

The α -Tubulin Gene Family Expressed during Cell Differentiation in *Naegleria gruberi*

Elaine Y. Lai, Stephen P. Remillard, and Chandler Fulton

Department of Biology, Brandeis University, Waltham, Massachusetts 02254

Abstract. Genes that direct the programmed synthesis of flagellar α -tubulin during the differentiation of *Naegleria gruberi* from amebae to flagellates have been cloned, and found to be novel with respect to gene organization, sequence, and conservation. The flagellar α -tubulin gene family is represented in the genome by about eight homologous DNA segments that are exceptionally similar and yet are neither identical nor arrayed in a short tandem repeat. The coding regions of three of these genes have been sequenced, two from cDNA clones and one from an intronless genomic gene. These three genes encode an identical α -tubulin that is conserved relative to the α -tubulins of other organisms except at the carboxyl terminus, where the protein is elongated by two residues and ends in a terminal glutamine instead of the canonical

tyrosine. In spite of the protein conservation, the *Naegleria* DNA sequence has diverged markedly from the α -tubulin genes of other organisms, a counterexample to the idea that tubulin genes are conserved.

α -Tubulin mRNA homologous to this gene family has not been detected in amebae. This mRNA increases markedly in abundance during the first hour of differentiation, and then decreases even more rapidly with a half-life of ~ 8 min. The abundance of physical α -tubulin mRNA rises and subsequently falls in parallel with the abundance of translatable flagellar tubulin mRNA and with the in vivo rate of flagellar tubulin synthesis, which indicates that flagellar tubulin synthesis is directly regulated by the relative rates of transcription and mRNA degradation.

MICROTUBULES are crucial participants in cell division in every eukaryote and in various other aspects of cell shape and motility in most eukaryotic cells, including flagellar motility in those cells that have flagella or cilia. Because these important functions involve specific interactions of α - and β -tubulin subunits with each other as well as with GTP and divalent cations to form heterodimers, with other heterodimers to form microtubules, and with numerous other proteins to form and use both transient and stable microtubular structures, it is not surprising that α - and β -tubulins have been conserved throughout the evolution of eukaryotes. The conclusion about conservation was extended from amino acid sequence to nucleotide sequence when it was found that tubulin genes of one organism would readily cross-hybridize with tubulin genes of other organisms (10). As an example the first cloned DNA probe for α -tubulin, a cDNA clone to mRNA from embryonic chick brain, has been reported to cross-hybridize readily to α -tubulin DNA or RNA from mammals, sea urchins, *Drosophila*, *Chlamydomonas*, *Trypanosoma*, *Physarum*, *Tetrahymena*, and *Stylonychia* (3, 7, 8, 10, 12, 24, 54, 57, 58, 68, 71).

Despite the conservation of tubulin and its genes, the number and arrangement of tubulin genes vary widely (9). The ciliate *Tetrahymena* appears to have a single α -tubulin gene (8), but other eukaryotes examined have two or more. In

most cases where multiple α -tubulin genes are present the DNA segments differ sufficiently in restriction sites that individual genes produce distinct restriction fragments. The segments also do not appear to be closely linked to one another except for two linked segments among 10–15 in sea urchins (1). In contrast, the trypanosomid flagellates *Leishmania* and *Trypanosoma* each have roughly 15 α -tubulin genes that are copies or near-copies of one another and are linked in closely spaced tandem arrays (34, 58, 68).

The rapid phenotypic change undergone by the unicellular eukaryote *Naegleria gruberi*, from amebae to flagellates, offers exceptional opportunities to analyze events during a 'simple' cell differentiation (15). Amebae, the vegetative form of *Naegleria*, can be grown for thousands of generations without any trace of flagella or any of the structural components of the flagellar apparatus. Such amebae, transferred from a growth environment to a dilute buffer, differentiate within 100 min to streamlined flagellates with a complex flagellar apparatus and two anterior flagella. Much of our study of this differentiation has been motivated by the finding that the tubulin which forms the flagella is synthesized as a programmed event during differentiation (29). The de novo synthesis and antigenic specificity of this flagellar tubulin led to the original formulation of the multitubulin hypothesis (17). In addition, translatable mRNA for flagellar tubulin, both α - and β -subunits, not detected in amebae, was found

to increase markedly in abundance during the first hour of differentiation and then to rapidly disappear (31). The abundance of translatable flagellar tubulin mRNA was observed to rise and fall during differentiation in parallel with the rate of flagellar tubulin synthesis *in vivo* (31).

Our continuing dissection of flagellar tubulin synthesis during differentiation led us to isolate DNA clones representing the tubulin genes of *Naegleria*. We cloned these genes in spite of an unexpected limited homology of these genes to the tubulin genes of other organisms. We here report the characterization of three members of the α -tubulin gene family expressed during differentiation, which also provides a first examination of genes from this evolutionarily enigmatic organism. This family reveals a novel architecture of α -tubulin genes, in this case about eight very similar but not identical genes that do not appear to be arrayed in a short tandem repeat. The three genes from this family that were sequenced contain only translationally silent substitutions and thus encode a single α -tubulin. We present evidence that although the α -tubulin protein of *Naegleria* is conserved in comparison to those of other studied eukaryotes, its genes are much less conserved. We used the clones to measure the abundance of α -tubulin mRNA homologous to this gene family. This mRNA becomes relatively abundant during the first hour of differentiation, but has not been detected in cells before the initiation of differentiation. The abundance measurements indicate that the rate of flagellar tubulin synthesis during cell differentiation is directly regulated by the physical abundance of its mRNA. In addition, the results provocatively reopen the question of whether a separate, divergent α -tubulin gene family may be used for mitosis in *Naegleria* amoebae. A preliminary report of some of these findings has been presented (32).

Materials and Methods

Cells

N. gruberi NEG (15) was used for all experiments. Amoebae were grown and differentiation to flagellates was obtained and evaluated as described (31).

Isolation of RNA and DNA from *N. gruberi*

Total cellular RNA of *Naegleria* was extracted from cells at various times during differentiation, and poly(A)⁺ RNA was prepared from these samples, as described (31). DNA was isolated from cells suspended in 0.15 M NaCl, 0.1 M EDTA, pH 8.2 at 4°C and lysed by the addition of 2.4% (wt/vol) SDS followed in rapid succession by 2.4% (vol/vol) diethylpyrocarbonate, mixing for 15 s with a glass rod, and immersion at 65°C for 10 min. All these steps were required to minimize degradation of the DNA by endogenous nucleases (Walsh, C., and C. Fulton, unpublished observations). Subsequently the lysate, at room temperature, was adjusted to 1 M sodium perchlorate and extracted repeatedly with chloroform-isoamyl alcohol (24:1, vol/vol). The DNA was spooled, ethanol precipitated, treated with RNase A and with pronase, re-extracted with chloroform-isoamyl alcohol, spooled from ethanol, and dialyzed vs. 0.01 M Tris, 1 mM EDTA, pH 8.2 according to standard procedures (39, 67). Undegraded high-molecular-weight DNA was obtained at the end of this procedure, but this DNA contained material that inhibited digestion by restriction enzymes. To remove this material, the DNA was further purified by centrifugation to equilibrium in potassium iodide gradients (5). This additional step yielded DNA of high purity and normal sensitivity toward restriction enzymes.

Construction and Screening of a cDNA Clone Library

cDNA clones were constructed from poly(A)⁺ RNA extracted from cells after 60 min of differentiation (31) after the method of Okayama and Berg

(47) and using their vectors. A library of 5444 recombinant DNA plasmids in *E. coli* HB101 was stored in an ordered collection according to the procedure of Gergen et al. (19). Filter replicas on Whatman 541 were screened using a nick-translated (39) heterologous α -tubulin probe, the Pst I-Eco RII fragment of chicken cDNA clone pT1 which included the coding region starting at codon 40 (10, 71). Clone pT1 was kindly provided by Dr. D. Cleveland (Johns Hopkins University, Baltimore, MD). The low-stringency hybridization conditions used were those of Neff et al. (45): 15% (vol/vol) formamide, 6× SSPE (44), 0.5% (wt/vol) SDS, and 50 μ g/ml of sonicated, denatured *E. coli* DNA, at 55°C. The filters were washed in 1× SSPE, 0.5% SDS at 50°C. The results of colony filter hybridization under these conditions were "hybrids" in a gradation of grays. Putative clones were partially sequenced to confirm their identities. Once the first *Naegleria* α -tubulin cDNA clone, pN α T1, was found, the library was rescreened at higher stringency (19) to obtain additional clones, including pN α T2.

