F-Actin Bundles in *Drosophila* Bristles Are Assembled from Modules Composed of Short Filaments

Lewis G. Tilney, Patricia Connelly, Stacey Smith, and Gregory M. Guild

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract. The actin bundles in *Drosophila* bristles run the length of the bristle cell and are accordingly 65 μm (microchaetes) or 400 μm (macrochaetes) in length, depending on the bristle type. Shortly after completion of bristle elongation in pupae, the actin bundles break down as the bristle surface becomes chitinized. The bundles break down in a bizarre way; it is as if each bundle is sawed transversely into pieces that average 3 μm in length. Disassembly of the actin filaments proceeds at the "sawed" surfaces. In all cases, the cuts in adjacent bundles appear in transverse register. From these images, we suspected that each actin bundle is made up of a series of shorter bundles or modules that are attached end-to-end. With fluorescent phalloidin staining and serial thin sections, we show that the modular design is present in nondegenerating bundles. Decoration of the actin filaments in adjacent bundles in the same bristle with subfragment 1 of myosin reveals that the actin filaments in every module have the same polarity. To study how modules form developmentally, we sectioned newly formed and elongating bristles. At the bristle tip are numerous tiny clusters of 6–10 filaments. These clusters become connected together more basally to form filament bundles that are poorly organized, initially, but with time become maximally cross-linked. Additional filaments are then added to the periphery of these organized bundle modules. All these observations make us aware of a new mechanism for the formation and elongation of actin filament bundles, one in which short bundles are assembled and attached end-to-end to other short bundles, as are the vertical girders between the floors of a skyscraper.

THERE is a bewildering array of actin-binding proteins which, at least in vitro, function to cross-link actin filaments into bundles or networks or gels, and to inhibit or to stimulate actin assembly. They also serve many other functions, such as stabilizing either the barbed, pointed, or lateral surfaces of the filaments, severing actin filaments, nucleating actin assembly, attaching actin filaments to membranes, moving particles along actin filaments, or providing motion by sliding actin filaments past one another. What controls the length of actin filaments is less clear. In some cases, e.g., in skeletal muscle, in erythrocytes (see Fowler, 1996), or in stereocilia of the ear (Tilney et al., 1992*a*), the length is rigorously regulated, while in other cases, actin filament length seems to vary considerably.

Currently, there is increasing interest in knowing how the length of actin filaments is controlled, since this information relates to the mechanism of motile events, such as pseudopodial activity or intracellular movement of pathogens, where assembly of actin provides much (in some cases all) of the force for movement. For example, it has been proposed that in locomoting fish keratocytes, there is a dynamic turnover of actin filaments by a nucleation-release mechanism (Theriot and Mitchison, 1991) similar to that described for the movement of the intracellular pathogen *Listeria* (Tilney and Portnoy, 1989; Tilney et al., 1992*b*). This model predicts the existence of short (<0.5 μm) filaments in keratocytes (Theriot and Mitchison, 1991). In contrast, others studying the same system (Small et al., 1995) have provided evidence that the filaments in the lamellipodia are at least several microns in length. This observation is inconsistent with a nucleation-release model for motility; instead, it favors a different mechanism that involves treadmilling.

In other systems, the actin filaments may exceed 2 μm in length, particularly if they are cross-linked into bundles. Thus, individual filaments in the microvilli of intestinal epithelial cells are the length of the microvillus, e.g., 1–5 μm in length (Hirokawa et al., 1982), and in stereocilia of the ear they can be even longer, e.g., up to 10 μm in length (Tilney et al., 1992*a*). The acrosomal process of certain invertebrate sperm probably holds the record for the length of individual actin filaments. In *Limulus* sperm, for example, the actin filament bundle is 60 μm long (Tilney, 1975). From thin sections, fast-frozen and deep-etched preparations, and from studies where the bundle is frayed apart, the filaments in the center of the bundle run the full 60 μm. This is a special case, however, because each actin

Please address all correspondence to Lewis G. Tilney, Department of Biology, University of Pennsylvania, Philadelphia, PA 19104. Tel.: (215) 898-6388. Fax: (215) 898-8780.

subunit in a filament is cross-linked to an adjacent subunit in the same filament by the 95-kD protein, scruin. This interaction not only doubles the diameter of the filament, but makes it unusually stable (Tilney, 1975; Way et al., 1995). In other invertebrate sperm, such as *Thyone*, where the acrosomal process is even longer, e.g., 90 μm in length, we know that the filaments do not extend the full 90 μm. Instead, the process is composed of filaments of a fraction of the final length (our unpublished observation).

We have been studying the actin filament bundles in *Drosophila* bristle cells, which we view as a model system for understanding how actin filaments are positioned in the cytoplasm of a cell and thus influence cell shape. What makes this system particularly attractive is that there are many *Drosophila* mutants that form aberrant bristles (FlyBase, 1994). Of the half dozen mutants so far examined, alterations in the actin cytoskeleton are apparent (see Appel et al., 1993; Cant et al., 1994; Verheyen and Cooley, 1994; Petersen et al., 1994, Tilney et al., 1995; and our unpublished observations), and in three cases, these mutants fail to produce the actin-binding proteins: fascin (Cant et al., 1994), profilin (Verheyen and Cooley, 1994), and the forked proteins (Petersen et al., 1994). Thus, we have in hand many tools useful for determining how actin filaments provide pattern to a cell, what proteins are involved, when must they be present, what happens if they are reduced in number or overexpressed, and so forth.

In this report, we have tried to determine the length of individual actin filaments in the bundles present in *Drosophila* bristle cells. In the large bristles or macrochaetes, the actin bundles can be up to 400 μm in length. In the more numerous smaller bristles or microchaetes, they are 65–70 μm in length. Each bundle is composed of a hexagonally packed bundle of actin filaments that are cross-linked together (Tilney et al., 1995), and at least two cross-links are used (Tilney et al., 1995).

What we report here is unexpected and gives us a new dimension as to how cell shape is determined by the cytoskeleton. What we find is that actin bundles in the bristles are made up of short filament bundles or modules that are attached end-to-end. The individual filaments within each module average 3 μm in length. Developmentally, a module forms by the cross-linking of tiny clusters of actin filaments together, first forming a poorly organized bundle that, as time progresses, becomes a highly organized, maximally cross-linked bundle. The newly forming module is attached end-to-end to an earlier formed module. The filament polarity in each module is identical, further indicating that during the earliest time, nucleation and filament elongation are rigorously controlled.

Materials and Methods

Drosophila Stocks

The Oregon-R strain of *Drosophila melanogaster* was used as the wild type in these studies. The *singed* stock (sn^3) and the forked stock (f^{36a}) were obtained from the *Drosophila* Stock Center (Indiana University, Bloomington, IN) and maintained as viable homozygotes. Flies were maintained on standard cornmeal-molasses-yeast food at 25°C, 60–70% relative humidity, with a 12-h/12-h day/night cycle. Complete descriptions of genes and symbols can be found in Lindsley and Zimm (1992) and on FlyBase (1994).

Developmental Staging

White prepupae (0 time) were collected and placed on double-stick Scotch tape in a petri plate that was put back in the incubator at 25°C. At the appropriate time, the petri plates were removed and the pupae were dissected.

Dissection of Pupae

We modified our earlier procedure (Tilney et al., 1995). Under ideal moisture conditions, the outer pupal case could be removed with a pair of fine forceps since it is crunchy and tears easily. First, we removed the operculum, and then with one tip of the forceps, we tore the outer pupal case down the length of the pupa. The torn sides of the outer pupal case were then stuck down to the double-stick Scotch tape. The pupae were then lifted free by grabbing the tip of the abdomen, and the pupae were then stuck down on another piece of double-stick Scotch tape, ventral side down. The pupae were then covered with PBS. With a 5-mm scalpel (Accurate Surgical and Scientific Inc./Kaiser Medical Industrial Co., Westbury, NY), a small incision was made between the developing eyes extending down to the mouth parts. With curved Gills Welsh Vannes micro scissors (Storz Co., St. Louis, MO), a cut was made from the incision down the sides of the head and along both sides of the thorax above the wing buds, and then down the sides of the abdomen. A cross-wise cut along the anterior top of the abdomen joined the two side cuts. The cut piece of tissue or dorsal surface of the thorax was removed, placed on its back, and the internal organs and fat bodies were removed with fine forceps. The flight muscles were not removed at this time, since they help the tissue retain its integrity. Once cleaned, the tissue was fixed for light or EM.

Fixation and Processing for Light Microscopy

The cut and cleaned thorax was transferred to 4% paraformaldehyde in PBS for 20 min. The tissue was then transferred to 4% paraformaldehyde containing 0.1% Triton X-100 in PBS for an additional 20 min, washed three times in 0.1% Triton X-100 in PBS, and then placed in a drop of 10 mg/ml BSA in PBS. Subsequently, the tissue was placed in PBS and 0.1% Triton X-100 containing 10^{-6} M phalloidin conjugated to rhodamine (Sigma Chemical Co., St. Louis, MO) in the BSA solution for 30 min. The sample was then washed, placed on a slide, and immersed in glycerol-Citifluor (Ted Pella, Inc., Redding, CA). A coverslip was added, and the preparation was sealed with nail polish. The slide was then examined either with a universal fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) or a model TCS 4D confocal microscope (Leica, Heidelberg, Germany). Favorable confocal images of phalloidin-stained bristles were used to estimate module length within actin bundles. The length of each module was measured using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA) and their length distribution was plotted using Microsoft Excel (Microsoft Corp., Redmond, WA).

Methods for Transmission Microscopy

The isolated and cleaned dorsal thoraces were fixed by immersion in 2% glutaraldehyde (from an 8% stock purchased from Electron Microscope Sciences, Fort Washington, PA) in 0.05 M phosphate buffer, pH 6.8; fixation was for 1–4 h. After fixation in glutaraldehyde, the tissue was placed in 1% OsO_4 in 0.05 M phosphate buffer, pH 6.2, at 4°C for 45 min. The tissue was then washed three times in water at 4°C to remove the phosphate, and was enbloc stained with 0.5% uranyl acetate overnight at 4°C. The tissue was then dehydrated in acetone and embedded in Epon. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with an electron microscope (model 200; Philips Technologies, Cheshire, CT). Serial sections were cut and examined using slotted grids that were covered with formvar and coated with a thin layer of carbon. For routine observation, uncoated grids were used. The methods for detergent extraction and decoration with subfragment 1 of myosin are described in Tilney et al. (1996).

Scanning Microscopy

Drosophila flies were fixed overnight by immersion in 70% ethanol. They were then dehydrated completely, air dried, placed upon stubs, sputter coated with tungsten-platinum, and examined with a (AMR 1000; Amray Inc., Bedford, MA).

