Observations on How Actin Filaments Become Organized in Cells

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There is general agreement that actin not only is ubiquitous but also is the most commonly encountered protein in eukaryotic cells. Actin, however, is seldom evenly dispersed throughout the cytoplasm, even in the unpolymerized state; instead it is present, often in great amounts, in one part of a cell yet totally absent in others. Much time and energy has been expended in trying to learn how the location of the actin filaments is determined, particularly because it now is clear that they play a major role in providing a cell with its cytoskeleton and that they are intimately involved in a number of motility mechanisms. It is perplexing that the actin filaments can be highly ordered, in fact packed into paracrystalline arrays, in certain cells, yet in other cells, or even elsewhere in the same cell, they seem to be oriented at random in relation to each other and to the cell surface, producing in essence a gelled region. The most reasonable explanation for the diverse distributions of actin filaments in cells is that there are multiple mechanisms regulating them. Presumably, the seemingly endless number of proteins that bind to actin with high binding constants are involved in this regulation and at the same time provide the diversity in actin filament organization that is so essential to the differences among cell types.

Our approach has been to concentrate our study on cells or portions of cells in which the actin filaments show the highest degree of order. Our rationale is that in such systems the order produces a repetitive pattern that may allow us to discover some of the rules that bring about this order. In a less ordered system, interactions of any one type are fewer, i.e., many different interactions occur, making it very difficult to determine what regulates what. Once we have begun to understand the rules governing actin filament distribution in the more precise systems, we can tackle the less ordered ones.

Most investigators, including ourselves, have looked for regions in the cytoplasm that organize the distribution of actin filaments, presumably by controlling actin assembly or actin filament nucleation. Our thinking has been influenced by the results of studies on microtubules showing that basal bodies, centrioles, or pericentriolar material nucleate the assembly of microtubules and thereby help control the distribution of microtubules. So far, however, there are only two convincing biological examples of organizing or nucleating centers for actin filaments. One is an organelle that we call the actomere that is active during the acrosomal reaction in the sperm of the sea cucumber Thyone (Fig. 1). The second is dense material attached to a membrane in differentiating spermatids of the horseshoe crab Limulus (9). There are numerous other cases in which nucleating centers may be operational, but they remain unsubstantiated. For example, in the reformation of microvilli in intestinal epithelial cells after pressure-induced disassembly there is some dense material (11), presumably a nucleating center for actin filaments, yet Begg et al. (1) and Tilney and Jaffe (15) have demonstrated that during de novo formation of microvilli in sea urchin eggs no such spots of dense material are present; instead, individual actin filaments elongate from the plasma membrane and then associate to form a bundle. A possible explanation for this apparent contradiction is that on membranes diffuse nuclei may exist that initiate the assembly of individual actin filaments. Once the actin filaments, e.g., those in a microvillus, have been joined by cross-bridges, these nuclei possibly come together to form discrete spots that in reality are just clusters of the formerly diffuse nuclei. If the filaments depolymerize, even transiently, these spots would remain, and, when the filaments reassembled, the clusters would behave as nucleating centers.

Even more limited is our understanding of what determines where actin filaments assemble. It is possible that diffuse nucleating centers, such as the spectrin 4.1 complex in red blood cells (5), exist. Equally plausible is that the "individual" actin filaments result from spontaneous nucleation of actin monomers when the concentration of monomeric actin is above the critical concentration and dimers of actin have come together, perhaps bound to each other by actin-binding proteins. But even in the most precisely organized systems, where nucleating centers exist, there are actin filaments that do not appear to be a product of these centers. For example, in Thyone sperm the actomere appears to be able to nucleate only 22 filaments, yet in cross sections through the acrosomal process we find an average of 50 and as many as 100 filaments (Fig. 2). Thus, during the acrosomal reaction of Thyone sperm at least two different mechanisms that order actin filament distribution are operational, one, of course, being the actomere. What regulates the assembly of additional filaments not associated with the actomere has not yet been discovered. We do know that they display the same polarity as those nucleated...
from the actomere (7), that they lie parallel to those assembled from the actomere, and that, when we detergent extract the acrosomal process or assemble actin from sperm extracts, all the filaments are found associated with one another, indicating that they are joined by cross-bridges (6, 10). We presume that these "additional" filaments are the result of spontaneous nucleation from the enormous amount of actin in the sperm, but exactly when they form relative to the actomere-nucleated filaments and how they come to have the same polarity remains unclear.

We now would like to present a brief account of some recent observations on the formation, elongation, and patterning of the actin filaments in differentiating hair cells of the vertebrate cochlea. More detailed reports are being prepared for publication. We chose to include a summary of this material here because it not only demonstrates how complex the regulation of actin filament distribution is but also because it emphasizes that this distribution is governed by many mechanisms, some of which are separated by developmental time. The beauty of the differentiation of the hair cell is that the final length of its actin filaments is predictable, as is the approximate number of filaments in each bundle (16). The precision with which this cell controls actin filament length and number, filament polarity, and the orientation of the bundles, forces us to rethink our ideas about how actin is organized in cells.

To study how the actin filaments become organized in hair cells, we fixed cochleae from chicken embryos from 9 d until hatching and examined the formation of the stereocilia on the hair cells by both scanning and transmission electron microscopy. Our goal was to discover how a cell accurately determines the number of actin bundles (equals the number of stereocilia), the number of actin filaments in a bundle (equals the width of each stereocilium), the length of the actin filaments in the bundle (equals the length of the stereocilium) (Fig. 3), and the packing of the actin filaments in the bundle.

We discovered at the beginning of our study that the total number of hair cells per cochlea is determined very early in development and that differentiation occurs simultaneously throughout the cochlea. This made our analysis considerably easier because by knowing the position of a cell on the cochlea, e.g., how far from the distal or proximal end it was, we could determine in a 12-d embryo what the number, length, and width of the stereocilia would be in a mature cell at that location, even though at 12 d of development the stereocilia on that cell were shorter and thinner. Furthermore, we found that the growth in stereocilia number, length, and width and the number, length, and packing of the actin filaments within the stereocilia do not vary from cochlea to cochlea and from hair cell to hair cell. Each hair cell differentiates in a predictable and stereotyped way. Nothing occurs randomly. This allowed us to come to tentative conclusions about how this occurs and, more importantly, to eliminate a large number of hypotheses that did not fit our observations.

We found that the number of stereocilia per cell is determined early in embryonic development, e.g., at 9–10 d, for all the hair cells of the cochlea, even though at this age the stereocilia are a fraction of their mature length and width (Fig. 4). Also striking in the early embryo is that the stereocilia are hexagonally packed and that the orientation of the stereociliary bundle as determined by the single kinocilium is the...
FIGURE 3 In the center is a scanning electron micrograph of an entire bird cochlea. Enlargements illustrate the length of the longest stereocilia. The total number of stereocilia is best determined by "shaving" off the stereocilia and looking at the scars. Such a shaved hair cell is illustrated in the enlargement on the lower right. Enlargements, $\times 3,111$.

FIGURE 4 Graph relating the number of stereocilia per cell to the position of the hair cell on the cochlea. At the origin is the proximal end of the cochlea. Since the overall length of the cochlea increases with developmental age, we have expressed the position of the hair cells as percent of the total length of the cochlea. In 10-d embryos the number of stereocilia per cell is already determined.

The stereocilia do not increase in width until they have attained their mature length. A hair cell at the proximal end of the cochlea of a 12-d embryo has stereocilia of mature length, but mature width is not reached until 17 d of incubation (Fig. 6). This increase in stereocilia width is closely correlated with actin filament number (filament number is proportional to the $(\text{width})^{1/2}$; i.e., there are approximately 100 actin filaments per stereocilium in a 12-d embryo, but by 17 d there are 350–400 (Fig. 7]). Thus, the elongation of the actin filaments, a slow process, is separated in time from an increase in filament number.

When we compared the packing of the actin filaments in the stereocilia in early and late embryos, we found that in 9- and 10-d old embryos, actin filaments aligned parallel to the long axis are present in every stereocilium. However, from transverse sections it was clear that these filaments are not hexagonally packed but are very poorly ordered relative to each other, and in longitudinal section no striations due to the cross-bridges that connect adjacent filaments to one another were seen. As the embryos increase in age, the actin filaments in the stereocilia become progressively more ordered, that is, increasingly cross-bridged. In a 12-d embryo the actin filaments lie on a hexagonal lattice, and in longitudinal section we see a striped pattern due to the cross-bridges (13). Only when the bundle is maximally crosslinked (day 12) does it begin to increase in width by the addition of new actin filaments. We conclude from these observations that the stereocilia are only 1.5 $\mu$m long, the mature length of a hair cell located at the proximal end of the cochlea (Fig. 3); however, at the distal end, the mature length is 5.5 $\mu$m. It takes the stereocilia of these distal cells until hatching (21 d) to attain their mature lengths (Fig. 5).

same as that of the adult. Thus, each hair cell "knows" how many actin bundles to make and where to assemble them on its apical surface. It is surprising that the elongation of the stereocilia is exceedingly slow, taking approximately 1 d for every 0.5 $\mu$m of growth. Thus, in a 12-d embryo, the longest
growth of a stereocilium involves two separate processes. Initially, a small bundle of actin filaments appears and the filaments elongate to their mature lengths. As this is occurring, the filaments become increasingly cross-bridged to form a compact bundle. When this is complete, the bundle begins the second phase, an increase in diameter by the addition of new filaments peripheral to the existing bundle. This second phase can account for up to four times as many filaments in the mature cell as are found in the first phase (100 in a 12-d and 400 in a 17-d embryo). Biologically and structurally, it makes sense for the cell to produce a small bundle of hexagonally packed filaments before increasing the width of the bundle. If the cell assembled all 400 filaments at the same time with a nearly random order and then tried to hexagonally pack them, it would be very difficult (impossible?) to rearrange the existing cross-bridges into cross-bridges between filaments that lie on a hexagonal lattice. More specifically, if the bundle began with a disordered pattern, or was even liquidlike (2, 3), it would mean that the cross-bridges would have to be broken, then remade, then broken, then remade, etc., until the adjacent filaments maximized the number of cross-bridges, at which point the bundle would show hexagonal packing. The larger the bundle the more reshuffling that would have to occur, since the number of filaments in the bundle would be squared as the diameter of the bundle doubled. This reshuffling of bridges would be more difficult than it might seem because the actin filaments are from 1.5 to 5.5 µm long. Thus, two adjacent actin filaments, in order to become uncoupled, would have to break a maximum of from 45 to 165 bridges but retain their bridges to other filaments, which could be as many as 225 to 825. Effectively, this means that if a large bundle begins as a disordered, yet crosslinked, bundle, it could never become hexagonal due to the sheer number of cross-bridges that would have to be made, broken, then remade, etc. Therefore, it makes sense for the cell to start with a small, disordered bundle, order it into a hexagonal bundle, and then increase the size of the bundle by adding new filaments to its periphery. This, in fact, is what occurs.

We stated that the number and packing of stereocilia are determined early in embryonic development, e.g., at 9–10 d. It was surprising to us that the positions of the developing stereocilia change. This can be seen in Fig. 8, which illustrates stereociliary bundles from a 10-d-old embryo and a 5-d-old chick. Both hair cells are from the same region of the cochlea (near the proximal end) and both are printed at the same magnification. Two separate changes occur. First, as the stereocilia increase in width, they move apart to accommodate this growth while at the same time maintaining the same packing arrangement. This spreading apart of the stereocilia must be carried out in large measure by changes in the cross connections between the rootlet filaments that extend down from the stereocilia into the apical cytoplasm. The second change, a change in the shape of the bundle, is much more radical. In 9- or 10-d-old embryos the bundle of stereocilia that extends from the apical surface of the hair cell is circular in profile, yet in the adult hen or rooster the bundle is nearly square (at the distal end) or rectangular (at the proximal end), as is illustrated in Fig. 8. This change in shape is accomplished by the elimination of stereocilia at the front (Fig. 9) and the formation of stereocilia at the lateral margins of the bundle. Thus, both transmission and scanning electron microscopy of embryos fixed at intervals between 10 and 13 d of incubation reveal the degeneration of the stereocilia in front and the

![Figure 5](image-url)  
**Figure 5** Plot relating stereocilia length to the position of the hair cell on the cochlea. At the origin is the distal end of the cochlea. This curve is not normalized. In early embryos (10–12 d) the stereocilia on all the cells of the cochlea elongate at the same rate. By 13 d the stereocilia of the cells at the proximal end have stopped elongating, but all the others continue to elongate. By 21 d (hatching) all the stereocilia have stopped elongating except those in the cells at the distal end.

![Figure 6](image-url)  
**Figure 6** Plot relating stereocilia width to the position of the hair cell on the cochlea. At the origin is the distal end of the cochlea. At 10 d of embryonic development the stereocilia on all the hair cells are the same width. By 12 d the stereocilia at the proximal end of the cochlea are increasing in width, and with time these progressively increase in width at the proximal end.
FIGURE 7 Transverse sections through stereocilia from embryos 10, 12, and 16 d old. In 10-d embryos each stereocilium contains a small number of actin filaments that are poorly ordered relative to one another. By 12 d of development there are about 100 actin filaments present, most of which lie on a hexagonal lattice. By 16 d there are approximately 350 actin filaments, all of which lie on a hexagonal lattice. × 140,000.

sudden appearance of short stereocilia at the lateral margins of the bundle (Fig. 9, arrows). While some stereocilia are increasing in length others are increasing in width and still others are degenerating. It is amazing that all these events occur in the same part of the same cell.

Thus far in our discussions of the formation and differentiation of the stereocilia we have shown that early in development the hair cell determines the number and packing of its stereocilia or, translated into terms of actin filaments, the cell determines the number of actin bundles and where they should appear. These bundles elongate by actin filament growth, and as they do so the actin filaments become progressively more cross-bridged. Only after the stereocilia have grown to their mature lengths do they begin to increase in width by the incorporation of new actin filaments at the periphery of the bundle. At the same time, as the stereocilia begin to grow in width an extensive reorganization occurs: stereocilia in the front of the bundle degenerate, the actin filaments in these stereocilia disassemble, and other stereocilia appear de novo at the lateral margins of the bundle and elongate as the actin filaments lying within them elongate.

It is striking that the regulation of actin filament distribution is so very complex and clearly does not depend on only one mechanism, such as the turning-on of a nucleating site; instead many mechanisms are involved, some of which can be separated in time from others. Our in vivo study has shown us that what we have learned about the regulation of actin assembly by in vitro study is really very primitive. We now know that in the same part of the cell some actin filaments are disassembling while others are elongating and still others are being added to the periphery of the bundle, the regulation cannot simply be based on the availability of actin monomers, i.e., not a control of the rate of synthesis of actin. This also applies to the growth of the entire bundle of stereocilia. In profile each bundle has a staircase pattern, with short stereocilia in the front and progressively taller stereocilia behind. In a 10-d-old embryo all the stereocilia are the same length, and as development proceeds those at the back of the staircase grow faster than those in the front. Since all this occurs within 1 μm of the same surface of the same cell, equal distances from the cell’s ribosomes, we conclude that rate of actin synthesis cannot play a significant role in this regulation. Third, although we have not substantiated this, actin filaments do not appear at random in the apical cytoplasm. It is true that the cuticular plate, an array of actin filaments located just beneath the stereocilia (4, 12, 13) begins to form in the 10-d-old embryo, but the actin filaments here are oriented (8) parallel to the apical surface, except for the rootlet filaments that extend basally from these stereocilia. That there do not seem to be actin filaments oriented at random indicates that the actin used in the assembly of the bundle present in the stereocilia must be kept unpolymerized until it arrives in a
Scanning electron micrographs of the stereociliary bundles of a 10-d-old embryo and a 5-d-old chick printed at the same magnification. The hair cells are located near the proximal end of the cochlea in each case. Notice that during development the small, round bundle in a 10-d embryo is transformed into a rectangular bundle. Bars, 1 \( \mu \text{m} \). X 21,000.
stereocilium that has been programmed to elongate or increase in width. Clearly, the cell does not make a huge excess of monomeric actin over whose assembly it has poor control. Fourth, the cross-bridging between filaments progressively increases from day 10 to day 12, so that first the bundles have very poor order (infrequent cross-bridging), followed by a gradual increase in cross-bridging until it is maximal at 12 d. From that time on, even though the total number of actin filaments can increase fourfold, cross-bridging continues to be maximal. These observations tell us that either the amount of cross-bridging protein is limited in 10- to 12-d embryos or that it is somehow sequestered. After that, the amount of cross-bridging material is unlimited. Fifth, different stereocilia in the same part of the same cell elongate at different rates, indicating that the signal for the filaments to assemble must be controlled locally, perhaps by a capping substance or substances located within the stereocilium proper. And sixth, an increase in stereociliary width or actin filament number is separated in time from elongation of stereocilia or actin filaments. This indicates that the control is carried out in different ways.

We do not yet know how these patterned arrays of actin filaments are regulated. It seems likely that the hair cells make use of many of the actin regulatory proteins already described, namely, cappers, cross-bridges, actin depolymerizing proteins, and inhibitors of assembly, such as profilin. The cell somehow controls the regulators, because in the same portion of the same cell actin filaments are elongating, depolymerizing, and initiating the formation of new filaments, all at the same time.

The way in which actin filaments become organized in cells is exceedingly complex and must involve, even in the simplest systems, such as the acrosomal reaction of Thyone sperm, multiple mechanisms. The hair cell has taught us that we are a long way from fully understanding how actin filaments become organized in cells.

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