Construction and Screening of a Genomic Library

Fragments of *Naegleria* genomic DNA with an average length of 20 kb, prepared by partial digestion with *Sau*3A, were cloned into the λ replacement vector EMBL3 (14) cut with Bam HI and Eco RI. A total of 45,000 recombinant phage were obtained and then amplified (39) on *E. coli* CES201 (44), which for clones containing *Naegleria* inserts gave twice the plating efficiency as strain NM539. These phage, screened by plaque hybridization using the insert of pN α T1, gave 23 phage that contained α -tubulin-like DNA sequences. To select an α -tubulin genomic clone comparable to pN α T1 these 23 phage were rescreened using an oligonucleotide specific to pN α T1, selected after comparing the partial sequences of pN α T1 and pN α T2. The 14-mer, 5'-d[AAAAGTGTCATGG]-3', was kindly synthesized by Dr. Rolf Heumann of the Max-Planck-Institut, Munich. The 14-mer was 5'-labeled using T4 polynucleotide kinase (39), and hybridized under the conditions described by Montgomery et al. (43). The phage that hybridized most strongly was chosen for further analysis, and subsequently designated λ N α T3.

Agarose Gel Electrophoresis and Filter Hybridization

Southern transfer of *Naegleria* DNA digested with restriction enzymes, separated by electrophoresis in an agarose gel, and then partially hydrolyzed by acid depurination (73) was accomplished using standard procedures (39, 63). Nitrocellulose blots were prehybridized, then hybridized and washed under stringent conditions as described (p. 326 in reference 39) except the final washes were at 50°C in 0.1× SSPE and 0.1% SDS. An estimate of the number of α -tubulin genes in *Naegleria* was accomplished by calibrating the annealing results using a dilution of cloned DNA by the method of Lis et al. (11, 38).

Total cellular RNA of *Naegleria* was fractionated on denaturing (formaldehyde-containing) 1.5% agarose gels and transferred to nitrocellulose using standard procedures (39). DNA-RNA hybridization was carried out for 16 h at 37°C in 50% formamide, 5× SSC (39), 5× Denhardt's solution (39), 0.1% SDS, 25 μ g/ml poly(A), 100 μ g/ml sheared and denatured salmon sperm DNA, 20 mM sodium phosphate, pH 7.0, and 2 × 10⁶ cpm/ml of nick-translated DNA probe prepared as the 1.6-kb insert of pN α T1 excised using Pst I and Pvu II. Blots were washed to a final stringency of 0.1× SSC, 0.1% SDS at 50°C.

For quantitative RNA dots, triplicate 2- μ g aliquots of total *Naegleria* RNA, pretreated with DNase, were dotted onto nitrocellulose. Conditions of hybridization and washing were as described for RNA blots. The hybridization, in DNA excess, was shown to be quantitative in that the relative amount of α -tubulin sequence detected (a) was linear with the concentration of 60 min RNA up to 3 μ g per dot, (b) was independent of the presence of nontubulin-containing RNA (e.g., 0 min RNA) up to at least 3 μ g, and (c) was reproducible. After autoradiography individual dots were excised and their radioactivity determined.

Hybrid Selection of RNA and Cell-free Translation

For hybrid selection we used 5 μ g of pN α T1 DNA, poly(A)⁺ RNA isolated at 60 min of differentiation, and the procedure of Riccardi et al. (52). The hybridized RNA, released from the filters, was used to direct translation in the cell-free wheat-germ system with L-[³⁵S]methionine as label (31). The cell-free products were analyzed by isoelectric focusing (65) followed by electrophoresis in the second dimension as described (46) except that the sample buffer also contained 1 mg/ml L-lysine.

DNA Sequencing

The α -tubulin DNA clones were sequenced by the dideoxynucleotide chain termination method (55) after subcloning into bacteriophage M13mp8, 9, 18, or 19 using *E. coli* JM103, 107 and 109 as hosts (75). Either selected restriction fragments were subcloned or, in most cases, deletion derivatives were generated by unidirectional digestion using exonuclease III (25). Analysis of DNA sequences was performed using a Hewlett-Packard 9845C computer and programs written by the senior author.

Results

Isolation and Characterization of *Naegleria* α -Tubulin DNA Clones

A library of cDNA clones was prepared using poly(A)⁺ RNA isolated from *Naegleria* at 60 min of differentiation, when translatable flagellar tubulin mRNA is most abundant (31). Based on the assumption that α -tubulin genes are conserved, the coding region insert of chicken α -tubulin cDNA clone pT1 (10, 71) was used to screen this library. To obtain any hybridization of pT1 to *Naegleria* DNA, we had to resort to very permissive conditions of hybridization, similar to those used by Neff et al. (45) to isolate a yeast β -tubulin DNA clone. In pilot experiments, these permissive conditions allowed detection by pT1 of bands of *Naegleria* DNA in genomic blots (these bands were later found not to represent the α -tubulin gene family described in this paper). In colony hybridization of pT1 to our library, the permissive conditions resulted in a high signal-to-noise ratio, and many false positives were picked. After various attempts we obtained a clone that was shown by partial DNA sequencing to encode α -

tubulin; this first *Naegleria* α -tubulin DNA clone was designated pN α T1.

Subsequent experiments revealed that the α -tubulin coding regions contained in the *Naegleria* pN α T1 and the chicken pT1 clones would not reproducibly hybridize to each other, even under the permissive conditions. In retrospect, these experiments indicated that pN α T1 probably was picked from the library by chance rather than selected by pT1. The limited homology, initially unexpected in view of emphasis on the conservation of tubulin genes, is explained by the results of sequence analysis, as described below. We have also isolated clones representing *Naegleria* β -tubulin genes expressed during differentiation, and found that these clones show limited homology to chicken and other heterologous β -tubulin DNAs (Lai, E., S. P. Remillard, and C. Fulton, manuscript in preparation).

RNA selected from total 60-min RNA by hybridization to clone pN α T1 was translated in the wheat-germ cell-free system and the product examined by two-dimensional PAGE and autoradiography. Fig. 1 shows that the selected RNA directed the synthesis of α -tubulin. The translation product could also be immunoprecipitated by using the antibodies specific to *Naegleria* flagellar tubulin (31) (not shown). On Northern blots of 60-min RNA, pN α T1 recognized RNA of a single size, estimated at \sim 1.7 kb.

When the insert of pN α T1 was used to rescreen the cDNA library under stringent conditions, we found 27 strongly hybridizing clones at a frequency of 0.5% of the library. After preliminary characterization of these clones, which had very similar restriction maps, an additional clone, designated pN α T2, that was found to have a Bst EII site not present in pN α T1, was selected for further study. A comparison of the similar restriction maps of these two cDNA clones is shown in Fig. 2, A and B. Both clones have \sim 1.6-kb inserts that include the complete coding region of α -tubulin.

We selected 23 phage with inserts complementary to pN α T1 from a library of *Naegleria* genomic DNA in λ vector EMBL3. We decided it would be desirable to characterize a genomic clone similar or identical to pN α T1, and based on the sequencing of pN α T1 and pN α T2 then in progress we selected a 14 nucleotide segment from the 3'-untranslated region that would discriminate between them. The 14-mer detected 3 of 23 genomic clones; the one that gave the strongest signal was selected. A single α -tubulin coding region was localized in the 13.2-kb insert of this clone (Fig. 2, C and D). Subsequent sequencing (described below) revealed that the selected genomic clone is not identical to pN α T1, so this clone is designated λ N α T3. For brevity the two cDNA clones are called α T1 and α T2 and the genomic clone α T3.

Architecture of Genomic Sequences Homologous to α -Tubulin Genes

Genomic blots of *Naegleria* DNA, digested to completion with restriction enzymes that have six-base recognition sites, revealed a small number of fragments homologous to α T1, as shown by the examples in Fig. 3 (left). Some enzymes, such as Bam HI and Pvu II, gave a single strongly hybridizing band, and other enzymes gave two bands (e.g., Hind III and Pst I in Fig. 3); of the enzymes tested in Fig. 3 only Eco RI gave more than two major bands. With the exception of the Eco RI fragments, all the major fragments shown in Fig. 3 hybridized to both the 5' and the 3' portions of the insert

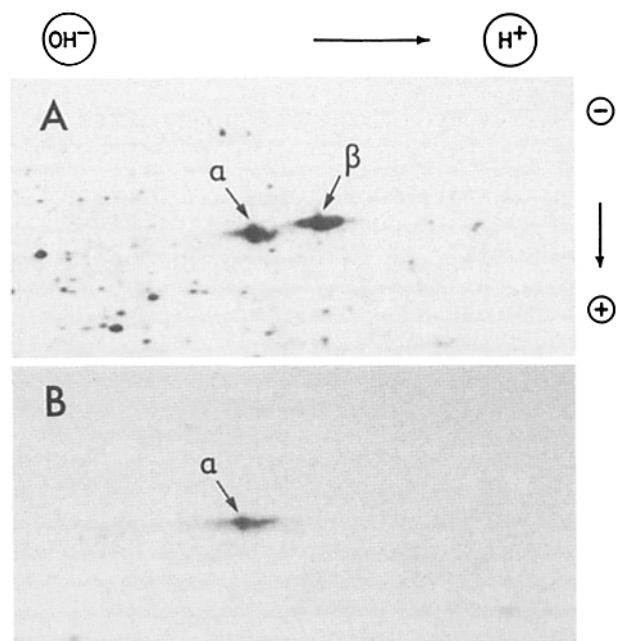


Figure 1. Translation products directed (A) by total 60-min RNA and (B) by the same RNA hybrid-selected using clone pN α T1. The RNA samples were used to direct translation in the wheat-germ cell-free system, and the [³⁵S]methionine-labeled products were analyzed by two-dimensional PAGE and autoradiography. Identification of the spots as α - and β -tubulin is based on our previous studies (31) and our unpublished results, including comigration with purified flagellar tubulin subunits.

