuous paths through the cytoplasm between the mitotic apparatus and cell cortex (Fig. 4). Thick filaments frequently appear in close proximity and parallel to mitotic microtubules (Figs. 5 and 6), especially in equatorial sections. The functional significance, if any, of this relationship is not known. At high magnification thick filaments appear "hollow" (Fig. 5), a feature shared by neurofilaments and glial filaments in other cells (23). Thick filaments bear no intimate relationship to the CR.

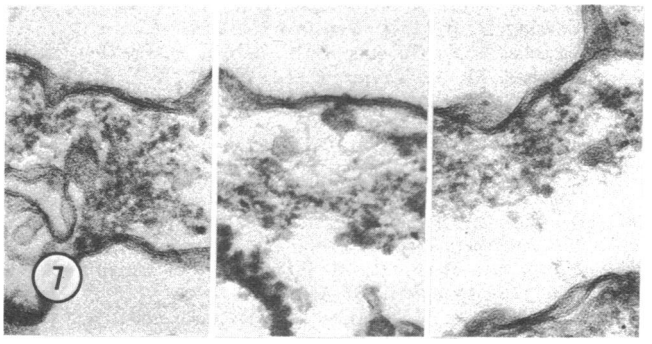


FIG. 7. Undecorated contractile ring microfilaments in controls incubated in HMM plus 10 mM ATP (*left*), egg albumin (*center*), and HMM plus 2 mM pyrophosphate (*right*). Sections compare precisely with Fig. 4 in plane, location, and magnification. All ×82,000.

DISCUSSION

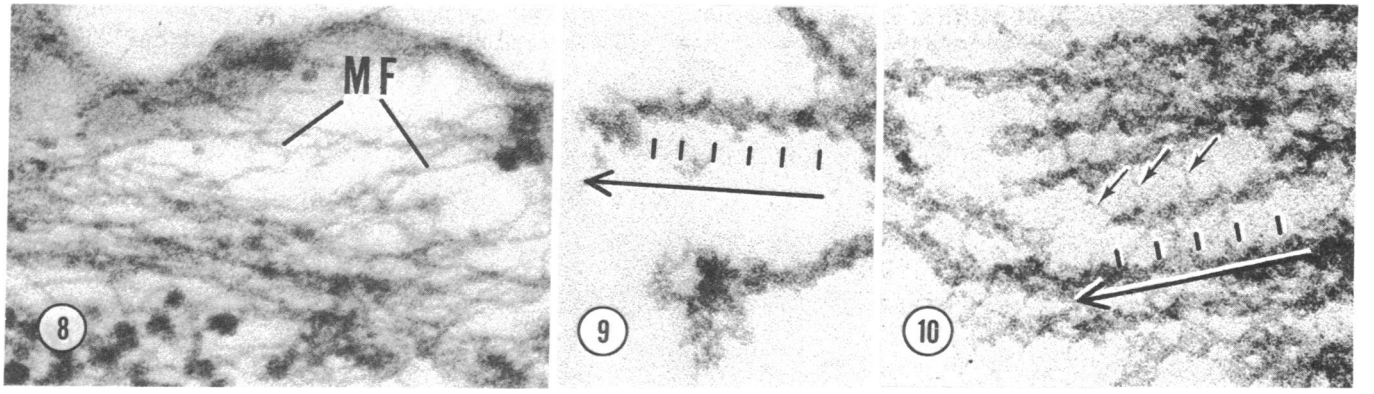
It has been shown that the contractile ring of cleaving HeLa cells survives glycerol-extraction and that its microfilaments display a specific and characteristic affinity for heavy meromyosin. Conditions that favor HMM-binding are identical to those that allow similar associations between HMM and actin (i.e., low ionic strength and absence of polyphosphates). These conditions, along with the repeating arrowhead substructure of the complex, strongly support the hypothesis that CR microfilaments are composed of actin or a closely related actin-like molecule. In this regard the preliminary report of this work (16) is accurate as published. Positive identity of actin in the CR must await more sophisticated biochemical evaluation.

