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Control of Initiation and Elongation of Cilia During Ciliary Regeneration in *Tetrahymena*

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Tetrahymena thermophila strain B could regenerate approximately 10% of its somatic ciliary mass in concentrations of cycloheximide believed to block all cytoplasmic protein synthesis. A quantitative study of the relative numbers and lengths of cilia regenerated in the presence and absence of cycloheximide under a variety of conditions suggested that specific initiation and elongation protein factors are involved in the control of ciliary morphogenesis in *Tetrahymena*.

The ciliated protozoan *Tetrahymena* can regenerate its cilia in both nutrient and non-nutrient medium. It has been shown in a number of laboratories that regeneration is completed within about a 2-h period after deciliation by the Rosenbaum and Carlson calcium shock procedure (1, 5, 8, 10, 13). It has also been shown by several laboratories that there is an induced synthesis of ciliary proteins associated with this regeneration (1, 5, 13; S. D. Guttman and M. A. Gorovsky, J. Cell Biol. **67**:149a, 1975). From the beginning, however, label dilution studies have consistently indicated that only part of the protein actually incorporated into the regenerating cilia is the result of this induced synthesis (5, 7, 13; Guttman and Gorovsky, J. Cell Biol. **67**:149a, 1975). This suggests that a significant proportion of the structural protein in regenerated cilia comes from preexisting cytoplasmic pools. It has also been found recently that tubulin induction occurs only after ciliary regeneration is well under way (1, 5), which leads to the same conclusion. The substantial use of stored proteins in ciliary regeneration by *Tetrahymena* suggests the operation of specific assembly control mechanisms which may be of general interest. For example, what keeps stored precursors from assembling in nondeciliated cells, and by what mechanism does deciliation derepress assembly of the precursors present at the time the cilia are removed?

In preliminary studies, we have recently found that strain B of *Tetrahymena thermophila* can regenerate a certain amount of ciliary material in the absence of protein synthesis. In the present study, we have examined this quantitatively under a variety of conditions in the hope of obtaining responses which might provide insight into the postulated assembly control mechanisms. Our results suggest that specific initiation and elongation factors may be involved in the control of ciliary assembly in *Tetrahymena*.

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MATERIALS AND METHODS

T. thermophila strain B stock cultures were maintained axenically in a tryptone medium (3) and grown for experiments in an enriched proteose-peptone medium (PPYGFe) containing yeast extract, glucose, and iron-ethylenediaminetetraacetic acid (15). Flask cultures containing 150 ml of growth medium were grown to a density of 3×10^5 cells/ml at 22°C. The cells were then rinsed by gentle centrifugation three times and suspended in 150 ml of the Carlsberg Institute inorganic medium (CBI-IM) (6). All experiments were begun after maintenance of the cells in inorganic medium for 4 h.

The cells were deciliated for regeneration experiments by the method of Rosenbaum and Carlson (10). In this method, the cells are concentrated in a small amount of a deciliation medium and briefly exposed to a high concentration of calcium. The cilia, weakened at the base by the calcium shock, are easily removed by drawing the cell suspension through a no. 16 needle fitted to a syringe. Immediately after the deciliation procedure, the cell suspension is diluted tenfold in inorganic medium, rinsed once, and resuspended in 150 ml of inorganic medium. The deciliated cells were allowed to regenerate their cilia with gentle shaking at room temperature. Deciliation and regeneration in the presence of cycloheximide was carried out as above, using 5 to 20 μg of cycloheximide per ml (Sigma Chemical Co.) in inorganic medium. A concentrated solution of cycloheximide in inorganic medium (1.5 mg/ml) was added to cells in the process of regenerating cilia in some experiments to achieve a final concentration of 10 or 20 μg/ml. In other experiments, the cells were deciliated and kept in the presence of 1 mM serotonin (Sigma Chemical Co.) or 5 mM dibutyryl adenosine 3′,5′-phosphate (cAMP) (Sigma Chemical Co.) in a 0.2 M sucrose solution in 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.2, throughout the regeneration period.

Samples of cells for scanning electron microscopy were fixed in 2% osmium tetroxide, dehydrated in an ethanol series, and critical point dried by the methods of Ruffolo (11). Micrographs were taken with a JEOL JSM 35C scanning electron microscope.

All scanning electron micrographs were photographically enlarged to a standard magnification of ×4,500. The relative numbers of somatic cilia in each sample were estimated by determining the mean number of somatic cilia per cell which could be seen in the micrographs. This considerably underestimates the true number of somatic cilia but permits comparisons between samples. The relative lengths of somatic cilia in samples were compared by measuring the lengths of ciliary images in the standard micrographs with a plastic rule. Ciliary mass was computed as the product of the numbers and relative average lengths of somatic cilia.

RESULTS

Regeneration in untreated cells. Scanning electron micrographs of *Tetrahymena* cells fixed immediately after deciliation showed that both oral and somatic cilia were effectively removed by the deciliation procedure (Fig. 1). After a lag period of approximately 20 min at 22°C, both oral and somatic cilia began to regenerate. However, it was observed that the oral cilia initiated regrowth synchronously, whereas somatic cilia initiated regrowth and elongated asynchronously. Cells fixed at 65 min after deciliation showed that only a fraction of the somatic basal bodies had started to reciliate, and these were apparently distributed randomly over the cell surface (Fig. 2). Oral cilia, on the other hand, appeared to be of uniform length. Cells with the full complement of cilia appeared as illustrated in Fig. 3.

The regrowth of somatic cilia was studied quantitatively by determining the relative numbers and lengths of regenerating cilia as a function of time at 22°C. The results (Table 1) indicate a continuous increase in the number of initiated basal bodies per cell from the end of the 20-min lag period until about 100 min after deciliation. This suggests that initiation is a controlled process in these cells; i.e., a specific initiation event must occur for each basal body to begin reciliation, and this event occurs at different times in different basal bodies. Eventually all basal bodies are initiated, elongation follows the initiation of each, and in this way the full complement of mature cilia is restored.

Regeneration in cycloheximide. *T. thermophila* strain B cells deciliated and kept in the presence of 5 to 20 μg of cycloheximide per ml regenerated about 15% of their cilia to approximately 67% of the final average length, or about 10% of the total ciliary mass (Table 1 and Fig. 4). Thin sections of cilia regenerated in cycloheximide appeared normal (not shown). These cilia were able to move; however, the beating was not coordinated over the cell surface, and the cells were therefore unable to swim.

Even though the effectiveness of cycloheximide in blocking protein synthesis in *Tetrahymena* at the concentrations used is well established (4, 16), we allowed cells to regenerate cilia in the presence of 5 μg of cycloheximide per ml and 0.83 μCi of [^{14}C]leucine per ml. No radioactivity could be detected in any of the proteins of these cilia in tests involving autoradiography after protein separation in 10% polyacrylamide gels. To the extent that we are able to determine, it therefore appears that the limited amount of regeneration which can be obtained in the presence of cycloheximide in *T. thermophila* occurs in the complete absence of protein synthesis.

The results obtained also suggest that the initiation of ciliary regrowth by basal bodies is more sensitive to cycloheximide than the process of elongation. The number of cilia in the 45-min cycloheximide sample was significantly less than the number in the 45-min control sample (Table 1), and no further increase occurred in cycloheximide after this time. In contrast, the mean ciliary length was the same in both 45-min samples, and there was a further increase in length in the presence of cycloheximide after 45 min. Elongation apparently continued to some extent after initiation was effectively stopped.

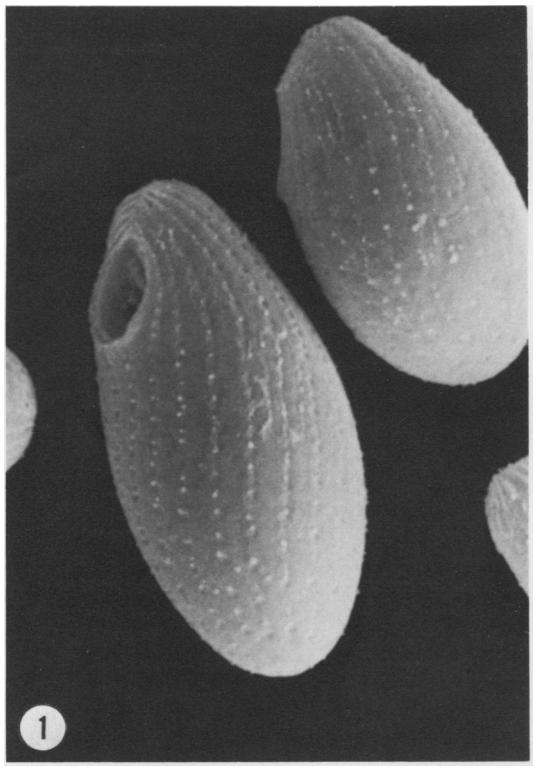
To test this further, we allowed cells to regenerate cilia for 45 min at 22°C, added cycloheximide at this time to a final concentration of 20 μg/ml, and then counted cilia and measured

FIG. 1. *Scanning electron micrograph of deciliated Tetrahymena cells. Cells were fixed for scanning electron microscopy immediately after deciliation by the method of Rosenbaum and Carlson (10). There is no evidence of the massive discharge of mucocysts reported after deciliation with dibucaine (12). Magnification for all figures, ×2,000.*

FIG. 2. *Micrograph of regenerating cells taken 65 min after deciliation. Both oral and somatic cilia have appeared, but the cells have not yet initiated the regrowth of all somatic cilia. Note the wide variation in ciliary lengths present.*

FIG. 3. *Micrograph of a Tetrahymena cell in non-nutrient medium showing the full complement of cilia.*

FIG. 4. *Micrograph of cells which have regenerated cilia in the presence of 20 μg of cycloheximide per ml (95 min). This is the maximal amount of ciliary regrowth that can occur in these cells in the absence of protein synthesis.*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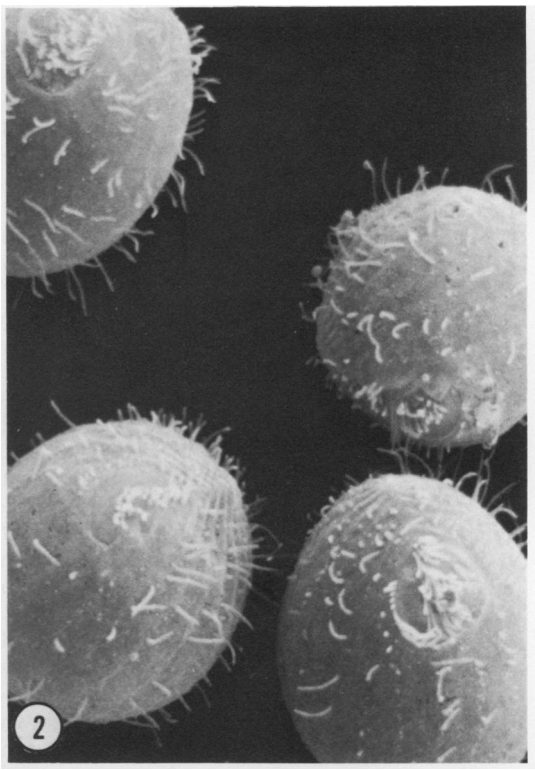
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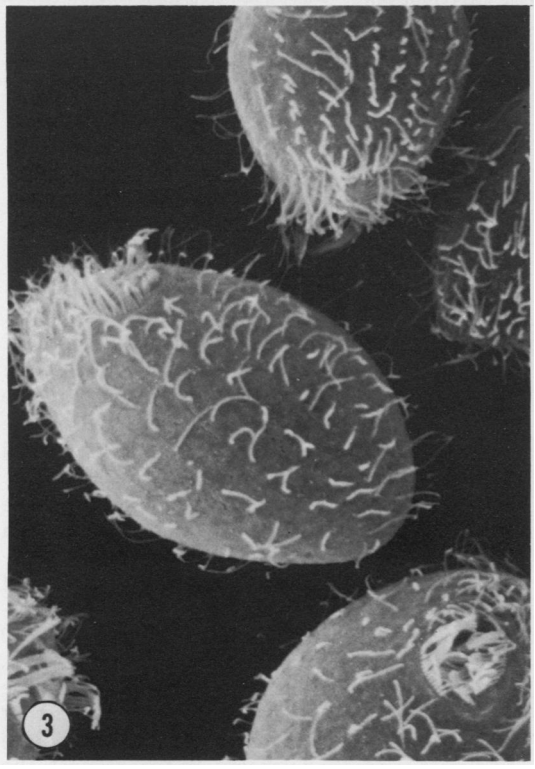
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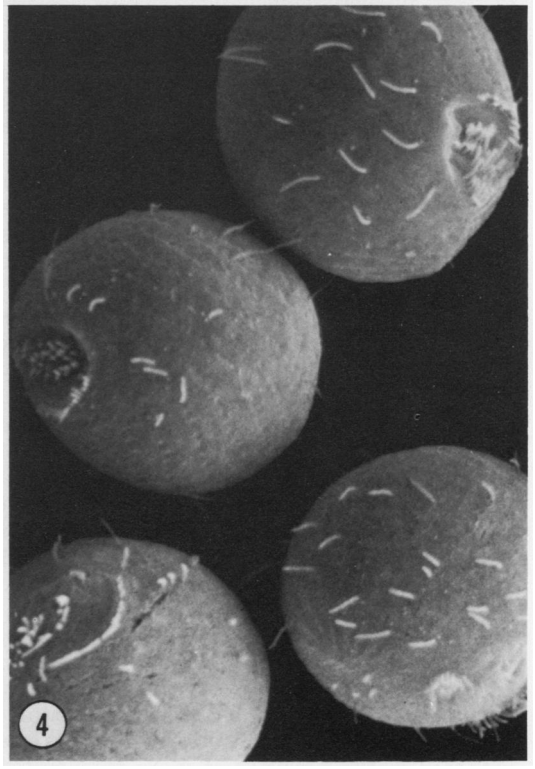
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lengths at 240 min. A comparison of these measurements with those obtained in the 45-min control sample (Table 2) shows that the initiation of cilia stopped immediately upon addition of the drug, but elongation of previously initiated cilia continued until they were as long as those in untreated cells. It seemed important to confirm this result in a separate experiment under different conditions. We therefore deciliated cells and regenerated cilia at a higher temperature (25°C) and used less cycloheximide (10 μg/ml). The mean numbers and lengths of cilia were greater at the time of drug addition than in the previous experiment (Table 2). However, the result was the same; there was no further increase in ciliary number, whereas cilia continued to elongate in the presence of cycloheximide. Figure 5 shows the condition of a typical cell at the time of cycloheximide addition in this experiment. The cell in Fig. 6 is representative of those seen 105 min later; although deficient in ciliary number, this cell has nearly full-length cilia.

We have shown previously that the amount of protein used in ciliary regeneration which is synthesized de novo during the process of regeneration depends on how the cells are grown before deciliation (13). Cells grown in a rich medium can apparently store ciliary precursors, with the result that the amount synthesized de novo will be less than for cells grown in a poor medium. This suggests the possibility that these cells might similarly regulate the factors limiting regeneration, in which case cells grown in a rich medium might regenerate more ciliary material in cycloheximide than cells grown in a poor medium. If so, it would also be of considerable interest to know whether they initiated more cilia, grew cilia to greater lengths, or both (Table 3). It is clear that the factors limiting for initiation and elongation were not regulated in this way; there was no significant difference in either the mean number of cilia or the mean ciliary length in cells grown in the two different media.

Effects of serotonin and cAMP. Regulatory roles in the control of ciliary regeneration in *Tetrahymena* have been postulated by Rodriguez and Renaud (9), who found that the onset of swimming behavior in regenerating cells was earlier in 1 mM serotonin, and also in 5 mM cAMP, than in control buffer. We attempted to confirm this conclusion by determining the numbers and lengths of regenerating cilia in control and treated samples at 45 min after deciliation at 25°C. Swimming behavior initiates shortly before this time in control cells, and any stimulatory effect of serotonin or cAMP should be reflected in the presence of more regenerated ciliary material in the treated cells. The results (Table 4) show that cells regenerating cilia in serotonin or cAMP had neither greater numbers nor longer lengths of cilia than control cells kept in inorganic medium or Tris buffer.

TABLE 1. *Time course of ciliary regeneration in normal and cycloheximide-treated cells at 22°C*

Sample (min)[a]	*n*	Ciliary no. ± SE[b]	Ciliary length ± SE[c]	Ciliary mass ± SE
45N	26	67 ± 4	3.7 ± 0.2	248 ± 19
65N	5	121 ± 6	6.9 ± 0.7	852 ± 5
95N	5	197 ± 4	8.0 ± 0.2	1,600 ± 3
240N	7	235 ± 12	9.2 ± 0.4	2,148 ± 180
Untreated	7	204 ± 3	10.2 ± 0.5	2,075 ± 123
45CHX	14	25 ± 3	3.5 ± 0.2	101 ± 11
240CHX	39	30 ± 2	6.8 ± 0.3	208 ± 13

[a] N following the time means that regeneration took place in non-nutrient medium. CHX following the time means that regeneration took place in non-nutrient medium containing cycloheximide (20 μg/ml).

[b] SE, Standard error.

[c] In arbitrary units from scanning electron micrographs at standard magnification.

TABLE 2. *Extent of regeneration after cycloheximide treatment at 45 min after deciliation*[a]

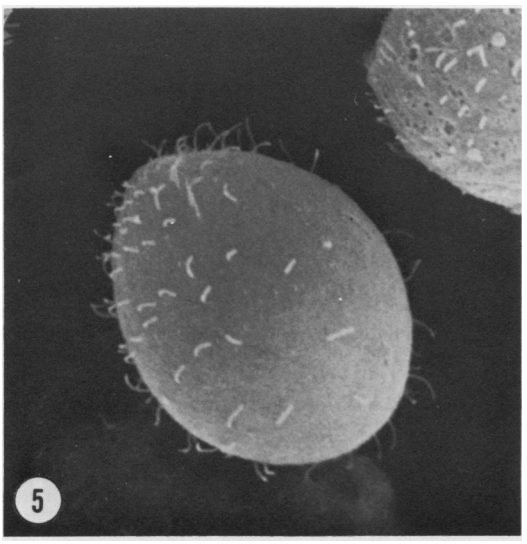
Temp (°C)	Time after deciliation (min)	*n*	No. of cilia/cell	Ciliary length
22	45	26	67 ± 4	3.7 ± 0.2
	240	15	59 ± 4	10.3 ± 0.4
25	45	12	97.5 ± 8.3	4.7 ± 0.36
	150	12	94.8 ± 10.3	9.9 ± 0.32

[a] Cycloheximide was added to a final concentration of 20 μg/ml (22°C) and 10 μg/ml (25°C).

DISCUSSION

Previous studies of deciliation and deflagellation in a wide variety of eucaryotic cell types have shown that these structures break off at a specific scission point near the base (2). In *Tetrahymena*, this has been shown to be just above the centrally located axosome and between the ciliary necklace and plaque particles within the ciliary membrane. After deciliation, the basal bodies with their very short ciliary stubs are covered over by surface membrane (12). These structures persist and serve to nucleate regrowth of the missing ciliary shafts.

We found in this study that the number of basal bodies in deciliated cells which had initiated ciliary regrowth increased steadily from 20 min until about 100 min after deciliation at 22°C. The sequential nature of activation within the cellular population of basal bodies indicates that the initiation of reciliation by any given basal body is a controlled process. In this respect, the



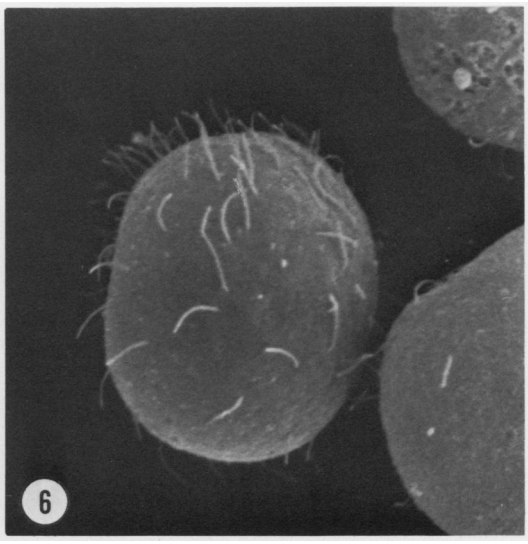


FIG. 5. *Scanning electron micrograph showing the extent of ciliary regeneration when cycloheximide (10 μg/ml) was added at 45 min after deciliation (25°C).*

FIG. 6. *Extent of ciliary regeneration at 105 min at 25°C after the administration of cycloheximide (10 μg/ml) at 45 min after deciliation. Although the number of regenerating cilia did not increase in cycloheximide (compare with Fig. 5), there was a considerable increase in the lengths of cilia in the process of elongating at the time the drug was added.*

TABLE 3. *Effect of growth conditions on the extent of ciliary regeneration in cycloheximide*

Medium	% of ciliary tubulin from pool[a]	*n*	No. of cilia/cell[b]	Ciliary length[b]
1% Proteose-peptone	33.7	28	46 ± 5	6.6 ± 0.4
PPYGFe	85.2	21	38 ± 16	6.9 ± 0.3

[a] Pool without cycloheximide. Data from Skriver and Williams (13).

[b] Determined at 150 min after deciliation in the presence of 5 μg of cycloheximide per ml at 25°C.

TABLE 4. *Effect of serotonin and cAMP on the rate of ciliary regeneration*

Medium	*n*	No. of cilia/cell[a]	Ciliary length[a]
CBI-IM[b]	10	54 ± 6	6.6 ± 0.4
Tris buffer	11	56 ± 6	4.6 ± 0.3
Serotonin (1 mM)	10	52 ± 4	5.1 ± 0.4
cAMP (5 mM)	10	48 ± 7	5.5 ± 0.4

[a] Numerical data from cells fixed at 45 min after deciliation.

[b] Inorganic medium of Hamburger and Zeuthen (6).

deciliated basal body is like the mitotic microtubule organizing center (MTOC) in eucaryotic cells generally; the mere presence of an unciliated basal body is not sufficient to promote assembly. Like the cell center in the mitotic cycle (14), it must achieve a competence to nucleate structural assemblies as a result of temporally regulated cellular control mechanisms. This general conclusion is also supported by recent results obtained by Frankel et al. (J. Frankel, E. M. Nelsen, and E. Martel, submitted for publication), who found that certain unciliated basal bodies formed relatively early in the cell cycle of *Tetrahymena* participate in a wave of ciliation which occurs later during furrow formation. The fact that this ciliation wave is restricted to a zone just posterior to the fission line suggests further that the control of basal body ciliation may also be spatially regulated under certain conditions.

The present study suggests that the attainment of nucleation competence by *Tetrahymena* basal bodies may involve specific initiator proteins which are synthesized only during the initiation process and are not stored by the cell. This is suggested by the response of cells regenerating cilia to the addition of cycloheximide; it was found that the initiation of regeneration by deciliated basal bodies stopped immediately upon addition of the drug, whereas the elongation of previously initiated cilia was apparently unaffected. The small amount of initiation which occurred in cells deciliated in the presence of cycloheximide may have been due to the presence of a limited amount of initiator protein associated with a normal replacement of cilia which may be going on in untreated cells. Top-

ographic considerations lead to the suggestion that the initiator protein may be a structural component of cilia which assembles into the scission zone. It is also possible that it is somehow responsible for the occurrence of differential scission in this region.

The results obtained with cycloheximide also lead to the suggestion that there may be specific protein factors limiting for ciliary elongation. Elongation, unlike initiation, did not stop immediately upon transfer of early regenerates to cycloheximide. Nevertheless, cilia regenerated in the presence of cycloheximide were never full length. The specificity of the postulated elongation factors is suggested by the results of the experiment using cells grown in the rich medium (PPYGFe). Our previous work has shown that *Tetrahymena* cells grown in this medium have enough stored ciliary tubulin to regenerate 85% of the cilia to full length (13). However, Tables 1 and 3 show that the 15 to 20% of basal bodies per cell which are initiated in cycloheximide in cells grown in this medium do not grow to full length (also evident in Fig. 4). This shows that major structural components like tubulin are probably not limiting for the elongation of cilia and leads to the suggestion of specific elongation factors. Together with the fact that few basal bodies initiated regrowth in these cells, the data suggest that major building blocks are ordinarily not limiting for ciliary regeneration and lead to the notion of specific control factors.

The indicated tight coupling between synthesis and utilization, a regulatory property apparently not shared by ciliary tubulin or dynein (13), may ultimately be of use in identifying and isolating the postulated ciliary initiation and elongation factors. Our results did not provide confirmation of the suggestion by Rodriguez and Renaud (9) that serotonin and cAMP may play a role in the control of ciliary regeneration.

ACKNOWLEDGMENT

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ADDENDUM IN PROOF

Several investigators have pointed out the potential usefulness of experiments involving two sequential deciliations in cycloheximide. So far we have not found a way of doing this which does not produce extensive cell damage.

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