

# mRNAs for Microtubule Proteins Are Specifically Colocalized during the Sequential Formation of Basal Body, Flagella, and Cytoskeletal Microtubules in the Differentiation of *Naegleria gruberi*

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**Abstract.** We have examined the distribution of four mRNAs— $\alpha$ -tubulin,  $\beta$ -tubulin, flagellar calmodulin, and Class I mRNA—during differentiation of *Naegleria gruberi* amebas into flagellates by in situ hybridization. Three of the four mRNAs— $\alpha$ -tubulin,  $\beta$ -tubulin, and Class I mRNA—began to be colocalized at the periphery of the cells as soon as transcription of the respective genes was activated and before any microtubular structures were observable. At 70 min after the initiation of differentiation, these mRNAs were relocated to the base of the growing flagella, adjacent to the basal bod-

ies and microtubule organizing center for the cytoskeletal microtubules. Within an additional 15 min, the mRNAs were translocated to the posterior of the flagellated cells, and by the end of differentiation (120 min), very low levels of the mRNAs were observed. Cytochalasin D inhibited stage-specific localization of the mRNAs, demonstrating that RNA localization was actin dependent. Since cytochalasin D also blocked differentiation, this raises the possibility that actin-dependent RNA movement is an essential process for differentiation.

**N**AEGLERIA *gruberi* amebas differentiate into swimming flagellates in less than 2 h after initiation of differentiation (6). During differentiation, *N. gruberi* changes its shape sequentially from an amorphous ameba to a sphere and then to a flagellate with a regular contour, forming two basal bodies, two flagella, and cytoskeletal microtubules (CSMT)<sup>1</sup> de novo (see Fig. 7). Using a monoclonal antibody against *Naegleria*  $\alpha$ -tubulin, Walsh showed that *Naegleria* amebas do not have microtubule-based structures except for the mitotic spindle fibers in dividing cells (25). Microtubule structures first appear in the cytoplasm of a cell ~50–55 min after initiation of differentiation, and then two basal bodies are formed on the cell periphery. Two flagella begin to appear from the basal bodies, and, at 70 min after initiation of differentiation, 50% of the cells have visible flagella. When the flagella reach about 10  $\mu$ m in length, 80 min after initiation, a complex array of CSMT is observed radiating from the base of the flagella at which the basal bodies are located. At 120 min after the initiation, the flagella reach full length (~15  $\mu$ m) and the

cytoskeletal microtubules elongate, reaching the distal end of the cell (25). Thus, the formation of basal bodies appears to be the key step in the formation of the flagella and the CSMT during the differentiation of *N. gruberi*.

The basal body is the microtubule organizing center for flagellar (or ciliary) axonemal microtubules and is structurally identical to a centriole. In a few organisms and cell types, including *Naegleria gruberi*, the basal body or its surrounding material seems to act as a microtubule organizing center in the formation of cytoskeletal microtubules (3, 12, 14). However, the mechanism of basal body formation and its function in the formation of the microtubule systems still remain to be elucidated.

While the composition and assembly of the basal body are poorly understood, it could be assumed that the local concentration of the component proteins should be high enough to initiate the assembly of the organelle and that the colocalization of the component proteins in a limited area of the cell would greatly facilitate this process.

Localization of mRNA is one of the possible mechanisms by which a cell can concentrate a protein in a specific area (13, 22, 26). Using in situ hybridization, we examined the distribution of four mRNAs that are transiently and coordinately accumulated during differentiation of *N. gruberi*, differentiation-specific (DS) mRNAs (19, 20). Two of the four DS mRNAs encode  $\alpha$ - and  $\beta$ -tubulin, the major components of flagellar axoneme and cytoskeletal microtubule system (21). The third DS mRNA, the flagellar cal-

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1. *Abbreviations used in this paper:* CSMT, cytoskeletal microtubules; DIC, differential interference contrast; DIG, digoxigenin; DS, differentiation-specific.

modulin mRNA, encodes a calmodulin (21) that is found in the flagellar axoneme but not in the cell body (7, 10). The protein product of the fourth DS mRNA that is ~7-kb long has not been identified (Class I mRNA [20]). We report here that these DS mRNAs are specifically localized in a specific area from which the flagella grow, and we present evidence that this specific localization of the DS mRNAs could have an important role in the formation of the microtubule systems during the differentiation of *Naegleria* amebas into flagellates.

## Materials and Methods

### Cell Growth and Differentiation

Growth and differentiation of *N. gruberi* strain NB-1 was carried out as described elsewhere (6, 19).

### Cell Fixation

Differentiating cells were fixed in a formaldehyde fixation buffer (1× fixation buffer; 25 mM sodium phosphate, pH 7.2, 1% formaldehyde, 63 mM sucrose, 1 mM Tris-HCl [25]). 200  $\mu$ l of differentiating cells ( $10^7$  cells/ml) and 200  $\mu$ l of 2 mM Tris-HCl, pH 7.6, at 20°C were added into 400  $\mu$ l of 2× fixation buffer and incubated for 5 min at 4°C. 70  $\mu$ l of the fixed cells were applied to a glass slide coated with gelatin-chromium potassium sulfate (27) and air dried for 3–4 h at room temperature. The fixed cells were treated with 0.1% NP-40 in the fixation buffer for 5 min at room temperature, washed four times with PBS buffer, and then dried at room temperature. The fixed and permeabilized cells were then soaked sequentially in methanol and acetone for 10 min at 4°C.

### Probe Preparation

A cDNA fragment of each mRNA (PstI restriction fragment of pcNg 8–5, 580 bp, for  $\beta$ -tubulin [20]; EcoRI fragment of  $\alpha$ 13, 450 bp, for  $\alpha$ -tubulin [4]; PstI fragment of pcNg 1–8, 520 bp, for Class I [20]; PstI fragment of pcNg 44, 375 bp, for flagellar calmodulin [20], and PstI fragment of pcNg 3–28, 700 bp, for a nonspecific mRNA [20]) was labeled with digoxigenin (DIG)-11-dUTP by using a DIG DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany).

