

# mRNAs for $\alpha$ - and $\beta$ -Tubulin and Flagellar Calmodulin Are among Those Coordinately Regulated when *Naegleria gruberi* Amebae Differentiate into Flagellates

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**Abstract.** Three of four mRNAs that are specific to the differentiation of *Naegleria gruberi* amebae into flagellates (Mar, J., J. H. Lee, D. Shea, and C. J. Walsh, 1986, *J. Cell Biol.*, 102:353–361) have been identified as coding for flagellar proteins. The products of these mRNAs, which are coordinately regulated during the differentiation, were identified by in vitro translation of hybrid-selected RNA followed by two-dimensional gel electrophoresis and antibody binding. Six cross-hybridizing clones complementary to a 1.7-kb RNA (class II) all selected mRNA that was translated into two  $\alpha$ -tubulins. The principal in vitro product,  $\alpha$ -1, comigrated with a cytoplasmic  $\alpha$ -tubulin, while the minor product with a more acidic pI,  $\alpha$ -2,

comigrated with flagellar  $\alpha$ -tubulin. While *Naegleria* flagellar  $\alpha$ -tubulin was found to be acetylated based on its reaction with a monoclonal antibody specific to this form, we suggest that  $\alpha$ -2 is not likely to arise due to acetylation in vitro but probably represents the product of a second  $\alpha$ -tubulin gene. The class III clone, also complementary to a 1.7-kb RNA, selected  $\beta$ -tubulin mRNA. In the course of this work it was found, using monoclonal antibodies to the  $\alpha$ - and  $\beta$ -subunits of tubulin, that *Naegleria*  $\alpha$ -tubulin migrated faster than  $\beta$ -tubulin on SDS-PAGE. The class IV clone, which hybridizes with a 0.5-kb RNA, selected an mRNA that was translated into a heat stable calcium-binding protein, flagellar calmodulin.

**T**HE rapid and synchronous differentiation of *Naegleria* amebae into flagellates is easily induced by washing amebae free of the bacteria or axenic medium that serves as a food source (reviewed by Fulton in references 7 and 8). When amebae differentiate into flagellates they form basal bodies (11), flagellar rootlets (24), and flagellar axonemes in just over 1 h (5, 37). By 120 min after initiation, 95–100% of the cells have full length flagella (10) and most have developed a microtubule-based cytoskeleton (42). In concert with the formation of cytoplasmic microtubules, the cells assume an asymmetric oval form, the flagellate shape (8). Neither tubulin (12, 13, 19) nor translatable tubulin mRNA (23) appear to be present in amebae in appreciable amounts. Yet within 10–15 min after the initiation of the differentiation, functional tubulin mRNA appears (23).

The formation of flagella by *Naegleria* amebae is not an obligate part of the life cycle (7). Apparently, amebae can grow and divide for an unlimited number of generations without ever forming flagella. This fact, combined with the complexity of the flagellar apparatus (the flagellar axoneme alone contains nearly 200 different proteins [29]), raises intriguing questions about the regulatory mechanisms involved in the differentiation. Based on studies using inhibitors of RNA and protein synthesis, the formation of flagella in *Naegleria* requires the synthesis of new RNA and protein (14). At least 70% (19) and probably >90% (12) of the tubulin

in the flagellar axoneme is included among the proteins synthesized de novo during the differentiation. A flagellum-specific calmodulin (CaM-1)<sup>1</sup> also appears during the differentiation (9). The requirement for RNA synthesis probably reflects, in part, the need for tubulin and CaM-1 mRNA synthesis, as judged by in vitro translation (9, 23). The report of an up to 80-fold increase in  $\alpha$ -tubulin mRNA during the differentiation (22) suggests that the changes in mRNAs measured by in vitro translation reflect changes in mRNA concentration and not changes in their ability to be translated.

Using a cDNA library prepared from differentiating cells, Mar et al. (31) have recently identified 10 plasmids containing sequences complementary to at least four poly(A)<sup>+</sup> RNAs specific to differentiating cells. Designated as differentiation-specific (DS) RNAs, they appeared transiently during the differentiation in a coordinate manner. The DS RNAs were undetectable until 10–15 min after initiation, reached a peak as flagella formed at 70 min, and then declined to ~20% of maximum by 120 min. The 10 DS plasmids fell into four classes based on cross-hybridization. The characteristics of the DS plasmids and their mRNAs are summarized in Table I. Although the class II and III mRNAs were expected to code for tubulin subunits based on their size and

1. Abbreviations used in this paper: CaM, calmodulin; DS, differentiation specific; 2D, two-dimensional.

**Table I. Characteristics of the DS cDNA Clones**

Class	Cross-hybridizing* clones	Complementary* poly(A) <sup>+</sup> RNA	Protein produced in vitro
		<i>kb</i>	
I	pcNg 1-8, pcNg 7-43	6-7	Unknown
II	pcNg 2-14, pcNg 2-22, pcNg 2-38, pcNg 3-30, pcNg 4, pcNg 6-18	1.7	$\alpha$ -tubulin
III	pcNg 8-5	1.7	$\beta$ -tubulin
IV	pcNg 44	0.5	Flagellar CaM

\* Reference 31.

abundance, they did not cross-hybridize with clones for chicken tubulin (31).