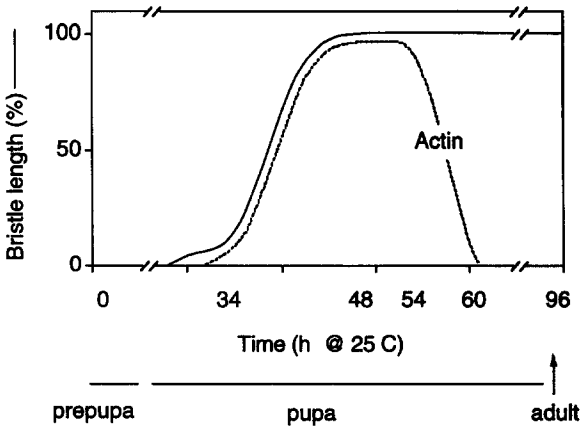


Figure 1. Graph illustrating when bristles begin to emerge and reach their maximum length in pupae. It is of interest to know that formation of actin bundles lags slightly behind bristle emergence. The bundles begin to break down in 53-h-old pupae. The 0-h time point corresponds to white puparium formation.

Results

Stages in Bristle Development

From the careful study conducted by Lees and Picken (1944), we know and have confirmed that bristles begin to emerge from pupae 32 h after puparium formation at 25°C. By 35 h, the bristles have attained their maximum diameter and ridges can be detected. After 35 h, the bristles grow rapidly in length, but not in diameter, and by 41 h, have reached about three quarters of their adult length. The final length is reached between 44 and 48 h. By 53–54 h, the actin bundles, located in the valley between the ridges, begin to break down (Overton, 1967; and our unpublished results) after the formation of a thickened inner cuticular layer. By 60 h, no traces of the actin bundles remain (our unpublished data). These staged events are depicted in Fig. 1.

Actin Bundles Break Down in an Unusual Way

In our earlier study (Tilney et al., 1995), we showed that in 45-h bristles, there are 7–11 actin filament bundles in each microchaete running from its base to its tip. The actin filaments in each bundle were hexagonally packed, maximally cross-linked, and displayed a 12-nm period in longitudinal sections. This period can be ascribed to the fascin cross-link encoded by the *singed* gene. One face of the bundle is closely applied to the plasma membrane and is likely to be connected to it.

When we examined phalloidin-stained bristles of increasing developmental age by fluorescence microscopy, what we saw was bizarre: the bundles appeared to have been "sawed" transversely into pieces 1–10 μm in length (Fig. 2, *a–c*). In addition to these apparent cuts, large gaps could be seen in the bundles of older bristles (Fig. 2, *c* and *d*). Initially, we thought that these transverse breaks might be caused by an artifact of specimen handling for light microscopy, but when the 54-h bristles were fixed, thin sec-

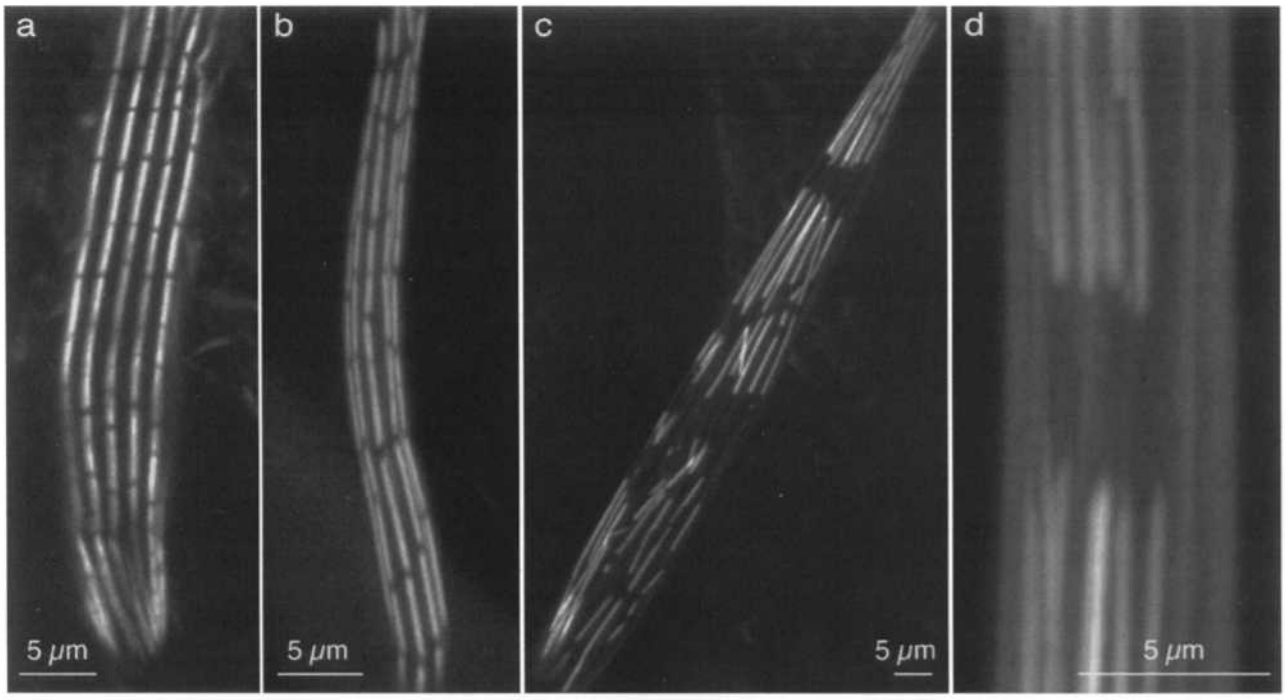


Figure 2. Portions of bristles from mature wild-type pupae stained with rhodamine-conjugated phalloidin and examined by confocal microscopy. The gaps within actin bundles increase in size as a function of developmental age and often occur in transverse register. (*a* and *b*) Optical sections showing some of the actin bundles in a 43-h macrochaete (*a*) and a 48-h microchaete (*b*). Note that the longitudinal gaps between modules are narrow in the younger bristle and wider in the older one. (*c*) Optical section through a 53-h macrochaete showing large gaps in the actin bundles. (*d*) A portion of a 56-h macrochaete showing large gaps in virtually all actin bundles in this region. Note that the bottom of the topmost modules are relatively flat when compared to the top of the bottommost modules which are pointed. The tips of all the bristles shown are at the top of the figure. Bars, 5 μm.

Figure 3. Longitudinal section through a wild-type 54-h bristle. By this stage, a layer of chitin (*C*) has been deposited. (*a*) The actin bundle depicted here has two gaps along its length. (*b*) These gaps cannot be caused by the bundle being cut obliquely, as individual microtubules can be followed for long distances, e.g., from one bundle segment to the next (*arrowheads*). Furthermore, the 120-Å periodicity of the fascin cross-bridge can readily be seen on the lower portion of the actin bundle. Bars: (*a*) 1 μm; (*b*) 0.1 μm.

tioned, and examined by EM, we also found transverse gaps in the bundles in longitudinal sections (Fig. 3 *a*). These gaps are not caused by grazing sections through the bundles, because individual microtubules that lay parallel to the bundles and immediately lateral to them are present throughout the section (see Fig. 3 *b*, *arrows*). Furthermore, each bundle segment shows the 12-nm period only observable in a near-perfect longitudinal section, which is indicative of the cross-link fascin. Thus, the bundle illustrated in Fig. 3 *b* must be cut in a true longitudinal section.

Transverse sections through the 54-h bristles revealed equally bizarre images of the bundles. As expected from the 12-nm period, the filaments in the bundles were hexagonally packed, just like the 45–48-h bristles (for an explanation see Tilney et al., 1995), but often we saw cuts across the filament bundles (see Fig. 4, *a* and *b*, *arrows*). From these images, it appears as if a bundle is composed of two subbundles closely applied to each other (Fig. 4 *b*). The gaps in these transverse sections are not randomly positioned, but always divide the bundle such that the portion

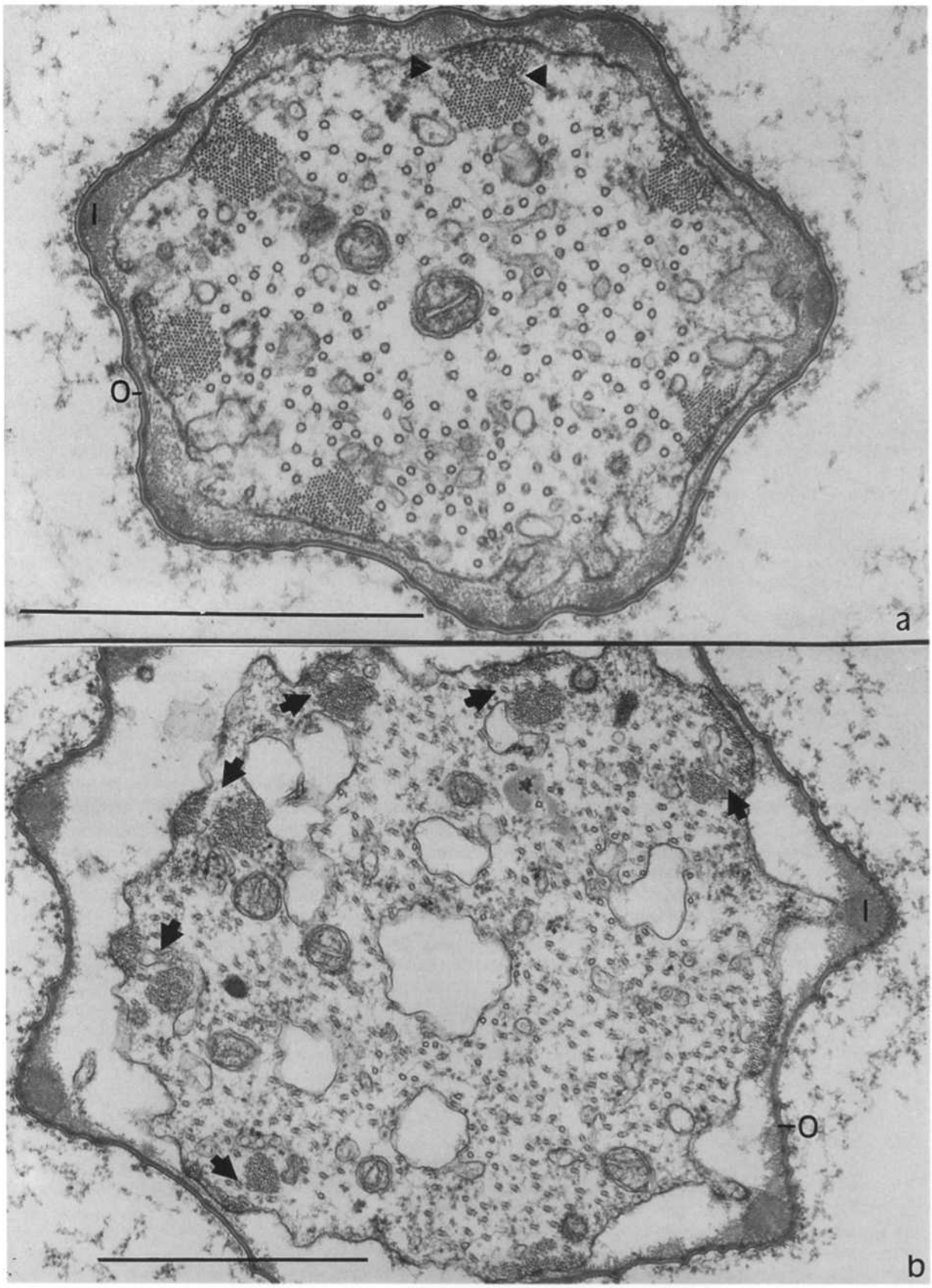


Figure 4. Transverse sections through two bristles from a wild-type 54-h pupa. A layer of chitin consisting of an outer (*O*) and inner (*I*) amorphous layer has been deposited. The inner layer lies between actin bundles such that the actin bundles lie in the groove between the ridges visualized by scanning microscopy. (*a*) The arrows point to a split or dislocation in an actin bundle. (*b*) Splits within actin bundles are common in this bristle (*arrows*). Bar, 1 μm.

of the bundle associated with the plasma membrane remains associated with it.

