New Poly(A)+RNAs Appear Coordinately during the Differentiation of *Naegleria gruberi* Amebae into Flagellates

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Abstract. We have examined the nature of the requirement for RNA synthesis during the differentiation of *Naegleria gruberi* amebae into flagellates (Fulton, C., and C. Walsh, 1980, *J. Cell Biol.*, 85:346–360) by looking for poly(A)+RNAs that are specific to differentiating cells. A cDNA library prepared from poly(A)+RNA extracted from cells 40 min after initiation of the differentiation (40-min RNA), the time when formation of flagella becomes insensitive to inhibitors of RNA synthesis, was cloned into pBR322. Recombinant clones were screened for sequences that were complementary to 40-min RNA but not to RNA from amebae (0-min RNA). Ten of these diffentiation-specific (DS) plasmids were identified. The DS plasmids were found to represent at least four different poly(A)+RNAs based on cross-hybridization, restriction mapping, and Northern blot analysis.

Dot blot analysis was used to quantify changes in DS RNA concentration. The four DS RNAs appeared coordinately during the differentiation. They were first detectable at 10–15 min after initiation, reached a peak at 70 min as flagella formed, and then declined to low levels by 120 min when flagella reached full length. The concentration of the DS RNAs was found to be at least 20-fold higher in cells at 70 min than in amebae. The changes in DS RNA concentration closely parallel changes in tubulin mRNA as measured by in vitro translation (Lai, E. Y., C. Walsh, D. Wardell, and C. Fulton, 1979, *Cell*, 17:867–878).

Naegleria gruberi amebae can differentiate into swimming flagellates (9, 11, 14). Flagellates are characterized by the presence of typical eukaryotic flagella (8, 37), basal bodies (15), cytoplasmic microtubules (43), a flagellar rootlet (25, 26), and a distinct flagellate shape (12), whereas amebae lack all of these structures (8, 37). The differentiation is rapid and synchronous; 50% of the cells develop visible flagella (T_{50}) in 60 to 70 min depending on the strain and the differentiation conditions. The T_{50} has been found to be highly reproducible for any given strain and set of conditions (11). For example, using strain NEG grown on bacteria and differentiated at 25°C, the T_{50} is 61 min with a standard deviation of 2 min (17). The T_{50} for strain NB-1, used in the current work, is typically 68 min under similar conditions (7, 44).

Cycloheximide inhibition of protein synthesis during the differentiation suggests that formation of flagella (17), formation of cytoplasmic microtubules (43), and formation of the flagellate shape (11) all require protein synthesis. The protein synthesis essential for formation of these structures is completed sequentially, in the order listed. The requirement for protein synthesis during formation of flagella has been verified by the demonstration that most, if not all, of the flagellar outer doublet tubulin is synthesized *de novo* during the differentiation (16, 22).

Similar experiments using Actinomycin D (AMD)[1] and Daunomycin to inhibit RNA synthesis suggest that RNA synthesis is also required for the formation of flagella and the flagellate shape (17). This conclusion is supported by studies of tubulin mRNA levels during the differentiation (24). As judged by an in vitro translation assay, tubulin mRNA is absent from amebae until 10–15 min after initiation of the differentiation. The amount of tubulin mRNA was found to increase in parallel with tubulin synthesis until flagella were formed and then declined to low levels by the time flagella reached full length, at 120 min.

These observations have led us to ask if the appearance of new mRNAs are required for the formation of flagella. To identify such mRNAs, we have cloned cDNA copies of poly(A)+RNA from differentiating cells and screened the resulting library in an attempt to identify sequences that are only expressed in differentiating cells. We report here the characterization of a collection of such sequences.

[1] *Abbreviations used in this paper:* AMD, Actinomycin D; bp, base pair; DS, differentiation-specific; kb, kilobase; NS, nonspecific; SSC, 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; 20× SSPE, 3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM Na_2EDTA, pH 7.4.

Materials and Methods

Cell Culture, Strains, and Differentiation

Naegleria gruberi strain NB-1 was grown and differentiated as previously described (43). *Escherichia coli* strain HB101 was grown in LB medium (28). Large-scale *E. coli* cultures for plasmid preparation were grown in M9 medium (28) supplemented with 0.5% (wt/vol) casamino acids, 0.2% (wt/vol) glucose, and 12 μg/ml tetracycline. Ampicillin was added at 20 μg/ml as required.

Preparation of DNA and RNA

Plasmid amplification was carried out in LB medium (28). The cells were lysed by the method of Katz et al. (21) as modified by Dr. N. D. F. Grindly, Yale University (personal communication). Cells washed with ice-cold TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were suspended in 8 ml sterile ice-cold SUTE (15% [wt/vol] sucrose in 50T5E [50 mM Tris-HCl, 5 mM EDTA, pH 8.0]) per liter of original culture. Lysozyme (10 mg/ml in SUTE) was added to 1.1 mg/ml, the suspension mixed by gentle inversion, and incubated at 23°C for 15 min. The cells were lysed by addition of 1.1 vol of 0.1% (vol/vol) Triton X-100 in 50T5E and incubation at 0°C for 10 min with occasional mixing by gentle inversion. Cell debris and chromosomal DNA were pelleted by centrifugation at 37,000 *g* for 60 min at 3°C. Plasmid DNA was isolated from the supernatant by banding in CsCl-ethidium bromide gradients followed by *n*-butanol extraction and ethanol precipitation (28). Recent plasmid preparations have used lysis with SDS (28) with equally good results.

RNA was prepared from *Naegleria* by a modification of the method of Jacobson (20). All glassware and plasticware were soaked overnight in 0.001% diethylpyrocarbonate, rinsed with sterile distilled water, and then autoclaved before use. When possible, solutions were sterilized by autoclaving.

Cells were collected by centrifugation and suspended in ice-cold distilled water at 2–4 × 10^7 cells/ml. An equal volume of 4% (wt/vol) SDS in 50 mM Tris-HCl, pH 7.4, was added while the suspension was rapidly mixed on a vortex mixer and then the lysate was immediately adjusted to 0.1% (vol/vol) diethylpyrocarbonate. 1 vol of water-saturated, freshly redistilled phenol was added and mixed, followed by the addition of chloroform/isoamyl alcohol (24:1) at one-half the volume of phenol. The extract was mixed vigorously for 20–30 s and then maintained as an emulsion for 2–5 min by occasional mixing. Subsequent phenol–chloroform extractions and ethanol precipitation were carried out essentially as described (20). In experiments in which multiple RNA samples were prepared over a 2–3-h period, the cells were lysed, mixed with phenol and chloroform/isoamyl alcohol, and kept at 0°C with occasional mixing until all samples had been collected. The average yield of RNA was 3–4 mg/10^8 cells. Poly(A)+RNA was isolated by two cycles of chromatography on oligo(dT)-cellulose (20).