### In Situ Hybridization

In situ hybridization with the DIG-labeled cDNA probes was carried out as suggested by the manufacturer. The cells were prehybridized for 3 h with 100  $\mu$ l of prehybridization buffer (50% formamide, 5× SSC, 5× Denhardt's solution, 4 mM EDTA, 100  $\mu$ g/ml wheat germ tRNA) per slide at 45°C in a moist chamber. The prehybridization buffer was removed carefully with filter paper, and 20  $\mu$ l of hybridization buffer (the same as prehybridization buffer without the tRNA) with DIG-labeled denatured cDNA probe (~2 ng/ $\mu$ l) was added to each slide glass. Each slide was covered with a siliconized cover glass and sealed with rubber cement to prevent evaporation of the hybridization buffer. Hybridization was carried out at 45°C for 6 h in a moist chamber. After hybridization, the slides were washed twice with 3× SSC, twice with 0.3× SSC, and then twice with 0.2× SSC (15 min for each wash).

### Determination of mRNA Location

After hybridization, location of each mRNA was determined by alkaline phosphatase-conjugated anti-DIG antibody with Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrates using a kit from Boehringer Mannheim.

### Propidium Iodide Staining

Nuclei were visualized by propidium iodide staining of the cells. After in situ hybridization with DIG-labeled cDNA probes and immunological detection of respective mRNAs, the samples were stained with propidium iodide (500  $\mu$ g/ml in 50% glycerol, 0.5 M Tris-HCl, pH 7.5, 2% 1,4-diazabicyclo-[2,2,2]-octane) for 10 min. The stained cells were washed three

times with PBS and examined by fluorescence microscopy with a rhodamine filter.

## Results

### Localization of the Tubulin mRNAs

Cells were taken at 0, 20, 40, 70, 85, and 120 min after initiation of the differentiation. The cells were fixed and hybridized with DIG-labeled cDNA probes, and the distribution of the DS mRNAs was determined by anti-DIG antibody conjugated with alkaline phosphatase as described in Materials and Methods.

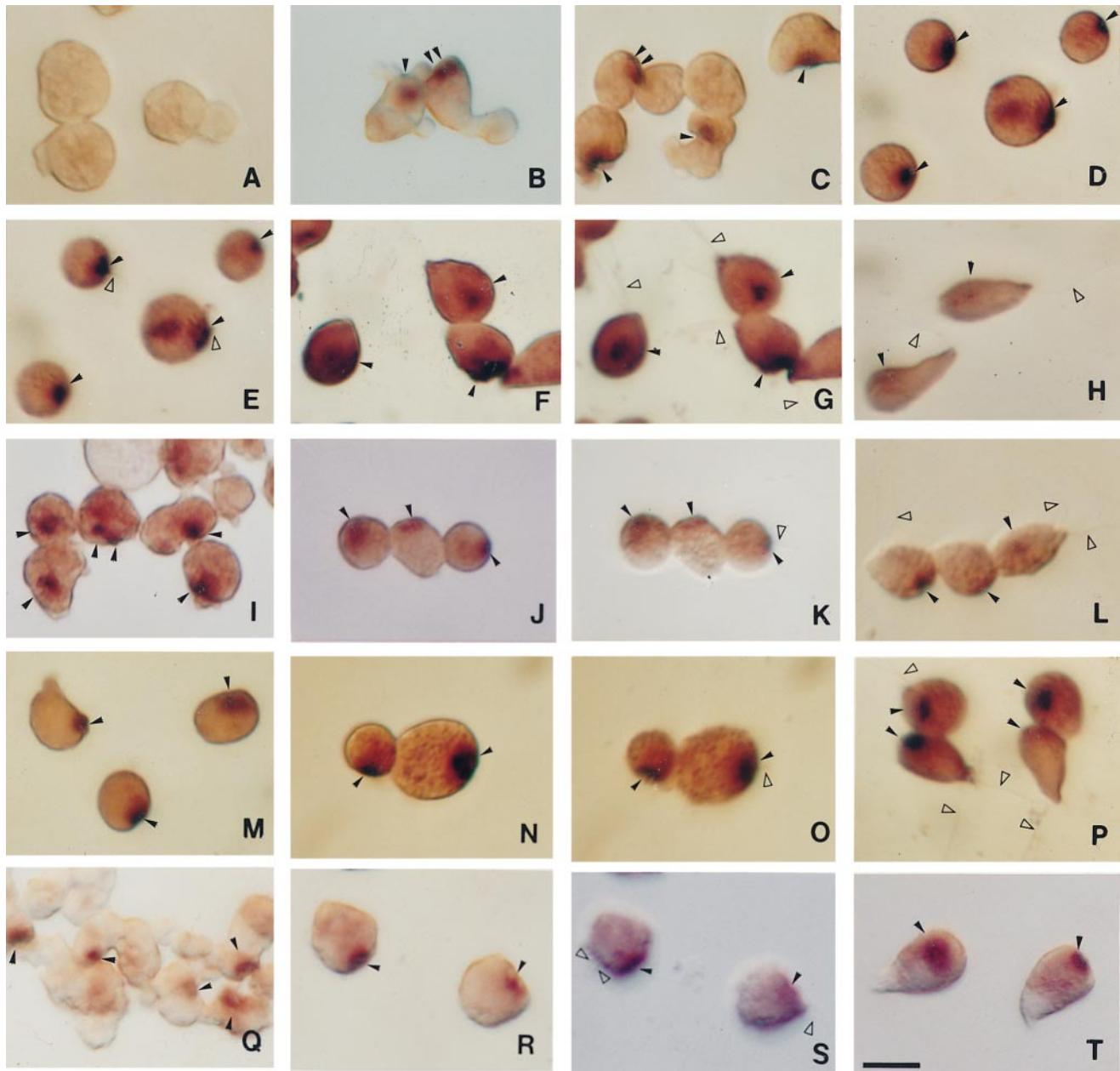
When a probe for  $\beta$ -tubulin mRNA was used, no  $\beta$ -tubulin mRNA was detected in amebas (0 min, Fig. 1 A) as expected from the previous reports (19, 20). At 20 min after the initiation,  $\beta$ -tubulin mRNA is actively transcribed (19), and the amount of this mRNA has reached about 20 to 30% of the peak value (20). At this stage, the  $\beta$ -tubulin mRNA was detected in 76% of cells, and in 74% (56 out of 76) of the labeled cells, the  $\beta$ -tubulin mRNA was concentrated at one location (Fig. 1 B; Table I). In the rest of the labeled cells, the tubulin mRNA was concentrated at two, or rarely three, close locations (Fig. 1, B and C; Table I; also see Fig. 1 I for  $\alpha$ -tubulin mRNA).