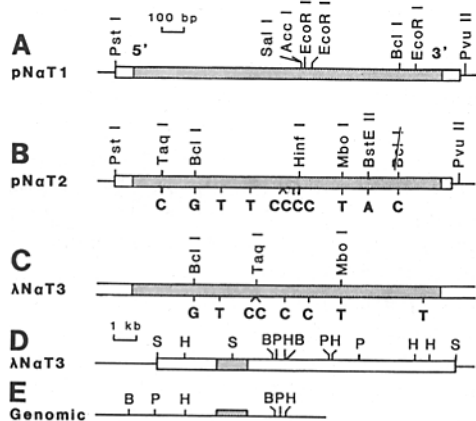


Figure 2. Restriction maps of *Naegleria* α -tubulin DNA sequences. The maps are oriented 5' to 3' with respect to the shaded coding region. (A and B) Inserts of cDNA clones, including their terminal Pst I and Pvu II restriction sites. (C and D) Genomic clone. (E) Map of restriction sites shared among most α -tubulin genes of *Naegleria*. (A–C) are drawn at the same scale; (D and E) are reduced to 0.1. Sites were determined by restriction mapping and, for A–C, DNA sequencing. A shows selected sites in the coding region which, except for Bcl I, are conserved in all three clones. B and C show only restriction sites that differ from those in clone pN α T1 (A); within the coding regions the nucleotide substitutions from A are shown below the line. D shows the coding region within the 13.2-kb insert of λ N α T3 with sites for (B) Bam HI; (H) Hind III; (P) Pvu II; and (S) Sal I. E shows a map of the major genomic α -tubulin genes (those that yield small prominent genomic fragments) deduced using single and double digestions of genomic DNA with these and other enzymes followed by Southern blots probed with the insert of pN α T1. The fragments mapped appear to be of similar abundance in each of the single and double digestions. Larger fragments of genomic DNA that contain α -tubulin-like sequences were seen in all digestions but could not be fitted into this map.

of α T1 (cut with Acc I; Fig. 2 A); therefore these fragments each contain at least the central portion of the α T1-like sequence. The sequences represented by the major Bam HI and Pvu II fragments have such conserved restriction sites that it was possible to deduce a single genomic map for these major fragments from double digestions and genomic blots (Fig. 2 E). This map was later found to concur with the map of genomic clone α T3 (Fig. 2 D). Overall these results suggest either a small number of homologous α -tubulin DNA sequences in the genome, such as two, or multiple copies of very similar sequences.

Quantitative genomic blots (11, 38) allowed us to estimate the number of homologous α -tubulin DNA sequences in the genome. In the example shown in Fig. 3, plasmid DNA of pN α T1 linearized by digestion with Pvu II was loaded on an agarose gel adjacent to 2.0- μ g aliquots of digested total *Naegleria* DNA, in amounts equivalent to from 1 to 16 copies of α T1 per 2 μ g of *Naegleria* DNA. (The total DNA content of haploid amoebae is 0.17 pg per cell (15), which is equivalent to 1.6×10^5 kb.) After transfer and hybridization, the signal gave a hybridization standard curve (Fig. 3, inset). Only fragments nearest in size to the 4.2-kb plasmid standard were quantitatively compared. The 5.4-kb Pvu II fragment was found as 7.2 copies in the genomic DNA; the

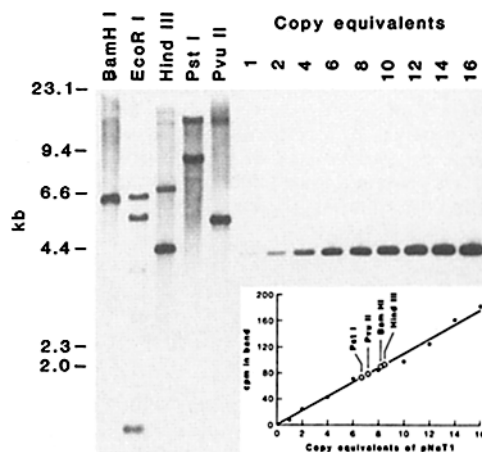


Figure 3. α -Tubulin sequences homologous to pN α T1 are repeated at least eight times in the genome of *Naegleria*. Total *Naegleria* DNA was digested to completion with the indicated restriction endonucleases, and aliquots of 2.0 μ g were placed in the indicated lanes of a 0.8% agarose gel. In addition, the indicated multiples of pN α T1, linearized by digestion with Pvu II, were placed in the lanes on the right. The amounts of pN α T1 were adjusted to be equivalent to 1 to 16 copies of the α -tubulin sequence in the lanes with *Naegleria* DNA. As a size standard λ DNA digested with Hind III was run in another lane. After electrophoresis, the DNA was transferred to nitrocellulose and hybridized to the 32 P-labeled 1.6-kb Pst I–Pvu II insert of pN α T1. Radioactivity was determined by excising and counting individual fragments. (Inset) Plot of the radioactivity found in various copy number equivalents of pN α T1 and its use to determine the number of copies found in selected genomic fragments.

6.4-kb Bam HI fragment, 8.2 copies; the 4.4 kb Hind III fragment, 8.4 copies; and the 8.8-kb Pst I fragment, 6.7 copies. Although the fragments measured are larger than the 4.2-kb standard, the observed range of copy number (6.7–8.4) is smaller than the size range of the measured fragments (4.4–8.8 kb). These estimates suggest a minimum of about eight α T1-like sequences per genome. Two experiments using the same probe with genomic dot blots also gave an estimate of 5–10 copies per genome. It would be desirable to refine this estimate using a single-copy gene as internal standard, but no such gene has yet been characterized in *Naegleria*. The multiplicity of copies is also strongly supported by the finding that probes containing only portions of α T1 3' to the Acc I site (Fig. 2 A) hybridize to eight separate Eco RI fragments (not shown).

These results probably underestimate the copy number. In particular, in all these digestions there were larger homologous fragments in the genomic blot that are not included in the copy number estimated for single bands. For example, the 4.4-kb Hind III fragment hybridized with an intensity expected if this band contained 8.4 copies of a α T1-like sequence, but the larger 7.0-kb Hind III fragment appeared to contain 2.6 additional copies. The 8.8- and 14.5-kb Pst I fragments appeared to contain 6.7 and 3.5 copies, respectively.

The α -tubulin genes of two flagellates, *Trypanosoma* and *Leishmania*, have been found to be organized in short tandem repeats of 2 and 3.7 kb, respectively (34, 58, 68). To test whether the major Bam HI and Pvu II fragments could arise

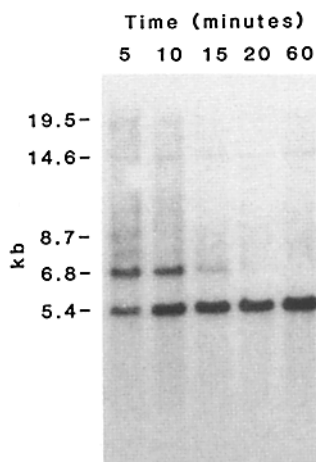


Figure 4. Partial digests of genomic DNA using Pvu II probed with pNaT1 insert DNA. 12 μ g of purified *Naegleria* DNA was digested with 24 U of Pvu II in 60 μ l of digestion buffer. At each indicated time a 10- μ l aliquot of the digestion mix was prepared for electrophoresis on a 0.5% agarose gel. The gel was subsequently blotted onto nitrocellulose and hybridized to nick-translated α T1 probe as in Fig. 3. A Hind III digest of λ DNA provided a size standard from which the sizes of the fragments were determined. Experiments using 0.4% agarose gels gave similar results.

from such repeats, we partially digested *Naegleria* DNA with each of these enzymes and looked for bands that formed a ladder of multiples of size. As shown in the example of Fig. 4, these partial digests have revealed larger bands, as would be expected, but no hint of multiples that could be explained as tandem repeats. These results indicate that the major α T1-like sequences are not in a short (<10 kb) repeat.

The picture that emerges from this description does not appear to conform to any previously described arrangement of α -tubulin genes, in which multiple tubulin genes have been found either to be dispersed and sufficiently divergent to readily yield separate restriction fragments or to be virtually identical in a tandem repeat. The majority of the multiple *Naegleria* α T1-like genes are so similar that they have conserved several restriction sites over a length of 6.8 kb (Fig. 2 E), yet other evidence, such as the multiple Eco RI fragments, indicate that even those genes included in the Bam HI fragment are not identical. Overall our results suggest that the major α -tubulin genes of *Naegleria* are present at roughly eight copies per genome, scattered or at least not closely arrayed as in a short tandem repeat, and exceedingly similar but not identical. The lack of identity is established for three of these very similar genes by DNA sequencing.

DNA Sequences of Three α -Tubulin DNA Clones

The inserts of two α -tubulin cDNA clones, α T1 and α T2, were sequenced completely using subclones in M13 derivatives and the dideoxynucleotide chain termination procedure. The genomic clone α T3 was sequenced through the comparable region. The coding region DNA sequences are given in Fig. 5. The three genes were found to differ from each other in a few nucleotide substitutions that are scattered over the coding region, as displayed in Fig. 2, B and C. The greatest difference, between α T1 and α T2, is 11 substitutions among 1359 nucleotides. It is remarkable that 6 of these 11 substitutions produce altered restriction sites (Fig. 2 B). If one derives a consensus sequence (as α T1 except G at nucleotide 276, T at 384, C at 673, and T at 918), α T1 differs from this sequence by 4 nucleotides, α T2 by 7, and α T3 by 4, indicating that the sequences differ from one another by an average of 0.4%. 13 of the 15 differences are C-T transitions.

The nucleotide sequence of α T3 was found to be congruent with those of the cDNA clones, so this genomic clone contains no intervening sequences in the coding region. Since the restriction maps of α T3 and of the majority of genomic α -tubulin genes concur in having, for example, a 4.4-kb Hind III fragment that spans the coding region (Fig. 2, D and E), it is unlikely that any of this group of genomic α -tubulin genes contain introns of any substantial size. The occurrence of introns in protistan α -tubulin genes is variable; the number ranges from zero in the tandemly repeated genes of *Trypanosoma brucei* and in one of two genes of *Schizosaccharomyces pombe* (28, 70), one or two introns in other yeast genes and in the *Chlamydomonas* genes (56, 62, 70), and seven in a *Physarum* α -tubulin gene (42).

Deduced Amino Acid Sequence

An identical α -tubulin is encoded by the three DNA sequences; all the nucleotide substitutions are translationally silent changes that occur in third-base positions (Fig. 5). The encoded amino acid sequence is typical of a conserved α -tubulin. The alignment of *Naegleria* α -tubulin is congruent with those of most sequenced α -tubulins through residue 451, so *Naegleria* α -tubulin contains no deletions or insertions except for an extension of two residues at the carboxyl terminus. The predicted molecular weight of *Naegleria* α -tubulin is 49,840. The predicted amino acid composition is in good agreement with an amino acid composition previously obtained for α -tubulin purified from flagellar outer doublets of *Naegleria* (Simpson, P. A., E. L. Cannon, and C. Fulton, unpublished data; cysteine and tryptophan were not determined).

The extent of similarity to other encoded α -tubulin sequences is given below the diagonal in Table I. This comparison indicates that *Naegleria* α -tubulin most closely resembles that of the algal flagellate *Chlamydomonas* and the trypanosome flagellate *Trypanosoma*, slightly less the ciliate *Stylonychia* and the true slime mold *Physarum*, and still less the α -tubulin sequences of metazoa. Even with its unusual carboxyl terminus, the *Naegleria* sequence is as conserved as those of other unicellular eukaryotes. For example, *Naegleria* α -tubulin shares 91% of its residues with *Trypanosoma* α -tubulin and 85% with those of rat and man, whereas *Chlamydomonas* shares 90 and 86%, respectively. In comparison to the other α -tubulin sequences available, *Naegleria* α -tubulin has few unique or distinctive residues except at the carboxyl terminus. The residues encoded in *Naegleria* α -tubulin but not found in one or more of the other α -tubulins listed in Table I are Leu₃₅ (variable, usually Gln), Ser₁₃₉ (variable, usually Asn or His), Ala₁₇₈ (Ser in the other α -tubulins), Ala₁₉₃ (usually Ser or Thr), Gln₂₂₀ (usually Glu), Cys₂₇₀ (variable), and nine COOH-terminal residues (in boldface in Fig. 6). This is comparable to the number of unusual residues encoded in α -tubulins of other unicellular eukaryotes.

The most unusual portion of the *Naegleria* α -tubulin sequence is the carboxyl terminus, a region which until recently has been thought to be quite conserved among α -tubulins and to end regularly in an encoded tyrosine (9). The COOH-terminal sequences available for α -tubulins are compared in Fig. 6. All the COOH-terminal regions are exceptionally acidic; each of those listed in Fig. 6 except the two