Preparation of cDNA Library

cDNA synthesis was carried out using the procedure of Wickens et al. (45) as modified by Wolfner (46) for use with small amounts of poly(A)+RNA. Poly(dC) tails were added to the double-stranded cDNA and poly(dG) tails were added to Pst I linearized pBR322 (2) with terminal transferase (34). The reactions were carried out so as to allow the addition of 20–30 nucleotides per 3′-end. Tailed DNA was recovered by chromatography through G-75 Sephadex and ethanol precipitation.

To anneal the cDNA to pBR322, ~200 ng of double-stranded, tailed cDNA was mixed with 400 ng of Pst I cut, tailed pBR322 in a total volume of 20 μl of 200 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.2 mM EDTA. The reaction was heated for 10 min at 60°C, incubated at 42°C for 2 h, and then cooled slowly to room temperature (2–4 h).

E. coli HB101 was transformed by a modification of the method of Mandel and Higa (27) as described by Tschumper and Carbon (42). Aliquots (200 μl) of "competent" cells were added to 0.1–1.0 μg of DNA in 100 μl of 10 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 10 μl of a solution of 0.1 M $MgCl_2$, 0.1 M $CaCl_2$. The mixture was incubated for 15 min on ice with occasional swirling. Uptake of DNA was initiated by warming the mixture at 42°C for exactly 2 min. The cell samples were then placed at room temperature for 10 min and 2.5 ml of LB medium were added. The cells were incubated at 37°C with shaking for 60 min to allow for the expression of the tetracycline gene. Cells were collected by centrifugation, resuspended in LB with tetracycline (12 μg/ml), and aliquots (0.05–0.2 ml) were spread onto LB agar plates containing tetracycline (12 μg/ml).

Gel Electrophoresis, Transfer to Nitrocellulose, and Hybridization

DNA was fractionated in horizontal 0.7–1.5% agarose gels run in the TAE buffer system (40 mM Tris, 20 mM acetic acid, 2 mM Na_2 EDTA, pH 8.1) (28). DNA fragments were visualized in UV light after staining the gel in 1 μg/ml ethidium bromide for 10–20 min. RNA was fractionated in gels containing formaldehyde using a modification of the method of Rave et al. (32). Gels contained 1.5% agarose, 2.2 M formaldehyde, 20 mM 3-(*N*-morpholino)propanesulfonic acid, 50 mM Na acetate, 1 mM EDTA, pH 7.0. RNA samples were suspended in 50% formamide/50% gel buffer, with 0.02% bromphenol blue and xylene cyanol, and denatured at 65°C for 10 min.

After visualization of the DNA, gels were soaked for 1 h in 500 ml of 1.5 M NaCl, 0.5 M NaOH, and then soaked in 500 ml of 1.0 M Tris-HCl, 0.9 M NaCl, pH 8.0, at room temperature for 1 h. DNA was transferred to nitrocellulose using the procedure of Southern (39), except that 10× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate, pH 7.0) was replaced with 20× SSPE (3.6 M NaCl, 200 mM NaH_2PO_4, 20 mM Na_2EDTA, pH 7.4). After transfer of the DNA, filters were rinsed with 2× SSPE, air dried, wrapped loosely on a glass plate with plastic wrap, and baked for 2 h at 80°C in a vacuum oven.

RNA gels were treated twice with 500 ml of denaturing buffer (50 mM NaOH, 100 mM NaCl), for 25 min each time, followed by two 30-min treatments in 500 ml of 20× SSPE. Nitrocellulose filters were pre-wetted with H_2O, soaked for 20 min in 2× SSPE, and the RNA was transferred to the filter as described for DNA.

Filters were hybridized as described by Davis et al. (4) using 50% deionized formamide in 5× SSPE, 5× Denhardt's solution (0.1% [wt/vol] each of Ficoll 400, polyvinylpyrolidone, and bovine serum albumin) (5), 1% (wt/vol) glycine, and 100 μg/ml of sonicated, denatured salmon sperm DNA.

Colony Hybridization

Growth, replication of recombinant colonies onto Whatman 541 filter papers, amplification of plasmids with chloramphenicol, and denaturation of bound DNA were carried out as described by Gergen et al. (18). Filters were pre-hybridized in plastic bags at 60°C in 6× SSC and 1× Denhardt's solution. Usually 6–10 8.2-cm diameter filters were pre-hybridized in 100 ml for 6–8 h at 60°C. Filters were hybridized in 6× SSC, 1× Denhardt's solution, and 150 μg/ml of sonicated, denatured salmon sperm DNA. The hybridization volume was 7–10 ml per six to ten 8.2-cm filters with 2–5 × 10^6 cpm of ^{32}P-labeled cDNA. After hybridization for 36–40 h, the filters were washed once with 500–600 ml of 6× SSC, 0.5% SDS at 60°C for 60 min with agitation. The filters were then washed with 500 ml of 0.2× SSC at room temperature for 30 min, dried, covered with plastic wrap, and autoradiographed on Kodak XAR-5 film with a Dupont Cronex intensifying screen at −70°C for 1–2 wk.

RNA Dot Blots

RNA was quantified by a modification of the RNA dot blot technique of Thomas (41) similar to that used by Schloss et al. (36). RNA (5–10 μg/100 μl) was denatured in 5× SSPE, 6% formaldehyde, 20% formamide at 65°C for 10 min. Denatured RNA was quickly chilled on ice, an equal volume of 20× SSPE was added, and the RNA concentration was diluted to 1.5 μg/100 μl with 10× SSPE, 3% formaldehyde. 3 μg of each RNA sample was applied to individual wells of a hybri-dot apparatus (Bethesda Research Laboratories, Gaithersburg, MD). The RNA solution was slowly drawn through a sheet of nitrocellulose pre-wetted with water and then 20× SSPE. The nitrocellulose filters were rinsed with 2× SSPE, air dried for 30–40 min, and baked in a vacuum oven for 3–5 h as described above. RNA dots were hybridized as described for Northern blots. After autoradiography, an equal area containing each dot was cut from the nitrocellulose and the radioactivity in the area determined by scintillation counting in 3 ml of Liquifluor (New England Nuclear, Boston, MA).