In the present work we have tried to determine if the DS RNAs code for one or more proteins of the flagellar apparatus. Using the DS plasmids to select poly(A)<sup>+</sup> RNA for in vitro translation, we have established that the class II and class III DS RNAs code for  $\alpha$ - and  $\beta$ - tubulin, respectively. This identification required that we determine the properties of the previously uncharacterized *Naegleria* tubulin subunits. The class IV DS RNA was found to code for flagellar calmodulin, while the product of the class I DS RNA has not been identified yet.

## Materials and Methods

### Growth and Differentiation of *Naegleria*

*Naegleria* strain NB-1 (7, 8) was grown on NM or PM agar with *Klebsiella pneumoniae* as previously described (10, 20, 42). Amebae were harvested by resuspension in ice-cold 2 mM Tris-HCl (pH 7.6 at 20°C) and then washed by centrifugation and resuspension in this buffer. The differentiation, initiated by suspending washed amebae in the washing buffer at 25°C, was carried out at 25°C in 125- or 250-ml Erlenmeyer flasks on a reciprocating shaker as described in detail previously (7, 10, 24).

### Tubulin Isolation

*Naegleria* flagellar tubulin was prepared as previously described (20). Rat brain tubulin was prepared by ammonium sulfate precipitation and DEAE cellulose chromatography using the procedure of Eipper (6). Rat brain tubulin was alkylated with iodoacetate as described by Stephens (39).

Fractions enriched in *Naegleria* cytoplasmic tubulin were prepared at 120 min after initiation of the differentiation, when cells contained an extensive array of cytoplasmic microtubules (42). Either whole flagellates or cell bodies obtained by deflagellation (20) were extracted by gentle suspension in a microtubule-stabilizing buffer (25% vol/vol glycerol, 0.1 M Pipes, pH 6.8, 1.0 mM EGTA, 0.1 mM MgCl<sub>2</sub>, 1.0 mM GTP, 50  $\mu$ g/ml phenylmethylsulfonyl fluoride (PMSF), 20  $\mu$ M leupeptin, 1.7 U/ml aprotinin, 0.5% wt/vol NP-40 at room temperature. After  $\sim$ 1 min, cell extracts were centrifuged in an Eppendorf microfuge for 2 min. The pellets were suspended in SDS gel sample buffer (21) and heated in boiling water for 2 min. The presence of numerous microtubules in these pellets was confirmed by fixation in formaldehyde followed by fluorescence microscopy using monoclonal antibodies against *Naegleria* tubulin (42).

### Hybridization Selection and In Vitro Translation

Hybridization selection was carried out by the method of Maniatis et al. (30) with minor modifications as described below. 10- $\mu$ g aliquots of plasmid DNA, prepared as previously described (31), were bound to nitrocellulose filters using a Dot Blot apparatus (Bethesda Research Laboratories, Gaithersburg, MD). Plasmid containing filters were hybridized with total poly(A)<sup>+</sup> RNA prepared from cells at 70 min after initiation, the peak of DS mRNA concentration (31). Hybridizations were carried out in heat-seal

bags with 200-300  $\mu$ g/ml of poly(A)<sup>+</sup> RNA at 44°C for 6 h. Hybridized RNA was released from washed filters into a solution of 100  $\mu$ g/ml of yeast tRNA by heating in boiling water for 1 min. RNA was recovered by precipitation in 0.2 M Na acetate-66% ethanol at -20°C. Total poly(A)<sup>+</sup> RNA prepared from *Naegleria* strain NEG at 60 min after initiation was a gift of Dr. E. Lai and Dr. C. Fulton, Brandeis University, Waltham, MA.

Poly(A)<sup>+</sup> RNA was translated in a cell-free system prepared from wheat germ (Bethesda Research Laboratories) containing 100 U/ml of RNasin (Promega Biotec, Madison, WI) and 333  $\mu$ Ci/ml of [<sup>35</sup>S]methionine (1,000 Ci/mmol, New England Nuclear, Boston, MA). Translations of total poly(A)<sup>+</sup> RNA contained 50  $\mu$ g/ml of RNA.

### Electrophoresis and Immunological Methods

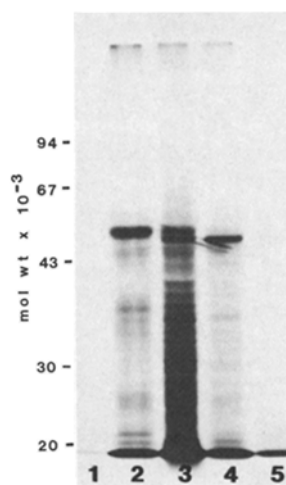
In vitro products were analyzed by SDS gel electrophoresis as described by Laemmli (21) and by two-dimensional (2D) gel electrophoresis as described by O'Farrell (33) using 40% pH 4-6 and 60% pH 6-8 ampholytes (Bio-Rad Laboratories, Richmond, CA) for the isoelectric focusing dimension and 7.5% polyacrylamide gels for the SDS dimension. Radioactivity in gels was visualized for fluorography after treatment with ENHANCE (New England Nuclear). Proteins were transferred to nitrocellulose sheets by electroblotting (41).