In 58-h pupae, the filament bundles were drastically reduced in diameter and, in some cases, missing altogether (Fig. 5). By 60 h, all traces of the bundles were gone (not shown). The cuticular layer surrounding the bristle was dramatically thickened by the time the actin bundles disappeared (Fig. 5). The microtubules remained and were abundant, as were other organelles, e.g., mitochondria and ER.

From these images, we suspected that individual bundles in bristles might be made up of a number of shorter bundles or modules attached end-to-end. In such a model, when the bundles start to break down, they would do so at the ends of the filaments that are located at the junction points between modules. As breakdown proceeds, gaps or spaces between the shorter modules should appear and increase in size, which they do (Fig. 2, *a–c*). Further evidence in favor of the modular idea comes by careful examination of the fluorescent phalloidin images. The ends of the modules nearest to the bristle tip are pointed, while those ends nearest to the base are flattened (Fig. 2 *d*). The significance of pointed tips and flattened bases to the modules is reviewed in the Discussion.

In many of the phalloidin-stained bristles, we saw that the gaps in the bundles within the same bristle often line up transversely as if in register, like the stripes on a tiger's tail. Thus, there must exist a mechanism to ensure that the gaps are lined up transversely.

Actin Bundles Show Transverse Cuts before Breakdown

Close examination of the actin bundles in full-length bris-

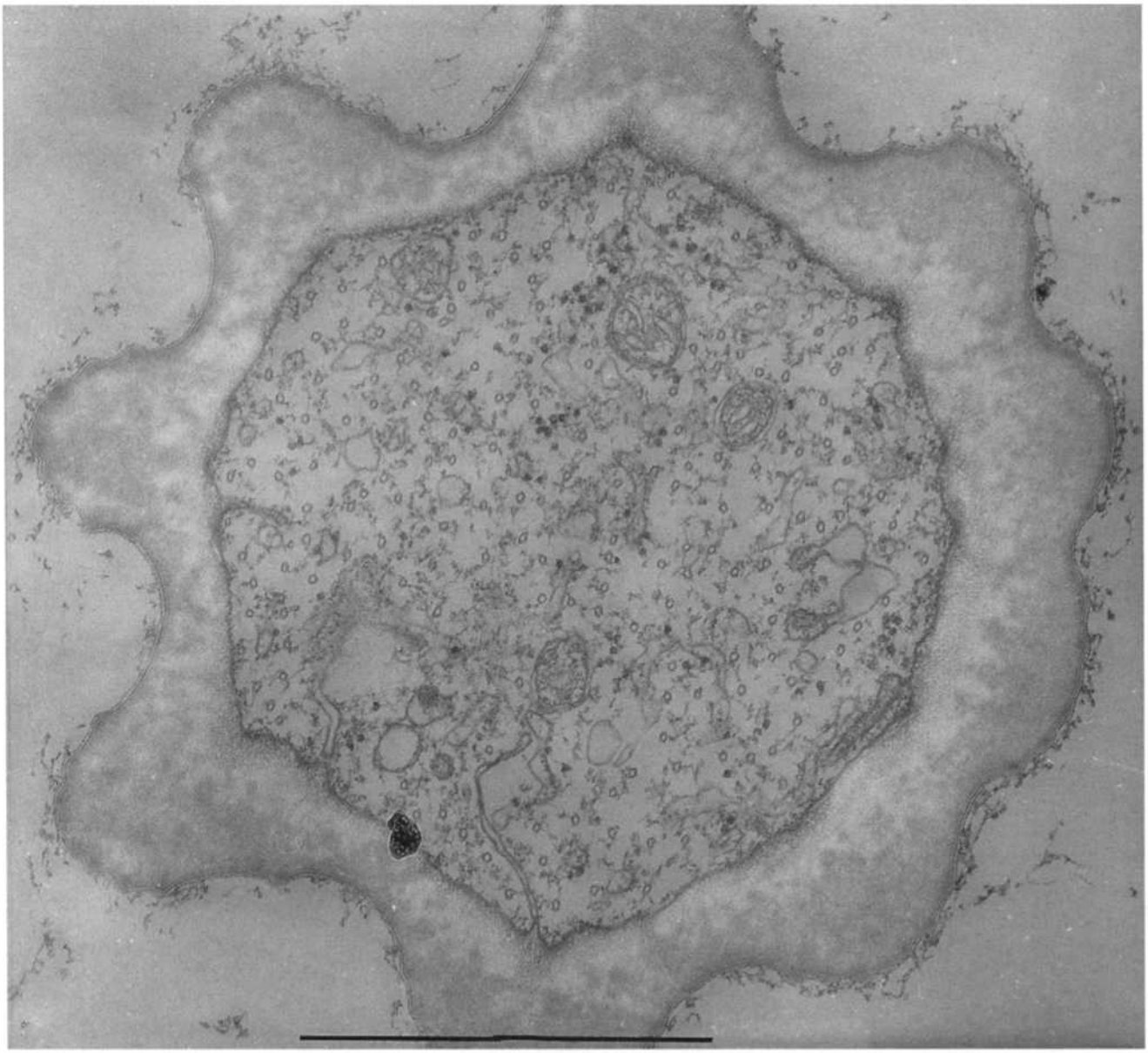


Figure 5. Transverse section through a bristle of a wild-type 58-h pupa. Although microtubules remain in this bristle, the actin bundles have disappeared completely. Note the fluted appearance of the chitin that surrounds the plasma membrane of the bristle. Bar, 1 μm.

tles in which the actin bundles have not yet begun to degenerate also showed the presence of transverse breaks (Fig. 6 *a*). Unlike the degenerating actin bundles, the width of the gaps were usually very small. In most cases, the gap within a single bundle was not perfectly transverse, but rather diagonal, as if each module had a pointed end nearest to the tip that overlapped with the flattened end of the module immediately above it (Fig. 6, *b* and *c*). As in later stages, the gaps of adjacent bundles within a single bristle are in transverse register (Fig. 6, *a–c*).

We should mention that the thorax was fixed with paraformaldehyde before detergent extraction, incubation with phalloidin, and placement on the coverslip. Thus, these gaps cannot result from crushing under the coverslip, a conclusion confirmed from thin-section analysis (Figs. 3, 4, and 7). What is interesting, however, is that when the bristles are examined in the fluorescent microscope, they are often bent. This bending seems to occur at gap sites, as if these are regions of instability (Figs. 6 *c* and 11).

To further test the idea that the actin bundles are of a modular construction before breakdown, we cut serial transverse sections of fully elongated but not degenerating bundles. One favorable set is illustrated in Fig. 7. In section 3, all 11 bundles in this microchaete showed no breaks or discontinuities. In section 5, three bundles (1–3) showed discontinuities or breaks in the bundles, and the other eight were intact with no breaks. By section 8, all the bundles, including bundles 1–3 were intact once again. Minimally, this means that the filaments in at least those three bundles do not extend the full 70 μm, the length of the bristle. And more realistically, the breaks suggest that this is the location of the overlap between modules.

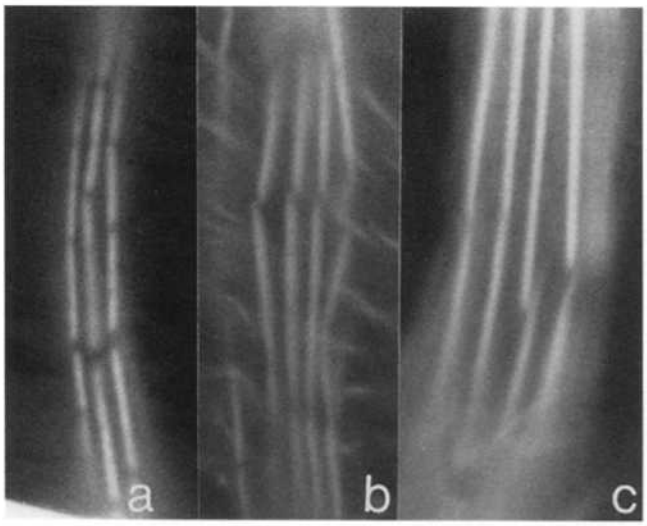


Figure 6. Portions of bristles from wild-type 48-h pupae stained with rhodamine-conjugated phalloidin and visualized with a fluorescent microscope. At this stage, the bristles are their final length, but the actin bundles have not yet started to break down. (*a*) At a low resolution, one can sometimes see that the bundles appear to have gaps within the bundles. (*b*) At a higher resolution, one can see two gaps in the bundles. They occur at the same position on adjacent bundles. A more careful examination of the gaps reveals that a gap consists of an overlap or splicing together of the bundles. (*c*) The splice is more obvious in the higher resolution picture.

It is of interest that these breaks occur in the same section for three adjacent bundles, again reinforcing the phalloidin-stained image where the breaks are in transverse register. In Fig. 8, we included a longitudinal section through the overlap regions of a bundle in a 44-h pupa. If serial sections were included, the filaments of adjacent modules would overlap. We have included this thin section because one end is flat with most of the filaments terminating at the same point (top module). The other end of the module, corresponding to the "pointed end" shows that the termination of filaments, is less regular (bottom module). This thin section reinforces our observations pertaining to the pointed and flat ends of the modules seen by fluorescence microscopy (e.g., Fig. 2 *d*) that reflect the polarity of the module. The few filaments from the lower module that connect to the upper module in Fig. 8 appear continuous in this section. Although this might be true, we cannot be certain of this conclusion because this thin section (minimally 500 Å) is many times thicker than an actin filament.

Examination of confocal images of phalloidin-stained actin bundles like those shown in Figs. 6 and 11 allowed us to determine the length distribution of modules in 43–48-h bristles. We find that mature actin bundles found in both macrochaetes and microchaetes contain modules of similar length. These modules have an average length of ~3 μm with a substantial length distribution (Fig. 9, *a* and *b*).

Actin Filament Polarity Is Unidirectional in All Bristle Modules

We immediately wondered whether or not the polarity of actin filaments in all the modules within the same bristle was unidirectional. This is crucial information in trying to understand how modules are formed developmentally. Accordingly, the isolated cortexes were detergent extracted and then decorated with subfragment 1 of myosin. We examined six different bristles and the actin filaments in these bundles at different levels in the same bundle, as well as in a number of different bundles in the same section of each of these six bristles. In all cases, the polarity of the filaments was identical. The barbed ends were located nearest to the tip of the bundle, and the pointed ends were located nearest to the base of the bristle. A section through one bristle decorated with subfragment 1 of myosin is illustrated in Fig. 10, and the arrowheads indicate the polarity of the filaments in those bundles. Thus, the polarity of the actin filaments in all modules is identical.

