

Flagellar Root Contraction and Nuclear Movement during Flagellar Regeneration in *Chlamydomonas reinhardtii*

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Abstract. When *Chlamydomonas* cells are deflagellated by pH shock or mechanical shear the nucleus rapidly moves toward the flagellar basal apparatus at the anterior end of the cell. During flagellar regeneration the nucleus returns to a more central position within the cell. The nucleus is connected to the flagellar apparatus by a system of fibers, the flagellar roots (rhizoplasts), which undergo a dramatic contraction that coincides with anterior nuclear movement. A corresponding extension of the root system, back to its preshock configuration is observed as the nucleus retracts to a central position. Anterior displacement of the nucleus and flagellar root contraction require free calcium in the medium. Nuclear movement and flagellar root contraction and extension are not sensitive to

inhibitors of protein synthesis (cycloheximide), or drugs that influence either microtubules (colchicine) or actin-based microfilaments (cytochalasin D). Detergent-extracted cell models contract and extend their flagellar roots and move their nuclei in response to alterations of free calcium levels in the medium. Cycles of nuclear movement in detergent-extracted models require ATP to potentiate the contractile mechanism for subsequent calcium-induced contraction. Flagellar root contraction and nuclear movement in *Chlamydomonas* may be causally related to signaling of induction of flagellar precursor genes or to the transport of flagellar precursors or their messages to sites of synthesis or assembly near the basal apparatus of the cell.

A model system for studying motility, precursor biosynthesis, assembly, structure, and genetics of flagella is *Chlamydomonas* (8, 9, 29). *Chlamydomonas* cells release their flagella in response to a variety of adverse environmental conditions (pH or temperature extremes, treatment with detergents, alcohols, and other irritants) through an active process called flagellar autotomy or "self-cutting" (cf. 12). Upon return to favorable conditions the cells rapidly regenerate new flagella using both a preexisting pool of flagellar precursors and newly synthesized flagellar proteins (11, 20, 28).

The flagellar apparatus of many green algal species, including *Chlamydomonas reinhardtii*, is physically linked to the cell nucleus by flagellar roots (rhizoplasts) composed in large part of a fibrous calcium-binding contractile protein of 20,000 mol wt (22, 24, 30). After detergent lysis of *Chlamydomonas* cells, basal bodies remain associated with the nucleus via this connecting system of fibrous roots (30). Similarly, centrioles of a variety of mammalian cells are spatially associated with the nucleus; centrioles often remain physically associated with interphase nuclei after detergent lysis and centrifugation (6, 13). Although much speculation is exercised in the literature, little is understood concerning the functional significance of the centriole/basal body-nuclear association.

Here, we demonstrate that flagellar roots linking the fla-

gellar basal apparatus to the nucleus of *Chlamydomonas* undergo a dramatic and rapid calcium-induced contraction in living cells at the time of flagellar excision. This contraction of the flagellar roots results in nuclear movement toward the flagellar basal apparatus. Subsequent reextension of the flagellar roots proceeds as the nucleus moves back to a more central position within the cell. The kinetics and duration of the flagellar root contraction are coincident with the period of flagellar precursor induction (9, 27). Both flagellar precursor induction and nuclear movement require free calcium in the medium. Although both phenomena could be independent responses to elevated intracellular calcium levels, we are led to question whether there is a causal relationship between flagellar root contraction and the signaling of nuclear events leading to flagellar precursor induction.

Materials and Methods

Cell Culture

Chlamydomonas reinhardtii, wild type strain 137c derivative N0mt+ (provided by Dr. J. Jarvik, Carnegie-Mellon University, Pittsburgh, PA), was maintained in Medium I of Sager and Granick (21) in 10-ml tubes under 12:12 h light/dark cycle at 24°C. All of the experiments described were carried out with gametes. These were obtained by transferring log-phase cultures into nitrogen-deficient medium and maintaining the cells under constant illumination for 24 to 36 h before experimentation.

