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Diversity and Abundance of Polyadenylated RNA from *Achlya ambisexualis*†

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ABSTRACT: The diversity, abundance, and DNA sequence representation of poly(adenylic acid) containing RNA derived from cells of *Achlya ambisexualis* cultured in defined and undefined media have been determined. The kinetics of hybridization of polyadenylated RNA with complementary DNA were the same for both culture conditions and revealed the presence of three frequency classes containing 29, 220, and 3000 different sequences of an average length of 1150 nucleotides. Complexity estimates derived from experiments in which polyadenylated RNA was hybridized to unique sequence DNA were in good agreement with these results. The kinetics of hybridization of complementary DNA with an excess of nuclear DNA indicate that approximately 10% of the RNA is transcribed from reiterated DNA sequences while the remainder is transcribed from single copy sequences.

Achlya ambisexualis is an aquatic fungus in which the development of sexual reproductive structures and the coordination of the mating process are regulated by steroid hormones (Barksdale, 1969; Gooday, 1974; van den Ende, 1976). This organism has been the subject of recent investigations in our laboratory because of its potential usefulness as a model system for studying the steroidal regulation of gene activity. Recently we have made two observations which suggest that the mechanisms controlling mRNA transcription and accumulation in *Achlya* may perhaps differ significantly from those operating in metazoan cells. First, we have found that, while *Achlya* possesses both unique and repetitive DNA sequence components, these are arranged in the genome in a pattern different from the characteristic short period interspersion pattern of higher eucaryotes (Hudspeth et al., 1978; Davidson & Britten, 1973; Davidson et al., 1975). Indirect calculations based on quantitative aspects of our data suggest that the repetitive and single copy DNA have average genomic lengths in the range of 2.7×10^4 and 1.4×10^5 nucleotide pairs, respectively, much longer even than those observed in higher organisms, such as *Drosophila,* with long period DNA sequence interspersion (Manning et al., 1975; Crain et al., 1976). Second, the processes of mRNA transcription and posttranscriptional modification in *Achlya* appear to be more straightforward than has been found in advanced eucaryotic forms. The nuclear and polysomal poly(A^+)RNA populations in *Achlya* are indistinguishable with respect to size distribution, DNA sequence representation, and sequence complexity (Timberlake et al., 1977). This is in contrast to animals in which the hnRNA is typically larger and considerably more complex than mRNA active in translation (Lewin, 1975a,b). However, in several general respects the mRNA of *Achlya* is similar to that of higher eucaryotes (e.g., polyadenylated and monocistronic).

The novel features of genome organization and mRNA synthesis which we have observed in *Achlya* raise the question of how the mechanisms of gene regulation in this organism are related to those in other eucaryotes and to those in procaryotes. Here we report the results of a study in which the distribution of poly(A^+) RNA into abundance classes was determined. This investigation was prompted by the observation that, in all eucaryotic cells thus far examined, the poly(A^+) mRNA has been found to occur in classes with different relative cellular concentrations (abundance classes) (Bishop et al., 1974; Hastie & Bishop, 1976; Monahan et al., 1976; Levy & Dixon, 1977; Hereford & Rosbash, 1977a; Galau et al., 1976). Different abundance classes may have specialized metabolic roles and

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may be separately regulated (Hereford & Rosbash, 1977b; Hastie & Bishop, 1976; Levy & Dixon, 1977). We find that, with respect to poly(A$^+$) RNA abundance classes, *Achlya* is qualitatively similar to other eucaryotic organisms.

Materials and Methods

RNA and DNA Extraction. Achlya ambisexualis strain E87 was cultured in liquid PYG or glucose/L-glutamic acid medium (M-1) as previously described (Timberlake, 1976) except that 3 L of culture medium in a 4 L Erlenmeyer flask was vigorously aerated for 17 h at 25 °C. Procedures for the isolation of DNA, polysomes, and RNA have been described (Timberlake, 1976; Timberlake et al., 1977; Hudspeth et al., 1978).