Tubulins were visualized using monoclonal antibodies prepared against *Naegleria* flagellar tubulin. The anti- $\alpha$ -tubulin antibody AA-4.3 has been previously described (42). The anti- $\beta$ -tubulin antibody AA-12.1 was obtained from the same fusion as AA-4.3. The acetylated form of  $\alpha$ -tubulin was visualized with monoclonal antibody 6-11B-1, specific to acetylated  $\alpha$ -tubulin (34), a gift of Dr. G. Piperno, The Rockefeller University, NY. Primary antibodies were located with goat anti-mouse IgG conjugated to horseradish peroxidase (Cooper Biomedical, Malvern, PA) using 4-chloro-1-naphthol as substrate (16). Tubulins synthesized in vitro were immunoprecipitated with a polyclonal antiserum against *Naegleria* outer doublet tubulin (20) (a gift of Dr. C. Fulton, Brandeis University) and fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring Corp., San Diego, CA) as described by Kessler (17) except that samples were incubated with antiserum for 1 h at 20°C, and then with Pansorbin for 1.5 h at 20°C. After washing three times by centrifugation, bound protein was eluted by heating at 100°C with SDS sample buffer for 2 min.

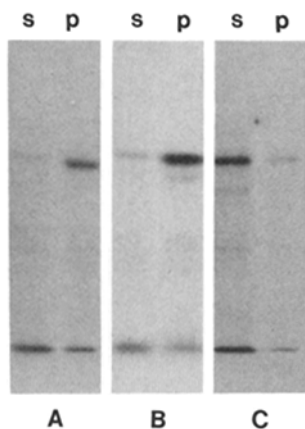
## Results

### Class II and Class III Sequences Code for Tubulin Subunits

In vitro translation of mRNA selected by any of the six class II plasmids produced proteins that migrated on SDS gels as



**Figure 1.** In vitro translation of mRNA selected by the class II and class III plasmids. RNA was selected by hybridization to the class II plasmid pcNg 4 and the class III plasmid pcNg 8-5 from total poly(A)<sup>+</sup> RNA prepared using cells at 70 min after initiation of the differentiation. RNA that hybridized to plasmid DNA was eluted and translated in vitro in a wheat germ cell-free system containing [<sup>35</sup>S]methionine. Products of the in vitro translation were analyzed by electrophoresis on a 7.5% SDS gel and visualized by autoradiography of the dried gel. Lane 1, no RNA added; lane 2, RNA selected by the class III plasmid; lane 3, total poly(A)<sup>+</sup> RNA from cells at 70 min after initiation; lane 4, RNA selected by the class II plasmid; lane 5, RNA selected by the vector, pBR322.

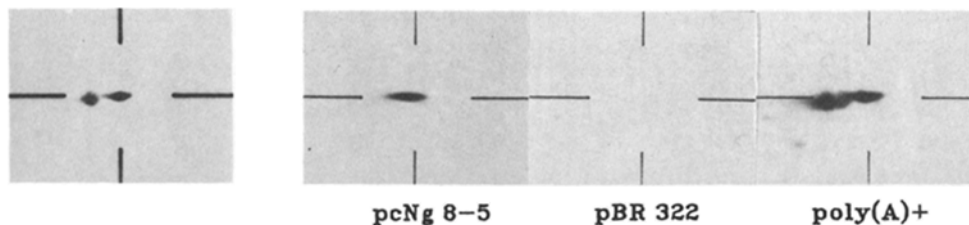


**Figure 2.** Immunoprecipitation of the in vitro translation products of class II and class III mRNA by antibodies against flagellar tubulin. The in vitro products of mRNA selected with the class II plasmid pcNg 6-18 or the class III plasmid were precipitated with a polyclonal antibody against *Naegleria* outer doublet tubulin. The supernatant (lanes s) and pellet (lanes p) from each reaction were fractionated on SDS gels and the in vitro products visualized by fluorography. (A) Class II products incubated with antitubulin; (B) class III products incubated with antitubulin; (C) class II products incubated with buffer instead of antibody. Incubation of the class III product with buffer produced a pattern similar to C, data not shown.