Nick Translation of DNA

The method used was based on that of Maniatis et al. (28, 29). The reactions (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 5 μg/ml BSA, 1 mM β-mercaptoethanol, 20 mM dATP, dGTP, and TTP, 15–50 μg/ml DNA, 100 U/ml of DNA polymerase I, 250 pg/ml DNase I and 32 μM ^{32}P-α-dCTP) were incubated for 60 min at 14°C. Specific activity of the nick translation product varied from 0.5–2.0 × 10^8 cpm/μg of DNA.

Materials

Oligo(dT)$_{12-18}$ and oligo(dT) cellulose were provided by Collaborative Research Inc. (Waltham, MA). Radiochemicals were supplied by New England Nuclear, (Boston, MA), and biochemicals, unless otherwise noted, were from Sigma Chemical Co. (St. Louis, MO). S1 nuclease was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); restriction endonucleases were from either Bethesda Research Laboratories or New England Biolabs Inc. (Beverly, MA). DNA polymerase I was a gift of Dr. W. S. Kelley, Carnegie-Mellon University, Pittsburgh, PA. Dr. J. Beard of the National Cancer Institute provided AMV reverse transcriptase. Terminal transferase was obtained from Dr. W. Salser, University of California, Los Angeles.

Results

Complementary DNA (cDNA) was prepared from poly(A)+RNA extracted from cells 40 min after initiation of the differentiation (40-min poly[A]+RNA). This RNA was chosen because experiments using inhibitors of RNA synthesis had suggested that cells complete the RNA synthesis essential for formation of flagella at 40 min after initiation (17). The cDNA was cloned into the Pst I site of pBR322 by the G-C tailing technique. *E. coli* strain HB101 was transformed with the recombinant DNA and colonies were selected for resistance to tetracycline and sensitivity to ampicillin. Colonies were screened by colony hybridization using ^{32}P-labeled cDNA prepared from 40-min poly(A)+RNA. Positive colonies were further characterized by colony hybridization using ^{32}P-labeled cDNA from 40-min poly(A)+RNA in the presence of a 100-fold excess of unlabeled poly(A)+RNA from amebae (0-min RNA). Colonies were also hybridized to ^{32}P-labeled cDNA prepared from 0-min poly(A)+RNA. A total of 10 colonies were found to hybridize strongly to 40-min cDNA while showing no hybridization to 0-min cDNA. These colonies were recloned and an example of each was chosen for further study. These plasmids or the *Naegleria* sequences they contain will be referred to as differentiation-specific (DS) plasmids or DS sequences. Four colonies showing approximately equal hybridization with both 40-min poly(A)+RNA and 0-min poly(A)+RNA were also chosen for study. These will be referred to as nonspecific (NS) plasmids or NS sequences.

The presence of an insert in the plasmid DNA from each DS and NS colony was confirmed by digestion with Pst I and agarose gel electrophoresis. Nine of the 10 DS plasmids and all four of the NS plasmids examined were found to have at least two Pst I sites, yielding a fragment equal to the original plasmid plus additional fragments ranging from 200 to 2,500 base pairs (bp). The remaining plasmid, pcNg 2-38, was found to have one Pst I site and gave rise to a single molecule ~180 bp larger than pBR322.

To determine the relationship, if any, between the various cloned sequences, they were tested for cross hybridization. Nick-translated plasmid DNA was hybridized to Southern blots of gels on which plasmid DNA cut with Pst I had been electrophoresed. Four classes of DS sequences were identified on the basis of their cross hybridization (Table I). Class I consisted of pcNg 1-8 and pcNg 7-43. Class II consisted of six members; pcNg 2-14, pcNg 2-22, pcNg 2-38, pcNg 3-30, pcNg 4, and pcNg 6-18. Class III and Class IV had one member each; pcNg 8-5 and pcNg 44, respectively. None of the NS plasmids cross-hybridized with each other or with the DS plasmids.

Table I. Characterization of DS-RNA Clones

Class	Cross-hybridizing sequences	RNA(kb)*
I	pcNg 1-8, pcNg 7-43	6-7
II	pcNg 2-14, pcNg 2-22, pcNg 2-38 pcNg 3-30, pcNg 4, pcNg 6-18	1.7
III	pcNg 8-5	1.7
IV	pcNg 44	0.5

Plasmids containing the cloned sequences were labeled with ^{32}P by nick translation. The labeled plasmids were hybridized to Southern blots of unlabeled plasmid DNA digested with Pst I. Cross-hybridization to the inserted sequences was detected by autoradiography.

* Estimated from hybridization of labeled plasmid DNA to Northern blots of poly(A)+RNA; see, for example, Fig. 2.

The relationship between the multiple members of Class I and Class II was examined by restriction endonuclease mapping. The two members of Class I had the same restriction map (Fig. 1 *d*), suggesting that they are probably derived from the same sequence. Four of the six members of Class II (Fig. 1 *b*) had the same restriction map as one end of the large member of this class (pcNg 4, Fig. 1 *a*). The sixth member of Class II, pcNg 2-38, which lacks a second Pst I site, was too small to provide a well-characterized restriction map (Fig. 1 *c*). However, this sequence is clearly somewhat different from the other members of Class II based on the closeness of the EcoR I and Hinf I sites.

When the poly(A)+RNAs complementary to the DS sequences were examined by Northern blot analysis (Fig. 2), Class II and Class III sequences hybridized to a single band of 1.7 kilobases (kb) (lanes *2–5* and *7–9*). The Class I sequences hybridized strongly to a band of 6–7 kb and much more weakly to RNAs between 0.5 and 2 kb (lanes *6* and *11*). The Class IV sequence bound to a single band of ~0.5 kb (lane *12*). These data are summarized in Table I. The DS plasmids also hybridized to a single band when total RNA was used for Northern analysis (Fig. 3). The bands were the same size as those seen when poly(A)+RNA was run on the gels (Fig. 2). No hybridization was seen when poly(A)−RNA was used in Northern blots (data not shown).