Preparation of Poly(A$^+$) RNA.[1] Poly(A$^+$) RNA was prepared by chromatography on poly(U)-agarose (P-L Biochemicals, type 6) or on oligo(dT)-cellulose (Collaborative Research, type T3). Poly(U)-agarose chromatography was as previously described (Timberlake, 1976). Oligo(dT)-cellulose chromatography was a modification of the procedure of Aviv & Leder (1972). RNA (10–20 mg) was suspended in 10 mL of NETS buffer (500 mM NaCl, 1 mM EDTA, 0.2% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 7.6) and treated for 30 min with 10 μg/mL of proteinase K at 25 °C. The RNA was incubated for 2 min at 60 °C and applied to a 5 mL oligo(dT)-cellulose column equilibrated with NETS. The column was washed with NETS until the A_{260nm} of the effluent was <0.02. The bound RNA was eluted in 10 mM Tris-HCl, pH 7.6, 0.2% sodium dodecyl sulfate at 50 °C, adjusted to 500 mM NaCl, and rechromatographed as above. This procedure was repeated until >90% of the RNA bound to the column. The poly(A$^+$) RNA was finally eluted in H_2O and precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 6.0, and 2 volumes of absolute ethanol. After 14–18 h at −22 °C, the precipitate was pelleted, dried, and suspended at approximately 1 mg/mL in H_2O.

Determination of Poly(A) Size. Cultures were labeled with 4 μCi/mL of [2-^{3}H]adenosine (20 Ci/mmol) for 0.4 generation (PYG, 20 min; M-1, 2 h). RNA was prepared as above, resuspended in 10 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, pH 7.6, and treated with RNase A (10 μg/mL) and RNase T1 (15 units/mL) for 1 h at 37 °C (Vournakis et al., 1974; Timberlake, 1976). The reaction mixture was adjusted to 0.5% $NaDodSO_4$, 500 mM NaCl, and poly(A) was isolated by chromatography on oligo(dT)-cellulose. Following concentration by ethanol precipitation, poly(A) was dissolved in 40 mM Tris-citrate, 0.1% $NaDodSO_4$, 10% sucrose, pH 8.0, and electrophoresed with known molecular weight markers (Miles Laboratories) as described by Kaufman & Gross (1974). Number average sizes were calculated as $L_N = \Sigma(N_iL_i)/\Sigma(N_i)$, where N_i is the number of molecules in gel slice i and L_i is the nucleotide length at gel slice i.

Synthesis of cDNA, Poly(dT), and Labeling of Single Copy DNA. cDNA was prepared from poly(A$^+$) RNA using a modification of the procedure of Dr. Henry Burr (personal communication). Poly(A$^+$) RNA (1–2 μg) was incubated at 43 °C for 1 h in a reaction mixture (10 μL) containing 20 μg/mL oligo(dT)$_{12-18}$, 100 μg/mL actinomycin D, 30 mM 2-mercaptoethanol, 0.5 mM dGTP, dATP, TTP, 0.5 mM [^{3}H]dCTP (20 Ci/mmol), 50 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 50 mM NaCl, and 3 units of AMV reverse transcriptase. Sheared calf thymus DNA (20 μg) was added, the mixture was made to 0.2% $NaDodSO_4$ and 0.1 M NaOH and incubated for 10 min at 100 °C. After neutralization, the cDNA was deproteinized with $CHCl_3$ and chromatographed on a Sephadex G-100 column developed in H_2O. The excluded fractions were pooled and the cDNA concentrated by ethanol precipitation. The synthesis of [^{3}H]poly(dT) was essentially the same except that poly(A) was used as template and only [^{3}H]TTP was added as substrate. The preparation of single copy [^{3}H]DNA was as previously described (Timberlake et al., 1977). The final specific radioactivity of foldback-free DNA was 3×10^6 cpm/μg.

Hybridization and Reassociation Reactions. cDNA was reacted with an excess of DNA or poly(A$^+$) RNA as described in the legends to Figures 5 and 6. The fraction of the molecules containing duplex regions was assayed by hydroxylapatite chromatography at 60 °C in 0.12 M NaP, 0.2% $NaDodSO_4$ (Britten et al., 1974). The A_{260} of a 1 mg/mL solution was considered to be 28.6 for RNA and 20.0 for DNA.

Single copy [^{3}H]DNA was reacted with an excess of DNA or RNA at 60 °C in a buffer containing 10 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid), 0.3 M NaCl, 0.1 mM EDTA, pH 6.7. The C_0t or R_0t of all reactions was corrected relative to 0.18 M Na^+ (Britten et al., 1974). The extent of hybridization was determined by treatment with single-strand specific nuclease (S1) using the conditions of Zimmerman & Goldberg (1977). Reactions were pipetted onto Whatman 3MM filter paper, washed with 5% trichloroacetic acid and 95% ethanol at 4 °C, dried, and counted.

[^{3}H]Poly(dT)-excess hybridizations were carried out in the same buffer used for single copy [^{3}H]DNA for 2 h at 25 °C. The hybrid content of the reactions was assayed by S1 nuclease resistance as above and compared with a standard curve constructed using poly(A).