Conditions for Deflagellation and Flagellar Regeneration

Cells were pelleted by centrifugation and resuspended in fresh culture medium buffered with 10 mM Tris HCl, 1 mM K_2HPO_4 , 2.2 mM Na-acetate, pH 6.8 at a cell density of 10^7 cells per ml. An aliquot, designated zero-time sample, was removed and fixed in 3% formaldehyde and 1% glutaraldehyde in PBS, pH 7.2. The remaining cell suspension was pH shocked rapidly by adding 1 N acetic acid dropwise to achieve a pH of 4.3. After 2 min the pH was adjusted to 7.2 using 1 N KOH. The suspension was returned to the light and aliquots were removed at 15-min intervals over a 2-h period and fixed as above. Cells were also deflagellated by mechanical shear by vortexing in a fluted tube.

Flagellar Length and Nuclear Position Determinations

Nuclei were stained with the DNA fluorescent probe 4,6-diamidino-2-phenylindole (DAPI)¹ at 1 μ g per ml and observed under UV epi-fluorescence (excitation 365 nm, barrier 420 nm). DAPI images were recorded on Tri-X film at exposures of 30 s and developed in D-19 for 3 min. Flagellar length and nuclear position were determined with a calibrated ocular micrometer in a Nikon Optiphot microscope using a 40 or 100 $\times$ oil immersion objective, respectively. Nuclear position was measured from the anterior-most end of the cell to the top of the nucleus. 30 cells for each treatment and time point were scored, and the results are expressed as the mean $\pm$ 1 SD.

Immunofluorescence of Flagellar Roots

Monoclonal antibodies (17E10, A. Baron and J. L. Salisbury) directed against a major 20,000-mol-wt component of fibrous flagellar roots was used as hybridoma culture supernatant to stain *Chlamydomonas* cells. Briefly, BALB/c mice were immunized with flagellar root protein isolated from *Tetraselmis striata*. Hybridomas were screened using an ELISA technique and subsequently checked by immunofluorescence using *Chlamydomonas*. Monoclonal 17E10 stains the flagellar roots of a variety of lower eucaryotes and reacts with a 20,000-mol-wt protein from *Tetraselmis* and *Chlamydomonas* when assayed by Western immunoblot techniques. For immunofluorescence studies, cells were fixed as above, except the glutaraldehyde concentration was reduced to 0.1%, washed in PBS, permeabilized in -20°C methanol for 5 min, followed by -20°C acetone for 5 min, and air dried. The cells were incubated in primary antibody (17E10) diluted 1:500 in PBS containing 5% FCS and 5% glycerol for 1 h, washed three times in PBS, and reacted with secondary fluorescein-conjugated goat anti-mouse IG (1:400; Cappel Laboratories, Cooper Biomedical, Inc., Malvern, PA) for 30 min. The preparations were washed for 15 min in several changes of PBS, mounted in a glycerol/PBS mixture containing 2% *n*-propyl-gallate (Kodak), pH 8.0, and observed using a Nikon Optiphot microscope equipped for epi-fluorescence (excitation 450–490 nm, barrier 520 nm). Immunofluorescence images were exposed for 10–20 s and recorded on Tech Pan 2415 film that was hypersensitized in a Lumicon device (model 300; Lumicon, Livermore, CA) according to the instructions given by the manufacturer and developed in D-19 developer for 4 min.

Reactivation of Detergent-extracted Cell Models

Cells were pelleted by centrifugation and resuspended in a solution containing 0.01% NP-40, 10 mM Tris HCl, 1 mM K_2HPO_4 , 2.2 mM Na-acetate, pH 6.8 with either 3 mM $CaCl_2$ (high calcium) or 3 mM EGTA (low calcium), and 3 mM ATP where indicated in the legend to Table I. Cell models were incubated for 5 min in each calcium treatment and 30 min in each EGTA treatment and transferred between high and low calcium buffers with two intervening wash steps in the same buffer without added ATP, calcium, or EGTA. Flagellar root morphology and nuclear position determinations were made as indicated above.