Northern blot analysis (Fig. 3) also provided a preliminary characterization of the temporal changes in DS sequence concentration during the differentiation. As expected from the criteria used in their selection, the DS sequences did not hybridize to RNA from amebae (Fig. 3, lane *1*). The DS sequences were first detected in RNA from cells 25 min after initiation (Fig. 3, lane *3*) and they continued to be present until at least 90 min (Fig. 3, lane *6*). This same pattern of temporal expression was seen for each of the four classes of DS sequences (Fig. 3, *a–d*). Temporal changes in 3 NS sequences were also examined by Northern blots analysis of poly(A)+RNA (Fig. 4). While there was some variation during the differentiation, the NS sequences were all present in amebae and persisted until 120 min. Each NS sequence hybridized to a single band that did not change in size during the differentiation. The pattern of NS sequences in total RNA has not been analyzed yet.

To obtain a more accurate picture of the temporal changes

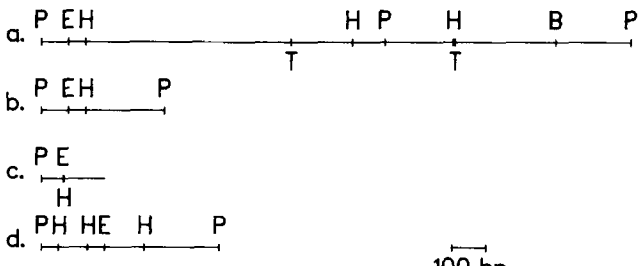

Figure 1. Restriction maps of Class I and class II DS cDNA sequences. Cloned cDNA sequences were digested with combinations of restriction enzymes and the products were analyzed by agarose gel electrophoresis. (*a*) cDNA from the Class II plasmid pcNg 4. (*b*) cDNA from the Class II plasmids pcNg 2-14, pcNg 2-22, pcNg 3-30, and pcNg 6-18. (*c*) cDNA from the Class II plasmid pcNg 2-38. (*d*) cDNA from the Class I plasmids pcNg 1-8 and pcNg 7-43. *B*, Bam HI site; *E*, EcoR I site; *H*, Hinf I site; *P*, Pst I site; and *T*, Taq I site.

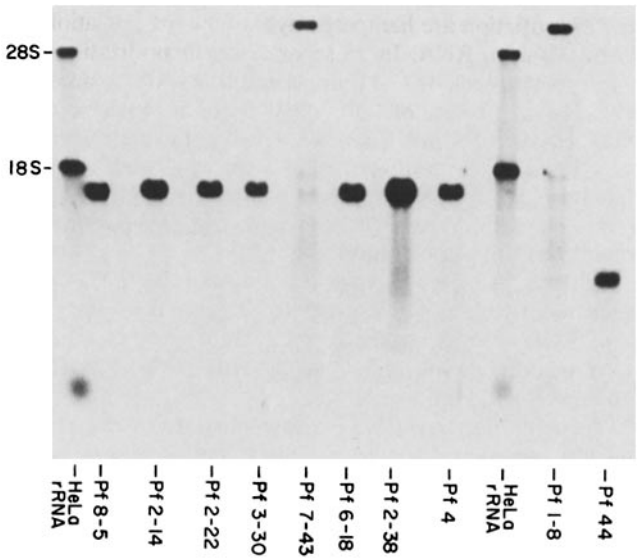

Figure 2. Northern blot analysis of the DS plasmids. 20 μg of denatured poly(A)+RNA prepared from cells at 70 min after initiation of the differentiation was loaded into each well of a 1.5% agarose-formaldehyde gel. After electrophoresis and transfer to a nitrocellulose filter, the blot was cut into strips and each lane was hybridized to a different ^{32}P-labeled DS plasmid. On completion of the hybridization and washing, the strips were autoradiographed together on one x-ray film. The intensity differences are not proportional to the abundance of the RNAs because the specific activity of the nick-translated probes varied. RNA sizes are calculated relative to size standards of HeLa cell rRNAs and pBR322 cut with Hinf I. The positions of HeLa cell 18S and 28S rRNAs are indicated on the left. Note that plasmids are indicated with a preliminary nomenclature as Pf 8-5, etc., while the text uses the current nomenclature, pcNg 8-5, etc. In all cases, the numerical part of the designation is the same. As defined in Table I, the plasmids are organized into the following classes: Class I, Pf 1-8 and Pf 7-43; Class II, pf 2-14, Pf 2-22, Pf 2-38, Pf 3-30, Pf 4, and Pf 6-18; Class III, Pf 8-5; and Class IV, Pf 44.

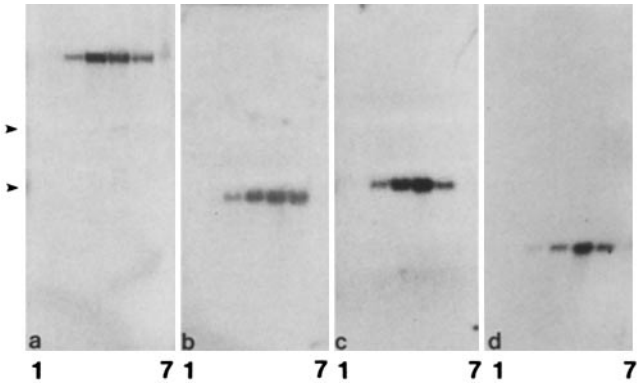

Figure 3. Analysis of total RNA from different times during the differentiation for the presence of DS sequences. 20 μg of total RNA isolated from differentiating cells at the times indicated was analyzed as described in Fig. 2. (*a*) Blot hybridized to the Class I plasmid pcNg 1-8. (*b*) Blot hybridized to the Class II plasmid pcNg 2-38. (*c*) Blot hybridized to the Class III plasmid pcNg 8-5. (*d*) Blot hybridized to the Class IV plasmid pcNg 44. Lane *1*, 0-min (amebae) RNA; lane *2*, 10-min RNA; lane *3*, 25-min RNA; lane *4*, 40-min RNA; lane *5*, 60-min RNA; lane *6*, 90-min RNA; and lane *7*, 120-min RNA.