Developing Bristles

Light Microscopy. In Fig. 11, (*a–e*) we show a series of five newly emerged bristles (macrochaetes) that are beginning to elongate. Each is stained with fluorescent phalloidin. Thin bundles of actin filaments are present at the base of the bristle. But strikingly, the bundles do not extend to the cone-shaped tip of the bristles. All that is present at the tip is some diffuse fluorescence. This is in contrast to mature bristles, where the bundles extend right to the pointed tip of the bristle (Fig. 10, *f–g*). In bristles that have elongated slightly (Fig 10, *b–e*), we see that the basally situated bundles show breaks, or to put this into context, a modular construction. Modules in adjacent bundles are in trans-

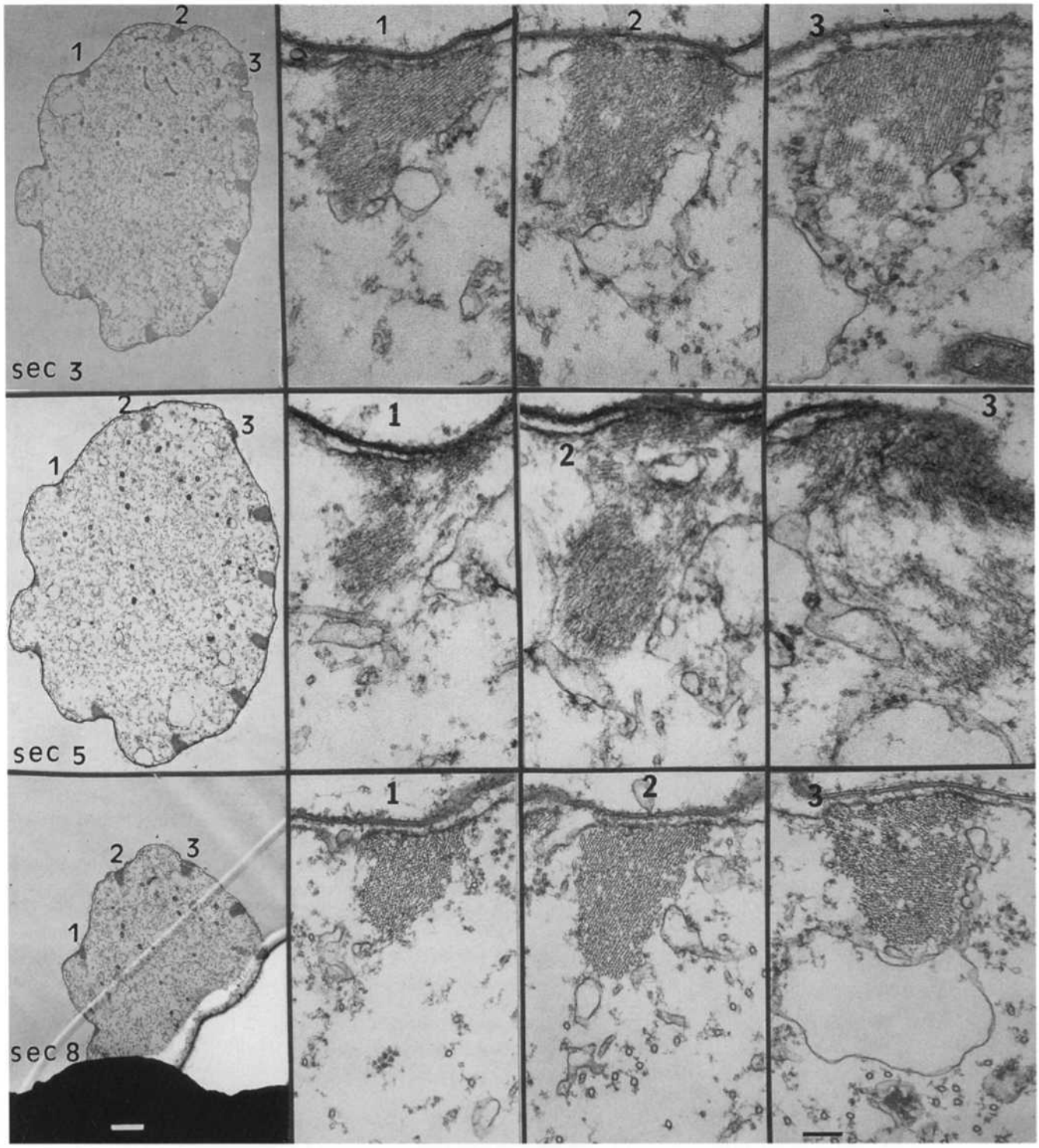


Figure 7. Serial sections of an individual bristle of a 48-h wild-type pupa. Illustrated on the first vertical lane are serial sections 3, 5, and 8. Bar, 1 μm. The bundles are numbered, and higher magnifications of each from the three sections illustrated here are positioned on the right of this vertical lane. Bar, 0.1 μm. What is interesting is that bundle number 3, seen in section 3, breaks into two bundles in section 5, and by section 8, a large bundle reforms.

verse register. Furthermore, these first modules are approximately the same length as those seen in mature bristles. Thus, modules form concurrently with elongation of the bristles. As the bristle elongates, the modules increase in fluorescence as if they are increasing in diameter. We also saw spots of fluorescence lined up between adjacent bundles in developing (Fig. 10 *c*, *arrowheads*) but not mature (Fig. 10, *f–g*) bristles.

Examination of sprouting macrochaetes allowed us to visualize newly forming modules. When we measured the

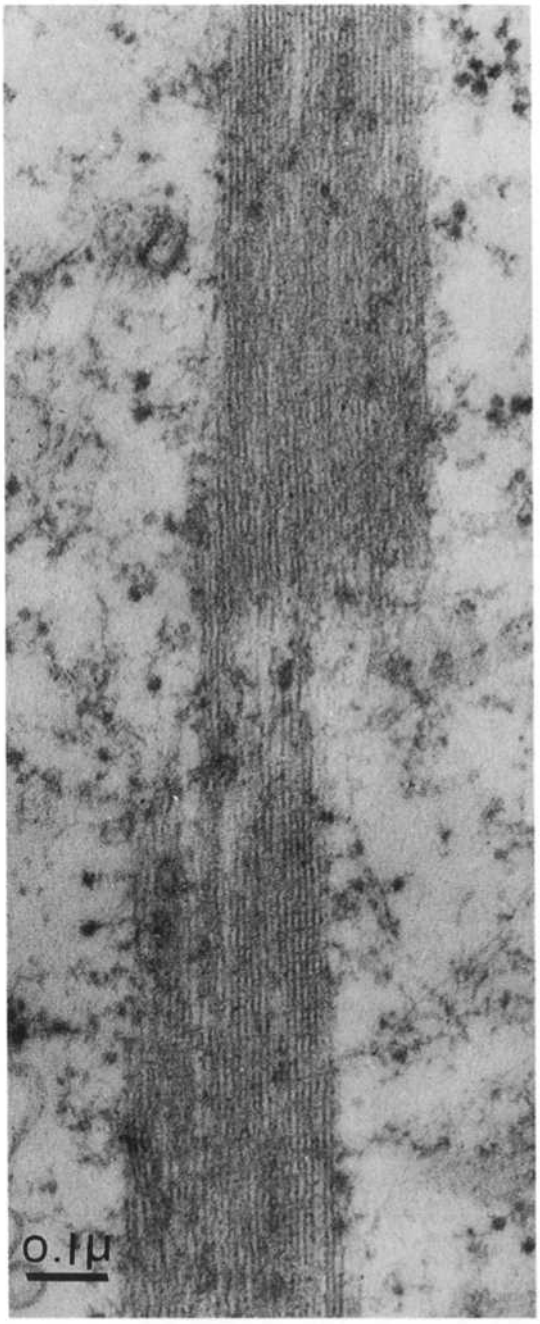


Figure 8. Longitudinal section through a filament bundle from a wild-type 44-h bristle. This section illustrates the end-to-end association of two modules. The print is oriented so that the tip of the bristle is upward. It is of interest to know that the base of the upper module is flat, with the component actin filaments terminating at approximately the same level. This image is comparable to the fluorescence images shown in Fig. 6. Bar, 0.1 μm.

basemost and the next tipward modules in actin bundles that were at least two modules long, we found a similar distribution of module lengths averaging ~3 μm (Fig. 9 *c*). A similar length distribution was also found in macrochaetes of intermediate developmental age (data not shown) and mature macrochaetes and microchaetes (Fig. 9, *a* and *b*). Thus, average module length is similar throughout development.

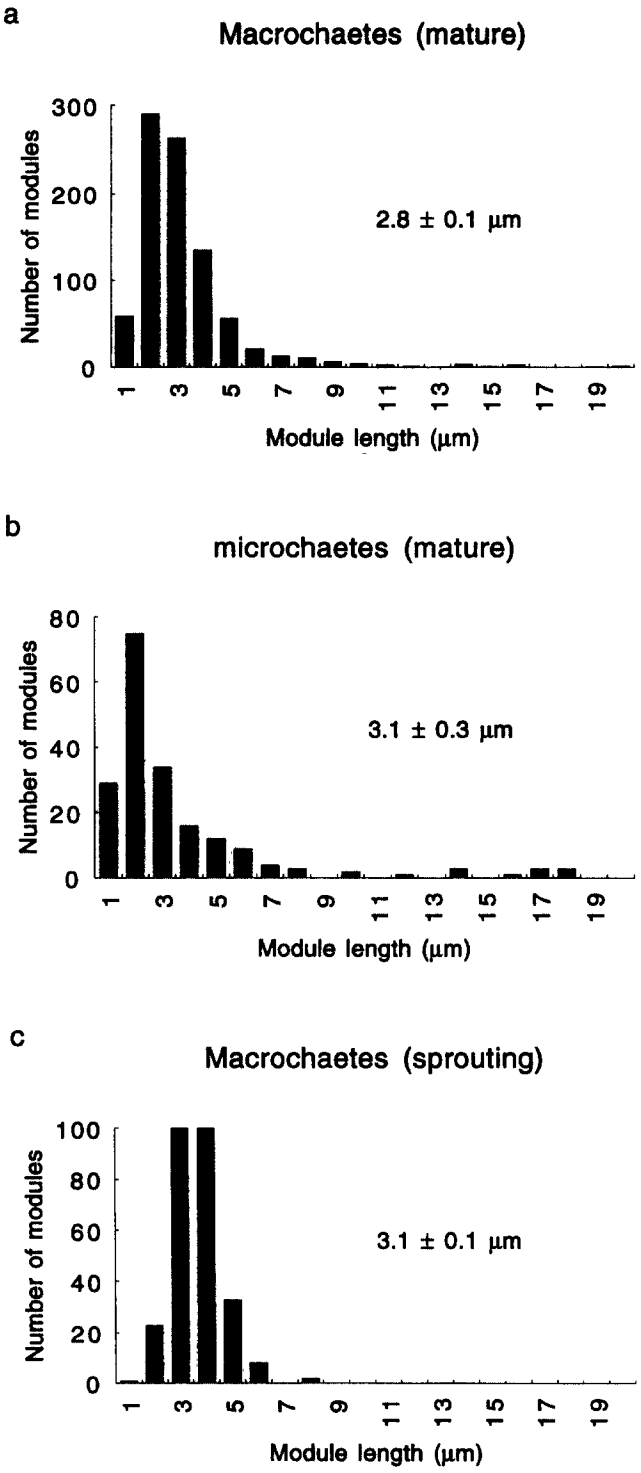


Figure 9. Module lengths in developing *Drosophila* bristles. (*a*) Confocal images of 10 mature macrochaetes (>100 μm) from 43–48-h pupae were used to measure the lengths of 877 modules. (*b*) Images of five mature microchaetes (>50 μm) from 44–48-h pupae were used to measure the lengths of 195 modules. (*c*) Images of 13 sprouting macrochaetes (14–32 μm) from 32–34-h pupae were used to measure the lengths of the basemost modules (n = 143) and the next modules located toward the bristle tip (n = 124). The length distribution of these two classes were nearly identical and are plotted together. The average module length (in micrometers) ± SEM is given in each panel.