Analysis of Hybridization Data. The RNA-excess data were fit, with the aid of a computer, to the expression $d/D_0 = \Sigma_{i=1}^{N} P_i[1 - \exp(-0.69R_0t/R_0t_{(1/2)i})]$ where d/D_0 is the fraction hybridized, i denotes the transition, P_i is the fraction of cDNA in transition i, and $R_0t_{(1/2)i}$ is the R_0t at which 50% of the cDNA in transition i is hybridized (Bishop et al., 1974; Pearson et al., 1977). DNA-excess data were analyzed assuming second-order kinetics.

Results

RNA Characterization. Association of Poly(A$^+$) RNA with Polysomes. The proportion of the cellular poly(A$^+$) RNA which was associated with polysomes was determined by labeling cells for four doublings with [^{3}H]uridine and extracting the polysomes. Polysomes were sedimented on sucrose density gradients and fractionated as shown in Figure 1. The total amount of RNA in each region of the gradient was determined by trichloroacetic acid precipitation, while the amount of poly(A$^+$) RNA was estimated by poly(U)-agarose chromatography following RNA extraction. The results of this experiment are summarized in Table I. Approximately 72% of the total RNA and 85% of the poly(A$^+$) RNA sedimented at >100 S. Less than 5% of the poly(A$^+$) RNA derived from >100S polysomes sedimented at > 80 S after treatment with EDTA (data not shown). These results indicate that the majority of the poly(A)$^+$ RNA of *Achlya* is mRNA associated with polysomes and probably active in translation.

Fraction of mRNA Which Is Polyadenylated. We have made several attempts to determine what proportion of the

[1] Abbreviations used are: poly(A$^+$) RNA, polyadenylated RNA; poly(A), poly(adenylic acid); cDNA, complementary DNA; NETS, 500 mM NaCl, 1 mM EDTA, 0.2% $NaDodSO_4$, 10 mM Tris-HCl, pH 7.6; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; NaP, equimolar Na_2HPO_4 and NaH_2PO_4; R_0t, moles of RNA nucleotides s L^{-1}; C_0t, moles of DNA nucleotides s L^{-1}.

TABLE I: Subcellular Distribution of Poly(A$^+$) RNA.

Fraction[a]	Total RNA cpm × 10^{-5}[b]	%	Poly(A$^+$) RNA[c] cpm × 10^{-4}	As % total poly(A$^+$) RNA	As % total RNA
I	1.3	9.5	0.08	2.6	0.06
II	2.5	18.2	0.38	12.5	0.30
III	9.9	72.3	2.58	84.9	1.90
Total	13.7	100	3.04	100	2.26

[a] The fractions are those shown in Figure 1. [b] A portion of each fraction was precipitated with cold trichloroacetic acid, collected on a Millipore filter, washed with 5% trichloroacetic acid and 95% ethanol, and counted. [c] RNA was extracted from the fractions and chromatographed on 1-mL columns of poly(U)-agarose (P-L Biochemical, type 6) as previously described (Timberlake, 1976).

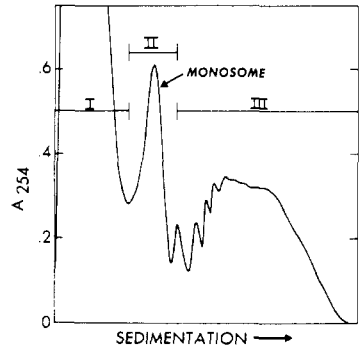

FIGURE 1: Fractionation of polysomes. Cells were labeled for four doublings with [^{3}H]uridine, harvested, powdered in liquid N_2, and suspended in TEMN buffer (Timberlake, 1976). A 15 000*g* supernate was prepared and sedimented on log-linear sucrose density gradients (Brakke and Van Pelt, 1970) in a Beckman SW41 rotor at 40 000 rpm for 70 min. The gradients were fractionated as indicated by the brackets (|—|). The distribution of radioactivity is given in Table I.