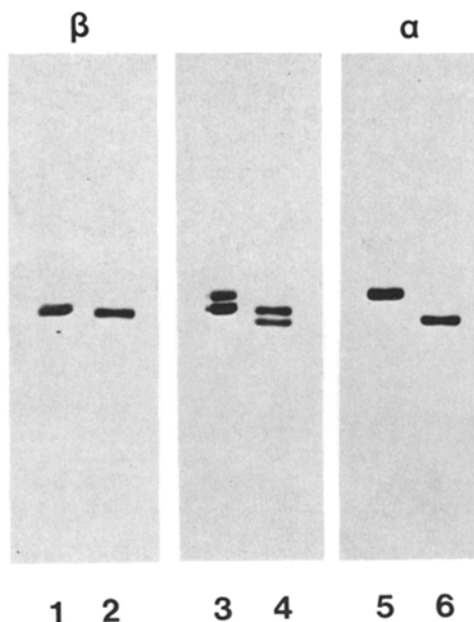
incubated with antitubulin; (B) class III products incubated with antitubulin; (C) class II products incubated with buffer instead of antibody. Incubation of the class III product with buffer produced a pattern similar to C, data not shown.

a single band with a molecular ratio ( $M_r$ ) of 52,000 (Fig. 1, lane 4). When the class III plasmid, pcNg 8-5, was used to select mRNA, the in vitro product migrated as a single band with an  $M_r$  of 54,000 (Fig. 1, lane 2). The class II and class III proteins comigrated with two major proteins produced when total poly(A)<sup>+</sup> RNA was translated in vitro (Fig. 1, lane 3). When the plasmid vector pBR322 was used to select mRNA, no in vitro products resolvable on this gel system were produced (Fig. 1, lane 5).

Based on in vitro translation, immunoprecipitation, and copolymerization with brain tubulin, the two major products of flagellate mRNA are known to be  $\alpha$ - and  $\beta$ -tubulin (23). The identity of the products of the class II and class III mRNAs as tubulin subunits was confirmed by immunoprecipitating the in vitro products of hybrid-selected mRNAs using an antiserum against *Naegleria* flagellar tubulin. As illustrated in Fig. 2, the product of both the class II and class III mRNAs was quantitatively precipitated by this antiserum. The tubulin subunits synthesized in vitro were further characterized by coelectrophoresis with *Naegleria* flagellar outer doublet tubulin on 2D gels. After electrophoresis, the proteins were transferred to nitrocellulose sheets and the flagellar tubulin subunits were located with monoclonal antibodies against *Naegleria*  $\alpha$ - or  $\beta$ -tubulin using a second antibody coupled to horseradish peroxidase.



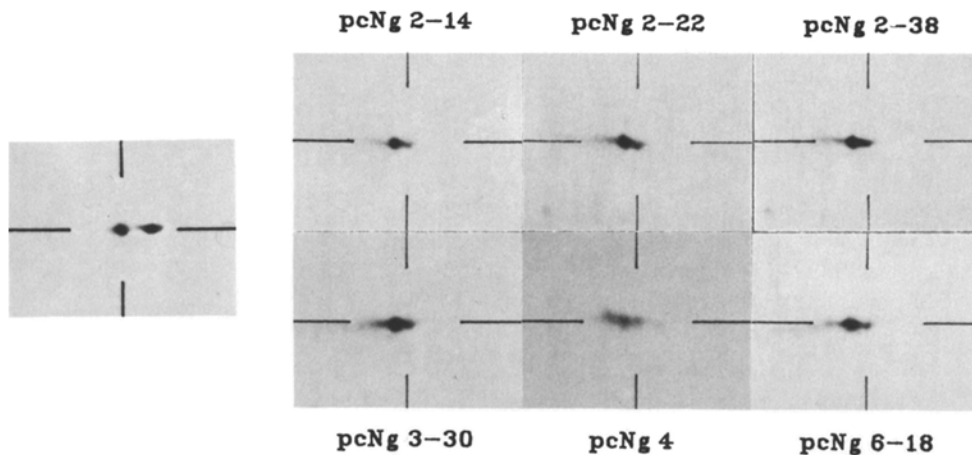
**Figure 4.** Characterization of the in vitro products coded for by the class III poly(A)<sup>+</sup> RNA. Hybrid-selected RNA was translated in vitro as described in Fig. 1. An aliquot of each reaction mixture was mixed with 1  $\mu$ g of *Naegleria* flagellar tubulin and fractionated by 2D gel electrophoresis. The separated proteins were transferred to nitrocellulose by electroblotting. *Naegleria* flagellar  $\alpha$ - and  $\beta$ -tubulin subunits were located using a mixture of monoclonal antibodies as described in Fig. 3. In vitro products were located by autoradiography of the immunoblots. Cross-hairs, on a transparent sheet, were located over the  $\beta$ -tubulin subunit on each immunoblot and then transferred to the autoradiograms using index marks of radioactive ink placed on the nitrocellulose. The left panel illustrates the location of  $\alpha$ - and  $\beta$ -tubulin on the immunoblots. The three right panels are autoradiograms of the in vitro products produced with poly(A)<sup>+</sup> RNA selected by pcNg 8-5 and pBR322 as well as the products of total poly(A)<sup>+</sup> RNA from cells at 70 min after initiation. Isoelectric focusing was carried out from left (-) to right (+) and SDS gel electrophoresis from top to bottom. Only the tubulin region of each gel is illustrated. No other spots were visible on immunoblots or on autoradiograms of hybrid selected RNA.