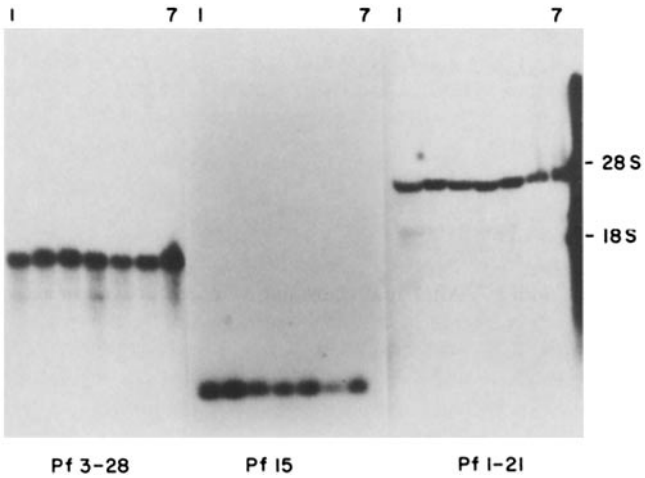

Figure 4. Analysis of poly(A)+RNA from different times during the differentiation for the presence of nonspecific sequences. 20 μg of poly(A)+RNA isolated from differentiating cells at the times indicated was analyzed as described in Fig. 2 using the nonspecific plasmids indicated. Note that plasmids are indicated with a preliminary nomenclature as Pf 3-28, etc., while the text uses the current nomenclature, pcNg 3-28, etc. In all cases, the numerical part of the designation is the same. Lane *1*, 0-min (amebae) RNA; lane *2*, 10-min RNA; lane *3*, 25-min RNA; lane *4*, 40-min RNA; lane *5*, 60-min RNA; lane *6*, 90-min RNA; and lane *7*, 120-min RNA.

in DS RNAs, total RNA was prepared from cells at 5–10-min intervals throughout the differentiation. The concentration of the DS sequences in total RNA was analyzed using the dot blot technique. Preliminary experiments using RNA from 70-min cells demonstrated that the extent of hybridization was a linear function of the total RNA in a dot up to 8 μg (Fig. 5). At higher levels of RNA input, the hybridization dropped sharply. As a consequence, all dot blot hybridizations were carried out at 3 or 4 μg of total RNA per dot. By using total RNA for these experiments we have avoided errors in estimating poly(A)+RNA concentrations introduced by variable amounts of contaminating poly(A)−RNA and by possible changes in the amount of poly(A)+RNA per cell. The fact that the total amount of RNA per cell does not change during the differentiation (17) combined with the presence of only one size class of RNA complementary to each class of plasmid means that the dot blot data are a direct reflection of the concentration of the DS RNAs in the cell.

Fig. 6 presents the results of dot blot analyses for all four classes of DS sequences. Fig. 6*a* illustrates the changes in the Class IV RNA using plasmid pcNg 44 to hybridize to RNA samples prepared from two separate differentiations carried out several months apart. The Class IV RNA was first detected at 15 min after initiation; its concentration increased linearly until ~60 min, reached a peak at 70 min, and then declined to ~10% of the peak value by 120 min. As illustrated in Fig. 6*a*, this pattern was very reproducible from one RNA preparation to another.

Fig. 6*b* illustrates the temporal changes in Class II RNA as measured using four different plasmids to analyze the same series of RNA samples. As expected, all of these cross-hybridizing plasmids gave the same pattern. This pattern was the same as seen for the Class IV RNA. Fig. 6*c* compares the average of the four curves in Fig. 6*b* with the pattern obtained by analyzing a series of RNA samples from a separate differentiation using another Class II plasmid, pcNg 3-30. Again, both curves show the same pattern, indicating that the changes in Class II RNA are reproducible from one differentiation to

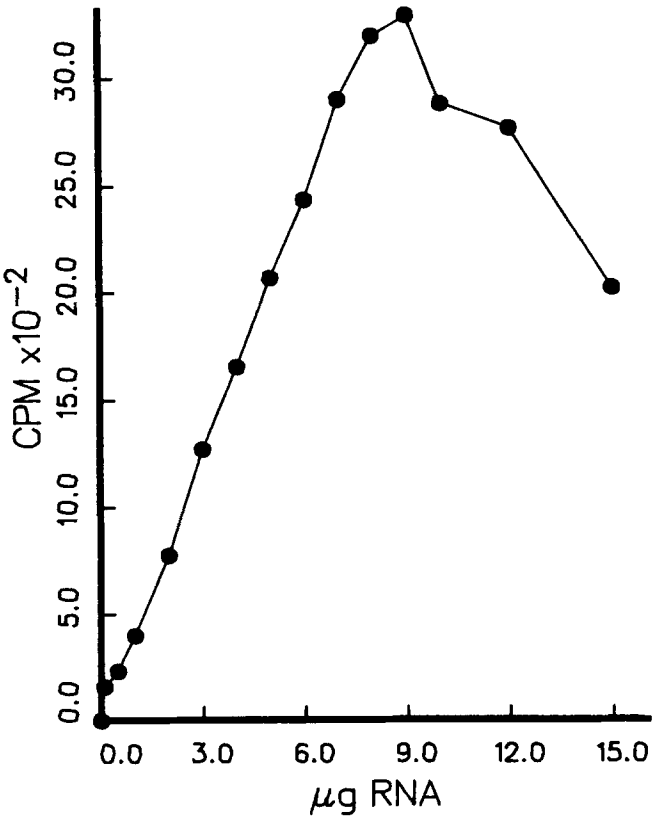

Figure 5. The effect of RNA concentration on dot blot hybridization. Increasing concentrations of total RNA, isolated from cells 70 min after initiation, were bound to a nitrocellulose filter using the dot blot apparatus. The filter was hybridized to the Class III plasmid pcNg 8-5 labeled with ^{32}P. After autoradiography, the radioactivity in each dot was determined by scintillation counting. Each point represents the average of duplicates after correcting for the background bound to 10 μg of yeast tRNA.

another. The concentrations of Class I RNA (Fig. 6*d*) and Class III RNA (Fig. 6*e*) were also found to follow the same pattern and to be reproducible from one RNA preparation to another.

Fig. 6*f* illustrates changes in the concentration of 2 NS RNAs during the differentiation. One RNA (pcNg 3-28) varied by <20% throughout the differentiation in one experiment while in a second preparation it declined by ~40% during the first 60 min of the differentiation and then returned to nearly the original concentration by 120 min. The second RNA (pcNg 2) showed a transitory twofold increase in concentration, reaching a peak at 70 min.

Changes in the concentration of the four classes of DS RNAs and the appearance of flagellates during the differentiation are summarized in Fig. 7. In all four cases, the DS RNAs first appear between 10 and 15 min after initiation and reach a peak at 70 min just as flagella first appear on the cell surface. The subsequent decline of these RNAs shows more heterogeneity, with the Class III sequences being lost at the fastest rate and the Class I RNA at the slowest rate. By 120 min after initiation, the time when cells have formed full-length flagella, three of the four classes of DS RNAs had declined to ~15% of their peak values while the Class I RNA had declined to ~30% of its peak value.