Results

Flagellar Regeneration

Fig. 1 A shows a typical flagellar regeneration curve for

1. Abbreviation used in this paper: DAPI, 4,6-diamidino-2-phenylindole.

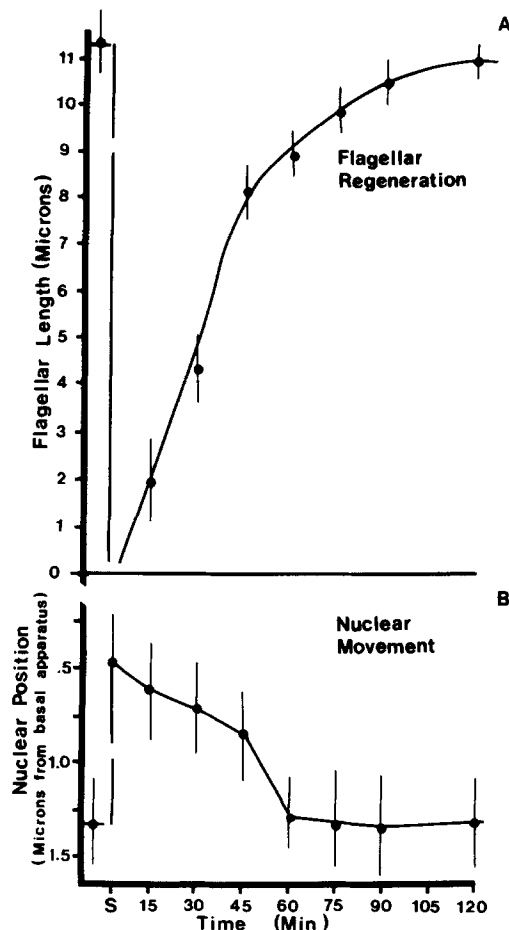


Figure 1. Flagellar regeneration and nuclear movement in *Chlamydomonas* after pH shock. (A) Kinetics of flagellar regeneration after pH shock. (B) Nuclear position after pH shock. Note change in scale. Initially, the nucleus is in a central position, ~ 1.5 μ m from the flagellar basal apparatus. Immediately after pH shock, the nucleus moves to the anterior-most region of the cell where it remains for ~ 30 –45 min. The nucleus then returns to a more central position. Each point represents the mean value for 30 individual cells. Standard deviation of the data is indicated with vertical bars.

Chlamydomonas, after pH shock deflagellation. *Chlamydomonas* cells normally have a pair of flagella, each ~ 11 μ m long. Although, this value may vary slightly for particular strains and culture conditions, it is generally characteristic for a given population of cells. Cells respond to adverse environmental conditions, such as pH shock, by shedding their flagella. If the cells are immediately returned to favorable conditions, they will regrow new flagella with rapid decelerating kinetics, such that by 90 min flagellar length has returned to normal preshock values. The kinetics of flagellar regrowth shown here are typical for *Chlamydomonas* (9).

Nuclear Movement Coincides with Deflagellation and Flagellar Regeneration

Fig. 1 B illustrates changes in nuclear position of the same cells studied in Fig. 1 A, as observed by fluorescence light microscopy. Each *Chlamydomonas* cell has a single nucleus which is ~ 3 μ m in diameter and is typically located ~ 1.3 – 1.7 μ m from the anterior end of the cell. Immediately after pH

shock, or mechanical shear of the flagella (data not shown), the nucleus rapidly moves to the anterior most region of the cell to lie just below the flagellar apparatus. This initial movement is extremely rapid since we have been unable to capture intermediate nuclear positions even by quickly fixing the cells after pH shock. The nucleus begins to move back to a more central position immediately on return to favorable pH conditions. The return movement is slow and appears biphasic. The nucleus moves $\sim 1/3$ of the way back to a central position during the first 45 min, then rapidly moves the rest of the way during the period between 45 and 60 min. The standard error bars for the data shown in this and subsequent figures reflect the limits of resolution of the optical methods used. All cells that survive pH shock or mechanical shear, and that rapidly regrow their flagella show nuclear movements as described here. Typically, living cells that have not been subjected to environmental stress show a centrally placed nucleus. However, occasional dead cells seen in otherwise healthy cultures often show anterior displacement of their nuclei.

Nuclear Movement Corresponds to Contraction and Extension of Flagellar Roots

Fig. 2 illustrates individual cells under phase contrast optics (a-c), stained for DAPI nuclear fluorescence (d-f), and stained with monoclonal antibodies to show flagellar roots (g-i). Cells are shown before and immediately after pH shock, and after 90 min of recovery. The top row of images shows flagellar length for the three treatment times. Initially, cells possess two flagella of equal length ($\sim 11 \mu\text{m}$; Fig. 2 a). After pH shock, cells drop or release their flagella and are no longer motile (Fig. 2 b). By 90 min after return to favorable culture conditions the flagella have regrown and attained normal length and motility (Fig. 2 c).