At 40 min after the initiation, most of the cells were still amoeboid in shape (6). At this stage, transcription of the  $\beta$ -tubulin mRNA is most active (19), and the amount of the mRNA has reached about 60% of its peak value (20). At this stage, the  $\beta$ -tubulin mRNA was detected in 81% of cells, and in 82% of the cells (66 out of 81), the tubulin mRNA was localized in one area. In most of these cells, the  $\beta$ -tubulin mRNA was located at the periphery (Fig. 1 C; Table I).

At 70 min, most of the cells have rounded up into spheres, about 50% of the cells have visible flagella on the surface (6, 25), and the amount of the tubulin mRNA is at its peak (20). At this stage, strong  $\beta$ -tubulin mRNA-specific staining was observed in 84% of cells (Fig. 1, D and E). In the majority of these cells (91%, 76 out of 84; Table I), the  $\beta$ -tubulin mRNA was concentrated in one area. This specific localization was more evident in the flagellated cells. The  $\beta$ -tubulin mRNA was localized in one area in 94% of the flagellated cells. When we examined the location of the tubulin mRNA in flagellated cells with one labeled spot, this  $\beta$ -tubulin mRNA-specific staining was located at the base of the growing flagella in 82% of the flagellated cells (Table II).

At 85 min after initiation, most of the cells have visible flagella, the amount of tubulin mRNA has decreased to 80% of the maximum (20), complex array of the CSMT is rapidly growing (25), and most of the flagellated cells have already started to elongate (6). At this stage, the  $\beta$ -tubulin mRNA was still found to be concentrated in a specific area in 90% of the cells (Table I). However, the  $\beta$ -tubulin mRNA was not found at the base of the flagella in most of the flagellated cells. Instead, the tubulin mRNA was found at the posterior or in the middle of the flagellated cells (Fig. 1, F and G; Table II).

To see this translocation of the mRNA from the base of flagella to the posterior of the flagellates and the relationship between the translocation and the change in the cell



**Figure 1.** Localization of DS mRNAs during the differentiation of *N. gruberi*. Differentiating cells were taken at various stages of differentiation and fixed as described in Materials and Methods. The fixed cells were used for in situ hybridization with cDNA probes labeled with DIG. Location of each mRNA probe was determined by using anti-DIG antibody conjugated with alkaline phosphatase. (A–H) Location of  $\beta$ -tubulin mRNA. (A) 0 min; (B) 20 min; (C) 40 min; (D and E) the same cells at 70 min at different focal planes to show the flagella; (F and G) the same cells at 85 min at different focal planes to show the flagella; (H) 120 min. (I–L) Location of  $\alpha$ -tubulin mRNA; (I) 40 min; (J and K) the same cells at 70 min at different focal planes to show the flagella; (L) 85 min. (M–P) Location of Class I mRNA. (M) 40 min; (N and O) the same cells at 70 min at different focal planes; (P) 85 min. (Q–T) Location of flagellar calmodulin mRNA. (Q) 40 min; (R and S) the same cells at 70 min at different focal planes; (T) 85 min. Black arrowheads indicate locations of the DS mRNAs, and open arrowheads point to the flagella. The pictures were taken using a microscope (model Optiphot-2; Nikon, Inc., Melville, NY) under differential interference contrast (DIC) conditions. Bar, 10  $\mu$ m.

shape, we quantitated the number of flagellates having one labeled spot based on their shape and on the location of the mRNA in the cells at 70 and 85 min (Table II).

At 70 min, 93% of the flagellated cells were round in shape. In 88% (82 out of 93) of these round flagellates, the  $\beta$ -tubulin mRNA was found at the base of the flagella. In the rest of the round flagellates, the  $\beta$ -tubulin mRNA was

located mostly in the middle of the cells (Table II). In the elongated cells (7%), the  $\beta$ -tubulin mRNA was not found at the base of the flagella but in the middle or at the posterior of the cells (Table II). At 85 min, the majority of flagellated cells (78%) had started to elongate, and 22% of flagellated cells were still round in shape. In the elongated flagellates, the  $\beta$ -tubulin mRNA was found in the middle

Table I. Localization of the DS mRNAs

Time after initiation	Percentage of cells that show specific localization of the DS mRNA			No signal (%)	Percentage of cells with flagella
	At one region (A)	At 2 or more regions (B)	A + B		
<i>min</i>					
<u><math>\beta</math>-tubulin</u>					
0	0	0	0	0	0
20	56 $\pm$ 8	20 $\pm$ 3	76 $\pm$ 8	24 $\pm$ 8	0
40	66 $\pm$ 2	15 $\pm$ 3	81 $\pm$ 2	20 $\pm$ 3	0
70	76 $\pm$ 1	8 $\pm$ 3	84 $\pm$ 3	16 $\pm$ 3	44 $\pm$ 2
85	82 $\pm$ 2	8 $\pm$ 3	90 $\pm$ 2	10 $\pm$ 2	83 $\pm$ 7
120	46 $\pm$ 10	12 $\pm$ 3	58 $\pm$ 12	42 $\pm$ 12	94 $\pm$ 3
<u><math>\alpha</math>-tubulin</u>					
0	0	0	0	0	0
20	51 $\pm$ 8	24 $\pm$ 4	75 $\pm$ 8	26 $\pm$ 8	0
40	53 $\pm$ 1	17 $\pm$ 3	70 $\pm$ 2	30 $\pm$ 2	0
70	75 $\pm$ 4	10 $\pm$ 2	85 $\pm$ 3	15 $\pm$ 3	48 $\pm$ 2
85	76 $\pm$ 7	16 $\pm$ 3	92 $\pm$ 3	8 $\pm$ 3	81 $\pm$ 4
120	24 $\pm$ 4	57 $\pm$ 24	82 $\pm$ 22	19 $\pm$ 22	96 $\pm$ 2
<u>Flagellar calmodulin</u>					
0	0	0	0	0	0
20	ND	ND	ND	ND	0
40	49 $\pm$ 7	29 $\pm$ 7	78 $\pm$ 3	22 $\pm$ 4	0
70	70 $\pm$ 5	15 $\pm$ 2	85 $\pm$ 3	15 $\pm$ 3	42 $\pm$ 4
85	72 $\pm$ 4	8 $\pm$ 1	80 $\pm$ 4	20 $\pm$ 4	85 $\pm$ 5
120	ND	ND	ND	ND	ND

After in situ hybridization as in Fig. 1, cells were observed under a DIC microscope. Percentage of cells that show specific localization of the mRNA was determined by randomly counting 100 well-separated cells for each time point. The numbers represent the average of percentage of five sets of independent experiments.