**Figure 3.** Characterization of *Naegleria* flagellar tubulin subunits. Tubulin prepared from *Naegleria* flagellar axonemes and from rat brain was fractionated by electrophoresis on a 7.5% SDS gel. The tubulin subunits were transferred to a sheet of nitrocellulose by immunoblotting.  $\alpha$ - and  $\beta$ -tubulin were located by incubating strips from the immunoblot with monoclonal antibodies against *Naegleria* flagellar tubulin subunits and then visualizing the monoclonals with goat anti-mouse IgG coupled to horseradish peroxidase using 4-chloro-1-naphthol as substrate. Rat brain tubulin was reduced and alkylated in order to better resolve the tubulin subunits. *Naegleria* tubulin, however, was not alkylated because this caused the subunits to run closer together, although it did not change the relative positions of  $\alpha$ - and  $\beta$ -tubulin. Lanes 1, 3, and 5 were loaded with 2  $\mu$ g of rat brain tubulin. Lanes 2, 4, and 6 were loaded with 1.5  $\mu$ g of *Naegleria* flagellar tubulin. Lanes 1 and 2 were incubated with the anti- $\beta$ -tubulin antibody, AA-12.1; lanes 5 and 6 were incubated with the anti- $\alpha$ -tubulin antibody, AA-4.3; and lanes 3 and 4 were incubated with a mixture of both monoclonals.

### *Naegleria* $\alpha$ -Tubulin Migrates Faster than $\beta$ -Tubulin on SDS Gels

In characterizing the monoclonal antibodies used in these experiments it became apparent that *Naegleria* tubulin subunits migrate differently on SDS gels than those of mammalian tubulins. Both the anti- $\alpha$ -tubulin antibody, AA-4.3, and the



**Figure 5.** Characterization of the in vitro products coded for by the class II poly(A)<sup>+</sup> RNA. The products of the RNA selected by the six class II plasmids were analyzed by 2D gel electrophoresis as described in Fig. 4 except that the cross-hairs were positioned over the flagellar  $\alpha$ -tubulin subunit on the immunoblots.

anti- $\beta$ -tubulin antibody, AA-12.1, bind to *Naegleria* flagellar, cytoplasmic, and mitotic microtubules (42), as well as to extensive networks of cytoplasmic fibers with the characteristics of microtubules in both monkey (BSC) and mouse (M-15) cells (Walsh, C., unpublished observations). Fig. 3 illustrates the specificity of these antibodies for tubulin subunits from both *Naegleria* flagella and rat brain tubulin. AA-4.3, as previously described (42), bound to the *Naegleria* tubulin subunits that migrated more quickly. However, AA-4.3 bound to the slower migrating or  $\alpha$ -subunit of rat brain tubulin. AA-4.3 also bound to all four of the  $\alpha$ -tubulin subunits of *Drosophila melanogaster* testis tubulin (35), (Fuller, M., personal communication). On the other hand, AA-12.1 bound to the *Naegleria* tubulin subunits that migrated slowly and the faster migrating or  $\beta$ -subunit of rat brain tubulin. When AA-4.3 and AA-12.1 were reacted with *Naegleria* tubulins separated by 2D gel electrophoresis, the subunit migrating more slowly in the SDS dimension proved to have the more acidic pI, a feature characteristic of most  $\beta$ -tubulins (e.g., Fig. 4).

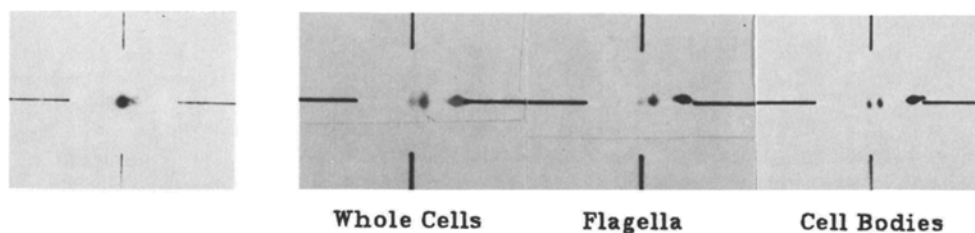
### Class III DS RNA Codes for $\beta$ -Tubulin

The product of in vitro translation using mRNA selected with the class III plasmid, pcNg 8-5, comigrated on 2D gel electrophoresis with  $\beta$ -tubulin from *Naegleria* flagella (Fig. 4). The cross-hairs in the figure were centered over the flagellar  $\beta$ -tubulin subunit on each immunoblot and then aligned on the corresponding autoradiogram using index marks of radioactive ink placed on the nitrocellulose sheet. This technique allows very precise location of the in vitro

product relative to the tubulin subunits. Fig. 4 also illustrates the pattern of proteins, with molecular masses and isoelectric points similar to tubulin subunits, synthesized by the cell-free system from wheat germ using total poly(A)<sup>+</sup> RNA prepared from cells 70 min after initiation of the differentiation. Both the hybrid-selected mRNA and the total poly(A)<sup>+</sup> RNA produced a product that substantially overlapped flagellar  $\beta$ -tubulin. However, the in vitro products are displaced slightly to the basic side in the isoelectric focusing dimension, i.e., the left side of the figure. The total poly(A)<sup>+</sup> RNA product had a major spot that corresponded to the pcNg 8-5 coded product and an elongation on the acidic (right) side which included all of the flagellar  $\beta$ -tubulin.