α T1 ATG AGA GAA GTT ATT TCA ATT CAC ATT GGT CAA GCT GGT GTT CAA GTC GGT AAC GCC TGT TGG GAA CTC TAC TGT TTG GAA CAT GGT ATT
1 Met Arg Glu Val Ile Ser Ile His Ile Gly Gln Ala Gly Val Gln Val Gly Asn Ala Cys Trp Glu Leu Tyr Cys Leu Glu His Gly Ile
 α T2
 α T3
91 CAA CCT GAT GGT TTG ATG CCA TCC GAT AAG ACT ATT GGT GTT GAA GAT GAT GCT TTC AAT ACC TTC TTC TCT GAA ACT GGT GCT GGT AAG
31 Gln Pro Asp Gly Leu Met Pro Ser Asp Lys Thr Ile Gly Val Glu Asp Asp Ala Phe Asn Thr Phe Phe Ser Glu Thr Gly Ala Gly Lys
 α T2
 α T3
181 CAC GTT CCA AGA GCT GTC TTC CTC GAT TTG GAA CCA ACT GTT GTT GAT GAA GTT CGT ACC GGT ACT TAT AGA CAA CTC TTC CAC CCT GAA
61 His Val Pro Arg Ala Val Phe Leu Asp Leu Glu Pro Thr Val Val Asp Glu Val Arg Thr Gly Thr Tyr Arg Gln Leu Phe His Pro Glu
 α T2
 α T3
271 CAA TTA ATC ACT GGT AAG GAA GAT GCT GCT AAC AAC TAT GCT AGA GGT CAC TAC ACT ATT GGT AAG GAA ATT GTC GAT TTG TGT TTG GAC
91 Gln Leu Ile Thr Gly Lys Glu Asp Ala Ala Asn Asn Tyr Ala Arg Gly His Tyr Thr Thr Ile Gly Lys Glu Ile Val Asp Leu Cys Leu Asp
 α T2
6
 α T3
6
361 AGA ATT AGA AAG TTG GCT GAC AAC TGT ACT GGT TTG CAA GGT TTC CTT GTC TTC TCA TCT GTT GGT GGT GGT ACT GGT TCT GGT TTG GGT
121 Arg Ile Arg Lys Leu Ala Asp Asn Cys Thr Gly Leu Gln Gly Phe Leu Val Phe Ser Ser Val Gly Gly Gly Thr Gly Ser Gly Leu Gly
 α T2
T
 α T3
T
451 GCC TTG TTG TTG GAA AGA TTG TCT GTT GAC TAT GGT AAG AAG TCC AAG TTG GGT TTC ACT GTC TAT CCA TCT CCT CAA GTT GCT ACT GCT
151 Ala Leu Leu Leu Glu Arg Leu Ser Val Asp Tyr Gly Lys Lys Ser Lys Leu Gly Phe Thr Val Tyr Pro Ser Pro Gln Val Ala Thr Ala
 α T2
T
 α T3
541 GTT GTT GAA CCA TAC AAC TCT GTC TTG TCC ACT CAC GCC TTG TTG GAA CAC ACT GAT GTT GCT GTC ATG TTG GAC AAT GAA GCT ATC TAT
181 Val Val Glu Pro Tyr Asn Ser Val Leu Ser Thr His Ala Leu Leu Glu His Thr Asp Val Ala Val Met Leu Asp Asn Glu Ala Ile Tyr
 α T2
C C
 α T3
C C
631 GAT ATC TGT AGA AGA TCA TTG GAT ATC CAA AGA CCA ACT TAT ACT AAC TTG AAC AGA TTG ATT GCT CAA GTC ATT TCC TCA TTG ACT GCT
211 Asp Ile Cys Arg Arg Ser Leu Asp Ile Gln Arg Pro Thr Tyr Thr Asn Leu Asn Arg Leu Ile Ala Gln Val Ile Ser Ser Leu Thr Ala
 α T2
C C
 α T3
C C
721 TCT TTG AGA TTT GAT GGT GCT TTG AAC GTC GAC GTT ACT GAA TTC CAA ACT AAC TTG GTC CCA TAT CCA AGA ATT CAC TTT ATG TTG TGT
241 Ser Leu Arg Phe Asp Gly Ala Leu Asn Val Asp Val Thr Glu Phe Gln Thr Asn Leu Val Pro Tyr Pro Arg Ile His Phe Met Leu Cys
 α T2
C
 α T3
C
811 TCT TAT GCT CCA GTT ATT TCT GCT GAA AAG GCT TAT CAC GAA CAA TTG TCT GTT GCT GAA ATT ACC AAC TCT GCT TTC GAA CCA GCC TCA
271 Ser Tyr Ala Pro Val Ile Ser Ala Glu Lys Ala Tyr His Glu Gln Leu Ser Val Ala Glu Ile Thr Asn Ser Ala Phe Glu Pro Ala Ser
 α T2
 α T3
901 ATG ATG GCT AAG TGT GAC CCA AGA CAC GGT AAA TAC ATG GCT TGT TGT TTG ATG TAC AGA GGT GAT GTC GTT CCA AAG GAC GTC AAT GCT
301 Met Met Ala Lys Cys Asp Pro Arg His Gly Lys Tyr Met Ala Cys Cys Leu Met Tyr Arg Gly Asp Val Val Pro Lys Asp Val Asn Ala
 α T2
T
 α T3
T
991 GCT GTT GCC ACT ATT AAG ACT AAG AGA ACT ATC CAA TTC GTT GAC TGG TCT CCA ACT GGT TTC AAG TGT GGT ATC AAT TAC CAA CCA CCA
331 Ala Val Ala Thr Ile Lys Thr Lys Arg Thr Ile Gln Phe Val Asp Trp Ser Pro Thr Gly Phe Lys Cys Gly Ile Asn Tyr Gln Pro Pro
 α T2
A
 α T3
A
1081 ACT GTC GTT CCA GGT GGT GAT TTG GCT AAG GTT CAA AGA GCT GTC TGT ATG ATT TCC AAC TCT ACT GCC ATT GCT GAA GTC TTC TCC AGA
361 Thr Val Val Pro Gly Gly Asp Leu Ala Lys Val Gln Arg Ala Val Cys Met Ile Ser Asn Ser Thr Ala Ile Ala Glu Val Phe Ser Arg
 α T2
 α T3
1171 ATT GAT CAC AAG TTC GAC TTG ATG TAT GCC AAG AGA GCT TTC GTC CAT TGG TAT GTC GGT GAA GGT ATG GAA GAA GGT GAA TTC TCT GAA
391 Ile Asp His Lys Phe Asp Leu Met Tyr Ala Lys Arg Ala Phe Val His Trp Tyr Val Gly Glu Gly Met Glu Glu Gly Glu Phe Ser Glu
 α T2
C
 α T3
C
1261 GCC AGA GAA GAT TTG GCT GCC CTT GAA AAG GAC TAC GAA GAA GTT GGT ACT GAA TCC CAA GAA GGT GAT GGT GAA GAA GGT GAA GAT GGT
421 Ala Arg Glu Asp Leu Ala Ala Leu Glu Lys Asp Tyr Glu Glu Val Gly Thr Glu Ser Gln Glu Gly Asp Gly Glu Glu Gly Glu Asp Gly
 α T2
T
 α T3
T
1351 GGT GAC CAA TAA
451 Gly Asp Gln STOP
 α T2
 α T3

Figure 5. DNA sequences of the coding region of three α -tubulin genes of *N. gruberi* and the single predicted amino acid sequence. The nucleotide sequence of pN α T1 is presented on the upper line, with the encoded amino acid sequence below it. On subsequent lines, for pN α T2 and λ N α T3 only nucleotide differences from α T1 are shown. α T1 and α T2 were sequenced completely on both strands; α T3 was sequenced on one strand except for the 5' region, up to nucleotide 110, which was sequenced on both strands.

Table I. Homology Matrix for α -Tubulins and Their Genes

| Gene | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | Species |
|------|----|----|----|----|----|----|-----|-----|----|--|
| 1 | | 72 | 73 | 78 | 72 | 71 | 72 | 72 | 68 | <i>Naegleria gruberi</i> pN α T1 (Fig. 5) |
| 2 | 92 | | 83 | 76 | 78 | 84 | 78 | 78 | 82 | <i>Chlamydomonas reinhardtii</i> α 1 (62) |
| 3 | 91 | 90 | | 75 | 75 | 79 | 76 | 76 | 78 | <i>Trypanosoma brucei</i> α (28) |
| 4 | 90 | 91 | 87 | | 73 | 74 | 73 | 72 | 71 | <i>Stylonychia lemnae</i> pmac C9 (24) |
| 5 | 88 | 87 | 85 | 84 | | 75 | 73 | 73 | 73 | <i>Physarum polycephalum</i> α (30) |
| 6 | 85 | 86 | 85 | 83 | 83 | | 83 | 82 | 87 | <i>Drosophila melanogaster</i> α 1 (66) |
| 7 | 85 | 86 | 85 | 84 | 83 | 97 | | 94 | 86 | Rat pIL α T1 (36) |
| 8 | 85 | 86 | 85 | 84 | 83 | 97 | 100 | | 85 | Human $\beta\alpha$ 1 (23) |
| 9 | 84 | 85 | 84 | 83 | 82 | 96 | >99 | >99 | | Chicken pT1 (71, 36) |

Values below the diagonal are percent identities of the encoded α -tubulins, and those above the diagonal are percent identities of the DNA coding regions. Percent identities were calculated from manually aligned sequences with gaps (deletions or insertions of codons) each counted as one amino acid residue or three nucleotides, respectively. Most α -tubulins aligned readily in the 451 codon format, with introduction of six codon deletions in the *Stylonychia* sequence. *Naegleria* α -tubulin required the addition of two codons at the 3' end. All sequences are complete except the chicken sequence begins at codon 40 and the *Physarum* sequence ends at codon 423. The sequences for the unusual α -tubulins of yeast (56, 70) are omitted because these sequences are quite divergent from all the other α -tubulin sequences, such that the identities for any of the encoded yeast α -tubulins to α -tubulins of other species range from 67 to 76% while the lowest among the tabulated α -tubulins is 82%.

| α -Tubulin Sequence | 431 | 436 | 441 | 446 | 451 |
|---|-------------------------|---------------------|-----------------------------|-------------------------------|-------------------|
| Consensus | Asp Tyr Glu Glu Val Gly | Ala Glu Ser Ala Gly | Gly Glu Gly Glu Glu Glu Tyr | | |
| <i>Naegleria</i> α 1- α 3 | Thr | Gln | Asp | Gly Glu Asp Gly Gly Asp (Gln) | |
| Mammals and a bird | Val Asp | Val | | | |
| Mouse M3/7, Human H2a | Val Asp | Val | Ala | Ala --- | |
| Hamster α 111, Mouse M3/6 | Asp | | Asp --- | --- Asp | |
| Mouse M3/4 | Ile Asp | Tyr | --- Asp --- | Asp | |
| Chicken α 2 | Asp | Ala Thr Asp Leu Phe | Asp | Asn Ala --- | Asp Ser |
| Sea urchin <i>Lytechinus</i> | Val Asp | Val | Ala | | |
| <i>Drosophila</i> α 1/3 | Met Asp | Gly Asp | | Gly Ala --- | |
| <i>Drosophila</i> α 2 | Ile Asp | Thr Thr Glu Leu | | Asp --- | |
| <i>Drosophila</i> α 4 | Phe | Leu Asp Asn | Glu Gly | Asp | Asp Phe Asp (Phe) |
| Alga <i>Chlamydomonas</i> α 1-2 | Phe | | Ala | | |
| Ciliate <i>Stylonychia</i> | Ile | Ile Val | | | Met |
| Trypanosome <i>T. brucei</i> | | Asp Met Asp | | Asp Val | |
| Slime mold <i>Physarum</i> | | Thr Val --- | Asp Asp | Ala Glu | Met |
| Yeast <i>S. pombe</i> α 1 | Gln Asp | Met Asp Asn | Met Tyr | Ala Asp | |
| Yeast <i>S. pombe</i> α 2 | Gln Asp | Met | Val Asp Tyr Met | --- | |
| Yeast <i>S. cerevisiae</i> TUB1 | Ile | Asp | --- | Tyr Ala Glu | (Phe) |
| Yeast <i>S. cerevisiae</i> TUB3 | Ile | Asp | --- | Tyr Ala --- | (Phe) |