mRNA in *Achlya* is polyadenylated by using antibiotics to preferentially inhibit rRNA synthesis. These experiments have been unsuccessful because the antibiotics tested were either not specific enough (actinomycin D), did not inhibit rRNA synthesis (5-fluorouracil), or drastically affected translation (cycloheximide). However, a rough estimation of this parameter can be made from the size distribution of polysomes and poly(A$^+$) RNA. Assuming that an average polysome consists of 8 ribosomes (Figure 1), that each ribosome contains 2.1×10^6 daltons of rRNA, and that the median size of poly(A$^+$) RNA derived from polysomes is 5.6×10^5 daltons (17 S, Timberlake et al., 1977; also see Figure 2) then 3.3% of the total polysomal RNA would be predicted to be poly(A$^+$) RNA if all of the mRNA were polyadenylated $[(5.6 \times 10^5)/(8)(2.1 \times 10^6) \times 100]$. In fact, we find that 2.6% of the polysomal RNA is polyadenylated (Table I). Thus, $[2.6/3.3 \times 100]$ or approximately 80% of the mRNA contains poly(A) sequences of sufficient length to bind to poly(U)-agarose. This calculation is considered only to indicate that a majority of the mRNA is poly(A$^+$) RNA because of the uncertainty of the several assumptions involved.

Quantitation of Poly(A$^+$) RNA Preparations. Hybridization studies were performed using poly(A$^+$) RNA derived from unfractionated cells because we have found (1) the complexity of polysomal RNA is identical with total cellular RNA and with nuclear RNA (Timberlake et al., 1977); (2) the majority of the poly(A$^+$) RNA is associated with polysomes; and (3) the majority of the mRNA appears to be polyadenylated. Large quantities of poly(A$^+$) RNA were isolated by chromatography on oligo(dT)-cellulose. While the vast majority of the rRNA and tRNA failed to bind to the column, the poly(A$^+$) RNA was nevertheless substantially contaminated with rRNA as indicated by a large 26S peak which was resolved by sucrose density gradient centrifugation. Rechromatography of the RNA which bound initially, until >90% was retained by the column, removed most, but not all, of the contamination. In order to accurately determine R_0t values, it was therefore necessary to ascertain the proportion of the oligo(dT)-cellulose-bound RNA which was actually poly(A$^+$) RNA. This was accomplished by first independently calculating the poly(A) content of poly(A$^+$) RNA and then directly determining the poly(A) content of each RNA preparation by [^{3}H]poly(dT)-excess hybridization.

The poly(A) content of poly(A$^+$) RNA was calculated from the average lengths of the poly(A$^+$) RNA and the poly(A) isostichs. Since the poly(A$^+$) RNA preparations obtained by oligo(dT)-cellulose chromatography could not be used directly to determine average molecular weights because of rRNA contamination, we adopted two alternative approaches. In the first, labeled poly(A$^+$) RNA was purified by poly(U)-agarose chromatography. Previous results have shown that small amounts of poly(A$^+$) RNA purified in this way are free from detectable contamination with rRNA (Timberlake, 1976; Timberlake et al., 1977). The sedimentation pattern of a representative poly(A$^+$) RNA preparation is shown in Figure 2A. The number average size of this poly(A$^+$) RNA population was calculated to be 1100 nucleotides. In the second approach, the average size was estimated using poly(A$^+$) RNA isolated by oligo(dT)-cellulose chromatography. Poly(A$^+$) RNA was sedimented on sucrose gradients and, after fractionation, aliquots of each fraction were hybridized to an excess of [^{3}H]-poly(dT). If the length of the poly(A) isostich is not a function of the length of the poly(A$^+$) RNA, then [^{3}H]poly(dT)-excess hybridization will score the relative number of molecules in each fraction. A representative result is shown in Figure 2B. Using this procedure, the number average size of the poly(A$^+$) RNA was calculated to be 1150 nucleotides. The excellent agreement between the first and the second approach suggests that the assumptions used are valid. Using either method, no significant differences were observed in the average sizes of poly(A$^+$) RNA derived from cells cultured in either defined or undefined medium (Table II).

The average lengths of poly(A) isostichs in poly(A$^+$) RNA were determined by polyacrylamide gel electrophoresis of RNase A + T1 digests of RNA labeled in vivo with [^{3}H]-adenosine. Electrophoretic profiles of poly(A) derived from both media are shown in Figure 3. The poly(A) migrates as a heterogeneous population with a modal size of approximately 45 nucleotides. The number average lengths were calculated to be 33 and 34 for undefined and defined media, respectively. These size estimates are similar to those reported for other fungi (Johnson et al., 1977; Groner et al., 1974), although

TABLE II: Quantitation of Poly(A$^+$) RNA.

Culture condition	Poly(A)[a] length (nucleotides)	Poly(A$^+$) RNA[a] length (nucleotides)	% poly(A) Calcd[b]	% poly(A) Actual[c]	% poly(A$^+$) RNA[d]
Undefined medium	33	1150	2.9	2.7	93
Defined medium	34	1190	2.9	2.7	93

[a] Number average sizes were obtained from Figure 2B or similar data and from Figure 3. [b] (Poly(A) length/poly(A$^+$) RNA length) × 100. [c] Determined by hybridization to an excess of [^{3}H]poly(dT). [d] (Actual poly(A) content/calculated poly(A) content) × 100.