(45%, 35 out of 78) or at the posterior (55%, 43 out of 78) of the cells. In the round flagellates, the tubulin mRNA was located at the base of the flagella or in the middle of the cells (Table II). By 120 min, the differentiation is completed and the concentration of the mRNA has fallen below 10–20% of the maximum value (20). At this stage, the  $\beta$ -tubulin mRNA was still concentrated at a specific area in about half of the cells (in most cases, at the posterior) but the staining was faint (Fig. 1 *H*; Table I).

These results show that the  $\beta$ -tubulin mRNA is concentrated at a specific area of a differentiating cell, that at 70 min this mRNA is localized at the base of the growing flagella, and that the mRNA is translocated from the base of the flagella to the posterior as the cells differentiate. These results also suggest that the translocation of the  $\beta$ -tubulin mRNA begins before the initiation of cell elongation.

The distribution of  $\alpha$ -tubulin mRNA during *N. gruberi* differentiation was essentially identical to that of the  $\beta$ -tubulin mRNA (Fig. 1, *I–L*; Tables I and II) except at 120 min. Even though the stainings were faint like those of the  $\beta$ -tubulin mRNA, we observed the  $\alpha$ -tubulin mRNA in 82% of cells at this stage, and the mRNA was concentrated at two or more regions in 70% of the stained cells (20). The distribution of the Class I mRNA, a DS mRNA of unknown protein, was similar to that of the  $\alpha$ - and  $\beta$ -tubulin mRNA. In amebas, the Class I mRNA was not detected (data not shown). However, after 40 min of differentiation, the Class I mRNA was found concentrated at the periphery of the cells (Fig. 1 *M*). In the later stages of differentiation, the Class I mRNA was distributed in the same way as the  $\alpha$ - and  $\beta$ -tubulin mRNAs (Fig. 1, *N–P*).

### Localization of the Flagellar Calmodulin mRNA

The distribution of the flagellar calmodulin mRNA that encodes a flagellar-specific calmodulin (7) was very similar to those of the other three mRNAs in early stages (up to 40 min), but it was quite different at later stages (at 70 and 85 min). This mRNA was not detected in amebas like the other three mRNAs (data not shown). At 40 min, at which time transcription of this gene is most active (19), the

Table II. Translocation of the DS mRNAs in Flagellated Cells

Time after initiation	Round-Flagellates (%)				Elongated-Flagellates (%)				Total (%)
	B	M	P	Sub total	B	M	P	Sub total	
<i>min</i>									
<u><math>\beta</math>-tubulin</u>									
70	82 $\pm$ 3	9 $\pm$ 2	2 $\pm$ 1	93 $\pm$ 2	0	4 $\pm$ 1	3 $\pm$ 1	7 $\pm$ 2	100
85	10 $\pm$ 2	10 $\pm$ 1	2 $\pm$ 1	22 $\pm$ 3	0	35 $\pm$ 4	43 $\pm$ 3	78 $\pm$ 3	100
<u><math>\alpha</math>-tubulin</u>									
70	76 $\pm$ 2	18 $\pm$ 3	1 $\pm$ 1	95 $\pm$ 2	0	3 $\pm$ 1	2 $\pm$ 1	5 $\pm$ 2	100
85	9 $\pm$ 2	11 $\pm$ 1	2 $\pm$ 1	22 $\pm$ 3	0	31 $\pm$ 3	47 $\pm$ 3	78 $\pm$ 3	100
<u>Flagellar calmodulin</u>									
70	16 $\pm$ 2	73 $\pm$ 1	6 $\pm$ 2	95 $\pm$ 2	0	2 $\pm$ 1	3 $\pm$ 1	5 $\pm$ 1	100
85	1 $\pm$ 1	16 $\pm$ 1	1 $\pm$ 1	18 $\pm$ 3	0	27 $\pm$ 1	55 $\pm$ 1	82 $\pm$ 1	100

The location of the tubulins and flagellar calmodulin mRNAs in flagellated cells were determined based on the cell shape. After each set of in situ hybridizations in Table I, 100 flagellated cells with one labeled spot were randomly chosen from 70- and 85-min samples. The shape of the cells and location of the tubulin and flagellar calmodulin mRNA in the cells were counted as in Table I. The numbers represent the average of percentage of five independent experiments. *B*, at the base of flagella; *M*, in the middle of the cell; *P*, at the posterior of the cell.

mRNA was detected in 78% of the cells (Table I). In 63% (49 out of 78) of these cells, the flagellar calmodulin mRNA was found in one area, and in most of the cells, the mRNA was located at the cell periphery (Fig. 1 Q; Table I).

At 70 min, the flagellar calmodulin mRNA was detected in 85% of cells, and in 83% (70 out of 85) of these cells, the mRNA was localized at one area (Table I). However, location of the mRNA relative to that of the flagella and to the shape of cells was quite different from those of the other three DS mRNAs. The flagellar calmodulin mRNA was localized at the base of flagella in only 16% of round flagellates. In 77% of the round flagellates (73 out of 95), the mRNA was located in the middle of the cells (Fig. 1, R–S; Table II).

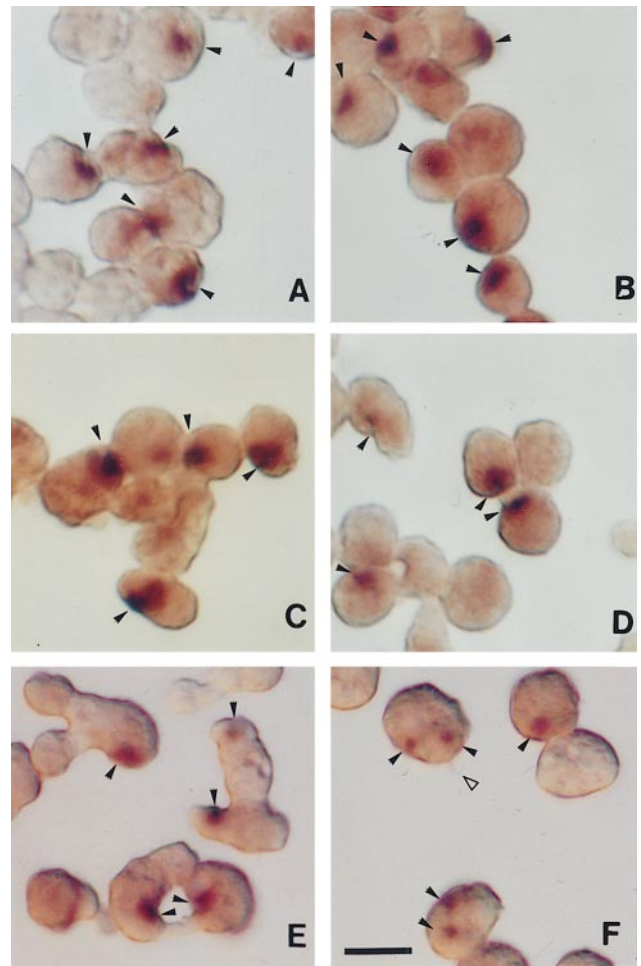
At 85 min, the flagellar calmodulin mRNA was still specifically localized in 80% of cells, and the mRNA was localized at one area in 90% of the cells (72 out of 80) (Fig. 1 T; Table I). In most round flagellates of this stage, the flagellar calmodulin mRNA was located in the middle of the cells (16 out of 18). In elongated flagellates, the mRNA was found at the posterior (67%) or in the middle of the cells (33%) (Table II). At 120 min, only a faint staining was observed (data not shown).