Figure 6. The carboxyl termini of α -tubulins. The consensus sequence was derived after manual alignment of the sequences, including the introduction of gaps (---). In subsequent rows only differences from the consensus sequence are shown. Residues in boldface indicate substitutions unique, among conserved α -tubulins, to *Naegleria* α -tubulin. The sequences other than *Naegleria* are from the following sources: pig (49), rat (21, 35), mouse (37, 72), chinese hamster (13), human (12, 23), chicken (50, 71), sea urchin (2), *Drosophila* (66), *Chlamydomonas* (62), *Stylonychia* (24), trypanosome (28), *Physarum* (42), *S. pombe* (70), and *S. cerevisiae* (56).

divergent α -tubulins of *S. cerevisiae* have 9 to 11 acidic residues among the 19 to 23 residues beginning at 431. Most of the α -tubulins end in an encoded Glu-Tyr, but recently several exceptions have been found: the α -tubulins of the ciliate *Stylonychia* and of the mouse testis-specific α 4 end in the Glu before Tyr, the divergent α -tubulins *Drosophila* α 4 and *S. cerevisiae* TUB1 and TUB3 end in Glu-Phe, a *Physarum* α -tubulin ends in Met, and a divergent chicken α -tubulin, α 2, ends in Ser. Although some of the other α -tubulins are shorter than the consensus sequence at the COOH-terminus, only that of *Naegleria* is longer.

DNA Sequence Homology

Since the α -tubulin protein sequence of *Naegleria* is conserved comparably to those of other unicellular eukaryotes whose α -tubulin DNA sequences hybridize readily to heterologous probes, why is there so little homology of the α -tubulin DNA sequences of *Naegleria* to the chicken α -tubulin cDNA clone pT1? The overall DNA sequence homologies of several α -tubulin genes are compared above the diagonal in Table I. It is notable that in every comparison the DNA sequence of *Naegleria* α T1 is less homologous to each of the others than they are to each other. In other words, the nucleotide sequence of *Naegleria* α -tubulin has diverged more rela-

tive to the amino acid sequence than have those of *Chlamydomonas*, *Trypanosoma*, *Physarum*, *Drosophila*, chicken, rat, and man.

As a consequence of this divergence of DNA sequence, the overall coding region homology of *Naegleria* α -tubulin genes to the chicken cDNA clone pT1 is only 68%, which happens to be the lowest homology in Table I and is near borderline for detection by hybridization (26). In addition to overall homology, the stability of molecular hybrids is also profoundly affected by the length of matches of contiguous nucleotides, and decreases rapidly with length below ~40 nucleotides (33, 74). Comparison of the sequences of *Naegleria* α T1 and chicken pT1 revealed that the longest contiguous matches are one each of 12 and 15 nucleotides.

The *Naegleria* α -tubulin DNA sequence lies at the extreme end of a continuum, as shown by the comparison of α -tubulin genes of four protists to the chicken pT1 sequence in Table II. All four of these protistan genes encode comparably conserved α -tubulin sequences, but their DNAs are increasingly divergent from the chicken sequence. At one extreme lies the *Naegleria* sequence, with insufficient similarity to hybridize reproducibly to the chicken sequence. At the other extreme lies the *Chlamydomonas* DNA sequence, which shows 82% identity to the chicken DNA sequence and contains 13 matches of from 20 to 33 contiguous nucleotides.

Evolutionary shifts in preferences for synonymous codons are sufficient to explain these differences. The sequenced *Naegleria* α -tubulin genes show extremely biased codon usage. Overall, 94% of the amino acids are encoded in pNaT1 by only 26 of the 61 possible codons. The codon bias seen in these *Naegleria* genes is opposite in direction to that found in chicken and *Chlamydomonas* genes. For example, 84% of the codons compared between chicken pT1 and *Naegleria* pNaT1 encode identical amino acid residues but of these only 30% use the same codon. The differences between the *Naegleria* and chicken DNA sequences, in addition to that produced by nonsynonymous codon changes, is quantitatively accounted for by codon bias. In a broader context, a comparison of the α -tubulin DNA sequences of the diverse eukaryotes listed in Table I revealed no segment of the coding region that is conserved beyond phyla except as required to encode conserved α -tubulin using the codon preferences of each organism.

5' and 3' Untranslated Regions

The 5' untranslated regions of the two cDNA clones contain only three nucleotide differences among the 70 nucleotides

Table II. Similarities of Protistan α -Tubulin Genes to Chicken pT1

| Gene compared with chicken pT1 | Identity | | | Nucleotides | | | Hybridize to chicken pT1 |
|---------------------------------|----------|-----|---------|---------------|----------------|-----------|--------------------------|
| | Protein | DNA | (G + C) | Longest match | No. of matches | | |
| | | | | | ≥ 20 | ≥ 15 | |
| Chicken pT1 | 100 | 100 | 63 | — | — | — | ++ |
| <i>Chlamydomonas</i> α 1 | 85 | 82 | 64 | 33 | 13 | 18 | ++ |
| <i>Trypanosoma</i> α | 84 | 78 | 58 | 29 | 6 | 10 | + |
| <i>Stylonychia</i> α | 83 | 71 | 49 | 20 | 2 | 4 | + |
| <i>Naegleria</i> α 1 | 84 | 68 | 42 | 15 | 0 | 1 | ± |

The coding region sequences used in this analysis, and the hybridization of these sequences to chicken pT1, are given in the references cited in Table I for organisms other than *Naegleria*.

A 5' Untranslated region

```

-70      -60      -50      -40      -30      -20      -10      1
αT1  ggg-AA-AAAAAAAAATCGCTCGTCTCCATTATAGAGAACTTGCAAAACGCTGTAATAATAATACAAA ATG
αT2  gggCAACAAAAAATCGCTCGTCTCCATTATAGAGAACTTGCAAAACGCTGTAATAATAATACAAA ATG
αT3  ACAAATTAATAAATCGCTCGTCTCCATTATAGAGAACTTGCAAAATAC-AAT-CCT--TAATAATACAAA ATG

```

B 3' Untranslated region

```

1363  1373  1383  1393  1403  1413  1423
αT1  AATAA ATTAATTACTCATTGAGACTTTTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
αT2  AATAA A-----TCCATTAAATC-----TCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
αT3  AATAA ATTAATTACTCATTGAGACTTTTCAAAAAAT

```

Figure 7. Nucleotide sequences of the untranslated regions of three *Naegleria* α -tubulin genes, read from 5' to 3' on the DNA sense strand. The figures show the sequences of two cDNA clones (α T1 and α T2) and the comparable segments of a genomic clone (α T3). Gaps, indicated by dashes, were introduced by eye to maximize identity. The arrows above the α T2 and α T3 sequences indicate differences between these sequences and α T1. (A) Sequences of the 5' untranslated region. The cDNA clones begin with lower-case g's which indicate the end of the oligo(dG) tract introduced during cloning (7 and 9 nucleotides in α T1 and α T2, respectively). All sequences end with the ATG start codon that begins Fig. 5. (B) Sequences of the 3' untranslated region, starting with the AATAAA that includes the TAA stop codon and, in the case of the cDNA clones, ending with the poly(dA) tract (39 and 34 nucleotides in α T1 and α T2, respectively) before the SV40 sequence in the vector. The underlined sequence in α T1 indicates the 14 nucleotides chosen for the oligonucleotide probe that was synthesized to the complementary strand and used to select α T3.

before the ATG start codon, whereas the genomic clone α T3 has a cluster of differences from both cDNA clones (Fig. 7 A). The cDNA clones begin with tracts of 9 to 11 A residues and overall these 5' untranslated regions are A+T rich to the extent that they contain 50% A residues. The 3' untranslated regions of the cDNA clones are exceptionally short, with 26 and 13 nucleotides before the poly(A) tails (Fig. 7 B). Each gene has an AATAAA that includes the TAA stop codon. Since these are the first gene sequences reported for *Naegleria* or any related organism, further analysis is deferred until more sequences are available.

The genomic clone α T3, selected using an oligonucleotide complementary to a portion of the 3' untranslated region of α T1 but not α T2, proved identical to clone α T1 in the 3' untranslated region up to the poly(A) tail of the cDNA clone (Fig. 7 B). In contrast, in the 5' untranslated region, α T1 and α T2 are similar whereas α T3 has a cluster of differences from both. In the coding region, α T3 is marginally more similar to α T1 than to α T2, but α T2 and α T3 share nucleotides at four positions where they differ from α T1 (Fig. 5). Overall the segmental similarities and differences among these three sequences suggest α -tubulin genes that have diverged from one another while being homogenized by some mechanism of intergene information exchange. It also suggests that these similar genes probably represent a single family with respect to expression and function. The three sequences clearly support the concept of a family of very similar but not identical α -tubulin genes in *Naegleria*.

Abundance of α -Tubulin mRNA during Differentiation

We previously showed that the abundance of translatable flagellar tubulin mRNA during differentiation is directly pro-

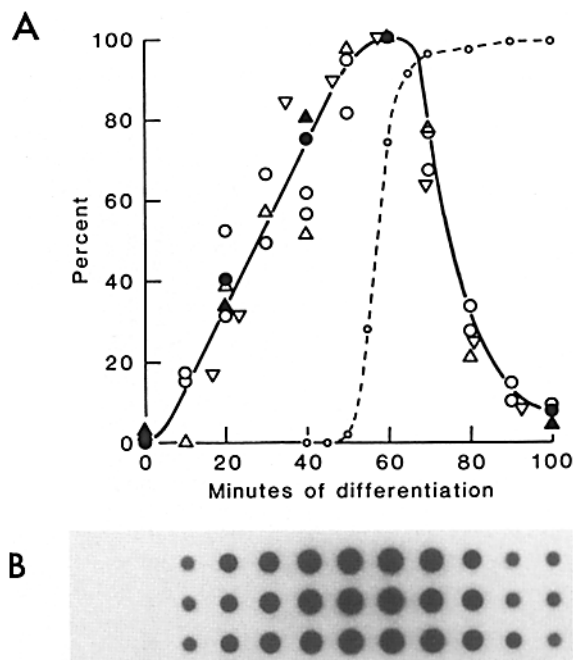


Figure 8. α -Tubulin mRNA abundance parallels translatable mRNA and in vivo synthesis. In A, α -tubulin mRNA sequence abundance during differentiation (circles) is compared to the abundance of translatable tubulin mRNA (triangles) and to the in vivo rate of flagellar tubulin synthesis (inverted triangles). All these values are expressed as the percentage of the maximum observed value. The kinetics of one of the differentiations used to isolate RNA is also shown, measured as the percentage of cells with flagella (dashed line). Quantitative RNA dots (B) were probed with the 32 P-labeled insert of pNaT1 as described in Materials and Methods. The open circles in A show two independent estimates of the amount of α -tubulin mRNA in samples isolated every 10 min during a single differentiation; where two circles are not shown the results were superimposable (e.g., there are two each at times 0 and 60). Measurements of the abundance of translatable flagellar tubulin mRNA were described previously (31). A new set of measurements of translatable flagellar tubulin mRNA by quantitative immunoprecipitation of translation products, using the procedure described (31), is compared with RNA dot measurements of α -tubulin mRNA sequences in the same set of RNA (solid triangles and circles). The measurements of the rate of in vivo synthesis of flagellar tubulin by pulse-labeling followed by determination of the specific activity of outer doublet tubulin are from Kowit and Fulton (29); the time points are adjusted to conform to the kinetics of differentiation at a lower temperature as previously described (31). The line is interpolated as described in the text.

portional to the rate of flagellar tubulin synthesis in the differentiating cells (31). We here provide evidence that the amount of α -tubulin mRNA sequence is proportional to the amount of translatable flagellar tubulin mRNA. Direct measurements of flagellar tubulin mRNA abundance by DNA-RNA hybridization are possible in this multigene family because all members of the family are so similar. Fig. 8 shows the results of several measurements, and compares these to the reproducible kinetics of differentiation under the conditions used (dashed line). This curve, which is measured as the percentage of cells with flagella, reveals the population heterogeneity in the time that individual cells differentiate (15). Although *Naegleria* differentiation is exceptionally syn-

chronous, the limited heterogeneity probably is also hidden in the curves for abundance of an mRNA and other quantitative variables. Quantitative RNA dots, in which the relative amount of α -tubulin mRNA sequence in total RNA is measured using the insert of α T1, are shown as circles, to be compared with the amount of translatable flagellar tubulin mRNA (*triangles*) and the rate of flagellar tubulin synthesis in vivo (*inverted triangles*). It is evident that the abundance of α -tubulin mRNA sequences and of translatable flagellar tubulin mRNA, and the rate of flagellar tubulin synthesis in the cells, all rise and fall together. Particularly striking are the measurements of physical mRNA and functional mRNA in a single series of RNA samples (*solid circles and triangles* at 0, 20, 40, 60, and 100 min). We conclude that the amount of functional mRNA and the rate of in vivo synthesis are directly controlled by the availability of the physical mRNA, i.e., by the rates of transcription and of degradation of this mRNA.

This change in abundance of α -tubulin mRNA is one of the most marked and rapid that has been described in a eukaryotic cell. The interpolated solid line in Fig. 8 shows one interpretation of the change. At the onset of differentiation, the abundance of α -tubulin mRNA, measured by RNA dots, has ranged from 0 to 1.2% of the abundance at 60 min (5 measurements), which indicates at least an 80-fold increase in abundance within 1 h. The curve (as interpolated) suggests that between 10 and 50 min α -tubulin mRNA increases at a rate of $\sim 2\%$ per min. The results indicate that α -tubulin mRNA begins to increase earlier in differentiation than was indicated by the less detailed and less precise measurements of translatable flagellar tubulin mRNA (*open triangles* in Fig. 8; reference 31). By 100 min, when the flagellates have full-length flagella, the abundance of α -tubulin mRNA, as well as the abundance of translatable flagellar tubulin mRNA and the rate of synthesis of flagellar tubulin in vivo, have decreased to less than 10% of the value at 60 min. If one assumes exponential decay of the α -tubulin mRNA, as is interpolated in Fig. 8, then during the decrease in abundance (70–100 min) the mRNA sequences have a half-life of ~ 8 min. A half-life of more than 9 min, or less than 7 min, cannot be fit to the data points. Of course if transcription of α -tubulin mRNA continues during the decay, or if the time that decay begins in individual cells is heterogeneous, the half-life would be less than 8 min. It is clear that the abundance of α -tubulin mRNA rises very rapidly during the first hour of differentiation, and falls even more rapidly during the next 30 min.

We were surprised by the low abundance of α -tubulin mRNA at time zero, i.e., in amebae. This low abundance is

shown even more dramatically in RNA gel-transfer hybridization (Fig. 9), which gives less background noise than dots. Even on deliberately overexposed Northern blots, as in Fig. 9 B, no trace of α -tubulin mRNA homologous to α T1 has been detected at 0 min, i.e., in RNA from amebae.

Discussion

The results presented in this paper provide a counterexample to the idea that tubulin genes are conserved. *Naegleria* happens to be the first among diverse organisms with strongly conserved α -tubulin in which detection of its genes using a chicken α -tubulin gene is borderline under permissive conditions of hybridization. Although *Naegleria* α -tubulin genes are extreme in their retention of encoded protein homology without comparable conservation of DNA homology, analysis of available α -tubulin sequences indicates that the *Naegleria* sequences represent one end of a continuous spectrum. No special explanation is needed. In addition to coding changes, silent evolutionary divergence over long periods, including in codon preference, with lack of requirement for conservation of any specific tracts of coding region DNA sequence, is sufficient to produce the observed results. In a sense, the remarkable finding was the initial one, that tubulin genes are often sufficiently conserved that a gene from one organism could readily be used as a probe in an evolutionarily distant organism (10), even to the extent, for example, that chicken tubulin genes could be used to measure tubulin mRNA in an alga (3). In a way the recurrent argument of conservation was always circular since the tubulin genes isolated using heterologous probes, perforce, were those that were "conserved." *Naegleria* provides a clear exception to the oft-stated generalization. In the case of β -tubulin genes, also frequently considered conserved, Neff et al. (45) noted that this conservation barely extends to yeast. Obviously, any genes encoding a conserved protein may diverge silently to the point that two divergent genes can no longer hybridize unless there are constraints other than codons to the evolution of the DNA sequences. Conservation of tubulin genes, although remarkable in its extent, is neither required nor universal among eukaryotes.

The architecture of this tubulin multigene family, a group of very similar but not identical genes, appears unique among the tubulin gene families described to date. Except for those examples in trypanosomid flagellates where a group of replicate genes are arrayed in tandem repeats, the multiple tubulin genes of other organisms are more divergent from one another than those of *Naegleria*. The homogeneity of the *Naegleria* genes suggests either very recent divergence or, more likely, exchange of information between members of the gene family in a manner that constrains their independent evolution. Such concerted evolution has been noted in other tubulin gene families (13, 64). In *Naegleria* there appear to be at least eight very similar genes; the three sequenced clones establish the presence of differences among at least three of these genes and the two different cDNA clones demonstrate that at least two are expressed. Although there are differences among these genes in the 5'- and 3'-untranslated regions and translationally silent differences in the coding regions, the three genes encode a single α -tubulin and it is likely that they belong to a single co-expressed gene family. Since the eight or more genes are all so similar, it is reason-

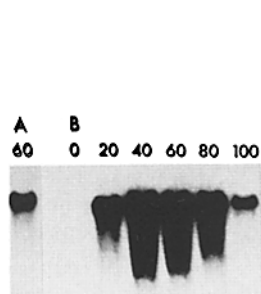


Figure 9. Northern blots of total RNA extracted at various times of differentiation probed with the insert of pN α T1. (A) 60-min RNA, exposed to show the single-sized fragment of α -tubulin RNA, estimated at 1.7 kb using *E. coli* rRNA as size standard. (B) Time series, overexposed to emphasize the amount of homologous RNA present at time zero.

able to infer that all eight may be co-expressed and that all may encode a single α -tubulin, but other possibilities are not precluded. The similarity of the restriction sites (Fig. 2 E) supports the conclusion that if there are any differences among the α -tubulins encoded by the unsequenced genes in the family, these differences are likely to be minor, such as single amino acid substitutions. Overall this is the largest number of nonidentical genes so far described in a unicellular eukaryote. One plausible explanation for the large number, considered previously on the basis of calculations of rates of transcription and translation (16), is that the multiple genes are required to synthesize sufficient flagellar tubulin during the rapid differentiation.

An important issue raised by our early work on flagellar tubulin synthesis during *Naegleria* differentiation was the multitubulin hypothesis, the proposal that organisms use different tubulins, made by different genes, for different microtubular organelles (17). Evidence currently indicates that organisms, including organisms that use microtubules for diverse functions such as mitosis and flagella or cilia, can get along with one gene for α -tubulin (*Tetrahymena*) or multiple copies of a single gene (*Leishmania*, *Trypanosoma*). It is also clear that both *Drosophila* and *Aspergillus* can use the product of a single tubulin gene for diverse microtubular functions (27, 41, 51). The issue of the multitubulin hypothesis is still unsettled in *Naegleria*, where the possibility of separate genes and gene products for "mitotic" tubulin in amebae and "flagellar" tubulin in flagellates was originally proposed (17). Evidence for multitubulins in *Naegleria* remains circumstantial in the absence of direct evidence for different genes or different primary structure in the mitotic and flagellar tubulins. The current study extends the evidence. mRNA homologous to the α -tubulin gene family expressed during differentiation has not been detected in amebae. One possibility is that the α -tubulin mRNA used for mitosis is degraded rapidly thereafter, as in *Chlamydomonas* and in *Physarum* (3, 57), and thus that little of this mRNA is found in a random population of amebae. However, a search of *Naegleria* amebae undergoing synchronous mitosis, using the coding region of α T1 as a probe, failed to reveal any time at which the level of α -tubulin mRNA was above background (Lai, E. Y., and C. Fulton, unpublished results). This leaves open the possibility that a separate family of α -tubulin genes, sufficiently diverged that they do not readily cross-hybridize to the α -tubulin gene family described here, could encode mitotic α -tubulin in *Naegleria*. We have reported herein an α -tubulin gene family for which the DNA sequences are so diverged from those of other organisms as to lack cross-species homology. We propose the possibility of another α -tubulin gene family in the same organism so diverged that the mitotic and flagellar α -tubulin gene families may not readily cross-hybridize.

The single α -tubulin encoded by the three sequenced *Naegleria* genes is strongly conserved except at the carboxyl terminus, which is two residues longer than those of other characterized α -tubulins, has its last seven residues peculiar to this tubulin, and lacks the COOH-terminal tyrosine encoded in most other α -tubulins (Fig. 6). Because of the number of acidic residues, all of the carboxyl termini including that of *Naegleria* probably are in an extended conformation (48). Recent evidence indicates that this region is exposed on tubulin dimers but not in assembled microtubules (6).

Terminal tyrosine is encoded on most α -tubulins and, at least in vertebrates, is posttranslationally removed and readded (reviewed in references 22 and 69). As a consequence the terminal tyrosine is not present on all tubulin molecules (48). The role of this post-translational modification has eluded understanding except for the finding that detyrosinated tubulin is found in a different subset of cellular microtubules than tyrosinated tubulin (20, 22). It is conceivable, of course, that the *Naegleria* protein is posttranslationally modified before the tubulin is used, although the COOH-terminal tyrosine is normally added to glutamic acid rather than to glutamine. *Naegleria* tubulin, synthesized in a cell-free system from wheat germ, apparently can polymerize with calf brain tubulin (31), so these COOH-terminal residues do not appear to produce a distinctive alteration in this assay. Recent work, using selective proteolytic cleavage of tubulin, has suggested that the carboxyl terminus of α -tubulin may play a largely regulatory role (53, 60). Removal of this segment does not prevent polymerization, but does remove the binding site for MAP2 (59) and the high-affinity calcium-binding site (61), and thus destroys the sensitivity of polymerization to MAP2 and to calcium. Since the C-terminus of *Naegleria* α -tubulin is so divergent, it is likely that some of these regulatory functions or interacting proteins may be altered in *Naegleria*.

Our interest in regulation of the synthesis and utilization of flagellar tubulin during *Naegleria* differentiation originally motivated this study. The marked programmed rise and fall in abundance of α -tubulin mRNA during differentiation (Fig. 8) is accompanied by parallel changes in abundance of β -tubulin mRNA, both translatable and physical (31; Lai, E. Y., and C. Fulton, unpublished results), which suggests coordinate regulation of the flagellar α - and β -tubulin genes. Concurrent regulation of *Naegleria* genes during differentiation has also been observed for translatable mRNAs of calmodulin and flagellar tubulin (18) and for four differentiation-specific RNAs (40). Since throughout differentiation the abundance of α -tubulin mRNA is directly proportional to the abundance of translatable flagellar tubulin mRNA and to the rate of flagellar tubulin synthesis in vivo, the conclusion seems inescapable that the amount of physical α -tubulin mRNA determines the rate of flagellar α -tubulin synthesis. Processing of α -tubulin transcripts to mature mRNA, if it occurs, must occur so rapidly that it does not influence this result. We conclude that expression is regulated at the level of RNA synthesis and/or stability. The decline in abundance of α -tubulin mRNA is so rapid that it is unlikely that the mRNA half-life is only 8 min throughout differentiation. It is possible that early in differentiation the mRNA has a substantially longer half-life and subsequently is somehow targeted for rapid decay. Obviously, a next step is to determine how the relative rates of transcription and decay of α -tubulin mRNA combine to produce the observed rise and fall in abundance.

The rise and fall in abundance of α -tubulin mRNA is striking. It is perhaps most comparable to the concurrent change in abundance of α - and β -tubulin mRNAs that occurs during flagellar regeneration in *Chlamydomonas* (4, 7). These two systems are quite different. In *Chlamydomonas*, what is induced is regeneration of flagella on a pre-existing flagellar apparatus following amputation of flagella. In *Naegleria*, the whole organelle complex—flagellar apparatus, flagellar tubulin, flagella—comes into being during an optional differentiation of amebae to flagellates. In *Chlamydomonas*, the

abundance rises about four to seven-fold during the hour after amputation of flagella, and then decreases more slowly than it does in *Naegleria*, with a half-life of 45–55 min. One other example of a marked rise and fall in abundance of tubulin mRNA has been reported; this occurs during the synchronous mitosis of *Physarum* plasmodia (57). In this organism, the rise, over a period of 10 h, is >40-fold, and the fall in abundance is rapid, with a half-life of 19 min. In each case, a primary objective is to determine what regulates the marked changes in abundance of tubulin mRNA. In *Naegleria*, the programmed rise and fall in α -tubulin mRNA abundance prompts the use of the cloned and characterized genes to seek insights into the causal sequence of events, beginning at the initiation of differentiation, that regulate the abundance of α -tubulin mRNA in relation to the synthesis of flagellar tubulin and its assembly into flagella.

We thank Dr. Don Cleveland for the chicken α - and β -tubulin cDNA clones, Drs. Paul Berg and Hiroto Okayama for their cloning vectors, Dr. Pieter Wensink for use of his apparatus for replicating the ordered library and for critical reading of the manuscript, Dr. Rolf Heumann for preparing the 14-mer, Dr. Allan Maxam for introducing us to DNA sequencing, Drs. Kathleen Karrer and Daniel Sussman for helpful suggestions about the manuscript, and Dr. Hayden Coon for profound encouragement.

This work was supported by grants from the National Science Foundation (DCB-8216357 and DCB-8616116) and the National Institutes of Health (GM24444 and RR07044).

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