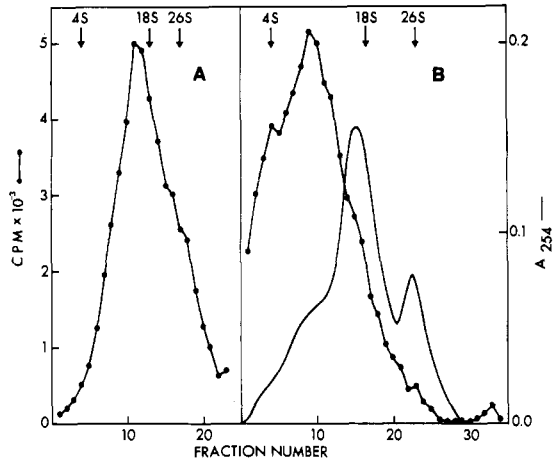

FIGURE 2: Size distribution of poly(A$^+$) RNA. (A) Sedimentation of steady-state labeled poly(A$^+$) RNA. Cultures were labeled as in Figure 1 and the poly(A$^+$) RNA was extracted and isolated by chromatography on poly(U)-agarose. The RNA was sedimented on 5-20% sucrose gradients in a Beckman SW 65 Ti rotor for 3.75 h at 60 000 rpm at 4 °C after heating for 2 min. The positions of molecular weight markers are indicated (↓). The number average size of the poly(A$^+$) RNA is 1100 nucleotides. (B) Sedimentation of unlabeled poly(A$^+$) RNA and poly(dT)-excess hybridization. Unlabeled poly(A$^+$) RNA was isolated by chromatography on oligo(dT)-cellulose and sedimented on sucrose gradients as in A. The gradients were fractionated and the A_{254nm} was recorded (—). An excess of [^{3}H]poly(dT) (5 × 10^6 cpm/μg) was hybridized to an aliquot of each fraction as described in the Materials and Methods section. The arrows show the position of molecular weight markers sedimented on parallel gradients. The number average size of the poly(A$^+$) RNA was calculated to be 1150 nucleotides (see text and Table II).

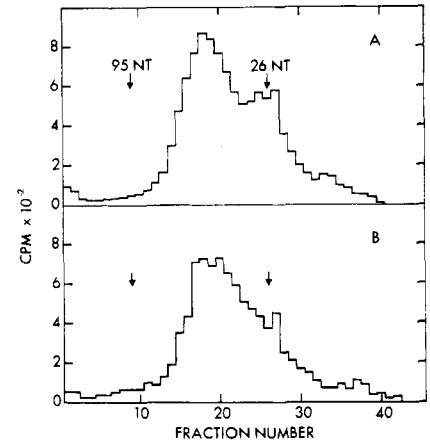

FIGURE 3: Electrophoresis of poly(A). (A) Size distribution of poly(A) isolated from cells cultured in defined medium. Cells were labeled for 0.4 generation with [2-^{3}H]adenosine and the poly(A) was prepared as described in the Materials and Methods section. Labeled poly(A) was coelectrophoresed with unlabeled poly(A) markers having modal lengths of 27 and 95 nucleotides on 12.5% acrylamide, 0.375% *N,N'*-methylenebisacrylamide gels. The electrophoresis buffer was 40 mM Tris-citrate, pH 8.0, 0.1% sodium dodecyl sulfate and electrophoresis was at 3 mA/gel until a bromophenol blue marker reached the bottom of the gel. Gels were soaked overnight in H_2O, scanned at 260 nm to locate the markers, sliced, and counted in a toluene-based scintillation fluid containing Protosol (New England Nuclear). (B) Poly(A) isolated from cells grown in undefined medium. Cells were labeled and the poly(A) isolated and analyzed as in A.

somewhat less than those reported previously for *Achlya* (Silver & Horgen, 1974; Timberlake et al., 1977). We feel that the present values are more reliable than the previous ones because they were obtained by direct comparison with poly(A) molecular weight markers. Based on the average molecular weights obtained as illustrated in Figures 2B and 3, we estimate that the poly(A) isostichs represent 2.9% of the poly(A$^+$) RNA (Table II).