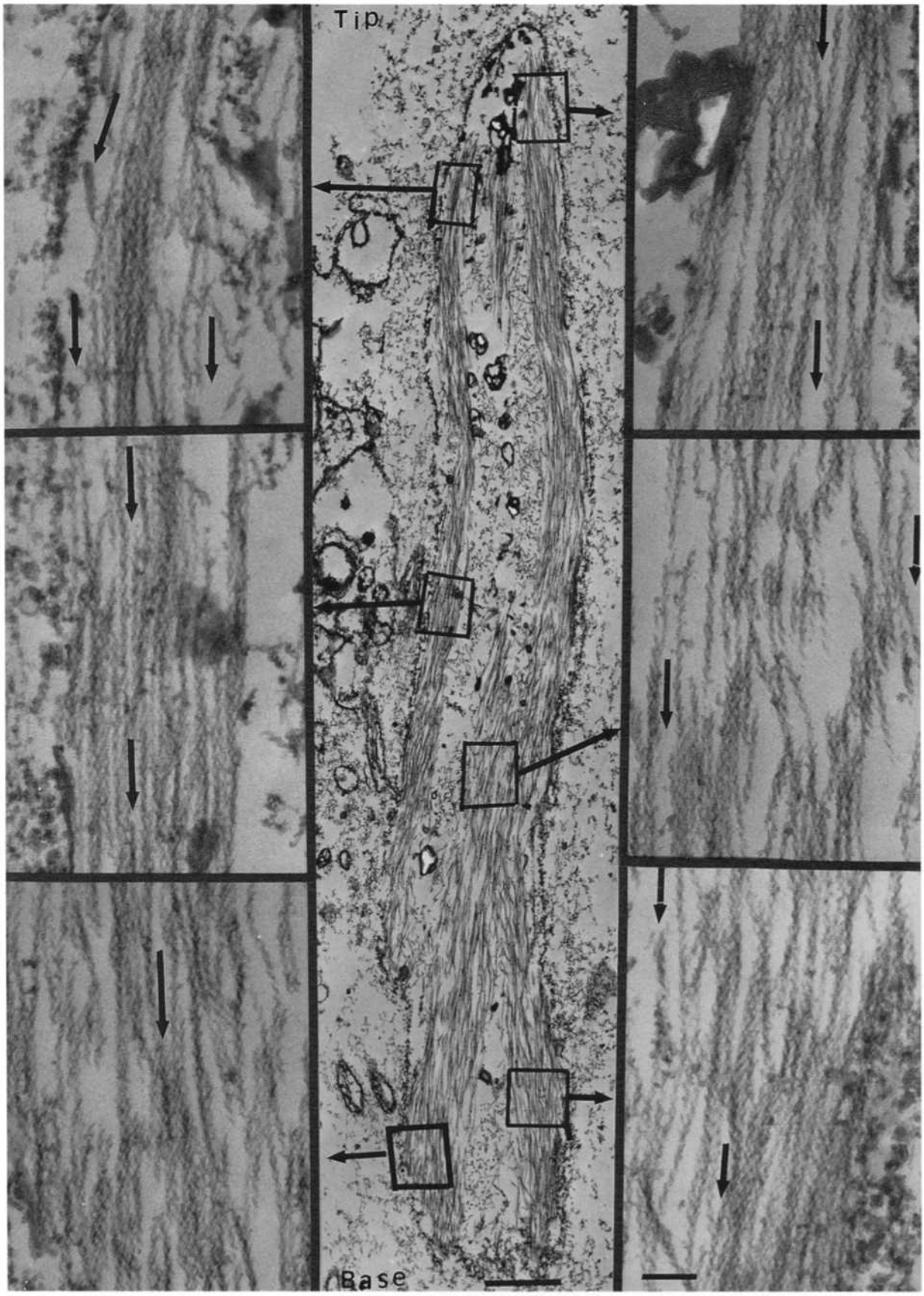


Figure 10. Thin longitudinal section of a 47-h wild-type bristle that had been detergent extracted and decorated with myosin subfragment 1 before fixation. A thin section through the entire bristle is shown in the center panel. The bristle tip is at the top. At least three separate bundles cut in longitudinal sections can be seen. Bar, 1 μm. The six side panels are shown at a higher magnification. Bar, 0.1 μm. The arrows reflect the polarity of the myosin subfragment 1 arrowheads (pointing toward the minus end of the actin filament) and indicate the polarity of the nearby decorated actin filaments.

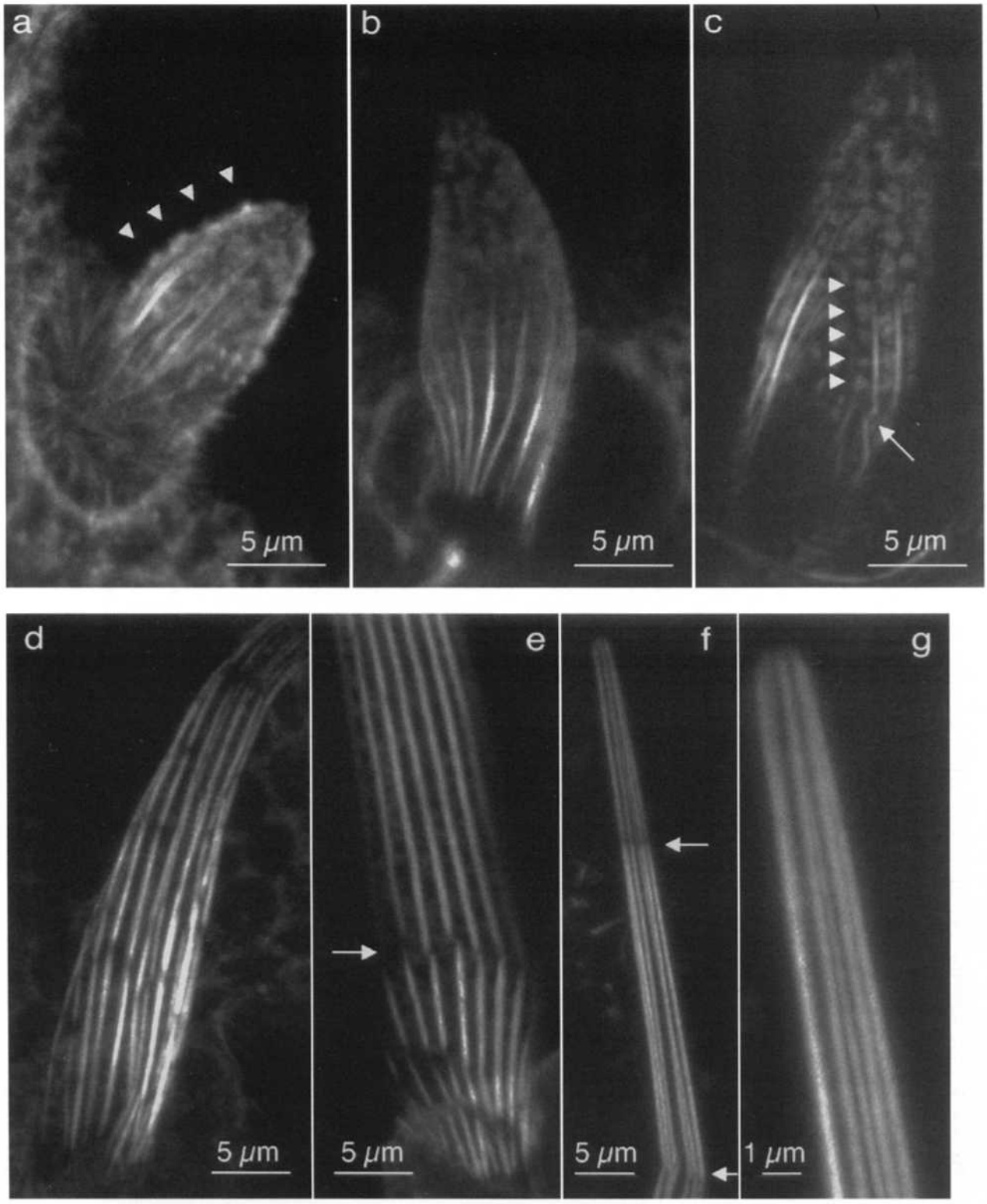
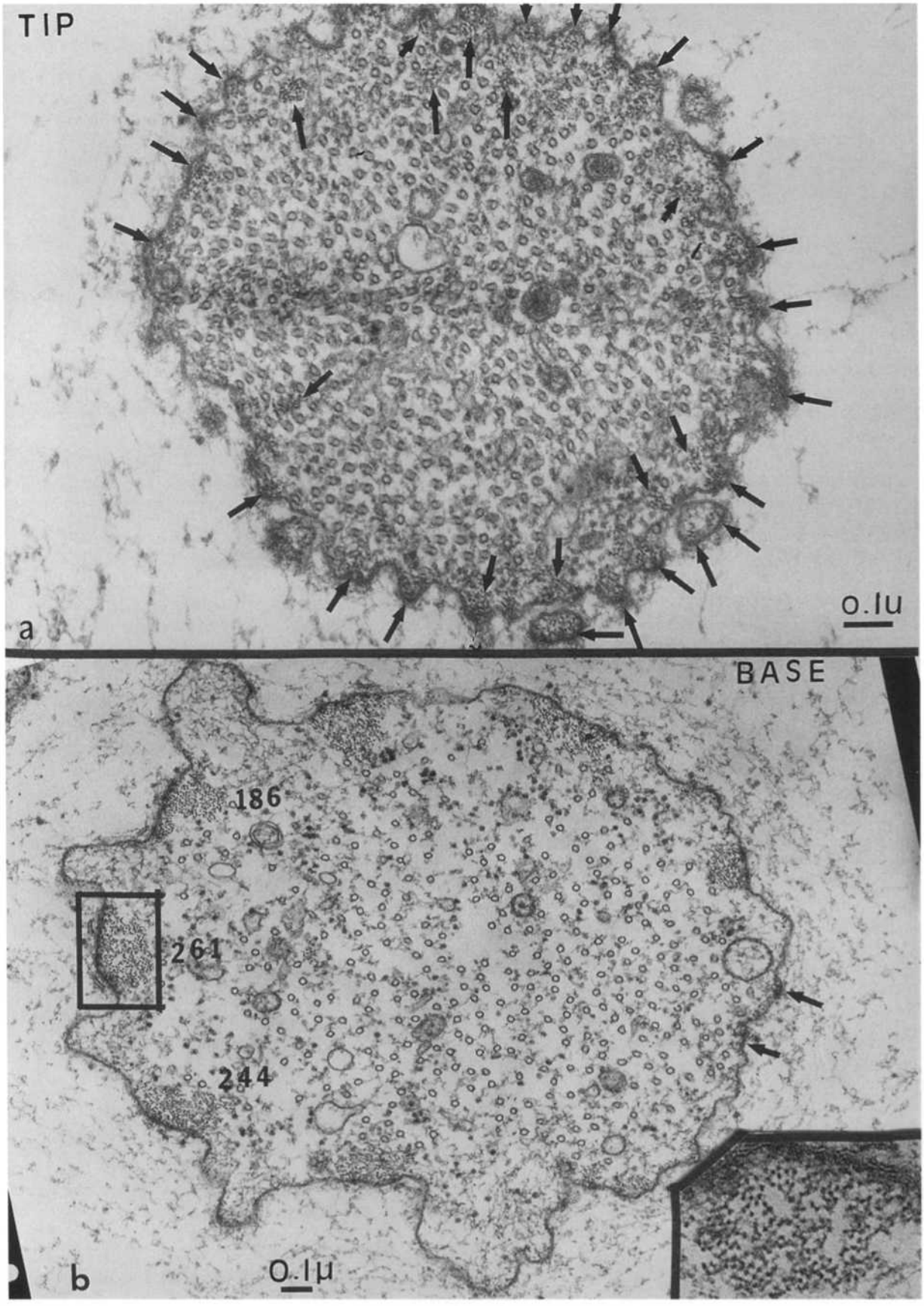


Figure 11. Formation of actin bundles in developing bristles. Wild-type bristles were stained with rhodamine-conjugated phalloidin and examined by confocal microscopy. (*a–e*) A series of macrochaetes of increasing length from 32–34-h pupae. Approximate base-to-tip lengths of these bristles are 14 μm (*a*), 18 μm (*b*), 26 μm (*c*), 40 μm (*d*), and 72 μm (*e*). Only a portion of the macrochaetes are shown in *d–e*. Note the numerous small and irregularly spaced actin filaments in the shortest bristle (*a*), as well as the actin-containing spots (some marked with arrowheads) in emerging bristles seen along the plasma membrane (*a*), and between adjacent actin bundles (*c*). End-to-end module association can be seen early in bristle development (arrows in *c* and *e*). The macrochaetes shown in panels *a–c* and *e* are scutellar bristles. (*f–g*) The tip of a mature microchaete from a 48-h bristle, a time before filament breakdown, is shown at two magnifications. In *f*, note the dramatic difference in actin content between the tipmost modules (less staining) and the penultimate modules and the transverse register of their ends (*upper arrow*). In addition, the connection between modules seems to be fragile, since this bristle was bent at the module junction (*lower arrow*). In *g*, note how the actin bundles extend to the tip of the bristle. Bars: (*a–f*) 5 μm; (*g*) 1 μm.


TIP
a
0.1u
BASE
186
261
244
b
0.1μ

Thin Sections. In Fig. 12 we illustrate transverse sections through the tip of a newly emerged bristle from a 35-h pupa (Fig. 12 *a*), a section near the base of the newly enlarged bristle (Fig. 12 *b*), and a section near the base of an elongating 38-h bristle (Fig. 13). These are representative sections.

At all levels and stages, there is a large population of microtubules that occupies the center of the bristle. At the tip of an elongated bristle (Fig. 12 *a*), the actin filaments are in tiny clusters of 6–10 filaments each (see arrows). In most cases, the actin filaments are associated with the limiting plasma membrane. There are also small clusters of filaments in the cortical cytoplasm that are not attached to the plasma membrane. Toward the bristle base we found eight filament bundles (Fig. 12 *b*) in addition to small filament clusters located on one margin of the bristle and in the cytoplasm proper (see arrows). These bundles are transverse sections of the modules seen in the fluorescent microscope (Fig. 11, *d–e*). It is of interest that the packing of the actin filaments in these bundles is poor. Although we see occasional portions of a bundle where the filaments are hexagonally packed, in most cases, the filaments are randomly ordered. By increasing the magnification of the bundles, it is possible to count the number of filaments per bundle (Fig. 12 *b*, *inset*). For the largest bundles in this micrograph, we counted 186, 244, and 261 filaments.

There are projections of cytoplasm between adjacent bundles (Fig. 12 *b*). These projections contain a tangled array of filaments between adjacent bundles. These tangled masses of filaments correspond to the fluorescent phalloidin spots that are highlighted in Fig. 10 (*a* and *c*).

In slightly older bristles (from 38-h pupae) cut transversely midway along their length, we see discrete bundles of filaments, but no small clusters either near the plasma membrane or within the shaft cytoplasm proper (Fig. 13). The bundles are no longer irregular in shape in the transverse sections, but they appear triangular in profile. Most interesting is the packing of the filaments within these bundles. The filaments are hexagonally packed with only an occasional tiny gap or space within the bundle proper (see Fig. 13, *inset*). In longitudinal sections, the filaments display the 12-nm period that is indicative of the fascin cross-link (data not shown; see Tilney et al., 1995, for an explanation of the 12-nm period, which is a product of the helical nature of actin filaments.) This cross-linking is also recognizable in transverse section by the festooned appearance of the bundle (see DeRosier and Tilney, 1982, for details). Not only are the filaments maximally cross-linked but the number of filaments per bundle has increased from 35-h bristles. Counts of the number of filaments per bundle for the largest bundles in Fig. 13 *b* are 383, 504, 527, and 628. Thus, on average, there are ~2–2.5 times as many filaments per bundle as in 35-h bundles.

Mutants

There are two particularly revealing mutants: *singed* and *forked*. The genes affected in these mutants both encode cross-linking proteins. The *singed* gene encodes an actin cross-linking protein similar to fascin (Cant et al., 1994), and the *forked* gene encodes at least one protein also involved in cross-linking actin (Petersen et al., 1994; Tilney et al., 1995). In subsequent communication, we will present additional information pertaining to the precise action of these proteins. We include here a generalized description which shows that mutants lacking each cross-link still form bundles composed of modules. Therefore, the modular design is not caused by a specific cross-link, but rather, reflects how modules are formed developmentally.

singed. The *singed* protein is a homologue of the actin-binding protein fascin (Bryan et al., 1993) and is normally present in bristle actin bundles (Cant et al., 1994). A number of *singed* alleles result in gnarled bristles characterized by an absence of singed protein in the actin bundles (Cant et al., 1994). By light microscopy using fluorescent phalloidin, we find that the bundles in *singed* bristles are twisted (Fig. 14 *a*), unlike the wild type, where the bundles are straight. Nevertheless, the bundles are composed of modules (Fig. 14 *b*), and the bundles, albeit twisted, usually extend the length of the bristle.

forked. The *forked* gene codes for six transcripts (Hoover et. al., 1993), and null mutants produce short, sometimes forked bristles (Petersen et al., 1994). Macrochaetes contain a variable number of very thin fluorescent bundles if stained with fluorescent phalloidin (Fig. 14 *c*). In addition, bundles are much thicker and better aligned near the base (Fig. 14 *c*). Actin bundles in macrochaetes (near the base) and microchaetes show modular construction, but are not twisted (Fig. 14, *c* and *d*). The diameter of the bundle appears small relative to the wild-type or *singed* mutants, corresponding to fewer filaments per bundle in thin section or ~1/50 of the number in the wild type (see Tilney et al., 1995). In some cases, it appears as if many of the small bundles normally found at the growing tip fail to associate into normal thicker bundles.

Discussion

Long Actin Filament Bundles Are Made Up of Filament Modules Attached End-to-End

The first clue that the 400-μm- (in macrochaetes) or 70-μm- (in microchaetes) long actin filament bundles are composed of a series of modules attached end-to-end came in studying the breakdown of the actin bundles in 54–58-h pupae (Fig. 2 *c*). Unlike what one would intuitively predict, the bundles looked as if they had been sawed transversely, as one would cut a fallen tree along its length with a chain saw. The small segments or modules then shorten when subunits are removed from their cut surfaces (Fig. 15 *b*). Subsequent examination of bundles that had not yet started to break down or bristles that were

Figure 12. Thin transverse sections through the tip (*a*) and the base (*b*) of a 35-h emerging wild-type bristle. The arrows indicate some of the numerous tiny clusters of actin filaments, most of which are associated with the limiting plasma membrane. In *b*, there are eight bundles of actin filaments, one of which is shown (*inset*) at a higher magnification (× 160,000). The number of filaments in three of the bundles is indicated in the micrograph. Also shown in *b* are membrane blebs (at 6 and 11 o'clock) containing tangled arrays of filaments. These correspond to the fluorescent spots seen in Fig. 10. Bar, 0.1 μm.

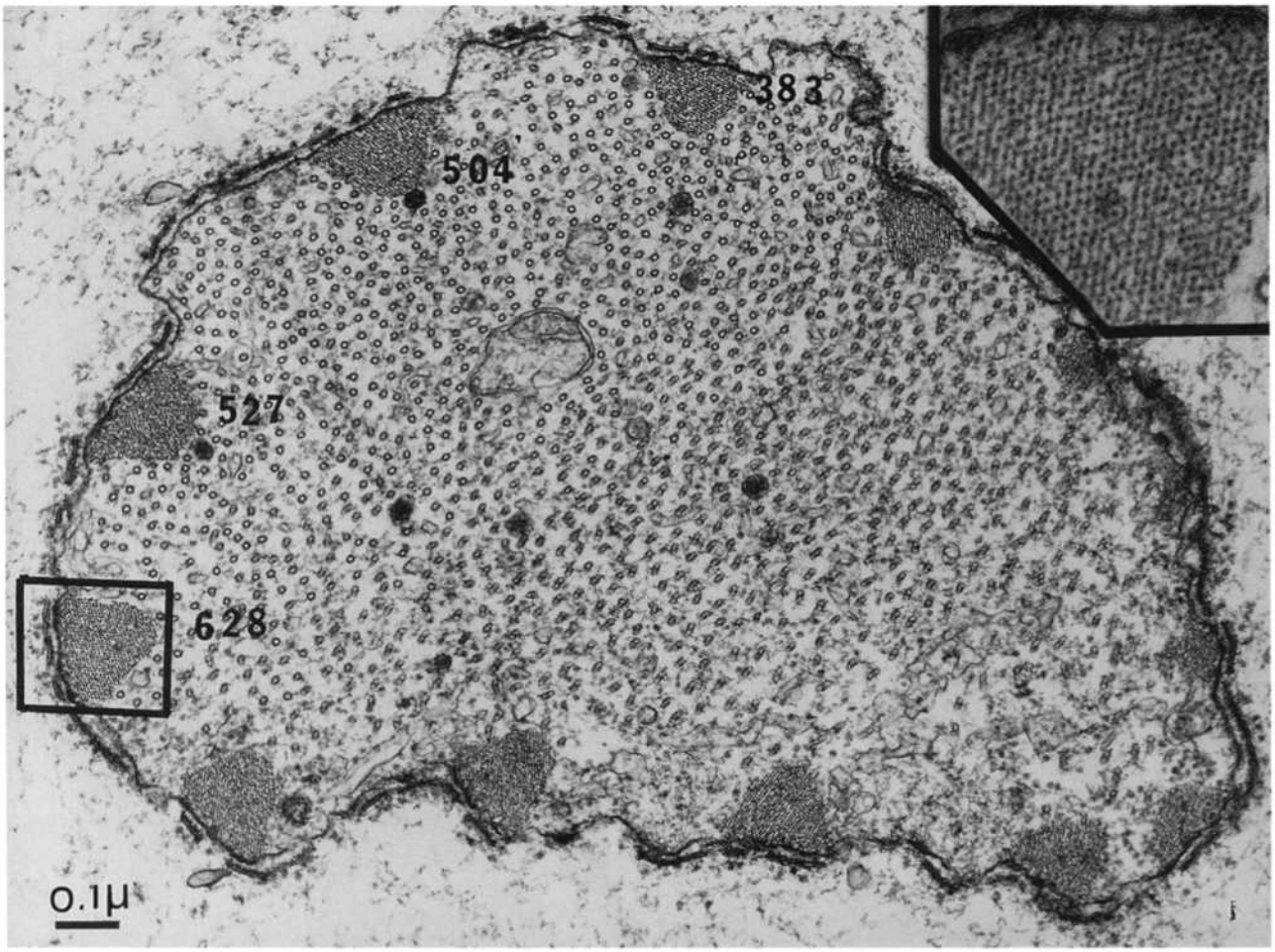


Figure 13. Thin transverse section through the base of a 38-h wild-type bristle. By this stage, the tiny clusters of poorly organized filaments seen earlier (Fig. 11 *b*) are no longer present. Instead, filaments are hexagonally packed into bundles. One of the bundles is enlarged in the inset (× 160,000). Although the cross-sectional area of the bundles is nearly the same as at the base of a younger bristle (Fig. 11 *b*), the number of filaments per bundle has increased two- to threefold. The number of filaments in three of the bundles is indicated in the micrograph. Bar, 0.1 μm.

in the process of elongating revealed that these bundles were also composed of modules (Figs. 2, 6, and 11). This cannot be an artifact of preparation for light microscopy, because serial thin sections examined by EM confirmed the modular design (Fig. 3). What is even more puzzling is that, from our confocal images and serial sections, the modules of all the bundles in a particular bristle are in transverse or quasitransverse register. Thus, modules are approximately the same length as if the initiation of one module in a bristle is tied to the initiation of other modules in adjacent bundles in the same bristle. Additionally, the termination of the modules in adjacent bundles must also synchronize.

Such a strategy of making long bundles out of a series of units that are attached end-to-end makes architectural sense. After all, this is how a skyscraper is built, one floor at a time, from the bottom up. The girders that support each floor are not only tied together laterally (cross-linked), but they are the same length (transverse register), and they are attached end-to-end.

Such a design philosophy eliminates problems that might be encountered if individual actin filaments, for one reason or another, do not extend the length of the actin bundle (400 μm in macrochaetes). Such problems would include gaps in a bundle, obligate tapering of a bundle, and so forth. Second, this design allows the rapid breakdown of the actin bundles that occurs late in development, since there are ~260 filament ends in a 400-μm-long bundle. Third, such a design philosophy gets around the problem mentioned in the Introduction, that actin filaments are generally unstable and seldom exceed 10 μm in length, except in special cases.

The fact that a long bundle is made up of filaments of varying lengths is interesting but not particularly exciting. What is mind boggling is that a long bundle is composed of a series of short bundles attached end-to-end, where the length of the filaments in the short bundles are <10 μm. This is a new facet of how a pattern is developed by the cytoskeleton, and one that we believe exists in other cytoskeletal contexts (see Conclusions in Discussion). What we still don't understand is precisely how a module is formed. The filaments that make up a module first appear in tiny clusters attached to the plasma membrane at the cone-shaped tip of a newly emerging or elongating bristle.

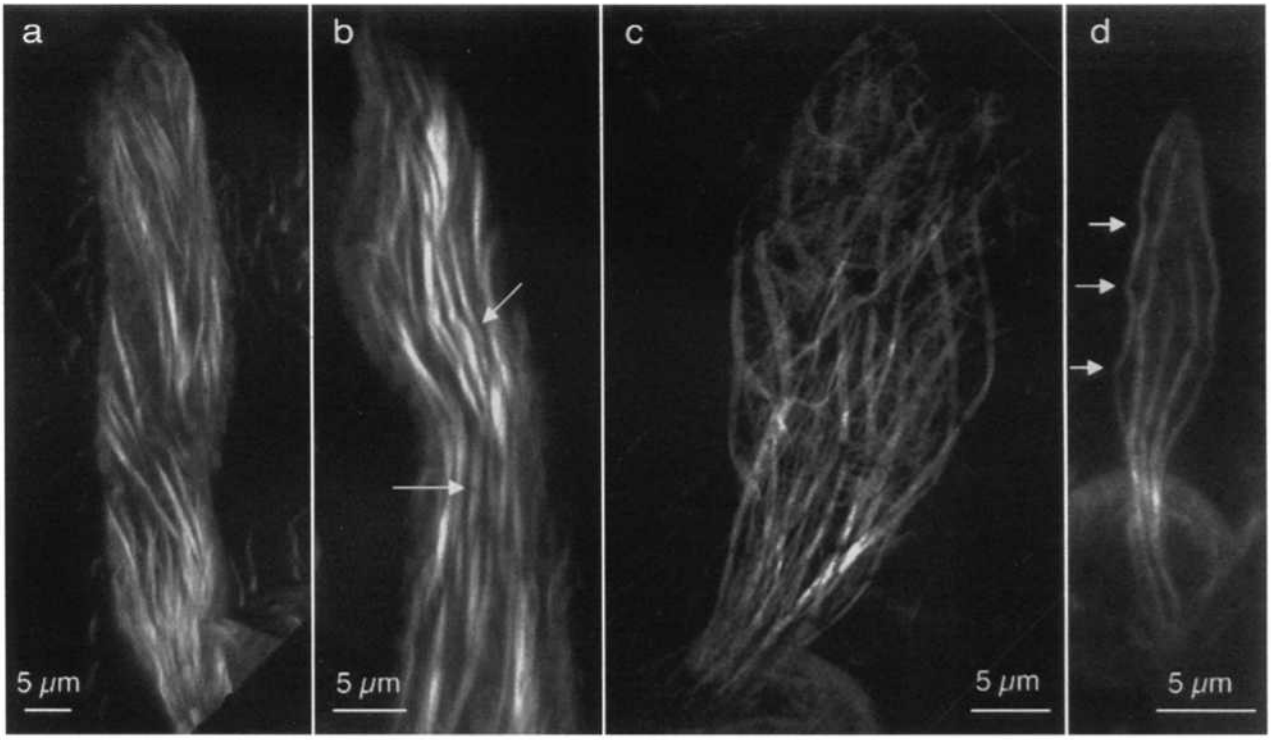


Figure 14. Actin bundles in mature bristles from mutants. Bristles were stained with rhodamine-conjugated phalloidin and examined by confocal microscopy. (*a–b*) Macrochaetes from 48-h *singed*^{3} animals. Note the twisted arrangement of the actin bundles indicated by the right-to-left sweep of the bundles (*a*). All bristles show some bundle gaps, indicating end-to-end module joining (*b*, *arrows*). (*c–d*) A macrochaete and a microchaete from 48-h *forked*^{36a} animals. Note the relatively parallel arrangement of bundles near the base of both bristles. The macrochaete (*c*) illustrates the failure of small actin bundles to coalesce into larger parallel bundles typically seen in the wild type (Fig. 10, *d* and *e*). Most end-to-end module associations in microchaetes result in junctions (*arrows*) characterized by angles (*d*). Bars, 5 μm.

These filaments are then pulled together into large, irregular, and poorly packed bundles. It is still unknown how the filaments in this module all end at the same length if they are attached (presumably nucleated) at many spots near the tip of the emerging or elongating bristle. Furthermore, once filament packing in the bundles becomes hexagonal, they increase two to three times in number by the lateral addition of more filaments to the bundle. Again, these new filaments all have the same length and polarity as the others. And finally, mistakes in the end-to-end attachment of modules are rarely encountered, and in fact, are only seen in mutants that lack or increase the number of specific modules (our unpublished observations).

Why Is it Important to Know That the Polarity of the Filaments in All the Modules Is Identical?

In our analogy of the attachment of girders that support the successive floors of a skyscraper, the polarity of each girder is irrelevant because the girder ends are identical. All that is necessary is that they are connected together end-to-end. But actin monomers assemble into a polarized filament whose ends are different. Furthermore, we know from earlier work that all the filaments within a particular bundle are hexagonally packed and maximally cross-linked and thus must have the same polarity (see DeRosier and Tilney, 1982, for a discussion of why this is so). To begin to understand how a module forms and how it is connected to modules directly below and above it requires knowledge about the polarity of each module. As is well known, the polarity of actin filaments can be readily determined by decoration of the filaments by subfragment 1 of myosin. What we found is that the filaments in a bundle are unidirectionally polarized, and this is the same for adjacent bundles (Fig. 10). Thus, the plus end of each module is located nearest to the tip of the bristle. What this means is that each module will probably be formed in the same way.

A careful examination of the junction of two modules revealed another interesting fact. The basal end of a module is flat in contrast to the tip end that is pointed (Figs. 2 *d* and 8). This confirms that each module has the same polarity. In addition, this observation is telling us something interesting about the construction of a module, since the minus end always corresponds to the flat end of the module. The fact that the apical end of the module is pointed, with filaments of different lengths, is not surprising because the bundle forms by the gathering and cross-linking of tiny filament clusters attached to the plasma membrane of the elongating bristle tip. The puzzle is how and why the basal end of the module (minus end of the actin filaments) is flat. This will be the topic of future investigation.