The middle row of images shows DAPI staining of nuclear and chloroplast DNA. The nuclear profile in the preshocked cell (Fig. 2 d) is circular (indicating a nearly spherical shape), $\sim 3 \mu\text{m}$ in diameter, and centrally positioned within the cell $\sim 1.3\text{--}1.7 \mu\text{m}$ from the flagellar apparatus. After pH shock (Fig. 2 e), the nucleus moves to the anterior-most region of the cell. In the example shown here, the nucleus shows a distinctly pyriform shape with the nuclear "apex" pointed toward the flagellar apparatus. Although the change in nuclear shape is observed in all cells immediately after pH shock, it can be clearly seen in only $\sim 1/3$ of the cells due to orientation on the slide. By 90 min after pH shock the nucleus has moved back to a central position (Fig. 2 f), again becoming nearly spherical in shape.

Indirect immunofluorescence localization using monoclonal antibodies directed against the 20,000-mol-wt flagellar root protein is shown in Fig. 2, g-i. The flagellar root system of a preshocked *Chlamydomonas* cell (Fig. 2 g) appears in an extended configuration and links the basal apparatus of the flagella to the nucleus. The distal fiber linking the two basal bodies is also distinctly labeled. The flagellar roots extend from the distal fiber into the cytoplasm toward the nucleus and branch into 10–20 distinct fimbria which surround the nucleus and embrace the nuclear envelope. After pH shock, or mechanical shear (data not shown), the fibrous flagellar root system undergoes a dramatic contraction (Fig. 2 h). Structural details are difficult to discern at this stage, how-

ever, it is apparent that the roots are drawn up toward the flagellar apparatus. In some instances individual extensions of the roots can still be seen projecting out from the contracted mass. By 90 min after pH shock and return to favorable conditions, the fibrous flagellar roots have reextended into the cytoplasm and appear similar to their preshock configuration (Fig. 2 i).

Nuclear Movement Requires Calcium

Fig. 3 shows that both flagellar regeneration (Fig. 3 A) and the initial nuclear movement (Fig. 3 B) require free calcium in the medium. When free Ca^{++} levels in the extracellular medium are maintained below $\text{pCa } 7$ with Ca/EGTA buffers, flagellar regrowth and anterior nuclear movements are inhibited. Although there is some variability in nuclear position around its typical central location in the cell, no gross nuclear movement is observed when the free calcium in the medium is held at these low levels.

Nuclear Movement Is Not Sensitive to Actin- or Tubulin-directed Drugs or to Inhibitors of Protein Synthesis

Fig. 4 illustrates that nuclear movement after pH shock is not sensitive to drugs that inhibit protein synthesis (cycloheximide, $30 \mu\text{g}/\text{ml}$), or to colchicine ($10 \text{ mg}/\text{ml}$), or cytochalasin D ($10 \mu\text{g}/\text{ml}$). Both the initial rapid nuclear displacement toward the anterior end of the cell and the subsequent slow recovery to the typical central nuclear position follow normal kinetics in each of these treatments. As expected, flagellar regeneration is reduced to approximately half normal levels by cycloheximide treatment. Flagellar regeneration fails to proceed at all in the presence of colchicine (Fig. 4 A), while cytochalasin D treatment results in no apparent affect on flagellar regeneration. These results indicate that neither actin nor microtubule-based cytoskeletal structures are involved in flagellar root contraction or extension and nuclear movement. Nuclear movement also does not require synthesis of new proteins.