In order to estimate rRNA contamination, the poly(A) content of each oligo(dT)-cellulose-bound RNA preparation was determined by hybridization of a known amount of RNA to an excess of [^{3}H]poly(dT) as described by Kaufman & Gross (1974). A standard curve prepared using poly(A) showed that the formation of S1 nuclease resistant hybrids was a linear function of the amount of poly(A) as long as poly(dT) was in excess. As shown in Table II, 2.7% of the RNA was poly(A). Thus, 93% of the bound RNA was in fact poly(A$^+$) RNA. This value was quite repeatable and did not vary more than a few percent in five independant isolations.

DNA Sequence Representation in cDNA. Previous results, based on DNA-excess DNA·RNA hybridization, have demonstrated that the majority of the poly(A$^+$) RNA sequences of *Achlya* are transcribed from single copy DNA, but that approximately 10% are transcribed from reiterated sequences. The repetitive elements are not covalently linked to single copy elements (Timberlake et al., 1977). We determined the kinetics of reassociation of cDNA with an excess of nuclear DNA in order to ascertain that the reiterated or single copy sequences were not preferentially amplified during the reverse transcriptase reaction and, furthermore, to determine if one class or the other was selectively transcribed and represented under the different growth regimes. The sedimentation of a representative cDNA preparation on an alkaline sucrose gradient is shown in Figure 4. Our reaction conditions routinely yielded cDNA with a modal size of 300-400 nucleotides. Figure 5A shows the reassociation of cDNA synthesized from poly(A$^+$) RNA isolated from cells grown in undefined medium. Approximately 80% of the sequences reacted with kinetics very similar to those expected for single copy DNA while about 10% annealed with kinetics which indicate that they are complementary to reiterated DNA sequences. From the ratio of the repetitive and single copy rate constants we calculate that the reiterated sequences are repeated an average of 20-25 times per haploid genome. This value is similar to the average reiteration frequency of middle repetitive DNA in *Achlya* (70; Hudspeth et al., 1978). The kinetics in defined media are shown in Figure 5B and are statistically indistinguishable from those shown in Figure 5A.

The Complexity and Abundance of Poly(A$^+$) RNA. The

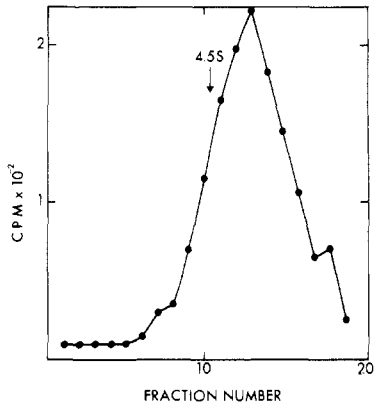

FIGURE 4: Size of cDNA. The size distribution of a representative cDNA preparation was determined by centrifugation on 5–20% alkaline sucrose gradients (Studier, 1965). Sedimentation was from left to right. All cDNA preparations used in this study were sized at least three times.