Estimates of the extent of increase in the DS RNAs during the differentiation are hampered by the low hybridization to amebae (0-min) RNA. In 28 separate dot hybridization experiments that included all four classes of DS RNAs, amebae hybridized an average of 1.34 ± 0.37 times as much as yeast tRNA used as a control. There were no significant differences between the hybridization to the different classes. For the Class I through Class IV RNAs, amebae contained an average of 3%, 4%, 2%, and 4% of the 70-min values, respectively. While these values obviously lack precision because of the difficulty of obtaining accurate levels for amebae RNA, they do suggest that amebae contain <5% of the peak level of any of the DS RNAs and therefore that there is at least a 20-fold increase in the concentration of these RNAs between 0 and 70 min.

Discussion

We have identified 10 cDNA-containing plasmids that hybridize to RNA from differentiating *Naegleria* (40-min RNA) but not to RNA from amebae (0-min) RNA. Based on cross-hybridization, restriction mapping, and Northern blot analysis, these 10 DS plasmids fall into four classes, representing sequences from at least four different poly(A)+RNAs. The two members of Class I, pcNg 1-8 and pcNg 7-43, contain inserts that have the same length and the same restriction map. This suggests that they are duplicates of the same cloning event rather than two independent cDNAs from the same or similar poly(A)+RNAs.

One of the six members of Class II, pcNg 4, is large enough to code for the entire 1.7-kb poly(A)+RNA to which it is complementary. Four other members of Class II, pcNg 2-14, pcNg 2-22, pcNg 3-30, and pcNg 6-18, have the same restriction map as one end of pcNg 4 and are all ~360 bp long, suggesting that these four plasmids could represent duplicates of a single cloning event. The sixth member of Class II, pcNg 2-38, contains an insert of ~180 bp and has a restriction map similar but not identical to the other members of Class II. We do not know if the difference between pcNg 2-38 and the other members of Class II represents a deletion that occurred during cloning or if this plasmid contains a sequence from another poly(A)+RNA. It seems likely that the six members of Class II represent clones of at least two, and perhaps three, independent cDNAs. Because of the short inserts in five of these plasmids, our data do not permit us to rule out the possibility that they are derived from three different poly(A)+RNAs with similar 3′-regions. We find, however, that all members of Class II are complementary to a 1.7-kb RNA in both poly(A)+RNA and total RNA and that the size of this RNA does not vary during the differentiation.

While both Class II and Class III sequences hybridize to the same size poly(A)+RNA, there is no cross-hybridization between any member of Class II and the Class III plasmid, demonstrating that these sequences are derived from different poly(A)+RNAs. Because the Class I and Class IV RNAs differ in both size and cross-hybridization from the other DS RNAs, it is clear that there are at least four different poly(A)+RNAs present in differentiating cells at 40 min that are rare or absent in amebae.

In addition to the DS sequences, the cDNA library was found to contain sequences that are present in both amebae and differentiating cells as well as sequences that are present in amebae but are less abundant or absent in differentiating