Nuclear Movement in Reactivated Cell Models

Reactivation of nuclear movement in detergent extracted cell models is shown in Table I. *Chlamydomonas* cells gently extracted in buffers containing the detergent NP-40 (0.01%) and the Ca^{++} -chelating agent EGTA (3 mM) possess a centrally placed nucleus. Transfer of these models into buffer containing CaCl_2 (3 mM) results in both excision of the flagellar axoneme and rapid movement of the nucleus to the anterior-most region of the cell. Subsequent transfer of the models back into EGTA (low calcium)-containing buffers results in reextension of the flagellar roots and movement of the nucleus back to a central position. However, if the models are returned for a second exposure to calcium-containing buffers flagellar root contraction and nuclear movement do not occur. Similarly, cell models made in buffers initially containing high calcium possess contracted roots and nuclei displaced toward the flagellar basal apparatus. After transfer of these models into buffers containing EGTA (low calcium) the flagellar roots extend and the nuclei return to a central position. At this stage, the flagellar roots again become refractory to subsequent treatment with high calcium. The contractile

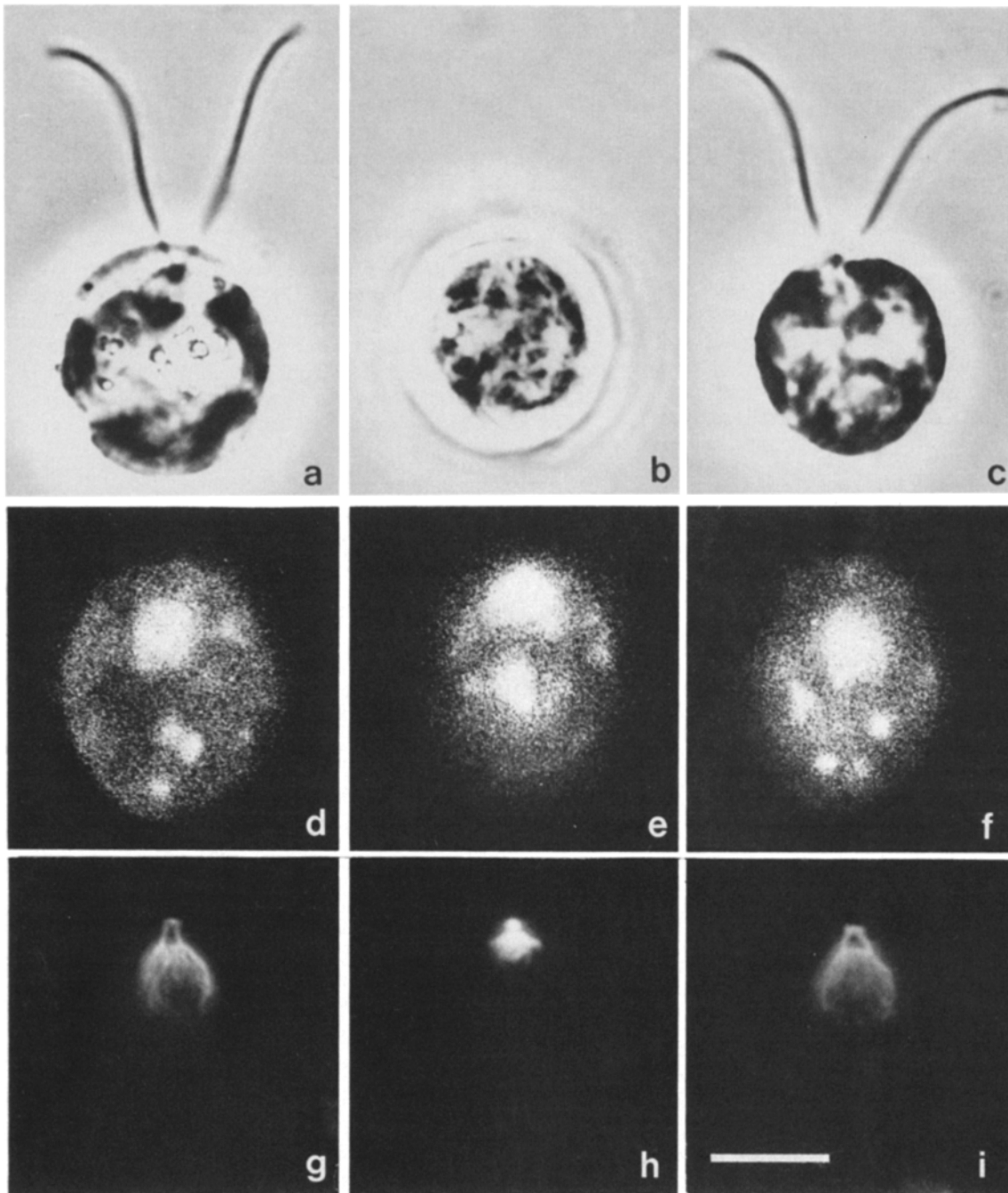


Figure 2. Flagellar length, nuclear position, and flagellar root morphology before pH shock (*a*, *d*, and *g*), immediately after pH shock (*b*, *e*, and *h*), and 90 min after pH shock (*c*, *f*, and *i*). The upper row shows phase contrast images of *Chlamydomonas* cells to illustrate the presence of flagella in (*a*), their excision after pH shock (*b*), and regrowth during flagellar regeneration (*c*). The middle row shows DAPI staining of nuclear DNA and illustrates nuclear position as central (*d*), anterior (*e*), and central (*f*). The lower row shows indirect immunofluorescence of flagellar roots using monoclonal antibodies directed against the major flagellar root protein. Before pH shock (*g*) the flagellar roots extend from the distal fiber of the flagellar basal apparatus into the central region of the cell where individual fimbria embrace the nuclear envelope. After pH shock (*h*), the flagellar roots contract drawing the nucleus toward the basal apparatus. The flagellar roots regain their extended configuration between 45 and 90 min after pH shock (*i*). Bar, 5 μ m.

system can again become potentiated for calcium-induced contraction and nuclear movement if the models are treated with ATP either before or during subsequent calcium treatments.

Discussion

Nuclear Movement and Contractile Flagellar Roots

We have demonstrated that the nucleus of *Chlamydomonas*

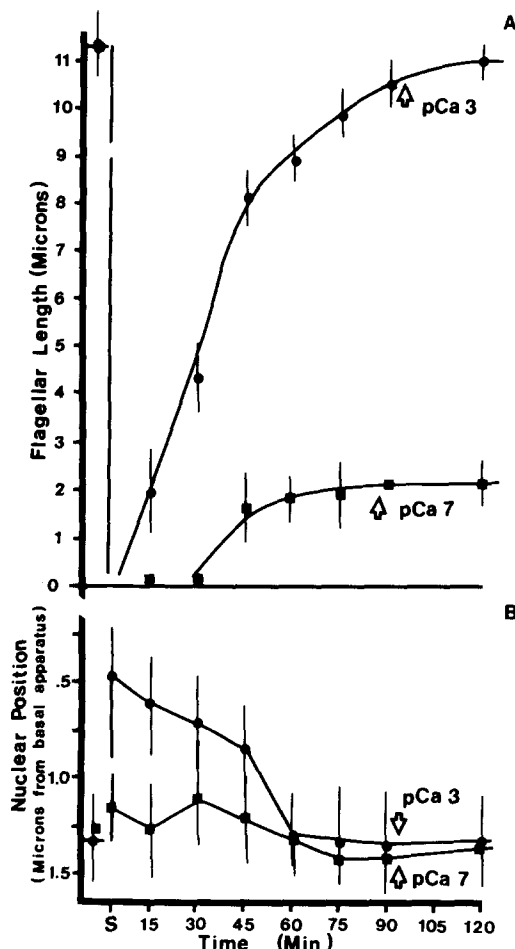


Figure 3. Flagellar regeneration and nuclear movement require free calcium to be present in the extracellular medium. (A) Flagellar regeneration at pCa 3 and pCa 7. Note that in low free calcium a few cells grow short flagella. (B) Nuclear movement is inhibited at low free calcium levels, pCa 7.

undergoes a rapid displacement toward the basal apparatus after flagellar excision, and that on return to favorable conditions the nucleus slowly returns to a more central position within the cell. These nuclear movements are mediated by the contraction and reextension of the fibrous flagellar root system which links the basal apparatus to the nucleus. We estimate that the fibrous roots branch to form 10–20 fimbria that embrace the nuclear envelope. Previously, we have demonstrated that fibrous flagellar roots are contractile organelles composed predominantly of low molecular weight calcium-binding phosphoproteins (22, 24, 30) which resemble, in many respects, *Vorticellid* spasmin (3). Nuclear movement during flagellar regeneration in *Chlamydomonas* is dependent on extracellular calcium and is not sensitive to inhibitors of protein synthesis or agents that perturb actin or tubulin-based cytoskeletal structures. These nuclear movements in *Chlamydomonas* occur within a restricted volume of cytoplasm and are subtle in that the entire nuclear displacement is $<1.5 \mu\text{m}$. For these reasons, nuclear movement in *Chlamydomonas* has not been noted by previous workers studying flagellar regeneration.

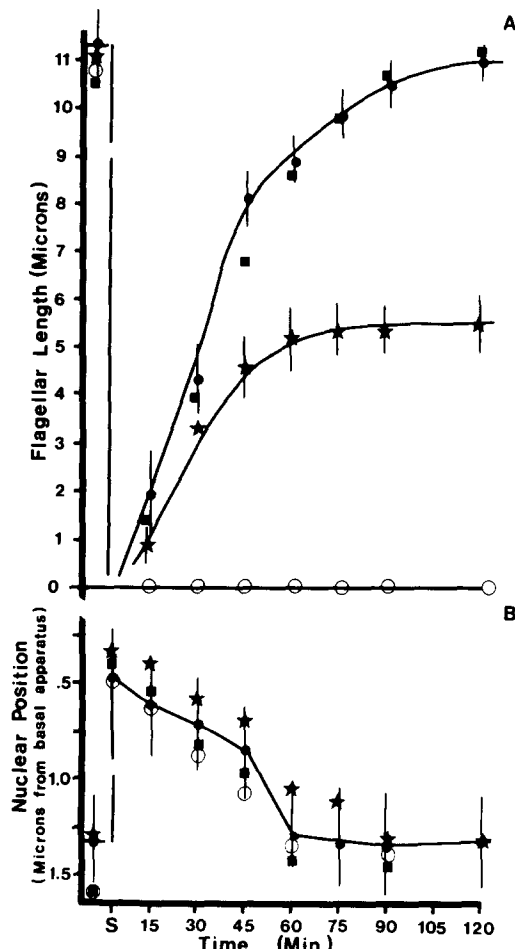


Figure 4. Nuclear movement is not sensitive to drugs that inhibit protein synthesis or influence microtubules or actin filaments. (A) Flagellar regeneration kinetics under control conditions (solid circles), in the presence of 10 $\mu\text{g/ml}$ cytochalasin D (solid squares), 30 $\mu\text{g/ml}$ cycloheximide (solid stars), and 10 mg/ml colchicine (open circles). (B) Nuclear movement under control conditions (solid circles), in the presence of 10 $\mu\text{g/ml}$ cytochalasin D (solid squares), 30 $\mu\text{g/ml}$ cycloheximide (solid stars), and 10 $\mu\text{g/ml}$ colchicine (open circles).

ATP Potentiates Flagellar Roots for Contraction

Our experiments with detergent-extracted cell models demonstrate that flagellar root contraction and extension and the corresponding nuclear movements are directly dependent on free calcium levels present in the solution. Cell models, initially extracted in buffers containing low levels of free calcium, show a central nucleus and extended flagellar roots. On subsequent treatment with millimolar free calcium, these models excise their flagellar axonemes, contract their flagellar roots, and move their nucleus toward the flagellar basal apparatus. Subsequent reextension of the flagellar roots and movement of the nucleus back to a more central location proceeds on chelation of the calcium by EGTA. A second round of contraction and nuclear movement, however, does not occur when the calcium levels are again raised; the contractile system becomes refractory to calcium. This refractory phase can be overcome by incubating the models in ATP under ei-

Table 1. ATP Potentiates Calcium-induced Nuclear Movement in Detergent-extracted Cell Models

Cell models processed through	Distance from basal apparatus ($\pm$ SD)	Nuclear position
	μm	
Lysis in 3 mM EGTA	1.81 (0.22)	Central
↓		
3 mM Ca^{++}	0.25 (0.19)	Anterior
↓		
3 mM EGTA	1.16 (0.32)	Central
↓		
3 mM Ca^{++}	1.20 (0.24)	Central
Lysis in 3 mM Ca^{++}	0.31 (0.20)	Anterior
↓		
3 mM EGTA	1.44 (0.35)	Central
↓		
3 mM Ca^{++}	1.43 (0.32)	Central
↓		
3 mM Ca^{++} and 3 mM ATP	0.12 (0.18)	Anterior
Lysis in 3 mM Ca^{++}	0.27 (0.19)	Anterior
↓		
3 mM EGTA	1.31 (0.41)	Central
↓		
3 mM Ca^{++}	1.47 (0.30)	Central
↓		
3 mM EGTA and 3 mM ATP	1.71 (0.23)	Central
↓		
3 mM Ca^{++}	0.20 (0.20)	Anterior

Each value represents the mean of 30 measurements for each treatment.

ther low or high free calcium conditions. On transfer of the ATP-potentiated models into solutions containing millimolar free calcium they will again contract their flagellar roots and move their nuclei. Our earlier studies show that the major 20,000 mol-wt flagellar root protein is phosphorylated during flagellar root contraction/extension cycles (22, 24). Based on the observations presented here, we would suggest that protein phosphorylation is a necessary step in the potentiation of the flagellar root protein for subsequent calcium-induced contraction.

Functional Implications of Nuclear Movements and Flagellar Root Contraction in *Chlamydomonas*

The significance of nuclear movement in small interphase cells such as *Chlamydomonas* is not immediately obvious. The size of individual *Chlamydomonas* cells precludes large scale nuclear movements as observed in algal and fungal filaments (2, 14, 16, 17) and in larger animal cells and syncytia (26). Nuclear movement in *Chlamydomonas* may facilitate the transport of flagellar precursors to the base of the flagellar apparatus. Although this possibility can not be discounted at present, this seems unnecessary because the distance involved is quite small and should not pose a significant barrier to diffusion of precursors. We have not discerned nuclear rotations (1, 4, 7) in *Chlamydomonas* cells because distinctive chromatin or nucleolar staining is not obvious at the level of investigation we have used.

The striking feature of nuclear movement in *Chlamydo-*

monas is that the initial movement coincides with flagellar excision, and the kinetics of recovery to a central nuclear location coincides with the timing of flagellar regeneration. Indeed, the period of time that the nucleus is held at the anterior-most region of the cell (the first 30–45 min after flagellar excision) coincides with the timing of induction of flagellar precursors including tubulin (cf. 27). Excision of *Chlamydomonas* flagella stimulates a rapid and coordinate synthesis of flagellar proteins (11, 27). The mechanism for this induction is unknown (9). Experiments in which the cytoplasmic pool of unassembled tubulin is raised by citrate induced resorption, or by colchicine treatment indicate that the pool size of flagellar precursors is not directly involved in the signaling of flagellar precursor induction (11, 28). Likewise, mutants that fail to regenerate their flagella after excision, yet induce tubulin synthesis under these same conditions, indicate that flagellar regeneration itself is not the signal for precursor induction (10). Flagellar root contraction and flagellar precursor induction may be independent responses to elevated intracellular calcium levels brought about by the conditions used to deflagellate the cells. However, since tubulin induction (11) and contraction of flagellar roots both require calcium, and since the kinetics of nuclear movement corresponds to that of flagellar precursor induction, we are led to question whether there is a causal relationship between root contraction and flagellar precursor induction. Contraction of flagellar roots might signal nuclear events which lead to induction of gene transcription. This is not a new concept (5, 15, 18, 19, 25). *Chlamydomonas* flagellar root contraction and flagellar regeneration may provide a model system uniquely suited for testing the hypothesis that signal transduction events propagated through the cytoplasm to the nucleus may, in certain cases, be mediated by a physical (cytoskeletal) linkage (18, 19).

Finally, nuclear movement toward the basal apparatus described here has general features similar to chromosome movement during mitosis. This is particularly relevant since we have observed flagellar root homologues at mitotic spindle poles of *Chlamydomonas* as well as other mitotic cells, including mammalian cell types (23; Baron, A., and J. L. Salisbury, unpublished observations). Analysis of flagellar root localization during the cell cycle and in flagellar root mutants, and studies of selective drug sensitivity are currently being used to further characterize the role of contractile flagellar roots and nuclear movement in *Chlamydomonas*.

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