### Class II DS RNA Codes for $\alpha$ -Tubulin

A similar analysis of the products of in vitro translation using the mRNAs selected by the six class II plasmids is presented in Fig. 5. In this case the major in vitro product,  $\alpha$ -1, migrated to the basic side of the flagellar  $\alpha$ -tubulin subunit while a minor in vitro product,  $\alpha$ -2, comigrated with flagellar  $\alpha$ -tubulin. This is similar to the behavior of the  $\alpha$ -tubulin of *Chlamydomonas reinhardtii* flagella (32). *Chlamydomonas* tubulin undergoes a posttranslational acetylation before incorporation into flagella (27, 28). If a similar processing takes place in *Naegleria*, we would expect to find a second form of  $\alpha$ -tubulin in the cytoplasm. Fig. 6 illustrates that an additional form of  $\alpha$ -tubulin was found when cytoplasmic microtubules were analyzed by 2D gel electrophoresis. This cytoplasmic  $\alpha$ -tubulin comigrated with the principal in vitro product of the class II mRNA.



**Figure 6.** Comparison of the class II products with cytoplasmic and flagellar  $\alpha$ -tubulin. Class II RNA was selected and translated as described in Fig. 4 except that the in vitro products were mixed with fractions containing microtubules from whole flagellates, flagel-

late cell bodies without flagella, or isolated crude flagella. Note: in this figure the cross-hairs were positioned over the principal in vitro product,  $\alpha$ -1, on the autoradiogram, left panels, and then placed on the respective immunoblot, three right panels, using index marks as described in Fig. 4.

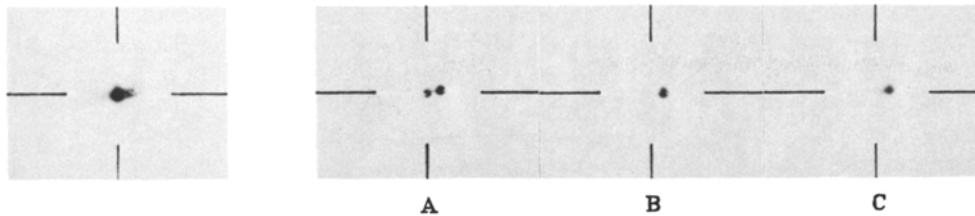


Figure 7. Location of acetylated  $\alpha$ -tubulin. Fractions enriched in cytoplasmic microtubules (A and B) or flagellar microtubules (C) were subjected to electrophoresis on 2D gels after mixing with the products of an in vitro translation of the mRNA hybrid section

lected using the class II clone pcNg 6-18. Proteins were transferred to nitrocellulose and then the immunoblots were incubated with AA-4.3, a monoclonal antibody to *Naegleria*  $\alpha$ -tubulins (A), or 6-11B-1, a monoclonal antibody specific to the acetylated form of  $\alpha$ -tubulin (B and C). Cross-hairs were positioned over the  $\alpha$ -1 tubulin spot on each autoradiogram, left panel, and then transferred to the immunoblots, three right panels, as described in Fig. 6.

Acetylation of the *Naegleria* flagellar  $\alpha$ -tubulin subunit was detected using a monoclonal antibody specific to the acetylated  $\alpha$ -tubulin of *Chlamydomonas* (34). As illustrated in Fig. 7, this antibody reacted with *Naegleria* flagellar  $\alpha$ -tubulin and with the more acidic form in the cell body but not with the more basic cell body form that comigrated with  $\alpha$ -1. The presence of acetylated  $\alpha$ -tubulin in the cell body fraction could be due to contamination of this fraction with flagella or it could be due to the presence of acetylated  $\alpha$ -tubulin in basal bodies or cytoplasmic microtubules as found in *Chlamydomonas* (25).

#### Class IV DS RNA Codes for Flagellar Calmodulin

In vitro translation of the mRNA selected by the class IV plasmid, pcNG 44, gave rise to a protein with an  $M_r$  of 18,800 on SDS gel electrophoresis in the presence of 2 mM EGTA. When this protein was analyzed on SDS gels in the presence of 2 mM  $\text{CaCl}_2$ , its  $M_r$  decreased to 15,600, a change characteristic of a calcium-binding protein. As with CaM (18), the product of the class IV mRNA was not precipitated when cell-free translation extracts were heated at 90°C for 2 min.

Fulton et al. (9) have demonstrated the existence of two CaMs in *Naegleria* strain NEG. CaM-1, with an  $M_r$  of 16,000 in the absence of calcium, accounted for ~75% of the total and was found in flagella, while CaM-2, with an  $M_r$  of 15,300 in the absence of calcium, was found in the cytoplasm. Both CaM-1 and CaM-2 were significant products in the heat stable fraction of an in vitro translation reaction using total poly(A)<sup>+</sup> RNA from differentiating strain NEG.