These results show that the flagellar calmodulin mRNA is also specifically localized and that the location of the flagellar calmodulin mRNA changes during the differentiation. These results also show that translocation of the flagellar calmodulin mRNA begins before the onset of translocation of the other three DS mRNAs.

### Colocalization of the DS mRNAs during the Differentiation

The distribution of the three DS mRNAs ( $\alpha$ -tubulin mRNA,  $\beta$ -tubulin mRNA, and Class I mRNA) at 70 and 85 min suggested that they are colocalized during the differentiation. To test this possibility, we performed two sets of in situ hybridization experiments. In one set of the experiments, locations of  $\beta$ -tubulin mRNA and  $\alpha$ -tubulin mRNA were examined simultaneously by adding the respective DIG-labeled cDNA probes in one hybridization reaction. If the two mRNAs were not colocalized, we would expect several (at least two) distinctly stained regions. As shown in Fig. 2, A (20 min) and B (40 min), the staining pattern was similar to that of Fig. 1, B and C, where only the  $\beta$ -tubulin cDNA probe was used. These results were summarized in Table III. We observed one stained region in 58% of stained cells at 20 min, 74% of the cells at 40 min, and 91% of the flagellated cells at 70 min. When the locations of  $\alpha$ -tubulin mRNA and Class I mRNA were examined in the same way, we obtained similar results (Fig. 2, C and D). These data suggest that the three DS mRNAs are colocalized during differentiation.

Because the distribution of the flagellar calmodulin mRNA was similar to that of the tubulin mRNAs at early stages, we examined whether the flagellar calmodulin mRNA was also colocalized with the other three mRNAs at early stages. In these experiments, we examined the location of the flagellar calmodulin mRNA and that of the  $\beta$ -tubulin mRNA simultaneously in 20-, 40-, and 70-min cells. We observed one stained region in 48% of the stained cells at 20 min and 73% of the stained cells at 40



**Figure 2.** Specific colocalization of the DS mRNAs. In situ hybridizations were carried out as in Fig. 1 except that two different DIG-labeled cDNA probes were added simultaneously to each hybridization. (A and B) Location of  $\beta$ -tubulin mRNA and  $\alpha$ -tubulin mRNA in 20- and 40-min cells, respectively. (C and D) Location of  $\alpha$ -tubulin mRNA and Class I mRNA in 20- and 40-min cells, respectively. (E and F) Location of  $\beta$ -tubulin mRNA and flagellar calmodulin mRNA in 40- and 70-min cells, respectively. Bar, 10  $\mu$ m.

min (Table III and Fig. 2 E). However, in flagellated cells at 70 min, we observed only 52% of the cells having one stained region. In the rest of the flagellated cells (48%), we observed two or more well-separated stained regions (two spots, 38%; three or more, 10%; Fig. 2 F). These results suggest that the flagellar calmodulin mRNA is colocalized with the other three DS mRNAs at early stages of differentiation. These results are also consistent with the observed fact that the flagellar calmodulin mRNA was found at the base of the flagella only in 16% of the flagellated cells at this stage (Table II) and support our rationale behind the method that we used to show the colocalization of the DS mRNAs.

### The Localization Is Specific to the DS mRNAs

This localization of the DS mRNAs could be the result of active transcription of the DS genes in the nucleus, especially at early stages of differentiation when the genes are

Table III. Colocalization of the DS mRNAs

Time after initiation	Percentage at one region	Percentage at two or more regions
<i>min</i>		
Localization of $\beta$ - and $\alpha$ -tubulin mRNAs		
0	0	0
20	58 $\pm$ 7	42 $\pm$ 7
40	74 $\pm$ 3	26 $\pm$ 3
70*	91 $\pm$ 3	9 $\pm$ 3
Localization of $\beta$ -tubulin and flagellar calmodulin mRNAs		
0	0	0
20	48 $\pm$ 8	52 $\pm$ 8
40	73 $\pm$ 4	27 $\pm$ 4
70*	52 $\pm$ 2	48 $\pm$ 2

Cells were simultaneously labeled with probes for  $\alpha$ - and  $\beta$ -tubulin mRNAs and for  $\beta$ -tubulin and flagellar calmodulin mRNAs. The percentage of cells that shows colocalization of the mRNA was determined by randomly counting 100 labeled cells. The numbers represent the average of percentage of two sets of independent experiments. \*Counted only in the flagellated cell.

actively transcribed (19). To test this hypothesis, we stained the 40-min cells, in which the DS genes are being most actively transcribed, with propidium iodide after in situ hybridization with the DS cDNA probes. As shown in Fig. 3, A and B, the location of  $\beta$ -tubulin mRNA was clearly cytoplasmic, distinct from that of the nucleus. This result showed that the specific localization was not the result of active transcription and of concentration of the mRNAs in the nucleus.