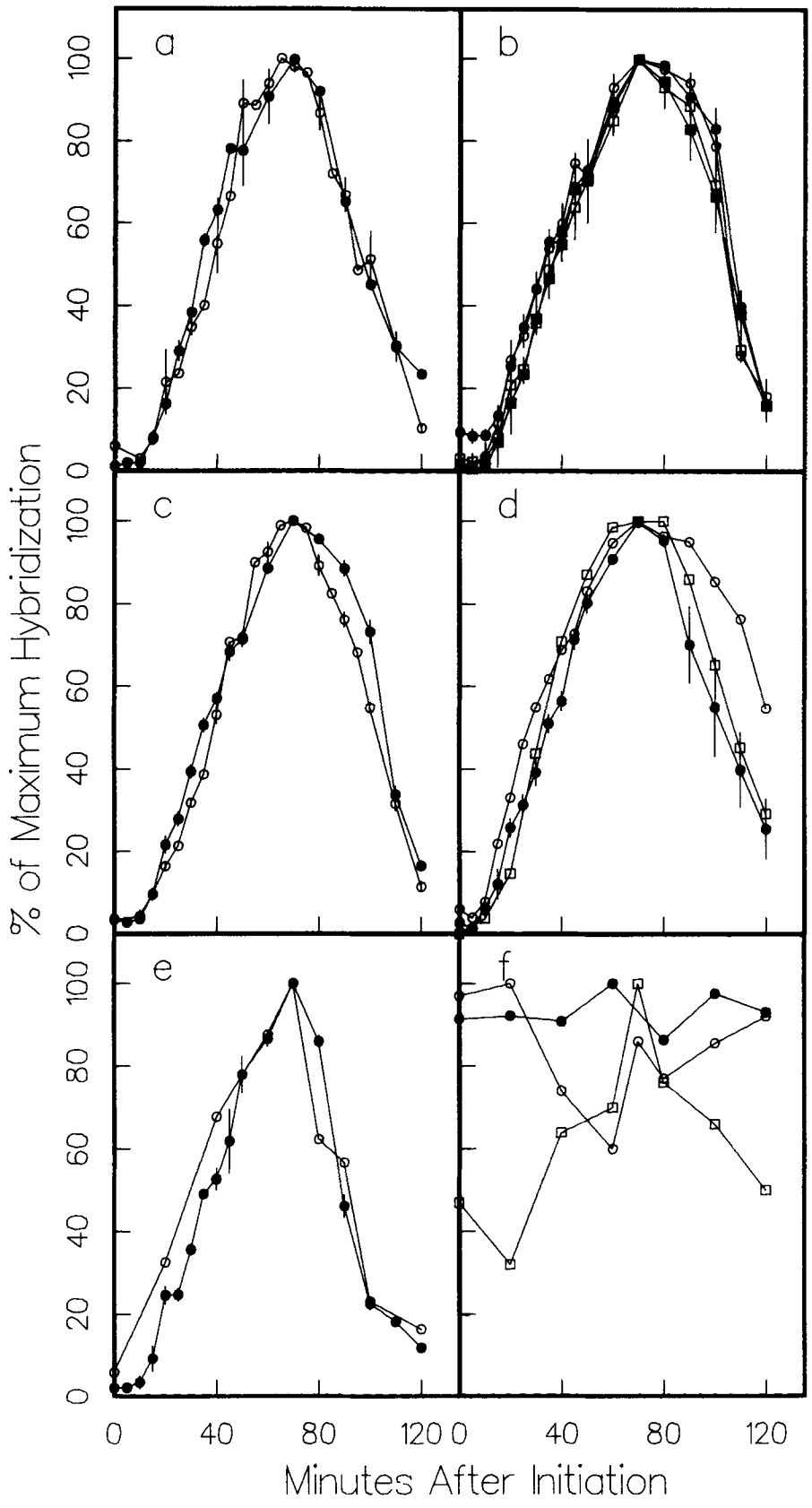

Figure 6. Concentration changes of DS and nonspecific sequences in total RNA during the differentiation. Total RNA was prepared from differentiating cells at the indicated times. The concentration of a given sequence in 3 μg of total RNA was determined by the dot blot technique. Each point represents the average of three or more replicates after correction for background hybridization to yeast tRNA. The vertical bars indicate the standard error of the mean. (*a*) Dots hybridized with the Class IV plasmid pcNg 44: (●) RNA preparation 1, (○) RNA preparation 2. (*b*) An RNA preparation hybridized with various Class II plasmids: (●) pcNg 4, (○) pcNg 2-14, (□) pcNg 2-22, (■) pcNg 2-38. (*c*) Two different RNA preparations hybridized with Class II plasmids: (●) the average of all four curves in *b*, (○) a second RNA preparation hybridized with pcNg 3-30. (*d*) Two different RNA preparations hybridized with the Class I plasmids: (●) pcNg 1-8 with RNA preparation 1, (○) pcNg 7-43 with RNA preparation 1, (□) pcNg 1-8 with RNA preparation 2. (*e*) Two different RNA preparations hybridized with the Class III plasmid pcNg 8-5: (●) RNA preparation 1, (○) RNA preparation 2. (*f*) Hybridization with the nonspecific plasmids: (●) pcNg 3-28 with RNA preparation 1, (○) pcNg 3-28 with RNA preparation 2, (□) pcNg 2 with RNA preparation 2. RNA preparations 1 and 2 are not the same in all panels. After correction for background as measured using yeast t-RNA, the average maximum hybridization in 28 dot blot experiments was 895 cpm.

cells. The limited data that we have on the NS sequences (Figs. 4 and 6) demonstrate that while the DS RNAs change in a coordinate and reproducible manner, the concentrations of the NS RNAs vary independently and less reproducibly. The fact that some poly(A)+RNAs do not follow the pattern of the DS RNAs shows that the coordination seen in the DS RNAs is not the result of some general property of poly(A)+RNAs. The variation in the pattern of pcNg 3-28

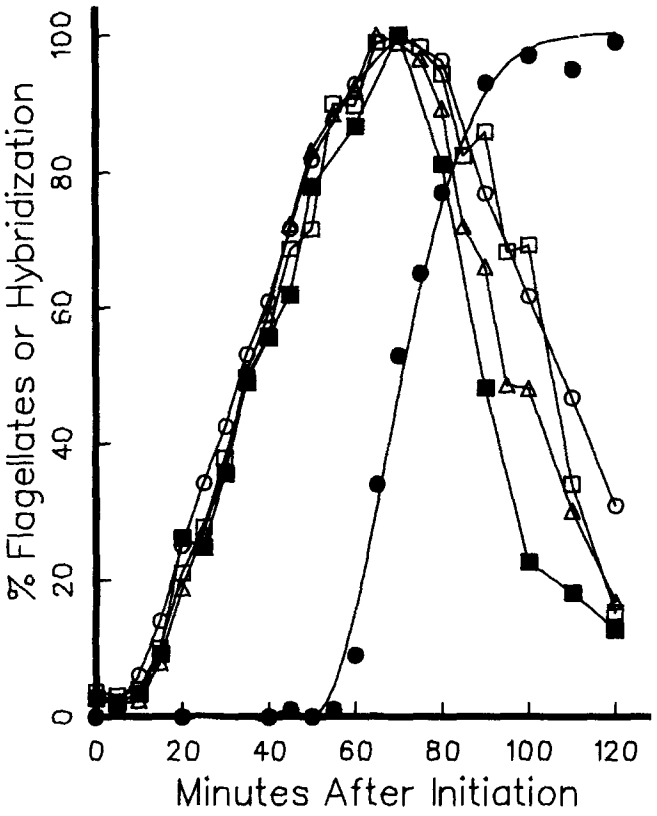

Figure 7. Summary of changes in the concentration of DS RNAs during the differentiation of *Naegleria* amebae into flagellates. The hybridization curves are the average of all the curves for a particular class using the data from Fig. 6. The data for percent flagellates are from a typical differentiation as determined by staining with Lugol's iodine. (●) % flagellates; (○) Class I sequences; (□) Class II sequences; (■) Class III sequences; (△) Class IV sequences.

could be a reflection of the state of the cells when the differentiation was induced.

There is a lag of ~10 min after initiation, before the DS RNAs begin to accumulate. This lag presumably includes the time when the cells are sensing and responding to the environmental changes that trigger the differentiation. In the present case, the differentiation is initiated by washing amebae free of bacteria (43). Although Fulton (10–13) has shown that the differentiation of *Naegleria* amebae can be influenced by changes in a variety of factors, including electrolyte concentration, changes in the concentration of a small organic molecule, and temperature, we do not understand how these changes control the differentiation. Fulton (10) has also found that the first 10 min of the differentiation can be induced by a number of subtle changes in the environment. Differentiation beyond this point, however, is dependent on more substantial changes. It will be interesting to determine if the lag in the appearance of the DS RNAs means that their accumulation is coupled to these later changes, or if their appearance may be triggered by subtle shifts which do not result in completion of the differentiation.

Changes in the concentration of all four classes of DS RNAs follow the same temporal pattern as changes in the level of functional flagellar tubulin mRNA (24). As determined by in vitro translation, tubulin mRNA appears after a lag of 10 min, reaches a peak at 60 min, and then declines to ~20% of maximum by 80 min. When corrected for the 7-min faster differentiation of strain NEG (T_{50} = 61 min) (17) that was used in the tubulin mRNA studies, our data for the DS RNAs in NB-1 are quantitatively identical. This observation lends support to our previous hypothesis that changes in flagellar tubulin mRNA as measured by in vitro translation are the result of changes in the concentration of these RNAs and not to changes in their ability to be translated.

