Introduction

During the past 20 years enormous progress has been made in describing the actin cytoskeleton of animal cells and characterizing the more abundant proteins involved. We are just now beginning to relate the in vitro properties of some of these actin-binding proteins to what is actually going on in cells. This is by no means a simple endeavor as cells contain 40-50 actin-binding proteins, some in huge amounts, and the problem is to try to determine how a cell functions as it does using all these proteins. In short, how does a cell inform the actin filaments and/or monomers to appear at the appropriate place and appropriate time, choose the appropriate actin-binding proteins to interact with, and do the appropriate thing? Recent studies on *Dictyostelium* mutants lacking one or more cytoskeletal proteins demonstrate how complex this problem really is. Some of these mutants have no observable phenotype, moving, dividing and phagocytosing normally, while others are only slightly affected (DeLozanne and Spudich, 1987; Noegel and Schleicher, 1991).

What is even more difficult to understand is that many actin assemblages form rapidly, eg. within seconds (Tilney and Inoué, 1982; Cassimeris et al., 1990; Symons and Mitchison, 1991), while others assemble at extremely slow rates, eg. days (Tilney et al., 1981). Although there may be exceptions, these differences in rates are usually linked to the precision in the length, number and arrangement of actin filaments located within a cell or a portion of it.

Our approach has been to select the most specialized cells that we can find, ones that have an entirely predictable cytoskeletal phenotype. Thus we know in advance where the cytoskeleton will appear, what it will look like, and what its constituents are. Then, by describing in detail stages in the formation of the cytoskeleton, we can at least know what is happening and from that try to relate what we know about the in vitro properties of the proteins that make up the cytoskeleton to what the cell is actually doing.

Actin filaments, stereocilia and hair cells of the bird cochlea

VI. How the number and arrangement of stereocilia are determined

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Summary

Beginning in 8-day embryos, stereocilia sprout from the apical surface of hair cells apparently at random. As the embryo continues to develop, the number of stereocilia increases. By 10 1/2 days the number is approximately the same as that encountered extending from mature hair cells at the same relative positions in the adult cochlea. Surprisingly, over the next 2-3 days the number of stereocilia continues to increase so that hair cells in a 12-day embryo have 1 1/2 to 2 times as many stereocilia as in adult hair cells. In short, there is an overshoot in stereociliary number. During the same period in which stereocilia are formed (9-12 days) the apical surface of each hair cell is filled with closely packed stereocilia; thus the surface area is proportional to the number of stereocilia present per hair cell, as if these features were coupled. The staircase begins to form in a 10-day embryo, with what will be the tallest row beginning to elongate first and gradually row after row begins to elongate by incorporation of stereocilia at the foot of the staircase. Extracellular connections or tip linkages appear as the stereocilia become incorporated into the staircase. After a diminutive staircase has formed, eg. in a 12-day embryo, the remaining stereocilia located at the foot of the staircase begin to be reabsorbed, a process that occurs during the next few days. We conclude that the hair cell determines the number of stereocilia to form by filling up the available apical surface area with stereocilia and then, by cropping back those that are not stabilized by extracellular linkages, arrives at the appropriate number. Furthermore, the stereociliary pattern, which changes from having a round cross-sectional profile to a rectangular one, is generated by these same linkages which lock the stereocilia into a precise pattern. As this pattern is established, we envision that the stereocilia flow over the apical surface until frozen in place by the formation of the cuticular plate in the apical cell cytoplasm.

Key words: actin filaments, stereocilia, hair cells, chick cochlea.
The key, of course, is the cell type. It must be absolutely predictable in time and space and the rest is just hard work.

The cell type we have chosen is the hair cell of the cochlea. To our knowledge this is the most precisely regulated actin machine so far described because it has a predictable number of cell extensions called stereocilia (Tilney and Saunders, 1983), they are of predictable length (Tilney and Saunders, 1983; Tilney and Tilney, 1988), and have a predictable width (Tilney and Saunders, 1983; Tilney and Tilney, 1988). Furthermore, from the length of the stereocilium we can easily determine the length of the actin filaments within each stereocilium and from the width of each stereocilium, we can calculate accurately the number of actin filaments present within (Tilney and Tilney, 1988).

In earlier papers in this series we described when the stereocilia elongate (Tilney et al., 1986), how actin filaments in the stereocilia become crossbridged together into compact bundles (Tilney and DeRosier, 1986), and how the staircase pattern of stereocilia in the bundle is generated (Tilney et al., 1988). In this paper we will consider how the cell determines the number of stereocilia to form and how it arranges them on the apical surface. We view this publication as the last of a series designed to describe what grossly happens during the differentiation of a hair cell. With this information in hand we can now logically progress to the next level, namely what proteins are involved in this organization (Shepherd et al., 1989; Tilney et al., 1989; Drenckhahn et al., 1991; Gillespie and Hudspeth, 1991), what are their relative concentrations, when are they made in development, how do they know what to do and where to go and what controls their expression?