We compared the heat stable in vitro translation products of total poly(A)<sup>+</sup> RNA from strain NB-1, used in this work, and those from strain NEG, used by Fulton et al. (9), with the product of the class IV mRNA. As illustrated in Fig. 8, the major heat stable product of NB-1 total poly(A)<sup>+</sup> RNA comigrated with the product of the class IV mRNA and both of these proteins comigrated with CaM-1, the flagellar CaM in EGTA and calcium. The apparent discrepancy in relative molecular mass between the values reported by Fulton et al. (9) and us are presumably due to differences in batches of reagents and to the values assigned to standards. The absence of a band corresponding to CaM-2, the cytoplasmic form of CaM in the total heat stable products of either total or class IV-selected poly(A)<sup>+</sup> RNA from strain NB-1, may reflect differences in the relative abundance of the mRNAs for CaMs between strains. In any case it is clear that the protein produced by the mRNA selected by the class IV plasmid

comigrates with CaM-1 both in the presence and absence of calcium.

In an effort to further characterize this protein, we have sequenced one strand of the central 234 bp of the class IV cDNA (Garofalo, G., and C. Walsh, unpublished observations). This is equal to 53% of the coding region of CaM. The single open reading frame predicts an amino acid sequence with an 81% homology to bovine brain calmodulin (43).

Attempts to obtain in vitro translation using the class I plasmids to select mRNA have, so far, been unsuccessful, perhaps due to the large size of this mRNA.

#### Discussion

Our data demonstrate that at least three of four coordinately regulated mRNAs specific to the differentiation of *Naegleria* amoebae into flagellates code for flagellar proteins. The prod-

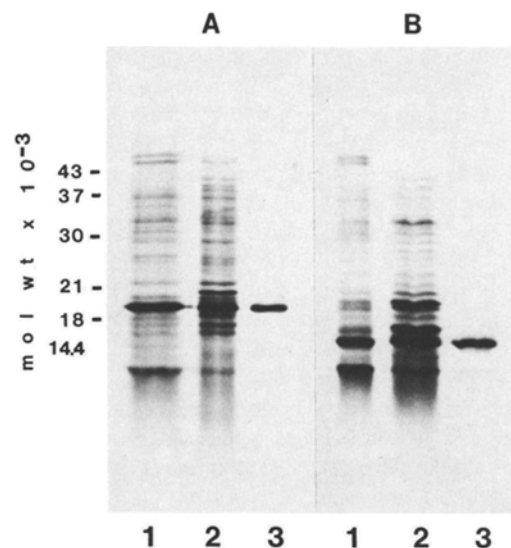


Figure 8. Comparison of the class IV mRNA product with flagellar CaM. In vitro translation products of total 70 min poly(A)<sup>+</sup> RNA from *Naegleria* strain NB-1 (lane 1), total 60 min poly(A)<sup>+</sup> RNA from strain NEG (lane 2), or the mRNA hybrid selected from NB-1 poly(A)<sup>+</sup> RNA using the class IV plasmid (lane 3) were heated at 90°C for 2 min and then the soluble proteins were fractionated on 15% SDS gels containing either 2 mM EGTA (A) or 2 mM  $\text{CaCl}_2$  (B). In vitro products were visualized by fluorography of the dried gels.

ucts of the class II and III mRNAs are tubulin subunits based on their comigration with tubulin during 2D gel electrophoresis and their binding to antibodies against *Naegleria* tubulin. The product of the class IV mRNA is a heat stable calcium-binding protein that comigrates with *Naegleria* flagellar CaM-1, both in the presence and absence of calcium, and shows significant nucleotide sequence homology with bovine brain CaM.

It is clear that the *Naegleria* tubulin subunits that migrate fastest on our SDS gels are  $\alpha$ -tubulins. This is demonstrated by the monoclonal antibody binding data and by their isoelectric points as well as by the fact that this tubulin subunit is acetylated in flagella. Further support for this identification is provided by the similarity, as noted below, between these *Naegleria* proteins and the  $\alpha$ -tubulins of *Chlamydomonas*. A reversal in the relative migration of  $\alpha$ - and  $\beta$ -tubulins has been reported in a number of unicellular organisms including *Physarum polycephalum* (3), *Crithidia fasciculata* (36), and *Tetrahymena thermophila* (40).

In vitro translation of the Class III mRNA produced a protein that migrated as a single somewhat elongated spot on 2D gels. This spot encompassed the region of  $\beta$ -tubulin. However, there was a slight extension of the in vitro product on the basic side, such that there was not complete correspondence with flagellar  $\beta$ -tubulin. This could reflect heterogeneity in the in vitro product that is only partially resolved in this gel system. In some autoradiograms there appeared to be a minor, more acidic product of this RNA. We cannot determine at this time if this heterogeneity is a contaminating nontubulin protein, an artifact of the translation system, or if it reflects the translation of more than one  $\beta$ -tubulin mRNA. The presence of an elongation of the  $\beta$ -tubulin spot when total poly(A)<sup>+</sup> RNA was translated in vitro could mean that there is an additional  $\beta$ -tubulin mRNA that did not hybridize with the class III plasmid or it may simply reflect the presence of another protein.