The specific localization of the DS mRNAs was not a general phenomenon of *N. gruberi* differentiation. We examined distribution of another *Naegleria* mRNA that is present at high concentrations both in amebas and in flagellates, a nonspecific mRNA (19, 20). Unlike the DS mRNAs, the nonspecific mRNA showed uniform distribution throughout the differentiation (Fig. 4, A and B; and data not shown). The specific localization of the DS mRNAs is fur-

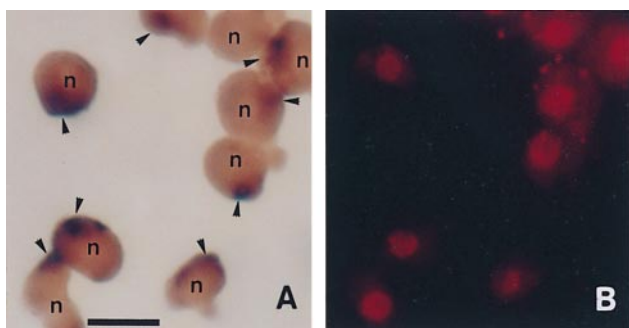


Figure 3. Localization of the  $\beta$ -tubulin mRNA in 40-min cells is not a result of concentration in the nucleus. After in situ hybridization with DIG-labeled  $\beta$ -tubulin cDNA and immunological detection of the mRNA with anti-DIG antibody as in Fig. 1, the samples were stained with propidium iodide and observed under DIC conditions and fluorescence microscopy. (A) Location of  $\beta$ -tubulin mRNA and nucleus in 40-min cells. Cells were observed under DIC optics as in Fig. 1. Faint circles (n) represent nuclei. (B) The same cells in A were observed using fluorescence optics with a rhodamine filter. Faint circles in A were specifically stained with the fluorescent dye. Bar, 10  $\mu$ m.

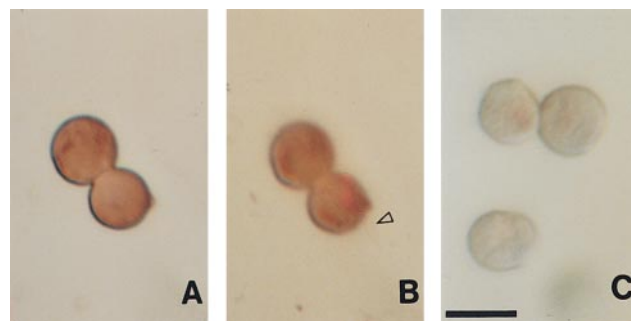


Figure 4. (A and B) Distribution of a nonspecific mRNA in 70-min cells. A PstI fragment of pcNg 3-28 (20) was labeled with DIG, and the location of this mRNA was determined as in Fig. 1. The same cells were observed using DIC optics at two different focal planes to show the flagella. (C) 70-min cells were stained with alkaline phosphatase-conjugated anti-DIG antibody after hybridization in the absence of a DIG-labeled probe. Bar, 10  $\mu$ m.

ther supported by the observed facts that the locations of flagellar calmodulin mRNA and the tubulin mRNAs are different in 70-min cells. When the cells were stained with the anti-DIG antibody without prior hybridization with the DIG-labeled probes or treated with RNase before the hybridization, no staining was observed (Fig. 4 C; and data not shown).

#### Involvement of Microfilaments in the Localization of DS mRNAs

Localization of mRNAs has been studied in many systems

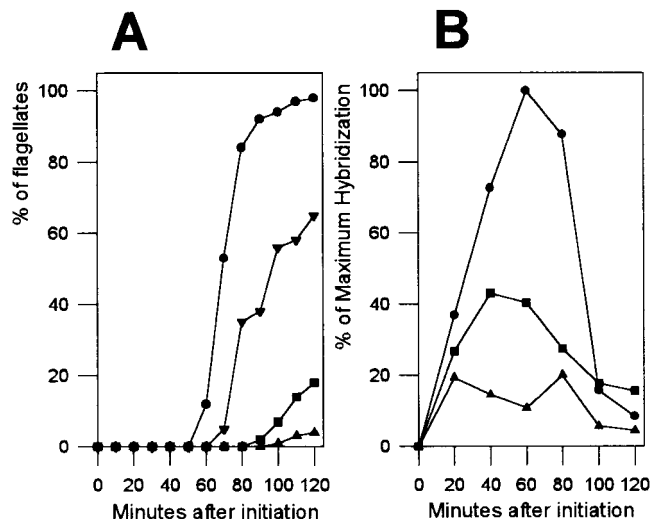
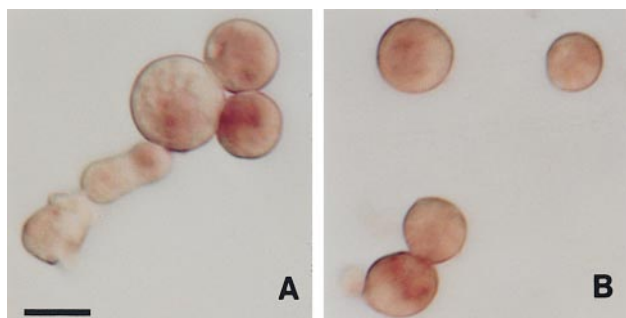


Figure 5. Effect of cytochalasin D on the differentiation of *Naegleria gruberi*. (A) Dose-dependent inhibition of *Naegleria* differentiation by cytochalasin D. Cytochalasin D (5 mg/ml in dimethylsulfoxide) was added at the beginning of differentiation to make a final concentration of 20, 50, or 100  $\mu$ g/ml. The differentiation was monitored as described in Materials and Methods.  $\bullet$ , control;  $\blacktriangledown$ , 20  $\mu$ g/ml;  $\blacksquare$ , 50  $\mu$ g/ml;  $\blacktriangle$ , 100  $\mu$ g/ml. (B), Effects of cytochalasin D on accumulation of  $\beta$ -tubulin mRNA. RNA was prepared from the control and cytochalasin D-treated cells, and the amount of  $\beta$ -tubulin mRNA was estimated by RNA slot blot hybridization (5  $\mu$ g RNA/slot) using  $^{32}$ P-labeled  $\beta$ -tubulin cDNA as a probe (2).  $\bullet$ , control;  $\blacksquare$ , 50  $\mu$ g/ml;  $\blacktriangle$ , 100  $\mu$ g/ml.



**Figure 6.** Cytochalasin D inhibited the specific localization of the  $\beta$ -tubulin mRNA. Cytochalasin D was added at the beginning of differentiation to a final concentration of 50  $\mu\text{g/ml}$ . Cells were taken at 20 and 70 min after the initiation and fixed. The fixed cells were in situ hybridized with DIG-labeled  $\beta$ -tubulin cDNA probe, and the location of the  $\beta$ -tubulin mRNA was determined as in Fig. 1. (A) 20 min; (B) 70 min. Bar, 10  $\mu\text{m}$ .