For the first time, it has been shown that cytochalasin-sensitive microfilaments (4) also have the capacity to bind HMM. These two properties, therefore, need not be viewed as mutually exclusive, as previously postulated (24).

The larger diameter of CR microfilaments in amphibian eggs may now be considered a curious anomaly, at least in the narrow context of the actin question. It is clear that both the larger CR microfilaments (8–10 nm) and the smaller ones (4–6 nm) bind HMM and therefore probably have an actin-like constituent in common. The insensitivity of cleaving amphibian eggs to cytochalasin B may reflect other physiological properties, such as impermeability (25), rather than fundamental differences with respect to the contractile ring.

With this new evidence, it becomes increasingly reasonable to generalize that all contractile rings probably contain something akin to actin. This further suggest that the actin-like protein is universally present in all animal cells, for all animal cells are either produced by or undergo the cleavage process, or both. Moreover, the actin-like molecule of the CR is probably as ancient as the cleavage mechanism itself.

If the contractile ring functions as a rapidly-assembled actomyosin system—working in ways roughly analogous to an intracellular muscle—then the location and nature of the myosin component is one of the major concerns. Contractile rings do not appear to possess 12- to 18-nm rod-like myosin filaments as they exist in skeletal muscle. Functionally, however, the presence of myosin is strongly indicated, and in this report it has been suggested that myosin is represented as an



FIGS. 8–10. Undecorated microfilaments of contractile ring (*MF*, Fig. 8) are thin and naked. Decorated microfilaments after HMM treatment (Figs. 9 and 10) display repeating arrowhead substructure. Polarity of individual filaments indicated by *large arrows*, periodicity by *short lines*. M-fibers are indicated by *arrows* in Fig. 10. All ×160,000.

oligomer, here designated as M-fibers. Such M-fibers strikingly resemble other suspected oligomeric myosin formations, such as: (*a*) the thinner, interconnecting, undecorated filaments from blood platelets (26, 27); (*b*) the cross-arms ("Querverbindungen") between bundles of decorated filaments in sections of slime molds (28); and (*c*) the "whisker extensions" (29) and cross-members of ladder-like aggregates (30) of natural and myosin-enriched actomyosin isolated from slime molds. Oligomeric myosins in some cells have been studied biochemically (31, 32). Certain physicochemical differences from muscle myosin have been noted.

As an organelle, the contractile ring consistently appears thicker after decoration by HMM than in any of the other preparations, including the CR of intact HeLa cells (4). Superficially, this suggests that HMM actually enhances preservation of the CR. Although this possibility is not disproved, it is perhaps more likely that CR microfilaments are merely pushed apart and made visually more conspicuous by the binding process of HMM. Forer and Behnke (21, 33) discuss at length the possible role of HMM in preserving microfilaments. In their hands, microfilaments of crane fly spermatocytes are only seen after incubation in HMM without polyphosphates. Although Forer and Behnke (33) do unquestionably demonstrate decorated microfilaments in the furrow region of cleaving cells, their inability to establish the preexistence of CR microfilaments makes interpretation difficult. Their arguments that HMM induces fixable microfilaments derives from this predicament, but such arguments are unnecessary in the present investigation with HeLa cells.

It has recently been claimed that actin is present in the isolated mitotic apparatus of HeLa cells (34), thus enlarging upon related findings based upon the use of fluorescein-labeled HMM as a marker for actin (35). Other studies involving cell fractionation of HeLa cells (15) show that actin can be detected in mitotic apparatuses, but only to the extent they are contaminated with surrounding cytoplasm. Such findings are consistent with present results that demonstrate that actin-like material surrounds the mitotic apparatus in the form of the contractile ring but that very little of it is actually within or a part of the mitotic apparatus.

I thank Dr. G. Kenny and Mr. F. Cartwright for supplying cell cultures, Dr. R. G. Yount for HMM, Drs. T. D. Pollard and H. Ishikawa for illuminating discussions, and Mr. D. Strickland for excellent technical assistance. This investigation was supported by Grant E-633 from the American Cancer Society and Grant GM-19464 from the National Institutes of Health.