The presence of DS RNAs raises the question of whether any of these sequences code for flagellar proteins. This is especially true of the Class II and III RNAs that resemble tubulin mRNAs in size. When we hybridized our Class II and III clones to the well-characterized α- and β-tubulin clones from chicken (3), we found no cross-hybridization even under conditions of low stringency. It has recently been reported, however, that the *Naegleria* tubulin genes cross-hybridize poorly if at all with these chicken tubulin clones (23). Using hybrid selection and in vitro translation, we have recently shown that the Class II sequences code for α-tubulin and the Class III sequence codes for β-tubulin. In addition, we found that the Class IV sequence codes for a flagellar calcium-binding protein (Shea, D., J. Mar, J. Lee, and C. Walsh, manuscript in preparation). The product of the Class I sequence has not yet been identified.

As measured using AMD, essential RNA synthesis for the formation of flagella is completed at 39 min (17), when the DS RNAs have reached 60% of their maximum concentration. Formation of the flagellate body shape, however, requires RNA synthesis until ~50 min (17), a time when the DS RNAs have reached nearly 80% of their maximum. Because AMD inhibition of RNA synthesis is not effective immediately and because it does not produce 100% inhibition of uracil incorporation (17), DS RNAs probably reach even higher levels before cells form flagella or the flagellate shape. This is not meant to imply that the limiting RNA(s), as detected by AMD studies, necessarily follow the same temporal pattern as the DS RNAs.

All four classes of DS RNAs reach their maximum concentration at the time when visible flagella appear at the cell surface, T_{50} = 68 min. Both the timing and coordination of these changes raise intriguing questions about the regulatory mechanisms involved. We are attempting to examine the degree to which changes in DS RNAs are coordinately regulated and coupled to the morphological changes by perturbing the timing of the differentiation. For example, when the appearance of flagella is delayed 35 to 40 min by heat-shock induction of multiple flagella (6, 44), we find that there is still close coordination in the appearance of the DS RNAs. The timing of their appearance is altered, however; they do not reach a maximum until 105 to 110 min, the time when flagella appear in heat-shocked cells (Mar, J., D. Shea, J. H. Lee, and C. Walsh, manuscript in preparation).

The rapid increase in the concentration of the DS RNAs between 15 and 70 min after initiation clearly represents a change in the rate of their synthesis relative to the rate of their degradation. Our data, however, do not permit us to determine the contribution of changes in the rate of synthesis or degradation to the observed increase in concentration. In preliminary studies (30), we have examined the effects of AMD on DS RNA concentration both during the increase and the decline. Addition of AMD at 40 min, during the period of linear increase in DS RNA concentration, resulted in a complete inhibition of accumulation within 10 min and

a subsequent decline with a half-life of ~56 min. While these data are useful in interpreting previous studies using AMD to determine the synthetic requirements for the differentiation (17), they must be interpreted with caution in estimating changes in the rates of synthesis and degradation because AMD only inhibits uracil incorporation into RNA by 82–91% in *Naegleria* (17). In addition, it is possible that AMD might induce changes in the rate of RNA degradation. AMD added during the decline in DS RNA concentration, however, had no effect on the half-life (30), suggesting that synthesis makes at most a small contribution after 70 min and that degradation is not increased by this drug.

After 70 min, the DS RNAs decline with half-lives of 16 to 33 min (Fig. 6 [30]), a rapid loss compared to the half-life of mRNA in many higher eukaryotic cells (see discussion in reference 40). These values are similar, however, to the 25-min half-life of actin mRNA during the first 60 min of the *Naegleria* differentiation (40). They are also similar to the 19-min half-life of tubulin mRNA after mitosis in *Physarum* (35).

The changes in DS RNA concentration during differentiation of *Naegleria* resemble changes in the levels of mRNA for tubulin and other flagellar proteins during flagellar regeneration in *Chlamydomonas* (33). The *Chlamydomonas* RNAs show transient increases, reaching their maximum levels between 10 and 50 min after deflagellation (36, 38). The maximum abundance of β-tubulin mRNA after deflagellation has been reported to be 7–10 (1) and 25 (31) times higher than in undeflagellated cells. The rate of tubulin mRNA synthesis increases four- to sevenfold within the first 10 min in response to deflagellation and then declines (1). Thus both deflagellation of *Chlamydomonas* and differentiation of *Naegleria* result in transient increases in a number of poly(A)+RNAs including those coding for tubulin.

The presence of at least three independent clones for the Class II DS RNA suggests that this poly(A)+RNA is present at high concentrations. In fact, in the current work we have analyzed only those clones which showed a strong differential signal on colony hybridization and therefore all four DS RNAs probably represent abundant sequences. The absence of other patterns of change in RNA concentration during the differentiation of *Naegleria* may simply reflect this rather small sample size. Many of the 25 clones analyzed in deflagellated *Chlamydomonas* were identified by screening plasmid DNA with labeled RNA from regenerating cells (36). This approach can detect clones for RNAs present at too low a concentration to be picked up by colony hybridization. It should be noted, however, that we find very close coordination between the Class II and III sequences, now known to represent α- and β-tubulin mRNA, while there are some differences in the appearance of α- and β-tubulin mRNAs in *Chlamydomonas* (36). Current experiments designed to analyze the pattern of a larger population of poly(A)+RNAs during the differentiation of *Naegleria* should help clarify differences and similarities between these two systems.

The changes in DS RNA concentration during the differentiation of *Naegleria* also resemble changes in the levels of tubulin mRNAs during the myxamoeba to flagellate transformation of *Physarum polycephalum*. In *Physarum*, tubulin mRNAs increased five- to sevenfold, reaching a maximum between 130 and 160 min after induction, when 50–60% of the cells had formed flagella and then declined to near basal levels by 240 min after induction, when the number of flagellates plateaued at 80% (19).

While *Naegleria*, *Chlamydomonas*, and *Physarum* all form flagellar axonemes, there are some significant differences in the biology of these three systems. Flagellar regeneration in *Chlamydomonas* requires the reformation of a normally existing organelle from existing basal bodies and transformation of *Physarum* involves the formation of flagella from an existing pro-flagellar apparatus while the differentiation of *Naegleria* involves the *de novo* formation of basal bodies and flagellar axonemes as well as associated structures such as the flagellar rootlet. Despite these differences, all three systems presumably require the participation of the more than 200 gene products required for formation of the flagellar axoneme.

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