(13, 22, 26). In some of the systems, it has been suggested that translocation of mRNA requires cytoskeletal components (22). Because *Naegleria* amoebae have an actin-based microfilament system (24) but no organized microtubule system before 50 min into the differentiation (25), we tested the possible role of the microfilament system in the localization of the DS mRNAs by using cytochalasin D, which disrupts microfilament systems in many eukaryotic cells.

Addition of cytochalasin D at the beginning of differentiation inhibited the differentiation in a dose-dependent manner. In 20  $\mu\text{g/ml}$  of cytochalasin D, 60% of the cells formed flagella. In 50 or 100  $\mu\text{g/ml}$  of cytochalasin D, the differentiation was strongly inhibited (Fig. 5 A). In these experiments, most of the cells changed their shape into spheres less than 30 min after the initiation of differentiation and remained as spheres until the end of the differentiation (Fig. 6).

To better understand the nature of the inhibition of the differentiation by cytochalasin D, we measured the amount of the  $\beta$ -tubulin mRNA in cytochalasin D-treated cells. As shown in Fig. 5 B, cytochalasin D partially inhibited the transient accumulation of the mRNA. In the presence of 50  $\mu\text{g/ml}$  cytochalasin D, the amounts of  $\beta$ -tubulin mRNA at 20, 40, and 60 min after the initiation of differentiation were 73, 59, and 40% of that of the control cells, respectively. At 100  $\mu\text{g/ml}$  of cytochalasin D, accumulation of the  $\beta$ -tubulin mRNA was further inhibited. Cytochalasin D also inhibited the transient accumulation of the  $\alpha$ -tubulin mRNA and the Class I mRNA in a similar way (data not shown). Addition of cytochalasin D at a later stage (80 min) had no effect on the formation of flagella (data not shown).

Because addition of cytochalasin D (50  $\mu\text{g/ml}$ ) at the initiation of differentiation inhibited accumulation of the tubulin mRNAs by only  $\sim 50\%$ , we examined the effect of cytochalasin D on the localization of the mRNA by in situ hybridization. We added cytochalasin D at the initiation of differentiation and examined the effect of the drug treatment on localization of the DS mRNAs. In cytochalasin-treated cells (50  $\mu\text{g/ml}$ ), the  $\beta$ -tubulin mRNA was found evenly distributed or in patches in the cytoplasm of the cy-

tochalasin-treated cells (Fig. 6). Only less than 9% of the cells at 20 min and 5% of the cells at 70 min showed specific localization of the  $\beta$ -tubulin mRNA. The  $\alpha$ -tubulin mRNA, Class I mRNA, and flagellar calmodulin mRNA were also found dispersed in the cytochalasin-treated cells (data not shown).

Unlike the effect of cytochalasin D, taxol (up to 50  $\mu\text{M}$ ) or colchicine (up to 20 mM) treatment did not cause significant effects on *Naegleria* differentiation when added at the beginning of differentiation.

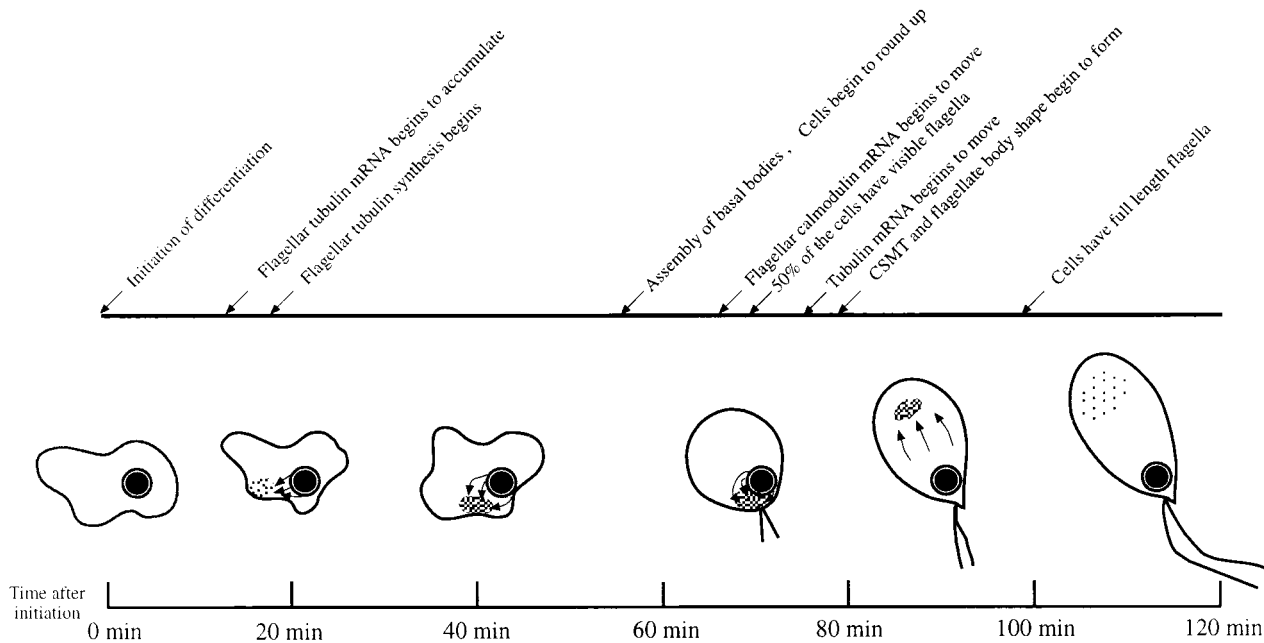
## Discussion

By in situ hybridization, we have examined the distribution of four mRNAs that are accumulated transiently and specifically during differentiation of *N. gruberi*;  $\alpha$ -tubulin,  $\beta$ -tubulin, Class I, and flagellar calmodulin mRNA. Our findings demonstrate that these DS mRNAs are specifically localized in a limited area of the differentiating cells at the early stages of differentiation, and then, as the differentiation proceeds, the DS mRNAs are translocated from the base of flagella to the posterior of the differentiating cells with a discrete order.