Our purpose here is to describe stages in the appearance of stereocilia and steps in the arrangement of these cell extensions into a compact bundle. Using this data we will then propose how a hair cell might solve the problem of ending up with the right number of stereocilia in an invariant arrangement by using very simple mechanisms. These mechanisms in turn make accurate predictions which are testable because the cochlea will differentiate in culture (Stone and Cotanche, 1991). What is fascinating to us is that our conclusions, based upon the data enumerated here, do and where to go and what controls their expression?

Materials and methods

Fertilized chicken eggs of the White Leghorn variety were obtained from Truslow Farms (Chesterton, MD) or from Spafas (Norwich, CN) and incubated at 37°C. Hatching occurred on day 22.

Preparation of the specimens for scanning microscopy

Cochleae of embryos 7-14 days of age were dissected as outlined by Tilney and Saunders (1983) and Tilney et al. (1986). Fixation was carried out by immersion of the cochlea, still surrounded on its basal and lateral surfaces with cartilage, in a freshly prepared solution of 1% OsO$_4$ in 0.1 M phosphate buffer at pH 6.3. Fixation was carried out at 0°C for 45 minutes. After fixation the cochleae were dehydrated in acetone to 75%. While still immersed in 75% acetone, the tegmentum vasculosum was removed with as much cartilage and connective tissue as possible. The sectorial membrane was then lifted off using fine forceps and needles. Dehydration of the cochlea was continued and the cochleae were rinsed in pure acetone several times before critical point drying, mounting on stubs and sputter coating. The mounting is crucial and the method has been documented in Tilney et al. (1986). The specimen was then examined in an AMR 1000 or JEOL scanning microscope with a LH$_6$ filament. Polaroid pictures were taken, the negatives were enlarged 2.6 ×, and the number of stereocilia on cells at different places along the cochlea was counted. The mean and standard error were calculated and the data plotted as a function of the % of distance from the proximal end of the cochlea.

Results

Background: the number and arrangement of stereocilia on the surface of mature hair cells of the chicken cochlea

Because the number of stereocilia per hair cell varies, depending on the location of the hair cell on the cochlea in two axes, it is essential at the outset to establish the terminology we will use. The chicken cochlea is sickle shaped (Fig. 1) with a broad, distal end tuned to low frequencies and a narrow, proximal end tuned to high frequencies. The broad convex margin populated by so-called “tall” hair cells is termed the superior edge and the concave margin the inferior.

A plot of the number of stereocilia per hair cell as a function of its position on the cochlea is presented in the lower part of Fig. 1. These values represent those that one would find on cells located on a midline down the cochlea, not those at the superior or inferior margin which would be greater or less respectively.

If we look down on the surface of a mature cochlea of a chicken we can easily and accurately identify the surface occupied by a hair cell as it is enclosed in a dense “hedge” of microvilli from supporting cells which surround and separate adjacent hair cells (Tilney and Saunders, 1983). The organization of the stereocilia in each bundle has been studied intensely. Briefly, in a climb directly up the staircase of this hexagonally packed bundle one follows a 1,0 lattice plane which we have referred to in earlier publications as...
Determination of number and arrangement of stereocilia

the 1,00 lattice plane (see Fig. 2) to distinguish it from 1,0 lattice planes located at 60° (Tilney et al., 1988). In climbing this lattice plane, not only is each stereocilium longer than the one immediately below it (unlike the other 1,0 lattice planes), but they touch each other and are attached to each other near their tips by extracellular connections called tip linkages (Pickles et al., 1989; Figs 2 and 3).

The number and arrangement of stereocilia in hair cells of embryonic cochleae

Differentiation of hair cells does not begin simultaneously throughout the cochlea, but starts near the distal end and sweeps rapidly towards the proximal end with those at the proximal end about a day behind those near the distal end in 7-day embryos. The extreme distal end is also slow in developing, being a day or two behind the mid distal end (for details see Cotanche and Sulik, 1984; Tilney et al., 1986). Although these are real differences, for simplicity we will describe the situation throughout the whole cochlea in 8-, 9-, 10-, 11-, 12-, 13- and 14- day embryos respectively.

8-day embryos

By 8 days, tufts of stereocilia can be found throughout the cochlea except at the extreme proximal end. In Fig. 4 we present three tufts or apical views of hair cells, one near the distal end (A), one near the middle (B), and one near the proximal end (C). These are all taken along the mid line of the cochlea. We have included these images here as this and the 9-day embryo are key stages and there are no detailed descriptions of these stages in the literature.

Between tufts of stereocilia are numerous randomly spaced microvilli. Whether these microvilli extend from supporting cells or hair cells is impossible to determine from surface views because the margins of these cell types cannot be distinguished. Usually there is a clear area surrounding each stereociliary tuft.

Each tuft contains a single kinocilium identifiable as such because it is thicker and longer than the stereocilia. The kinocilium is often located towards one margin of the tuft (Fig. 4B), although we just as frequently find it located in the center of the tuft (Fig. 4A and C). At this stage the kinocilium in one tuft shows no obvious relationship with those of adjacent tufts or with either the superior or inferior edges of the cochlea.

The stereocilia making up the tuft are not arranged in any discernable pattern and in general appear to be of equal lengths. Often there are irregular spaces between adjacent stereocilia. We have counted the number of stereocilia in each tuft. Although the absolute number is quite variable from tuft to tuft, there are on average more stereocilia per tuft at the proximal end than at the distal end (Fig. 5). This number is always smaller for all locations on the cochlea than that found in the mature cochlea.

9-day embryos

In low magnification images we generally find that the single kinocilium associated with each tuft of stereocilia tends to be on the same side of the cell as that on all the other tufts (Fig. 6A), in contrast to earlier stages when it shows no preferential orientation (Cotanche and Corwin, 1991). We do still find individual tufts whose kinocilium is located in the exact center of the tuft or on a tuft margin bearing no obvious relationship to the superior edge of the cochlea or to other tufts.

The tufts are round and each tends to bulge outwards, so that the stereocilia which generally project at right angles to the surface proper are slightly splayed like a short haircut. As one looks down on the head, the hair in the center extends towards you, that on the sides at an oblique angle to your line of vision.

The stereocilia all tend to be approximately the same length, but because of the bulging of the apical surface of the cell it is difficult to be completely sure. However, there is no systematic gradation in length as seen in later embryos and in the adult. The stereocilia are tightly packed and little or no empty space is found between their bases. At the same time one cannot discern any obvious pattern to the bundle (tuft; Fig. 6A).

Counts of the number of stereocilia per bundle reveal that there is a significant increase in number per tuft throughout the cochlea (Fig. 5). At the same time there exists less variation between adjacent bundles (Table 1). Interestingly the number of stereocilia per bundle in the distal 2/3 of the cochlea approaches that found in the mature cochlea (Fig. 5).
In 10-day embryos some of the stereociliary tufts begin to elongate, starting with what will be the tallest row or row immediately in front of the kinocilium (Tilney et al., 1988). This is commonly encountered in 10 1/2-day embryos and by this time several rows may be elongating (Fig. 6B). The elongating stereocilia tend to be wider than those in the front of the bundle and often the only way to distinguish the kinocilium from the elongating stereocilia is by the length: the kinocilium being longer than the stereocilia. Sometimes the kinocilium is not obvious either because it is the same length as the elongating stereocilia or because it has been broken off in the preparation of the cochlea for scanning electron microscopy when the tectorial membrane is mechanically pulled off the cochlea, examination of its lower surface reveals kinocilia still adhering to it (Tilney et al., 1989).

10 1/2-day embryos have two populations of stereocilia. One consists of those that are in the process of elongating located immediately in front of the kinocilium. Another, located in front of this elongating population, is a group of stereocilia all of which are the same length and diameter, e.g. short and thin.

At 10 1/2 days, most of the stereociliary tufts are round (Fig. 8), but a few are beginning to change into a semi-circular bundle with the tallest row or back row being straighter than those in the front of the bundle which remain convex. Thus the bundle appears to straighten as the stereociliary tufts elongate. This is commonly encountered in 10 1/2-day embryos and by this time several rows may be elongating (Fig. 6B). The elongating stereocilia tend to be wider than those in the front of the bundle and often the only way to distinguish the kinocilium from the elongating stereocilia is by the length: the kinocilium being longer than the stereocilia. Sometimes the kinocilium is not obvious either because it is the same length as the elongating stereocilia or because it has been broken off in the preparation of the cochlea for scanning electron microscopy when the tectorial membrane is mechanically pulled off the cochlea, examination of its lower surface reveals kinocilia still adhering to it (Tilney et al., 1989).

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Fig. 2. (A) View of a stereociliary bundle in a scanning electron micrograph of a hair cell located near the proximal end of the cochlea. The arrowheads indicate tip linkages. The longest stereocilia are aligned along the lower edge of this micrograph. Bar, 1 µm. (B) Diagrammatic representation of the arrangement of stereocilia in the bundle. Of interest is that in a climb directly up the staircase one follows the tip linkages up a 1,00 lattice plane. 1,0 lattice planes at 60° to this are indicated, as is the 1,1 view.

Fig. 3. A stereociliary bundle from a hair cell located near the distal end of the cochlea. This bundle is twisted advantageously to display the tip linkages clearly; the 1,00 plane is twisted. Bar, 1 µm.
Determination of number and arrangement of stereocilia

Stereocilia elongate. This feature is more apparent in older cochleae (Fig. 8).

In 10 1/2-day embryos the total number of stereocilia per bundle is about the same as that in the mature cochlea (Fig. 5), except at the two extreme ends of the cochlea which differentiate more slowly.

11-day embryos

Both the number of stereocilia that are elongating and the number of stereocilia per bundle has increased in embryos 1/2 day older (Fig. 5). At all but the extreme ends of the cochlea the number of stereocilia per bundle exceeds that in mature hair cells (Fig. 5). As will be discussed in detail in the next section, this increase in stereocilia number is not due to more stereocilia being packed into the same area. Instead the diameter of the bundle has increased leaving approximately the same amount of space between adjacent stereocilia. This can be best appreciated by comparing the total number of stereocilia per bundle (Fig. 5) with the surface area occupied by the bundle (Fig. 10). Thus between days 10 1/2 and 11 there is an increase in surface area, in stereociliary number, and in the number of stereocilia that are in the process of elongating.

12-day embryos

One day later the staircase pattern of stereocilia is now easily recognizable (Fig. 6C). There are two populations of stereocilia in each tuft, those that make up the staircase proper and a large population in front of the staircase. We counted the number of stereocilia in each population. Whereas in 10 1/2-day embryos the staircase population constitutes only about 45% of the stereocilia present on a mature cell, by 12 days this number is about 20% of that of the mature cochlea (Fig. 7; Table 2). Counts of those in the staircase proper are probably an underestimate because
Fig. 6. Scanning electron micrographs of individual hair cells located at the proximal (left column) and distal (right column) halves of the cochlea of 9 (A), 10 1/2, (B), 12, (C), and 14-day (D) embryos. Bar, 1 µm.
Determination of number and arrangement of stereocilia

the number of staircase rows may still increase. Furthermore, at this stage and in 13- and 14-day embryos, it is impossible to determine if a particular stereocilium at the foot of the staircase is a “staircase stereocilium” or an “extra”. In contrast the total number of stereocilia per tuft greatly exceeds the number present in tufts of mature hair cells at the same position on the cochlea. In fact, in the distal 2/3 of the cochlea on average there are 1 1/2 to 2 times as many stereocilia in these immature tufts as are found in the mature tufts (Fig. 5).

Careful examination of the bundle reveals an additional feature. Unlike earlier stages, eg. 9-day embryos (Fig. 8) where the bundle is circular, by 12 days the bundle is semi-circular (Fig. 8). The flattened half always contains the staircase with the tallest row of stereocilia located nearest the flattened or straight surface. This change in shape of the bundle can be best appreciated by orienting the cochlea so that one is looking directly down on the tuft (Fig. 8).

When we compare some of the more immature bundles located at the extreme proximal end (recall that hair cells at the extreme ends of the cochlea are a day behind the rest), with those that are more mature, located 20-40% from the extreme proximal end, we find that the change in shape of the bundle from perfectly spherical to that of a half sphere is correlated with the number of rows in the staircase. Thus if the number is small, eg. 2 (a tall row and a somewhat shorter row), the bundle is nearly spherical; if the number of rows is larger, eg. 4, the bundle is flattened on the side containing the staircase, and if the number of rows is even larger, eg. 7, then the back of the staircase is perfectly straight.

Careful examination of the packing of the stereocilia in preparations in which the stereocilia are not splayed apart at the extreme ends of the cochlea is also included. The standard error of the mean for each point is presented in Table 2.

Careful examination of the packing of the stereocilia in preparations in which the stereocilia are not splayed apart

Table 1. Variations in stereociliary numbers/cell plotted in Fig. 5

<table>
<thead>
<tr>
<th>Embryonic age</th>
<th>Position</th>
<th>Stereocilium number</th>
<th>Number of cells</th>
<th>s.e.m.</th>
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<td>89.0</td>
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<tr>
<td></td>
<td>2</td>
<td>71.3 (45-111)</td>
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<td>48.1 (30-80)</td>
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<td></td>
<td>4</td>
<td>31.1 (16-58)</td>
<td>37</td>
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</tr>
<tr>
<td></td>
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<td>36.4 (14-72)</td>
<td>14</td>
<td>5.8</td>
</tr>
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<td>3.6</td>
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<tr>
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<td>5</td>
<td>45.9 (32-59)</td>
<td>11</td>
<td>3.4</td>
</tr>
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<td>77.6 (59-94)</td>
<td>20</td>
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The 5 positions for each stage indicate the points on each curve in Fig. 5 - position 1 is the most proximal, position 5 the most distal. s.e.m. refers to the standard error of the mean.

Table 2. Variations in stereociliary numbers/staircase plotted in Fig. 7

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<th>Embryonic age</th>
<th>Position</th>
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<td>13 day</td>
<td>1</td>
<td>138.0 (122-154)</td>
<td>7</td>
<td>5.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>114.4 (80-173)</td>
<td>12</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>82.1 (69-98)</td>
<td>15</td>
<td>2.0</td>
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<td></td>
<td>4</td>
<td>68.8 (57-93)</td>
<td>19</td>
<td>1.9</td>
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<td></td>
<td>5</td>
<td>45.4 (29-56)</td>
<td>10</td>
<td>3.3</td>
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<tr>
<td>14 day</td>
<td>1</td>
<td>173.0 (162-184)</td>
<td>2</td>
<td>1.1</td>
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<tr>
<td></td>
<td>2</td>
<td>144.3 (129-169)</td>
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<td>115.9 (91-144)</td>
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<td>50.5 (37-57)</td>
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<td>2.6</td>
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</table>

“Position” and “s.e.m.” are the same as in Table 1.
(Fig. 8) reveals that they are arranged hexagonally with the 1,00 plane oriented up the staircase, the situation seen in the adult (compare Figs 2 and 8).

13-day embryos
At this stage the staircase proper can be more easily distinguished from the population of tiny stereocilia located at its foot. We should emphasize that the number of stereocilia included in the staircase proper (Fig. 7) is probably an underestimate, because at the foot of the staircase it is impossible to determine if a particular stereocilium will be incorporated into the staircase proper or if it will be reabsorbed. This will be particularly true at the proximal end of the cochlea where the first step of the staircase is small and thus those located on the bottom rung of the staircase are the same size as the stereocilia that will be reabsorbed (see Tilney et al., 1988).

14-day embryos
With an additional day of embryonic development we find a number of cells with only one population of stereocilia, those in the staircase proper. This is particularly true in the proximal 20% of the cochlea (Fig. 5). The second population in front of the staircase no longer exists in this region, presumably having been reabsorbed. The surface is smooth where they used to be located. This loss of stereocilia in the front of the staircase is also occurring at other locations in the cochlea as judged by gaps or spaces between the stereocilia in front of the staircase (Fig. 6D), and will be complete in 17-day old embryos (see Tilney et al., 1986).

The appearance of tip linkages
In an 11-day embryo we find a number of cells in which a staircase of 4 or 5 rows has begun to form. If the stereocilia in these are not splayed apart, we see that they are organized into rows following the 1,00 lattice plane or the plane that runs directly up the staircase, an organization called a "column" of stereocilia by Pickles et al. (1989) all attached together by tip linkages. This observation by itself indicates that the stereocilia lying within the 1,00 lattice plane must be somehow connected. Tip linkages can occasionally be found (Fig. 9A) in this lattice, but they are right at the limit of resolution of this technique. Tip linkages between stereocilia in other lattice planes were not found, nor were there tip linkages connecting the stereocilia in the 1,00 lattice plane in the staircase with those at the foot of the staircase.

By 12 days tip linkages are common (Fig. 9B). They connect stereocilia that are arranged in the 1,00 lattice plane just as in mature hair cells. This distribution is predicted by the work of Pickles et al. (1991). In fact when one finds, at low magnification, stereocilia aligned in the 1,00 lattice plane these are the tufts that contain the tip linkages visible at higher magnification. Again these linkages are found only in the staircase proper not between the stereocilia in the staircase proper and those at the foot of the staircase.

Surface area occupied by stereocilia
We measured the surface area occupied by the stereociliary tuft of each cell in which we had counted the total number of stereocilia per cell. We did this by carefully outlining these surfaces on tracing paper, cutting them out and weighing them. After adjusting for differences in magnification we could plot the surface area occupied by the tufts as a function of position and developmental age (Fig. 10).

By combining material from Figs 5 and 10 we can see in Fig. 11 that the surface area corresponds closely to the number of stereocilia present on that hair cell at all developmental stages until the short stereocilia in the front of the staircase disappear. Furthermore, the slopes of the curves for both stereocilia number and surface area are remarkably similar and thus the increase in surface area and stereocilia.
Determination of number and arrangement of stereocilia

Number are proportional to each other. This situation could only be true if the stereocilia are always packed closely together with little free space, a conclusion borne out by our observations, and if the stereocilia remain approximately the same width, which is true until 12 days (see Tilney et al., 1986).

Because a proportionality exists between surface area and stereocilia number during the period in which stereocilia are being produced (day 8 - day 12) and because stereocilia are tightly packed together and are approximately the same width, one way that a cell may control stereocilia number is by accurately regulating the dimensions of the apical surface of a hair cell and then, by filling up this surface with stereocilia, the requisite number could be determined. Unfortunately what we have measured is the surface occupied by stereocilia and what we need are measurements of the apical surface of the hair cell. Since it is not possible by scanning microscopy of these early stages to identify a hair cell surface from the surfaces of supporting cells that surround it, it is necessary to examine serial, thin sections of the cochleae.

Accordingly, we cut serial sections of a 9-day embryo and made reconstructions for three positions on the cochlea, the distal, mid and proximal ends. Representatives of one series are included here (Fig. 12). There are 3 observations that deserve particular attention. First, the bulk of the cochlear surface in a 9-day embryo is supplied by supporting cells, not hair cells, in contrast to the mature cochlea where hair cells provide most of the surface. Accordingly, in 9-day embryos the surface area of the hair cell is surprisingly small. Second, the junctional complexes, a tight junction and beneath it a large intermediate junction or zona adhaerens, are not oriented perpendicular to the apical surface, but at an angle as if the cell proper has contracted relative to the surface. This results in a bulging outwards of the apical surface of the hair cell. This may explain why the stereocilia viewed by scanning electron microscopy are splayed apart (Fig. 6A). Third, and most important, the bases of stereocilia, which can readily be identified in our serial sections, are seen extending from the entire apical surface, except for a tiny region directly over the junctional complex that is angled inwards. Thus at this stage the surface area occupied by the stereociliary tufts is nearly identical to the entire apical surface of the hair cell.

We have not cut serial sections of hair cells at other stages because the amount of effort does not seem proportional to the information gained. We have cut innumerable sections through these cochleae, however, and from these the following conclusion can be drawn, namely that until the stereocilia at the front of the staircase are reabsorbed (from day 14 and over the next couple of days), the stereociliary tuft that we see in our scanning photographs just about fills up the apical surface of the cell.

Fig. 9. (A) Scanning electron micrograph of a portion of the stereociliary bundle of an 11-day embryo. Tip linkages can be seen between many of the stereocilia in the staircase (arrowheads). (B) Scanning electron micrograph of a stereociliary bundle of a 12-day embryo. The stereocilia tend to be aligned in the 1,00 lattice planes (large arrowhead). Between individual members of a 1,00 lattice plane are tip linkages (small arrowheads). Bar, 0.2 µm.

Fig. 10. The surface area (µm²) occupied by the bundle of stereocilia is plotted as a function of the location of the hair cell.
Fig. 11. Data from Figs 5 and 11 are plotted together in this figure which indicates that from 9-12 days the number of stereocilia closely corresponds to the surface area occupied by the stereocilia. The stereocilia are packed tightly together at these stages - there is no "empty" space. ● = curve of the area; □ = curve of the number.
**Discussion**

*The change from a circular bundle to a rectangular one*

This change occurs in two stages. First, the circular bundle in a 10 1/2-day embryo is converted to the semicircular shape of a 12-day embryo. The flat surface of the semicircle is located behind the tallest row of the staircase. Second, the tiny stereocilia located at the front of the staircase are reabsorbed and thus the semicircular bundle is converted into a rectangular bundle. This phase begins in a 13-day embryo and is complete in a 16-day embryo. From our counts of total stereocilia numbers in 13-day embryos, 25-50% of the stereocilia must be reabsorbed.

The second stage is easy to understand conceptually as all that is required is the resorption of the stereocilia not in the staircase in a 13-day embryo. The first stage is more difficult, particularly as the stereocilia in both the circular form, a 10 1/2-day embryo, and the rectangular bundle, mature cells, are closely packed. What we think occurs during this stage is a rearrangement of the existing stereocilia as the staircase forms. To begin to understand how this rearrangement might take place, it is necessary to examine the mature bundle and then “work back” to earlier and earlier embryos. In the mature hair cell the stereocilia are connected together by both extracellular and intracellular linkages. The intracellular linkages occur in the apical cell cytoplasm, a region called the cuticular plate. These linkages are mediated by actin filaments that attach the rootlets (basal extensions into the cuticular plate of the central most actin filaments in the stereocilia) of adjacent stereocilia together (DeRosier and Tilney, 1989). These connectors are likely to be made of the actin crossbridging protein, spectrin (Drenckhahn et al., 1991).

The extracellular linkages consist of three types. The most publicized are the tip linkages that connect stereocilia that lie on the 1.00 lattice plane (see Figs 2 and 3 and Pickles et al., 1989). These linkages connect the tips of shorter...
sensory stereocilia to the sides of the longer ones situated directly above. These are thought to be located at or near the transduction channels (Hudspeth, 1985). The second type connect stereocilia together at their bases (Hirokawa and Tilney, 1982), and the third type are fine fibrils that connect adjacent stereocilia all along their lengths (Hirokawa and Tilney, 1982). The last are usually destroyed by fixation as they can only be seen in unfixed preparations.

The question becomes, when do these extracellular and intracellular linkages appear in development as they must solidify the arrangement of stereocilia into its mature pattern. The intracellular linkages in the cuticular plate begin to appear in 12-day embryos (Tilney and DeRosier, 1986), a time when a miniature staircase has already formed but before the tiny stereocilia at the base of the staircase have been reabsorbed. In contrast tip linkages (this report and Pickles et al., 1991) appear when the staircase is forming, e.g. in 10 1/2- to 12-day embryos. These tip linkages connect stereocilia present in the 1,00 lattice plane in the staircase, but not the stereocilia in front of the staircase that are closely packed but do not appear in the 1,00 lattice plane. There is no information available about when the other two types of extracellular linkages form.

Before describing how a circular bundle might be transformed into a semicircular one, we should reiterate information presented in the last paper in this series on how the staircase pattern is generated. What occurs is the sequential initiation of elongation of stereociliary rows (Tilney et al., 1988). Thus in a 10 1/2-day embryo, what will be the tallest row of stereocilia in the staircase elongates. Then in slightly older embryos (11-day), not only is the tallest row elongating, but now the next to tallest row is elongating as well and so forth. We proposed (Tilney et al., 1988) a simple model of how the sequential initiation of elongation might occur using the transduction machinery known to be present in stereocilia. Briefly, we suggested that the channels in the stereociliary membrane would transport ions into the stereocilia proper, that in turn would “uncap” the actin filaments so they could increase in length by the addition of monomers. Essential to this simple model are the timed appearance in development of tip linkages connecting stereocilia in the developing staircase.

Using this background information, what will follow is a geometric model on how the bundle is changed from a circular bundle to a semicircular one. In a 10 1/2-day embryo, what will be the tallest row has begun to elongate along half of the perimeter of the bundle. Because the bundle is round, the outermost row will have a longer perimeter or contain more stereocilia than the row immediately inside it. It is analogous to an onion in which the outermost layer is larger than the next innermost layer and so on. If each elongating stereocilium from this outer row wants to connect to a stereocilium farther inside the bundle by a tip linkage, there are not enough stereocilia in the inner row for each member of the elongating row to contact. For example in Fig. 13A, there are 12 in the outermost row, yet only 9 in the next inside row. For every member of the outer row to contact a stereocilium in an inner row requires the migration of 3 stereocilia from further in the bundle to the second row. The addition of 3 more stereocilia to the inner row will tend to straighten the back side of the bundle slightly (Fig. 13B). The two outer rows will elongate and will then connect by tip linkages to what will be a third row. Again more stereocilia will have to be incorporated into the third row from within the bundle proper as this row will have too few to begin with. This incorporation into the third row will straighten the first 3 rows even more (Fig. 13C). As more rows form, straightening of the staircase will continue until in a 12-day embryo the bundle appears semicircular with the back of the bundle perfectly straight. The second stage now begins with the resorption of the stereocilia at the front of the staircase. As this is happening the cuticular plate will form underneath the existing staircase stabilizing the staircase pattern further.

Thus the change from a circular bundle to a rectangular one is a consequence of linking together a closely packed population of stereocilia by tip linkages which connect stereocilia of increasing lengths. These tip linkages in turn form and maintain the profile of the staircase as the whole staircase elongates. This model, we feel, is substantiated by our observations. For example in the 10 1/2- and 12-day embryos of Fig. 8, we count 20 stereocilia in the back row, the row that is elongating. In a mature bundle (Fig. 2) at approximately the same location, we count 26. (Actually the bundle in Fig. 2 is located more proximally than the round bundle at 10 1/2 days or the 12-day bundle seen in Fig. 8). Thus, from our data, the number of stereocilia in what will be the tallest row of a hair cell in the embryo is approximately the same as that in the mature bundle near that location. Our observations here are complicated because in the round bundle the tallest row (back row) has a smooth contour (Fig. 13A). As the bundle straightens the back row begins to zigzag as it really consists of two 1,1 rows as illustrated in Figs 2 and 13D. Thus a count of the number of individual stereocilia in the back row is really of two 1,1 rows. What is impressive is that our counts substantiate our model.

With this in mind, we believe our model gives an explanation of how hair cells throughout the cochlea determine stereocilia number. It is due to a combination of the surface area of the cell, a time window for stereocilia formation, and selective resorption of the stereocilia not incorporated into the staircase.

**Actin filament nucleation by itself is not the signal to assemble stereocilia**

New stereocilia form in 8- to 12-day embryos except at the extreme proximal end (where development is a little slower than over the rest of the cochlea). After 13 days, the total number of stereocilia per cell decreases and at the same time the width of the stereocilia increases in 12- to 17-day embryos (Tilney et al., 1986). An increase in width means that many new actin filaments must be nucleated (Tilney and DeRosier, 1986; Tilney and Tilney, 1988). For example, in an 11-day embryo, the average number of actin filaments per stereocilium is 100, but by 17 days or in hatching chicks up to 700 can be found (Tilney and Tilney, 1988). The cuticular plate forms during the same period (Tilney and DeRosier, 1986) which also necessitates actin filament nucleation, yet no new stereocilia are produced during the same 5-day period. Thus actin nucleation by
itself does not produce new stereocilia, only an increased number of actin filaments.

**What might determine the number of stereocilia per cell**

Two observations pertain. First, stereocilia sprout at random from the apical surface of the cell beginning in an 8-day embryo. By 10 1/2 days, the number of stereocilia is the same as that found at the same relative position in a mature hair cell. Thereafter the number increases 1 1/2 to 2 times (in a 12- to 13-day embryo) and then this number diminishes to that in the mature cochlea by the resorption of stereocilia in front of the staircase. Second, the stereocilia are tightly packed until day 13 and located over the entire apical surface of the cell. In fact, the apical surface area of the hair cell correlates precisely with the number of stereocilia present in 9- to 13-day embryos.

Our idea is that between 8 and 13 days, the time window when stereocilia form, the hair cell tries to fill up all the available apical cell surface with stereocilia. Once this surface has been filled, no more stereocilia can appear until the surface increases. After the time window has elapsed, the apical surface can increase still further but no new stereocilia will appear. At the same time stereocilia that are not part of the staircase, eg. those located at the foot of the staircase, will be reabsorbed. Thus, according to this idea, a combination of a time window for stereocilia emergence, the cell surface, and stereocilia resorption are responsible for determining the number of stereocilia per cell.

It is worth emphasizing that stereociliary number varies in a predictable way depending upon the location of the hair cell on the cochlea, eg. 50 at the distal end, 300 at the proximal end, and also relative to the margins of the cochlea, eg. superior and inferior edges. Thus, nearly every hair cell has a different number of stereocilia from any other, but the number correlates precisely with the position the hair cell occupies on the cochlea. What this means is that the mechanisms which determine stereociliary number have to be flexible to make every cell different from every other one. The appealing feature of our idea is that it is simple and by having three control factors working in concert one can easily make every hair cell different from any other, yet predictable because two of the factors, namely the time window and the resorption are the same for every hair cell. In contrast, the third is a mechanism that will be different for every hair cell as it is related to the surface area of the cell. When the three mechanisms are combined, the final number of stereocilia can be amazingly variable, yet at the same time quite predictable.

**To build a complex cytoskeleton does not require prepattern etched into the cell membrane or cell cortex, but instead is assembled by a sequence of small steps**

As alluded to in the introduction, the hair cell is one of the most specialized cells of the body in which the cytoskeleton is entirely predictable, yet each hair cell is different from its neighbors in a predictable way. Thus depending upon the location of the hair cell on the cochlea in two axes we can predict how many stereocilia will form, what their lengths will be, what their arrangement will be, and what their widths will be. These features can be translated directly into how many actin filaments will be found in each stereocilium (its width), how long these actin filaments will be in each stereocilium (its length), and how the filaments are packed (Tilney et al., 1983). In the last four papers in this series we considered how a predictable, yet complex cytoskeleton is generated. The conclusion that we have arrived at in each paper is that the cytoskeleton is formed not by the cell synthesizing all the essential components at once and letting it self-assemble but rather, the differentiating hair cell builds its complex cytoskeleton in a series of discrete steps separated from each other by developmental time. Thus, we demonstrated that elongation of stereocilia or the lengthening of existing actin filaments by addition of monomers occurs in two phases, eg. 10 - 12 days and 17 - 21 days, while the growth in stereocilia width or nucleation of more filaments occurs during a period, 12 - 17 days, when growth in length is not occurring (Tilney and DeRosier, 1986; Tilney et al., 1986). The formation of the staircase occurs in four stages; they sprout, they sequentially begin to elongate with what will be the tallest row first, then they all elongate, and then they cease elongating sequentially (Tilney et al., 1988), with, of course, the hiatus in elongation of all the stereocilia between days 12 to 17. Stereocilia formation and their arrangement on the apical surface also occurs in discrete steps as indicated by this report. Thus in building this enormously complex actin machine what the hair cell is doing is first one job, eg. sprouting stereocilia from its surface. Then, using the results of this first job the hair cell starts a second job, eg. the elongation of the stereocilia beginning with what will be the tallest row of the staircase. At the same time the shape of the bundle begins to change from a circular one to a rectangular one. These two steps are followed by an increase in width of the stereocilia and the formation of the cuticular plate. These three steps are followed by the elongation of all the stereocilia and finally these 4 steps are completed by the cessation of growth which we believe is regulated by using up existing components (Tilney and Tilney, 1988).

Having shown that the cytoskeleton is made in discrete steps separated by developmental time, times that are predictable, the next logical undertaking is to determine how each step is, in turn, regulated and how the next step in the sequence is initiated. In short we need to relate assembly of the cytoskeleton to when the proteins are being made and how the hair cell knows when and how much to make of a given protein. We have started to do just this by trying to determine when the actin-binding proteins that are known to be present in the hair cell (see Drenckhahn et al., 1991) appear in the cytoplasm and how they know where to go. Furthermore, we are attempting to see when the mRNA for these and other proteins are made by the cell. Ultimately we want to see how gene transcription and/or translation is regulated.

Because the arrangement and number of the stereocilia on the cell surface are so precise as are the dimensions of the stereocilia (Tilney and Tilney, 1988), when we first examined a hair cell some 12 years ago, we suspected that to develop such a pattern would require a prepattern that somehow exists in or near the apical cell surface. In retrospect of course this idea is ludicrous for two reasons. First, as mentioned before, every hair cell is different, so how are these differences coded in a prepattern and second, if any
trauma occurs to the hair cell precursors and the prepattern is somehow altered or lost, the organism could not hear (cochlea) or stand erect (vestibular system) or detect movement (lateral line). Instead what has evolved is a whole series of simple steps which take place in order to generate the complex actin cytoskeleton present in hair cells. This cytoskeleton appears to be the most complex, yet most predictable of any present in animal cells.

Special thanks go to Pat Connelly for painstakingly cutting the serial sections. We would like to thank Bob and Linda Golder and Laura Moorhouse for the artwork. Supported by NIH grant HD 14474 (L.G.T., M.S.T.) and NIDCD-00412 (D.A.C.).

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(Accepted 4 June 1992)