1. Pollard, T. D. (1973) in *Biology of Amoeba* (Academic Press, New York), chap. 9.
2. Ishikawa, H., Bischoff, R. & Holtzer, H. (1969) *J. Cell Biol.* **43**, 312–328.
3. Rappaport, R. (1971) *Intn. Rev. Cytol.* **31**, 169–213.
4. Schroeder, T. E. (1970) *Z. Zellforsch. Mikrosk. Anat.* **109**, 431–449.
5. Schroeder, T. E. (1972) *J. Cell Biol.* **53**, 419–434.
6. Schroeder, T. E. (1968) *Exp. Cell Res.* **53**, 272–276.
7. Perry, M. M., John, H. A. & Thomas, N. S. T. (1971) *Exp. Cell Res.* **65**, 249–253.
8. Bluemink, J. G. (1971) *Z. Zellforsch. Mikrosk. Anat.* **121**, 102–126.
9. Selman, G. G. & Perry, M. M. (1970) *J. Cell Sci.* **6**, 207–227.
10. Bluemink, J. G. (1971) *Cytobiologie* **3**, 176–187.
11. Hammer, M. G., Sheridan, J. D. & Estensen, R. D. (1971) *Proc. Soc. Exp. Biol. Med.* **136**, 1158–1162.
12. Forer, A., Emmerson, J. E. & Behnke, O. (1972) *Science* **175**, 744–776.
13. Sanger, J. F. & Holtzer, H. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 253–257.
14. Spudich, J. A. & Lin, S. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 442–446.
15. Weihing, R. R., Osborn, D. C. & Chiu, R. W. (1972) *J. Cell Biol.* **55**, 275 abstr.
16. Schroeder, T. E. (1970) *J. Cell Biol.* **47**, 183 abstr.
17. Yount, R. G. & Koshland, D. E. (1963) *J. Biol. Chem.* **238**, 1708–1713.
18. Huxley, H. E. (1963) *J. Mol. Biol.* **7**, 281–308.
19. Moore, P. B., Huxley, H. E. & DeRosier, D. J. (1970) *J. Mol. Biol.* **50**, 279–292.
20. Behnke, O., Forer, A. & Emmersen, J. E. (1971) *Nature* **234**, 408–410.
21. Forer, A. & Behnke, O. (1972) *Chromosoma* **39**, 145–174.
22. Gawadi, N. (1971) *Nature* **234**, 410.
23. Wuerker, R. B. (1970) *Tissue & Cell* **2**, 1–9.
24. Wessells, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T. & Yamada, K. M. (1971) *Science* **171**, 135–143.
25. de Laat, S. W., Luchtel, D. & Bluemink, J. G. (1973) *Develop. Biol.* in press.
26. Adelstein, R. S., Pollard, T. D. & Kuehl, W. M. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2703–2707.
27. Behnke, O., Kristensen, B. I. & Nielsen, L. E. (1971) *J. Ultrastruct. Res.* **37**, 351–369.
28. Allera, A., Beck, R. & Wohlfarth-Bottermann, K. E. (1971) *Cytobiologie* **4**, 437–449.
29. Nachmias, V. T., Huxley, H. E. & Kessler, D. (1970) *J. Mol. Biol.* **50**, 83–90.
30. Nachmias, V. T. (1972) *J. Cell Biol.* **52**, 648–663.
31. Nachmias, V. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2011–2014.
32. Pollard, T. D. & Korn, E. D. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, in press.
33. Forer, A. & Behnke, O. (1972) *Chromosoma* **39**, 175–190.
34. Chu, L. K., Stetson, J. R., Hardiwidjaja, M. & Sisken, J. E. (1972) *J. Cell Biol.* **55**, 43 abstr.
35. Aronson, J. F. (1965) *J. Cell Biol.* **26**, 293–298.