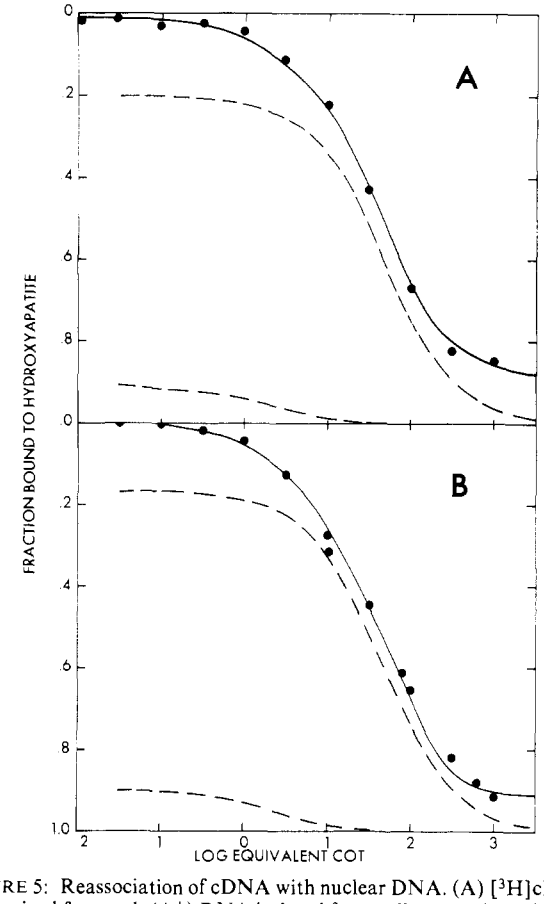

FIGURE 5: Reassociation of cDNA with nuclear DNA. (A) [^{3}H]cDNA synthesized from poly(A$^+$) RNA isolated from cells grown in undefined medium was annealed in a 100 000-fold excess of sheared nuclear DNA having an average length of 200 nucleotide pairs. Annealing was performed in 0.12 M NaP at 60 °C or 0.41 M NaP at 68 °C and the reactions were analyzed by hydroxylapatite chromatography. All C_0t values are expressed as equivalent C_0t (Britten et al., 1974). The solid line through the data points represents the best least-squares solution for two second-order kinetic components. Approximately 9% of the cDNA annealed with a K of 0.5 M^{-1} s^{-1} while 80% annealed with a K of 0.021 M^{-1} s^{-1}. (B) [^{3}H]-cDNA from cells grown in defined medium was annealed as in A. Approximately 11% of the cDNA reacted with a K of 0.5 M^{-1} s^{-1} and 83% reacted with a K of 0.023 M^{-1} s^{-1}.

complexity of the poly(A$^+$) RNA of cells cultured in either defined or undefined medium was determined by an analysis of the kinetics of hybridization of cDNA to an excess of template poly(A$^+$) RNA. Initial analysis showed that the kinetics

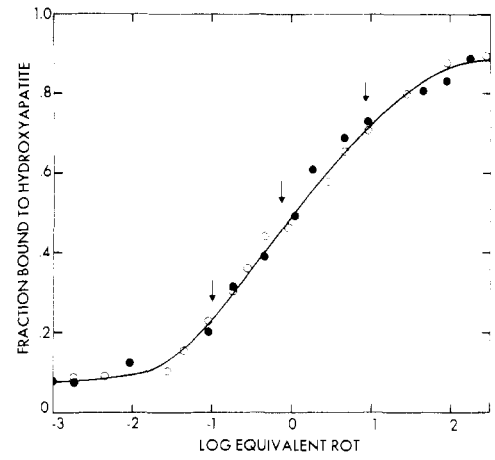

FIGURE 6: Hybridization of poly(A$^+$) RNA to cDNA. A 10- to 150-fold excess of poly(A$^+$) RNA was incubated with cDNA at 60 °C in 0.12 M NaP or at 68 °C in 0.41 M NaP. Reactions were terminated by freezing in dry ice–acetone and fractionated on 1-mL hydroxylapatite columns after the addition of 2 μg of sheared calf thymus DNA. All R_0t values are expressed as equivalent R_0t. The solid line through the data points represents the best least-squares solution for three first-order kinetic components. The arrows indicate the $R_0t_{1/2}$ of the three components. Poly(A$^+$) RNA and cDNA from cells cultured in undefined medium (●); poly(A$^+$) RNA and cDNA from cells cultured in defined medium (○). The data are summarized in Table III.

of the reactions could not be statistically differentiated and, therefore, the data are presented together in Figure 6. As shown in Figure 6, we found that hydroxylapatite fractionation of the hybridization reactions was a satisfactory method of analysis. In a control reaction using mouse globin mRNA (α + β chains) hybridized to 300 nucleotide globin cDNA, 21% of the cDNA bound to hydroxylapatite at the lowest R_0t measured (1×10^{-5}) and 85% bound at R_0t 1×10^{-2}. The $R_0t_{1/2}$ of the reaction was 1.1×10^{-3} (data not shown). Recently it has been shown that the rate of RNA-excess RNA·DNA hybridization is very close to that predicted from DNA·DNA reassociation (Galau et al., 1977). Our data are in agreement with this observation. Galau et al. (1977) found that the estimated pseudo-first-order reaction constant (pure) for the hybridization of ϕX174 RF DNA and (+) strand RNA was 200 M^{-1} s^{-1}. Since the size of the tracer in their experiments was the same as in ours (300 nucleotides) as were the conditions for hydroxylapatite fractionation, it is possible to predict directly the $R_0t_{1/2}$ of the globin mRNA·cDNA reaction. The genome size of ϕX174 is 5374 nucleotide pairs (Barrell et al., 1976) and the complexity of globin mRNA is 1200 nucleotides (Williamson et al., 1971). Thus, the predicted $R_0t_{1/2}$ (pure) of the globin mRNA·cDNA reaction is 8×10^{-4} [(0.69/200) × (1200/5374)]. We actually obtained a $R_0t_{1/2}$ of 1.1×10^{-3}. Because this value does not take into account the fraction of the RNA which is reactable, and due to slight uncertainties in size estimations, the predicted and actual rates are in good agreement.