Cross-links between Actin Filaments Do Not Determine or Influence the Presence of Modules

In our earlier publication on the actin bundles in *Drosoph-*

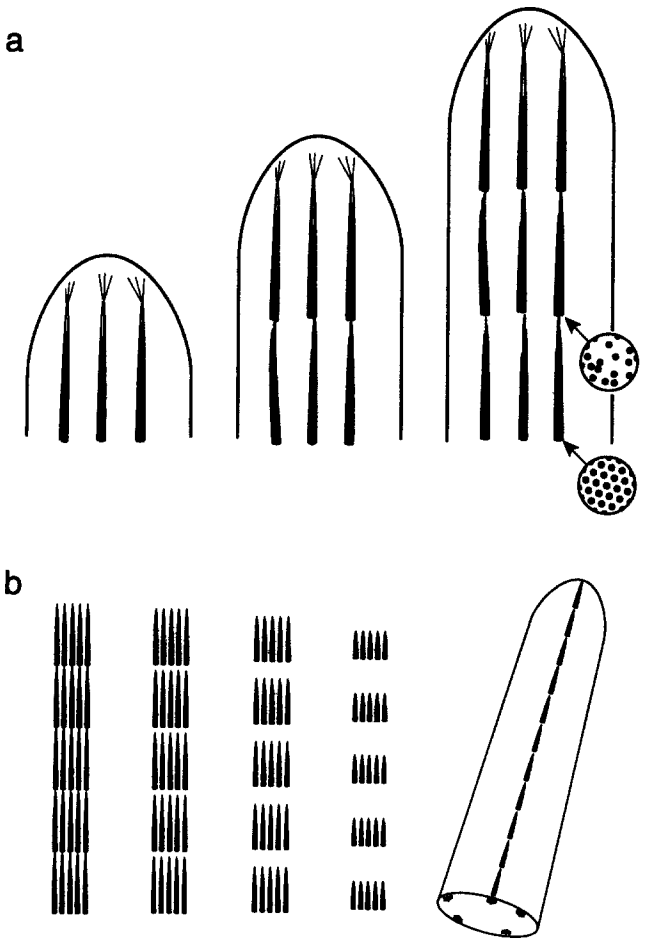


Figure 15. Formation and breakdown of actin bundles during *Drosophila* bristle cell development. (*a*) Drawing illustrating stages of module formation and differentiation. Tiny clusters of actin filaments (*thin lines*) are bundled together into modules (*tall-lined triangles*) and aligned with the more basal modules (*center panel*). Filament packing in newly formed modules, although cross-linked, shows random ordering (*right panel, upper inset*). As modules mature, filament packing becomes hexagonal and 2–2.5-fold more actin filaments are added to the periphery of the bundle (*right panel, lower inset*). (*b*) Drawing illustrating module breakdown. Each module in this simplified drawing consists of one triangle that represents ~500 actin filaments. Five vertical actin bundles are represented by modules attached end-to-end. The ends of the modules are in transverse register. With time (from left to right) modules shortened by the removal of actin subunits from one or perhaps both ends. The rightmost panel represents one bristle and highlights one bundle that is composed of modules. The circumferential positions of the other actin bundles (not drawn) are indicated at the base of the bristle.

ila bristles (Tilney at al., 1995), we concluded that the reason there are two cross-links in actin bundles (in bristles, microvilli, and stereocilia) is that in all three cases, there is a two-step process in bundle formation and maturation. First, filaments must be brought together into poorly packed clusters. These will be subsequently zippered together and maximally cross-linked by the second cross-link (see Tilney et al., 1995 for details and references). Because the *forked* transcripts are most abundant during bristle emergence (Hoover et al., 1993; Petersen, N., personal communication) and before the accumulation of singed protein (Wulfkuhle, 1996), we concluded that the *forked* protein is involved initially in forming irregularly packed bundles, and that the *singed* protein (fascin) then acts to zipper together and maximally cross-link the filaments in the bundles into a hexagonal bundle. This contention is further supported by this study.

The next question is whether the formation of modules is somehow related to the behavior of cross-links between filaments in a bundle. One could imagine therefore that the availability and/or synthesis of cross-links was periodic, or somehow that the *singed* and *forked* proteins interact in some way to produce modules. From our data on mutants that fail to produce one or the other cross-link, this possibility is unlikely. In the *singed* mutant that lacks fascin, modules of approximately the same length are present in the actin bundles. In the *forked* mutant, the filament bundles are slender and often short. In fact, they are also modular (Fig. 14, *c* and *d*). Occasionally, the modules are not "bolted" together properly and bundle junctions appear kinked (Fig. 14 *d*).

In short, we conclude that the formation of modules is not determined by the availability of cross-links, but instead, has to be determined by the nucleation and elongation of filaments at the apical tip of the developing bristles.

There is an additional observation concerning mutant bundles that should be mentioned. The *singed* bristles are twisted (Fig. 14 *a*). This mutant lacks the fascin cross-link that provides the 12-nm period and induces hexagonal filament packing. In the presence of the *singed* protein, the bundle is straight and not twisted because it makes use of the helical symmetry of the actin filament. In the *forked* mutant, the modules, albeit thin and poorly attached, are not twisted because fascin (the *singed* protein) is present, but the *forked* protein is absent (see Petersen et al., 1994; Tilney et al., 1995).

How Are Modules Formed Developmentally?

Because the number of filament bundles in a bristle is approximately the same (7–11 in microchaetes), and they are asymmetrically arranged with large bundles on one side of the bristle and small ones on the other side (see Tilney et al., 1995), one would intuitively predict that each module is formed by the nucleation of the actin filaments in each module from a "magic" spot attached to the tip of an elongating bristle. As the filaments in the module elongate, they become zippered together by the 12-nm bridge of fascin. Unfortunately, this conception is not supported by the evidence uncovered here.

Instead, what we found by confocal microscopy is that during bristle elongation, there are no bundles located at the cone-shaped tip of a bristle (Fig. 11, *a–c*). Instead, the bundles first appear ~5 μm more basally, and after formation, the filaments become hexagonally packed and then the bundles gradually increase in width. Thin sections cut through the elongating tips show that there are numerous tiny clusters of 5–10 filaments at the tip, most of which are associated with the limiting plasma membrane (Fig. 12 *a*). In sections cut more basally, we see bundles composed of 50–250 filaments, as well as very small clusters (Fig. 12 *b*). The filaments in the bundles at this early stage in elonga-

tion are not hexagonally packed, but show a random spacing with frequent gaps in the bundles. Shortly thereafter, the bundles become progressively more ordered and are hexagonally packed. In longitudinal sections, the 12-nm period indicates that the *singed*-encoded fascin cross-link is present. With developmental time, the bundles increase in diameter with the accumulation of more filaments to the lateral surfaces of the bundles, so that in 38-h pupae, bundles of mature diameter are present at the basal end of the bristle (Fig. 13).

From these images, we conclude that the initial formation of a module is by the clustering together of filaments, and that the ordering of the filaments in the module and its increase in filament number after the small bundle becomes hexagonally packed (Fig. 15 *a*) is remarkably similar to the sequence of events that occurs during the growth and differentiation of the stereocilia of hair cells of the ear, a subject that we have written about extensively (see Tilney et al., 1992 *a*). The difference is that in the stereocilia, we have no evidence of modules, although they may exist, particularly in the case of long stereocilia that are present in the reptile cochlea (Tilney et al., 1980; DeRosier et al., 1980). But our advantage in studying *Drosophila* bristles is that we know what the cross-links are, we have mutants whose phenotypes help us decide what each cross-link does (see below) and mutants that affect the association of the bundles with the plasma membrane (studies that will be submitted subsequently), and so forth.

What Determines Module Length?

Actin bundles in both macrochaetes and microchaetes are built from 3-μm modules (Fig. 9). It is also clear, however, that module length is distributed over a wide range. Therefore, actin filament length in the developing bristle cell is not as tightly regulated as actin filament length in muscle cells (see Fowler, 1996). In addition, there is transverse coordination of module length between adjacent actin bundles.

It is not always easy to visualize the modular nature of bristle actin bundles. Confocal views of longitudinal module-to-module junctions, characterized by partial module overlap or very close module-to-module juxtaposition, results in a uniform staining pattern along an actin bundle. An example of this can be seen in the top of Fig. 11 *e*.

It is difficult to evaluate the length of the tip-most modules in growing bristles. Although these modules seem longer than the more basal modules, their poor staining does not allow us to recognize putative module ends very well (e.g., Fig. 11 *c*) by confocal microscopy. Although we cannot say that 10-μm modules never form, we don't think that long modules are very stable. This conclusion is based on knowing how fast actin filaments grow and how fast modules form. We observe that macrochaetes grow to their mature length of ~400 μm in 16 h (Fig. 1), and surmise that bristle actin filaments must grow at an average rate of 25 μm/h. Very short (14–32 μm) macrochaetes often contain at least two stacks of 3-μm modules (Fig. 11 *c*). Since these sprouting macrochaetes are likely to be 0.6–1.3-h old, it appears that at least two sequential modules can form within that time, making it unlikely that extraordinarily long modules are stable for longer than 30 min.

Conclusions

The observations we have reported here have fundamentally changed our thinking on how actin bundles are assembled in cells. We hope that we have accomplished the same for the reader. Bundles of actin filaments are important in providing cells with a cytoskeleton, yet until now, these bundles were thought to be made up of long filaments. Instead, this study suggests that actin filaments, with few exceptions (*Limulus* sperm is the most obvious exception), are short, and to make a long cytoskeletal rod, one needs to make up the rod by units of short filaments that are glued together. At the same time, it focuses our attention on how a pattern is organized by the cytoskeleton. At this point, we clearly do not know how the length of a module, with one flat end and one pointed end, forms, nor do we know how modular length in adjacent bundles is synchronized so that they are in transverse register. The beauty of the *Drosophila* bristle is that even with existing mutants, we think we can uncover how this is determined.

In retrospect, perhaps the growth of the *Listeria* tail first made us aware that short actin filaments became associated together to form a long structure. The filaments become nucleated on the surface of the bacterium, cross-linked, and released only to be connected to new filaments being nucleated (reviewed in Tilney and Tilney, 1993). Once one realizes that this is how long bundles can be built, a number of other systems come to mind that probably use a similar philosophy. For example, we (Tilney et al., 1996) recently reported on how the ring canals of *Drosophila* follicles increase in diameter during development. We showed that as the ring canals increase in diameter, new bundles of actin filaments appear and are connected to the existing actin filaments that line the canal. The modules that we have described here are really just a slight variant to the philosophy that a particular module is attached to another module end-to-end rather than laterally. We also know of a third system where modular associations of actin bundles occur. This system can be seen in the nurse cells of stage 11 *Drosophila* follicles just before the dumping of nurse cell cytoplasm into the oocyte. Thus, we anticipate that what we have uncovered here is not unique to bristles, but will be found in a wide array of biological systems.

We would like to express our thanks to Mark Mooseker for opening his lab in Woods Hole to one of us (L.G. Tilney) so we could learn how to dissect and isolate the pupal thorax. We would also like to thank Dr. Warren Ewens for his help with the statistical analysis of module lengths; Victoria Mukovozov, Rachel Dubroff, and Shanika Samarasinghe for experimental help; and Gladys Gray-Board for patient instruction on the confocal microscope.

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