All six of the class II plasmids selected mRNA that directed the production of two proteins. The major protein,  $\alpha$ -1, comigrated with the  $\alpha$ -tubulin subunit from *Naegleria* cytoplasmic microtubules. The minor in vitro product,  $\alpha$ -2, comigrated with flagellar  $\alpha$ -tubulin. Although the monoclonal antibody specific to the acetylated form of  $\alpha$ -tubulin indicates that *Naegleria* flagellar  $\alpha$ -tubulin is acetylated it seems unlikely that  $\alpha$ -2 arises as the result of acetylation in vitro. It is more likely that  $\alpha$ -1 and  $\alpha$ -2 represent the products of two mRNAs that are both selected by the class II clones.

There are two  $\alpha$ -tubulin genes in *Chlamydomonas* and their sequences predict that they will produce proteins that differ by one charge (38). These proteins are thought to correspond to two  $\alpha$ -tubulins seen when *Chlamydomonas* mRNA is translated in vitro or in *Xenopus laevis* oocytes; alpha 1, with a pI of 5.7, and alpha 3, with a pI of 5.5 (26, 38). *Chlamydomonas* flagellar  $\alpha$ -tubulin comigrates with alpha 3, the minor more acidic  $\alpha$ -tubulin synthesized in vitro (26, 32), but flagellar  $\alpha$ -tubulin is derived from alpha 1 by acetylation (27, 28). Our in vitro translation data and this close correspondence between the  $\alpha$ -tubulins of *Chlamydomonas* and *Naegleria* suggest that there are at least two functional  $\alpha$ -tubulin genes in *Naegleria*. This is consistent with the report that there may be as many as eight  $\alpha$ -tubulin-like sequences in *Naegleria* strain NEG and that at least two of these are transcribed (22).

The presence of minor  $\alpha$ -tubulins with the same electrophoretic migration relative to both flagellar  $\alpha$ -tubulin and the major  $\alpha$ -tubulin synthesized in vitro, in organisms as evolutionarily distant as *Naegleria* and *Chlamydomonas*, suggests that they have an important functional role. This idea is supported by the fact that a similar minor form of  $\alpha$ -tubulin is seen when RNA from *Polytomella agilis* (32) or *Physarum polycephalum* myxamebae (15) is translated in vitro. In *P. polycephalum*, this  $\alpha$ -1 satellite, as it is referred to, is a product of RNA from myxamebae that are forming flagella, but apparently not of RNA from vegetative myxamebae (2). The  $\alpha$ -tubulin of *C. fasciculata* also seems to fit this general pattern with a minor, more acidic form (36), although the acetylated flagellar tubulin migrates at an even more acidic pI than the minor form.

Although we would like to know how synthesis of the nearly 200 different proteins of the axoneme is regulated, studies of the regulation of cilia and flagella formation have, understandably, concentrated on the synthesis of tubulin, the most abundant and best characterized protein in the axoneme (29). It is clear from studies in many organisms (see recent review by Cleveland [4]) that the synthesis of tubulin is coupled to the formation of cilia and flagella. In *Naegleria*, changes in the levels of tubulin are the result of changes in the concentration of tubulin mRNA. Initial studies using in vitro translation demonstrated that *Naegleria* amebae lack detectable tubulin mRNA and that RNA capable of producing tubulin in a cell-free extract appears between 10 and 15 min after initiation (23). The data presented above, coupled with our previous studies of changes in the DS RNAs (31), demonstrate that the appearance of the ability to synthesize  $\alpha$ - and  $\beta$ -tubulin is the result of at least a 20-fold increase in the concentration of  $\alpha$ - and  $\beta$ -tubulin mRNA during the first 70 min of the differentiation. A similar conclusion has been reached regarding changes in  $\alpha$ -tubulin mRNA in *Naegleria* strain NEG (22).

It has recently been reported that mRNAs for four nontubulin axonemal proteins—two radial spoke proteins and two dynein chains—are among those that are elevated after deflagellation of *Chlamydomonas* (44). Our data demonstrate that in *Naegleria* the mRNA for another nontubulin protein of flagella, CaM-1, is regulated coordinately with  $\alpha$ - and  $\beta$ -tubulin mRNA. This confirms the report (9), based on in vitro translation, that CaM-1 mRNA appears transiently during the differentiation. We think that it is likely that the as yet unidentified class I protein will also prove to be a component of the *Naegleria* flagellar apparatus.

The identification of a 16-bp consensus sequence in the 5' untranslated region of all four of the *Chlamydomonas* tubulin genes (1) suggests that they may all respond to a common regulatory element. We are attempting to obtain genomic clones for the four DS RNA sequences in order to determine if they share common sequences. This possibility is strengthened by the demonstration that the coordinate changes in the concentration of the *Naegleria* DS RNAs are a reflection of coordinate changes in the rate of their transcription (Lee, J. H., and C. J. Walsh, manuscript submitted for publication). Thus, in both *Naegleria* and *Chlamydomonas*, flagellum formation is associated with a transient increase in tubulin mRNA that is a result of changes in transcription, and at least some nontubulin axonemal proteins are regulated in the same way.



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