The hybridization kinetics exhibited by *Achlya* cDNA reacted with poly(A$^+$) RNA are complex in that they occur over several log units of R_0t (Figure 6). A small fraction of the cDNA was retained by hydroxylapatite even at very low R_0t values (6% at R_0t 1×10^{-3} and 1×10^{-4}). At R_0t 250, approximately 90% of the cDNA had reacted. This value did not increase even at much higher R_0ts (89% at R_0t 1500). Least-squares analysis of the combined data showed that they are adequately fit by three first-order kinetic components (Hastie & Bishop, 1976). Using these results in relationship to the hybridization kinetics displayed by globin mRNA reacted with

TABLE III: Sequence Complexity of Poly(A$^+$) RNA Determined by Hybridization to cDNA.[a]

Transition	% cDNA hybridized	% in transition	Obsd $R_0t_{1/2}$	Corr $R_0t_{1/2}$	Complexity as nucleotides[b]	No. different sequences[c]	Copies per nucleus[d] Defined	Copies per nucleus[d] Undefined
1	25	32	0.098	0.03	3.3×10^4	29	1000	440
2	25	32	0.74	0.23	2.5×10^5	220	140	58
3	28	36	8.62	3.1	3.4×10^6	3000	11	5

[a] The data are obtained from Figure 6. [b] Calculated from the $R_0t_{1/2}$ of mouse globin mRNA hybridized to cDNA under identical conditions. This was 1.1×10^{-3} mol s L^{-1} and was considered to represent 1200 nucleotides (Williamson et al., 1971). [c] A number average mRNA sequence was considered to be 1150 nucleotides. [d] Calculated using the following assumptions: the diploid genome size of *Achlya* is 4.4×10^{10} daltons. The mass ratio of DNA to RNA is approximately 1:35 in undefined medium and 1:15 in defined medium (Griffin et al., 1974). Poly(A$^+$) RNA represents 2.3% of the total cellular RNA (Table I). Therefore, there are about 94 000 and 40 000 average sized poly(A$^+$) RNA molecules per nucleus in undefined and defined medium, respectively.

TABLE IV: Sequence Complexity of Poly(A$^+$) RNA Determined by Hybridization to Single Copy DNA.

Culture condition	% of [^{3}H]DNA resistant to S-1 nuclease: Total[a]	DNA·DNA[b]	RNA·DNA[c]	Corr saturation value[d]	Complexity[e]: Nucleotides	Sequences
Undefined medium	5.68 ± 0.21	0.15	5.53	6.66	3.6×10^6	3100
Defined medium	5.96 ± 0.32	0.15	5.81	7.00	3.8×10^6	3300
Undefined medium + defined medium	6.08 ± 0.26	0.15	5.93	7.14	3.9×10^6	3400

[a] Values are the average of four determinations ± 1 SD. Incubations were to R_0t 750. [b] DNA/DNA values were determined from parallel reactions containing only single copy DNA. [c] Total less DNA·DNA. [d] Corrected for the fraction of the single copy DNA which reacted with a 100 000-fold excess of nuclear DNA by C_0t 1400 (0.83). [e] Calculations were made assuming asymmetric transcription. The complexity of *Achlya ambisexualis* single copy DNA was considered to be 2.7×10^7 nucleotide pairs (Timberlake et al., 1977).

its cDNA, it was possible to estimate the complexity of each abundance class as shown in Table III. These data suggest that *Achlya* cultured in either medium expresses approximately 3200 average size poly(A$^+$) RNA sequences distributed into three frequency components.

The estimate of sequence complexity derived from poly(A$^+$) RNA·cDNA hybridization kinetics is somewhat uncertain because of the assumptions involved and the difficulty in assessing the rate constants of the individual components with great accuracy. Small differences in sequence complexity would not be reliably detected using this approach. We therefore also estimated the complexity by hybridization of an excess of poly(A$^+$) RNA to single copy [^{3}H]DNA. In this approach, the fraction of the single copy DNA hybridized at saturation provides a direct estimate of the complexity of the RNA population transcribed from unique sequences (Galau et al., 1974). Single copy DNA was isolated and labeled in vitro as previously described (Timberlake et al., 1977) and hybridized to an excess of poly(A$^+$) RNA to R_0t 750. The hybrid content of the reactions was then assayed by treatment with S1 nuclease. In the experiment shown in Table IV, the poly(A$^+$) RNA derived from cells cultured in defined and undefined media were used individually as well as mixed together. We found that 6.7% and 7.0% of the single copy DNA were complementary to the RNA. