FREEZE-FRACTURE ANALYSIS OF MEMBRANE EVENTS DURING EARLY NEOGENESIS OF CILIA IN TETRAHYMENA: CHANGES IN FAIRY-RING MORPHOLOGY AND MEMBRANE TOPOGRAPHY

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SUMMARY
A freeze-fracture analysis of early neogenesis of somatic and oral cilia of *Tetrahymena* was conducted using exponentially grown cultures and also cells induced to undergo oral reorganization. In this report, presumptive ciliary domains (PCDs), sites of future outgrowth of somatic cilia, are identified and their membrane structure is described in detail. The fairy ring, an array of membrane particles that occurs within the PCD and appears to be a precursor of the ciliary necklace, is described. A sequence of early stages in the formation of the ciliary necklace of somatic cilia is deduced from topographical information and membrane particle arrangements and numbers. Evidence is presented that basal bodies are seated at the cell surface prior to initiation of necklace assembly and a possible role for the basal body in necklace assembly is suggested. In dividing cells, new oral cilia grow out prior to orientation of cilia-parasomal sac complexes relative to cell axes. In dividing cells and during oral reorganization, new cilia also develop prior to their alignment into membranelles. Thus, growth of cilia is independent of their spatial orientation. Fairy rings were not observed during oral reorganization. During cell division, proliferation of new cilia is accompanied by the formation of a network of junctions between a cortical system of membranous cisternae, the cortical 'alveoli'. These interalveolar junctions may serve as tracks for early positioning and orientation of new oral basal bodies.

INTRODUCTION
A freeze-fracture ultrastructural analysis of changes in membrane organization, cell surface topography and organelle orientation during early neogenesis of somatic and oral cilia of *Tetrahymena*, a protozoon, has been conducted. The study was carried out to establish a logical sequence of morphological events during early ciliogenesis, in order, eventually, to learn how the ciliary membrane is assembled, and how the patterning of the ciliated cortex is regulated. *Tetrahymena* is inherently interesting for studies on development of cell structure and pattern for many reasons, but especially because: (1) it has a complex cellular architecture, which is accurately reproduced during cell division; (2) phenomenologically, ciliate morphogenesis is strikingly similar to morphogenesis in multicellular systems (Frankel, 1974); and (3) the cellular properties of *Tetrahymena* are in principle the same as those of all eukaryotic cells. Freeze-fracture analysis provides one way to examine organization within membranes, providing information on the localization and ordering of some membrane...
proteins, which are distinguished from others because they project from the fracture face as discrete particulate elevations (intramembranous particles; IMPs), and by their unique topographical arrangements, such as clusters, strings, rectilinear arrays and rosettes. In the present study, such IMP arrays have been used as indicators of membrane organization. In addition, freeze-fracture replicas provide information on cell surface topography, which may reflect either intrinsic membrane structure or organization of the underlying cytoskeleton.

Freeze-fracture analysis of ciliary membranes has revealed several types of organized particle arrays, including the ciliary necklace (Gilula & Satir, 1972), plaques (Wunderlich & Speth, 1972; Plattner, 1975; Plattner, Miller & Bachmann, 1973), rosettes (Bardele, 1980, 1981; Hufnagel, unpublished data) and rows (Sattler & Staehelin, 1974; Bardele, 1980). These indicators of ciliary membrane order, plus those provided by the fluorescent hydrocarbon probe studies of Nozawa & Thompson (1979), suggest that the ciliary membrane has a highly ordered structure, making it a good system for analysis of assembly of membrane structure.

How ciliary membrane IMP arrays are assembled has been of some interest. One experimental approach has been to characterize changes in membrane structure accompanying ciliary regeneration following dibucaine-clipping of mature cilia (Satir, Sale & Satir, 1976). Some ultrastructural events during early neogenesis of cilia have also been observed (Satir, Schooley & Satir, 1972; Satir & Satir, 1974). The present study extends these observations.

The overall patterning of the cell cortex of ciliated protozoa results in part from the positioning, orientation and substructure of individual ciliary units, which are visible units of organization in the cortex of some ciliates, such as *Paramecium* (Hufnagel, 1969), and can be proposed to exist in all ciliated protozoa. These units (also known as territories; Pitelka, 1969; Hufnagel, 1969) are essentially similar, and consist of one or two cilia and associated basal bodies, fibrous and microtubular cytoskeletal structures, a portion of the plasma membrane and flat membranous cisternae known as alveoli. Cortical units arise by growth and subdivision of pre-existing cortical units (Dippell, 1965). As Beisson & Sonneborn (1965) have shown experimentally, the ciliary unit is the cytotactic unit of the ciliate cortex. That is, its position relative to other units and rotational orientation about an axis perpendicular to the cell surface are usually determined by the position and orientation of the pre-existing unit from which it arose.

Nevertheless, the size, shape, orientation and internal organization of ciliary units varies, in parallel with regional variations in organization of the cortex. Thus to understand how cortical patterning is regulated, it is necessary to understand how the pattern of an individual unit is established and how the units are oriented relative to each other. Light and electron microscopy have shown that ciliary basal bodies appear very early in the development of a unit and that cytoskeletal elements are gradually added by nucleated assembly (Lwoff, 1950; Dippell, 1968; Allen, 1969). While membranous components were observed to grow and subdivide (Dippell, 1965), the detailed events in assembly of cortical unit membranes have not been analysed. The present study attempts to extend our understanding of cortical membrane assembly
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by examining changes in membrane structure and membrane–cytoskeleton interactions during early stages of cortical unit development.

Some of the observations reported here have been summarized briefly in an earlier publication (Hufnagel, 1979).

MATERIALS AND METHODS

Observations were made on Tetrahymena pyriformis, strain GL-C, originally obtained from M. Gorovsky. The cells were grown on enriched proteose peptone (EPP) medium as described elsewhere (McGwin & Hufnagel, 1979).

Cells used in freeze-fracture studies were in a late phase of exponential growth or were synchronized for oral replacement (OR). To obtain cells synchronized for OR, methods adapted from Frankel (1970) were used. Log-phase T. pyriformis were washed three times with sterile modified amino-acid-free medium (AAFM-IV: 0.5% glucose, 0.03% MgSO₄, 0.04M-Hepes buffer, pH 6.8, with 1 M-NaOH), and resuspended in the original volume of sterile AAFM-IV. Following overnight incubation with shaking at 26–28°C, the cells were washed, resuspended as before in AAFM-IV, and subjected to alternating 4-h treatments at 33.8 ± 0.3 deg. C and 27 ± 1 deg. C, while being aerated by means of a Pasteur pipette attached by hoses to an aquarium pump. Following the seventh treatment at 33.8°C (end of heat-shock treatment; EST) the cells were returned to 27°C and sampled at intervals up to 2 h following EST. To determine the timing of OR, cells in 5 ml portions were stained by the Chatton–Lwoff method (Frankel & Heckmann, 1968). Similar portions were fixed with glutaraldehyde, glycerinated, frozen, fractured and replicated without etching. Unfixed, glycerinated cells were also studied. Freezing was on gold specimen-supports in freshly thawed Freon. The cells were subsequently fractured and replicated in a Denton freeze-etch device. Details of the methods for specimen preparation and replication are given elsewhere (Hufnagel, 1981a).

Dividing cells were encountered by chance in exponentially growing cultures, prepared for light microscopy by the Chatton–Lwoff method or for freeze-fracture electron microscopy as described above.

Replicas were cleaned with dichromate cleaning solution (Fisher), followed by several changes of distilled water; mounted on Formvar-coated copper grids and examined with Philips model 200 and 300 electron microscopes at 60kV. Particle diameters were measured at right angles to the direction of shadowing.

RESULTS

Structure of the somatic and oral ciliary membrane

As described by many others, both oral and somatic ciliary membranes are characterized by the presence of a necklace of IMPs. The necklace is found at the base of the ciliary shaft and is composed of two irregular rows of 9–12 nm particles, which are evident on both the protoplasmic face (PF) (Fig. 1) and external face (EF) (Fig. 2) of the ciliary membrane. Other IMPs, both ordered and random, are present in the ciliary membranes but their properties are not relevant to the present study.

Membrane topography in the vicinity of a mature somatic cilium

Fracture planes that follow the plasma membrane or outer alveolar membrane for long distances reveal extensive areas of the cortex. Mature cilia are cross-fractured close to their junctions with the cell surface and can be recognized by the abrupt discontinuities that occur as the fracture plane leaves the plasma membrane (PM) and
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enters the ciliary shafts (axonemes; see Fig. 3). Even when the fracture plane has mainly followed the outer alveolar membrane (Fig. 4A) it generally passes through a portion of the PM before entering the axoneme. This region of the PM has a characteristic topography, which appears as a ridge encircling the ciliary shaft when the EF of the PM is revealed (Fig. 4A, a). The ridge usually has a characteristic anteriorly situated elevation, which is a manifestation of the parasomal sac (PS). The PS lies just anterior to the cilium and is a specialized invagination of the plasma membrane, which communicates with a system of alveoli and coated vesicles surrounding the basal body. The fracture plane sometimes follows the entire invagination (Fig. 4A) and sometimes produces a cross-fracture through the neck of the parasomal sac (Fig. 3, arrows). This suggests that the parasomal sac is a dynamic structure, which extends to the internal alveoli and coated vesicles at certain times but becomes disconnected from them at other times.

All figures: in all cases except as indicated cells were fixed with glutaraldehyde and dehydrated to 50% glycerol. Abbreviations: ps, parasomal sac; PF, protoplasmic face; EF, external face; pm, plasma membrane; fr, fairy ring; oam, outer alveolar membrane; a, anterior; p, posterior; pcd, presumptive ciliary domain. Arrows in circles indicate direction of shadowing.

Fig. 1. Freeze-fractured cilium of Tetrahymena, revealing the PF of the ciliary membrane. Note the irregularly spaced particles of the ciliary necklace (arrows) and the larger tetragonally arranged particles of the ciliary plaques. The more distal regions of the ciliary membrane PF are characterized by numerous randomly dispersed particles similar in size to those of the necklace. X71 100.

Fig. 2. EF of the ciliary membrane of Tetrahymena, showing the ciliary necklace particles and the relatively infrequent occurrence of membrane particles distal to the necklace. Note that necklace EF particles are uniform in size. Turn page 180° for proper viewing. X75 400.

Fig. 3. EF of the plasma membrane of Tetrahymena; the topography in the vicinity of several mature cilia within a single ciliary row is shown. Note that some parasomal sacs (ps) are cross-fractured, while in other cases the fracture plane follows the contours of the sac membrane (arrows). Pos always lie anterior to somatic cilia. X28200.

Fig. 4. The topography around mature cilia, as viewed from inside the cell. a. Fracture plane has followed the outer alveolar membrane (oam) and entered the plasma membrane only, close to the ciliary shaft. Particulate ridge extending anterior (a) and posterior (p) to the cilium is where two different alveolar membranes join (ciliary meridian) – see Fig. 5. Similar interalveolar membrane junctions are found about half-way between ciliary rows (secondary meridians). X29 300. b. Fracture plane does not pass through the outer alveolar membrane; thus a large portion of the EF of the pm near the cilium is revealed. Turn page 180° for proper viewing. X36 600.

Fig. 5. Enlarged view of membrane junction connecting two different cortical alveoli revealing the PF of the oam. The junction is the particulate ridge that separates the two membrane faces. This junction may be a barrier to movement of molecules or organelles to the pm from the underlying cytoplasm. However, organelles such as mucocysts do penetrate this barrier, forming specialized circular patterns such as that seen in this figure (arrow). Such circular profiles are identified as mucocyst penetration sites by their characteristic distribution along interalveolar junctions. X49 200.

Fig. 6. Two ciliary rows seen from inside the cell. Note that cilia occur in pairs. In the upper and lower left (arrows), the anterior member of each pair is seen to differ from the appearance typical of cross-fractured cilia. X21 400.
In certain freeze-fracture preparations, the fracture plane seems to stay preferentially within the outer alveolar membrane (OAM), except near the ciliary shaft (Fig. 4A). In such cases, the ciliary shaft can be seen to be located on a longitudinal meridian (polar coordinate) along which other ciliary shafts are also localized. These meridians (ciliary meridians) generally alternate with meridians having no cilia associated with them (secondary meridians). Both types of meridians represent locations where adjacent cortical alveoli are contiguous and junctionally associated. The junctions between alveoli give rise to a particulate ridge whenever the fracture plane reveals the PF of the OAM (Fig. 5). No evidence of specialized membrane particle array organization in the PM overlying interalveolar junctions in the vicinity of cilia has ever been observed.

Membrane topography in the vicinity of nascent cilia

Frequently, cross-fractured cilia appear to occur in pairs (Fig. 6). This pairing is common in dividing cells (see Fig. 17, inset) but is also observed in non-dividing cells. Upon closer inspection (Fig. 7), the more anterior member of each pair is often seen to differ somewhat from the appearance typical of a cillum. The plasma membrane is continuous across the shaft region and usually contains membrane particles arranged in a crude ring. These regions of the PM are interpreted to be the presumptive sites of new cilia, and will hereafter be referred to as 'presumptive ciliary domains' (PCDs). The PCD is here defined to include the region containing specialized arrangements of membrane particles, as well as membrane topographical features related to the cilium, the surrounding cortical depression and the parasomal sac.

PCDs are usually but not always paired with mature cilia, and lie on the ciliary meridians and never on secondary meridians; the distance between PCDs and mature cilia can vary, but the closest mature cilium is almost always posterior to the PCD.

From a series of low-magnification survey pictures of log-phase cells, the total numbers of mature cilia and PCDs were determined. Of a total of 168 ciliary domains, 24 were PCDs, 120 contained mature cilia and 24 could not be identified. Thus PCDs constituted about 14% of the total ciliary domains. This figure is likely to be somewhat higher than the actual situation in log-phase cells, since there was some bias toward photographing areas with evident PCDs. Paired ciliary domains were numerous in some cells (see Fig. 6) but were isolated occurrences in other cells.

Fairy rings and other membrane substructure in PCDs

The most apparent feature of PCDs is the presence in the EF of the PM of a 0.25 μm wide ring of irregularly arranged IMPs (Fig. 7). These particles vary in diameter from 6–18 nm and are somewhat randomly distributed to form a broad circular track, which distinctly resembles the mushroom rings commonly found on lawns and in fields; hence the name 'fairy rings' has been applied to this particle array (Hufnagel, 1979). The fairy ring lies upon a plateau-like or ring-like elevation of the PM (as seen from inside the cell looking outward), which actually represents a depression of the cell surface. Usually, in the centre of the fairy ring several isolated membrane particles, ranging in diameter from 6–18 nm, are also found.
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Fig. 7. Enlarged view of a ciliary pair; the anterior member of the pair is actually a presumptive ciliary domain (pcd), characterized by a continuation of the pm across the space where a cilium should be. Note ring of IMPs (fairy ring) on the EF of the pm and central cluster of particles. ×61 400.

Fig. 8. Two examples of the PF appearance of the plasma membrane at a pcd. The particle distribution on the PF of the pcd appears random. In A, a central bulge in the pcd is clearly apparent. Such bulges are commonly seen in some preparations and may reflect the presence of a portion of the underlying basal body, possibly the axosome. ×53 000.

Fig. 9. The PF of a pcd located far from mature cilia. Note the concentric rings of IMPs. This may be a location where a mature cilium was broken off. Similar patterns have been seen in both fixed and unfixed cells. Unfixed; glycerinated. ×57 700.

In the region of the PCDs, the particle pattern of the complementary PF of the PM usually resembles the rest of the PF of the PM (Fig. 8A, B). The particles are about 4–8 nm in diameter, numerous and randomly arranged. However, in some instances, PF particles in the PCD appear to be arranged in as many as three distinct concentric circles (Fig. 9). Such circular patterns have generally been seen in domains that are not closely associated with a mature cilium, so these cases may be examples of mature cilia that have broken off. Often the centre of the PF of a PCD appears to have a small bump (Figs 8A, 9), which is sometimes represented on the EF by a central depression.
Fig. 10. A–D. Examples of pcds having EF fairy rings with small numbers of particles. The parasomal sacs (ps) of these domains are typically shallow and the PM is not significantly invaginated in the region of the fairy ring. Fairy-ring particles are frequently in short strings, which are oriented tangentially to the ring. A, ×48 500; B–D, ×41 000.

Variations in membrane topography and IMP distributions related to early neogenesis of somatic cilia

The detailed membrane morphology of PCDs was examined in replicas from exponentially growing, glutaraldehyde-fixed cells. Considerable variation was observed in the number of particles composing the EF fairy ring and located centrally in the ring. Fairy ring particles varied from as few as 23 to as many as 80. Central particles varied from 2 to 13. When the number of ring particles was compared with the number of central particles, there appeared to be an inverse relationship.

The frequency of fairy rings with different numbers of particles was also examined. Small numbers of particles (between 20 and 50) were found to be less frequent, while larger numbers (between 50 and 80) were more frequent. No case was encountered where a clearly evident PCD contained fewer than 20 particles. (However, most of these observations were made on preparations in which the fracture plane tended to follow the OAM and passed into the PM only in the vicinity of a PCD or mature cilium. It may be that the fracture plane was more likely to jump into the PM when ring particles were more numerous, perhaps due to changes in the hydrophobicity or topography of the PM in the area immediately surrounding the fairy ring. Alternative explanations are that early stages of ring formation occupied shorter times than later stages or were difficult to identify.)
Fairy rings with smaller numbers of particles were often incomplete (Fig. 10). Ring particles were clustered or in short linear groupings, which sometimes appeared to fan out from the outside margins of the ring (Fig. 10A-D). The rings were usually associated with a flat topography of the PM, and the parasomal sac was usually represented by a shallow depression of the PM (a low hill when viewed from inside the cell looking outward; Fig. 10). In one case, a cluster of particles was observed on the EF of the parasomal sac membrane (Fig. 11). In several other cases, one or more isolated particles were observed on the PS membrane (Fig. 10b, c).

Fairy rings with larger numbers of EF particles were usually located on plateaus formed by invagination of the PM (Fig. 12A-D). In several cases in which the plateau was very pronounced, a central depression was observed in the plateau and the fairy ring seemed to lie on the slopes of the depression (Fig. 13A, B). In a few cases, the depression was quite pronounced (Fig. 13B). This depression appears to represent a very early stage in growth of the cilium itself, while the plateau (surface invagination) may be a stage in the formation of the ring-like depression that surrounds each mature cilium.

In one case, a small cluster of six irregularly spaced particles was observed on the EF of the plasma membrane, just anterior to a mature cilium and its PS (Fig. 14). These particles were about 8–16 nm in size. The cluster lay close to a curved section of the particulate ridge, which is a portion of the alveolar meridian extending anterior to the mature cilium (double arrow, Fig. 14). This cluster of particles did not resemble the mucocyst rosette, whose EF aspect consists of seven particles, six of them arranged symmetrically around the seventh (see Fig. 14, inset), which also occurs along ciliary meridians.

Early neogenesis of oral cilia: oral replacement

During oral replacement (OR), the existing oral ciliature is dismantled and replaced with new cilia. These develop in part from basal bodies of the old oral structures and partly from new basal bodies arising in a proliferative zone just posterior to the old oral region (Frankel, 1970). During OR, there is an anterior–posterior gradient of pattern development, in which basal bodies near the anterior end are organized into oriented arrays to become the basis for oral membranellar organization, while the more posterior basal bodies are the last to become aligned (Fig. 15, top inset).

Freeze-fracture replicas were obtained from cells fixed at 45 min and 60 min after the end of heat-shock treatment (EST). Analysis of silver-stained preparations indicated that at these times cells in all stages of OR are present and that a large percentage of OR cells are in stages of basal body proliferation and early ciliogenesis (stages 3 and 4 of Frankel & Williams, 1973). An examination of many replicas revealed about two dozen cells in various stages of OR. Fig. 15 shows a low magnification view of a developing oral region at a stage that may be slightly later than that shown in Fig. 15, inset. The PF of the PM is revealed. Some alignment of cross-fractured cilia may be noted at the anterior end of the ciliary field, whereas the more posterior cilia are non-aligned. Aligned cilia are paired, with the pairs arranged in
curved fields representing early stages of development of the first and second oral membranelles. Some pairing of cilia to form the third membranelle is also evident. Within the aligned regions are spaces where cilia are absent. These locations have a low mound-like topography and are the most likely location of PCDs. Oriented particle arrays have not been discerned in such areas. The same is generally true when the EF of developing oral regions is examined (Fig. 15, bottom inset). PCDs appear as slight depressions of PM topography. In only one case (Fig. 15, bottom inset arrow) has evidence of IMP organization been observed within a PCD.

Early neogenesis of oral cilia: cell division

During cell division, new oral cilia for the posterior daughter develop from basal bodies that proliferate next to one of the post-oral ciliary rows, about midway between the anterior and posterior ends of the cell. This proliferation is accompanied by a spinning out of interalveolar junctions to form an irregular net, which is punctuated by small dots representing basal bodies (see Fig. 16, inset; this cell was stained by the...
Fig. 15. For legend see p. 147.
Fig. 16. For legend see p. 147.
Chatton–Lwoff method, which does not actually stain basal bodies but rather the ring-like borders of alveoli that surround the basal bodies. A freeze-fracture replica was obtained through a cell at a stage similar to that in Fig. 16, inset, revealing the PF of the PM (Fig. 16).

The IMPs of this region were numerous and not unusual in their arrangement. Some were oriented into short rows (see area marked by double arrow, Fig. 16) and some particle-free areas were observed, but these had no consistent relationship to membrane topography or organelle positions. Many cross-fractured mature or growing cilia were observed, but recognizable PCDs were not seen. Cross-fractured cilia were arranged randomly or in short, linear groups (see Fig. 16, upper left). Almost every cross-fractured cilium had associated with it a dimple, most likely an early stage in the formation of a parasomal sac (PS). The rotational symmetry established by the association of a PS with a cilium was completely random. Where some suggestion of ciliary alignment was evident, as in the short strings, there was no consistent symmetry relationship between the cilia and parasomal sacs. A few PS dimples were not associated with ciliary shafts and some ciliary shafts did not appear to have PS dimples close to them. The corresponding EF was not observed.

**DISCUSSION**

*Development of somatic cilia*

Since it has been shown for *Tetrahymena* that new basal bodies arise just anterior to ciliated basal bodies (Allen, 1969), it is assumed in this paper that within pairs the more posterior mature cilia are the parents of the more anterior nascent cilia predicted by PCDs. (A similar assumption was made by Nanney (1975) in interpreting Protargol-stained cells viewed by light microscopy.) An alternative interpretation is that the apparent nascent cilia are sites of recent ciliary breakage and that they predict regeneration of cilia following such breakage, as described for dibucaine-sheared *Tetrahymena* (Satir et al. 1976). However, the frequent location of PCDs close to and anterior to mature cilia does not seem likely if these domains resulted from random removal of mature cilia. Thus, PCDs paired with mature cilia are likely to represent sites of neogenesis of cilia rather than reciliation following clipping.

The variations in topography and the number and distribution of particles within presumptive ciliary domains suggest a morphogenetic sequence of events during early neogenesis of cilia. This sequence, which is based on reasonable logic rather than absolute proof, is presented diagrammatically in Fig. 17. While there is no way to prove that the scheme presented in Fig. 17 is true, the proposed sequences of topographical changes and changes in membrane particle distribution are consistent with what one would expect starting from an uncontoured and particle-free membrane. The proposed scheme in Fig. 17 is not meant to imply anything regarding the length of time spent at each stage.

The earliest stage (1) is hypothetical, but may correspond to Fig. 14. The cluster of particles may presage the formation of either the parasomal sac or the particle cluster found central to the fairy ring. Stage (2) is characterized by a partial fairy ring,
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Numerous central particles and a shallow cell-surface depression, which is to become the parasomal sac. During the third stage, ring particles accumulate, central particles are lost and both types of particles come to lie within a shallow circular invagination of the cell surface. In stage (4), the central particles have become reduced in number and the fairy ring is becoming incorporated into an evagination of the floor of the original surface depression. Stage (5) is a hypothetical stage during which major elongation of the cilium occurs.

The similarity in size of particles of the fairy ring and those of the ciliary necklace, as well as their ring-like arrangement, suggest that the fairy ring is a precursor of the necklace. This idea was first put forward by Satir *et al.* (1972). The sequence of topographical and membrane-structural changes described here in detail (summarized in Fig. 17) confirm in general the observations of Satir & Satir (1974) on necklace assembly in log-phase cells and provide additional topographical evidence for a sequence of assembly from fairy ring to necklace. Thus, in newly forming somatic cilia the necklace appears to assemble prior to the outgrowth of the cilium.

The EF fairy ring appears to be composed of particles of several different sizes, with the largest particles about two to three times as large as the smaller ones. Necklaces appear to be composed of particles of more uniform size, similar to the intermediate-sized particles of the fairy ring. Thus a sequence of assembly of larger particles from smaller ones or of disassembly of the largest into smaller ones may take place within the ring during its formation.

Satir & Satir (1974) have reported that one strand of the necklace assembles before the other. No evidence to this effect has been seen in the present study. However, the observations do suggest that EF particles may be added in short strings, as suggested by Satir & Satir (1974). In the present study the single broad fairy ring was not observed to subdivide into the two separate rings of IMPs characteristic of the mature ciliary necklace. This event may therefore occur during later stages of ciliogenesis or as a sequel to ciliary outgrowth.

The presence of particles central to the fairy ring has not been reported before. The evidence presented here indicates that central particles appear very early during formation of the PCD and suggests that they become somewhat less numerous as

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Fig. 17. Proposed sequence of events during early neogenesis of somatic cilia. Stages (1)–(5) are described in the text. Upper figures illustrate membrane topography and IMP arrangements of the EF of the pm, as seen from inside the cell. Lower figures illustrate in profile the topographical changes thought to accompany changes in particle number and distribution.
development of the fairy ring progresses. These observations suggest that the central particles may be precursors of the fairy-ring particles. Alternatively, they may represent a membrane attachment site for the basal body, or for the axosome, whose presence is suggested in some preparations by the occurrence of a small membrane evagination in the centre of each PCD (see Figs 8, 9). The presence of the axosome at similar stages needs to be verified by thin-section analysis. Some central particles are still present toward the end of fairy-ring assembly and the beginning of outgrowth of the ciliary shaft. This suggests that central particles may also have some role in the establishment of structural attachments between the tip of the growing axoneme and the ciliary membrane, perhaps related to the complex of attachment structures described in mature cilia by Dentler (1980).

PF particle rings do not occur prominently during early neogenesis of somatic cilia, while they are present following dibucaine shearing of mature cilia (Satir et al. 1976). Possibly, their presence is masked by the generally high density of PF IMPs in the region of the nascent PCD. Alternatively, properties of the fully mature ciliary membrane may result in a different partitioning coefficient of its particles (Satir & Satir, 1974).

The ciliary necklace is located where fibrous bridges connect the ciliary membrane to the microtubules of the axoneme (Dute & Kung, 1978; Wunderlich & Speth, 1972; Satir et al. 1972). However, the observations reported here indicate that it develops before the proximal end of the ciliary shaft begins to form. Thus, its particles appear not to be placed into position by an interaction of the assembling ciliary shaft with the plasma membrane. Either there is an earlier cytoskeletal mechanism that directs assembly of the fairy ring or the pattern of the fairy ring is determined wholly by molecular events occurring within the membrane itself.

Nanney (1975) has reported that in *Tetrahymena* about 50% of the somatic basal bodies of log-phase cells are naked. In the present study, only 14% of ciliary domains were found to be PCDs, while most of the remainder contained mature (or growing) cilia. Taken together, these observations suggest that presumptive ciliary domains develop long after naked basal bodies become positioned at the cell surface. Therefore, fairy rings could develop under the direction of the underlying basal bodies and their ring-shaped pattern might reflect the cylindrical arrangement of basal body microtubules. In support of this idea, the diameter of the fairy ring (0.25 μm) is close to that of the basal body deduced from measurements on ciliary axonemes in the same freeze-fracture preparations (Hufnagel, unpublished data).

The earliest membrane topographical change in the vicinity of a PCD is the development of a surface depression that will become the parasomal sac. Presumably, the basal body has already become established at the cell surface, and therefore it is most likely that proliferation of cytoskeletal structures near the basal body helps to direct membrane invagination. The invagination is always at the anterior end of the PCD, suggesting that the rotational symmetry of both the PM and underlying basal body are established prior to any of the events described here. However, the observations on developing oral cilia in a dividing cell suggest that the development of rotational symmetry need not always be an early event in ciliogenesis. It is not clear,
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though, whether basal bodies and PSs are disoriented as a unit, or whether they can be disoriented relative to each other.

Since all structures of the existing parental ciliary unit are already symmetrically determined, any one or more of them could participate in the establishment of symmetry within the newly developing ciliary unit. However, because of its proximity to the newly formed basal body, the alveolar meridian may have a particularly important role in the establishment of rotational symmetry of the PS-basal body unit. In this regard it is interesting to note that, during the early development of a new oral apparatus in dividing cells, a network of alveolar meridians develops in association with the proliferation of new oral basal bodies and cilia. These meridians assume many different orientations relative to the anterior–posterior axis of the cell, as do the parasomal sacs relative to nascent cilia. Since PSs must be able to invaginate through the alveolar sac layer, it is likely that the parasomal sac locations are somehow related to locations of alveolar meridians and one may in fact determine the other.

The complex changes in membrane topography that accompany early neogenesis of somatic cilia raise questions about the mechanisms involved. Changes in lipid or protein composition of one half of the lipid bilayer can result in membrane curvature; this could explain the events observed during ciliogenesis. Alternatively, membrane topography may be determined largely by an underlying cytoskeletal scaffold upon which the membrane is largely draped, with occasional protein rivets to hold it in place. One can argue for both explanations, but more information about the composition and local organization of the PM is needed, as well as further information about changes in cytoskeletal organization accompanying the membrane events described here.

Development of oral cilia during OR

The observations on OR reported here confirm those of Williams & Frankel (1973), from analyses of thin sections of OR cells, that: (1) ciliogenesis precedes ordering of basal bodies; and (2) that in each oral membranelle two rows of cilia develop at an earlier stage than the third row found in mature membranelles. Although at early stages new membranellar cilia are paired, both members of the pair do not always develop synchronously, so that gaps in the ciliary pattern were evident in replicas. These gaps are likely to represent sites where basal bodies are present, and one would expect to observe fairy rings at such locations. It was therefore surprising to find that at such locations, as well as in other regions of the developing oral apparatus, no consistent evidence of fairy-ring development was present.

The cells identified as being in the process of making new cilia could instead have been at an earlier stage at which ciliary resorption is the major event. This might explain the observed absence of fairy-ring assembly. However, Williams & Frankel (1973) have reported that resorbing cilia are typically found in deep depressions of the cell surface. Such depressions were not present in the areas identified as being engaged in ciliogenesis. Furthermore, ciliary development should have been the major event in OR cells at 60 min. Nevertheless, only one possible example of oriented particles was encountered in the region of the cell surface likely to be active in
ciliogenesis. This suggests either that during OR the assembly of fairy rings is a relatively rapid event immediately followed by ciliary outgrowth, or that the ciliary necklace does not form on oral cilia until after they have begun to grow out from the cell surface.

In the present study, replicas of hundreds of cells from OR cultures were surveyed, but the observations were limited to two time samples, 45 min and 60 min EST. More extensive analyses, including times later than 60 min EST, will be required to map completely the membrane events during OR and possibly determine the sequence of necklace assembly for oral cilia.

**Development of oral cilia during cell division**

As in OR, outgrowth of oral cilia during cell division appears to precede their alignment into linear arrays. Furthermore, growth of oral cilia also precedes the establishment of a consistent rotational relationship between the cilium and its parasomal sac. Therefore, ciliary growth appears not to be dependent on the prior establishment of structural connections between basal bodies.

As mentioned earlier, the early rotational disarray of cilium–PS units coincides with the proliferation of a disorganized network of alveolar meridians, revealed as dark lines in silver-stained preparations viewed by light microscopy. Freeze-fracture preparations reveal that these lines of contiguity between separate alveolar membranes are actually membrane junctions (Plattner et al. 1973), which clearly provide a structural barrier between the interior of the cell and the cell membrane. However, silver staining indicates that the alveolar meridians become punctuated by openings that permit newly synthesized basal bodies to attach to the cell membrane prior to the start of ciliogenesis. The alveolar meridian may thus have an important role in guiding the movement and positioning of new basal bodies during early stages of oral development, serving, in a sense, as a track along which nascent basal bodies may migrate to new locations. It is of interest, in this regard that, in *Tetrahymena* subjected to near-lethal temperatures (Rosenbaum, Erwin, Beach & Holz, 1966; Hufnagel, unpublished data), or abnormal fatty acid diets (Lo, Jasper & Erwin, 1976), both basal body arrangements and alveolar meridians are disrupted. A similar situation has also been found in Disorganized, a morphogenetic mutant of *T. thermophila* (Hufnagel, 1981b). The proliferation of alveolar junctions may thus be an important early event in the restructuring of the cortex during cell division. Future studies on ciliate morphogenesis should be directed toward identifying the mechanisms leading to localized proliferation of interalveolar junctions and the coordination of junctional proliferation with the assembly of new basal bodies.

In summary, the observations on somatic ciliogenesis reported here provide a logical sequence of events for the formation of the ciliary necklace and cell surface topographical changes accompanying de novo ciliogenesis. The observations make it likely that the fairy ring, the precursor of the ciliary necklace, develops under the influence of a pre-existing basal body. Ciliary necklace formation for oral cilia during oral reorganization may occur either more rapidly than for somatic cilia or at a different time. Development of new oral cilia in dividing cells occurs prior to the
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establishment of rotational symmetry or linear organization of the cilia into membranelles. Proliferation of new oral cilia is accompanied by the formation of a network of inter-alveolar junctions, which may serve as tracks for early positioning and orientation of new basal bodies.

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