The specific localization of the four DS mRNAs became evident 20 min after the initiation of differentiation, at which time transcription of the genes was just activated (19). Until 40 min, the four DS mRNAs were colocalized, and the location was clearly distinct from that of the nucleus (see Figs. 1 and 3; Table III). However, at 70 min the location of three of the four DS mRNAs, the tubulin mRNAs and Class I mRNA, was different from that of the flagellar calmodulin mRNA (Fig. 3; Tables II and III). In  $>80\%$  of flagellated cells, the tubulin mRNAs were localized at the base of the growing flagella, very close to the nucleus, where the basal bodies are located (Fig. 7) (25). On the contrary, the flagellar calmodulin mRNA was found at the base of flagella only in 16% of the flagellated cells at this stage. After this stage, the tubulin mRNAs also began to move away from the basal body area toward the posterior of the cells. By 120 min, the differentiation was completed; the DS mRNAs were still specifically localized in some cells, but the staining was very faint. In a small portion of cells, the DS mRNAs were localized at more than two areas. This result might be related to the fact that as many as 20% of cells can have more than two flagella (8).

This localization of the mRNAs could be mediated through the microfilament system. Because the amount of tubulin in the cell is very low (18) and no organized microtubular structures are observed at early stages of differentiation (20 and 40 min [25]), it is difficult to imagine that the initial transport of the DS mRNAs is mediated by microtubules. The fact that cytochalasin D treatment at the beginning of differentiation inhibited localization of the DS mRNAs is consistent with this hypothesis, but the effect of this drug on the accumulation of these DS mRNAs makes a firm conclusion impossible.

Addition of cytochalasin D (50  $\mu\text{g/ml}$ ) at the beginning of differentiation caused *Naegleria* amoebae to become spherical more quickly than control cells. This might be a result of the disruption of the microfilament system, the main cytoskeletal system in *Naegleria* amoebae. In this condition, the specific localization of the DS mRNAs, the for-



**Figure 7.** A schematic presentation of localization of the DS mRNAs during differentiation of *N. gruberi* amebas into flagellates. This diagram is drawn based on data of our own and from references 6, 10, and 25. Formation of CSMT from the base of flagella is not shown. The dotted area represents the site of localization of the DS mRNAs.

mation of flagella and cytoskeletal microtubules, and hence differentiation itself were almost completely inhibited. Even though cytochalasin D partially inhibited the accumulation of the DS mRNAs (~50% of the control), this partial inhibition alone does not explain the complete prevention of the differentiation. About 50% of cells can form flagella when actinomycin D is added at 40 min after the initiation of differentiation, at which time the amount of  $\beta$ -tubulin mRNA has only reached ~50% of the maximum (9, 20). When we examined the synthesis of  $\alpha$ -tubulin during the differentiation of cytochalasin-treated cells (50  $\mu$ g/ml, at the beginning of differentiation) and of control cells using a monoclonal antibody (25), we did not find significant decrease (Cho, H.I., and J.H. Lee, unpublished result).

Even though we do not understand the underlying mechanism, this specific colocalization and presumably translation of the DS mRNAs in one area could greatly increase the local concentration of the protein products. This high concentration of  $\alpha$ - and  $\beta$ -tubulin at the area could facilitate formation of microtubules for the basal body. The continuous translation of the tubulin mRNAs and flagellar calmodulin mRNA at or near the base of the growing flagella and subsequent transportation of the proteins to the growing end (11) could also facilitate the elongation of the flagella.

The localization of the tubulin mRNAs could also be important for the formation of the CSMT. Walsh observed the CSMT radiating from the base of the flagella (25). He also showed that inhibition of protein synthesis before 52 min of differentiation inhibited formation of both flagella and CSMT. However, addition of cycloheximide between 52 and 62 min of differentiation inhibited the formation of the CSMT only. Addition of the drug after 62 min did not block formation of either organelles (25). These facts im-

ply that further protein synthesis is required for the formation of the CSMT even after the completion of protein synthesis required for the formation of flagella and that some of the proteins are organized around the basal bodies to form a microtubule organizing center (15–17, 23) for the CSMT. Even though we cannot rule out the possibility that the amount of tubulin (and/or other component proteins) synthesized until 62 min of differentiation is not enough to form both organelles, these data suggest that the whole events required for the formation of flagella and CSMT are accomplished by 70 min after the initiation of differentiation (25), before the onset of translocation of the tubulin mRNAs from the base of the flagella.

The ordered translocation of the DS mRNAs, first the flagellar calmodulin mRNA then the tubulin mRNAs, to the posterior of the flagellated cells is quite intriguing. Based on the data in Table II, we may guess that the translocation of the flagellar calmodulin mRNA starts between 60 to 65 min after the initiation of differentiation. Unlike the tubulins, which are the component of the flagellar axonemes and CSMT, the flagellar calmodulin is specific for the flagellar axoneme (7), and the cellular events required for the formation of the flagella are completed before the completion of the events for the formation of the CSMT (see above). Based on these facts, we might assume that the translocation of the flagellar calmodulin mRNA begins after the completion of the cellular events for the formation of flagella, and then, after the completion of the events for the formation of the CSMT, translocation of the tubulin mRNAs ensues. These results implicate that the four DS mRNAs are transported to a specific area after being transcribed in the nucleus at early stages of differentiation and that there might be another mechanism that determines the order of translocation of different groups of mRNAs (e.g., mRNAs for microtubule proteins and



mRNAs for flagellar specific proteins) toward the posterior as the differentiation proceeds. This ordered translocation of the mRNAs also suggests that this translocation is a regulated process and not a result of CSMT elongation.

The cellular function of the translocation of the mRNAs is not clear. However, one interesting possibility is that the translocation might be related with specific degradation of the mRNAs in later stages of differentiation. This translocation of the DS mRNAs began just after the completion of protein synthesis, which is required for the formation of flagellar apparatus and CSMT (Table II and reference 25). At the same time, the amount of the DS mRNAs began to decrease rapidly (19, 20), and we have shown previously that the stability of the  $\beta$ -tubulin and Class I mRNAs is specifically regulated in these stages of differentiation (2). It is also possible that the translocation of the localized mRNAs at the posterior of the cells might be important for the later stage of cell differentiation, e.g., formation of the flagellate shape (25).

It should be noted that the Class I mRNA, which could encode a protein with a molecular mass up to 200 kD, is colocalized with the tubulin mRNAs. A few large microtubule-associated proteins have a molecular mass of 160–200 kD (1, 5, 15–17). We do not know the protein product of the Class I mRNA, but it is possible that the mRNA encodes a component of the basal body or of the microtubule system. If this turns out to be true, the specific localization of the DS mRNAs might give a reasonable account of a mechanism by which the formation of basal bodies and flagella, and later the formation of the CSMT, are coordinated.

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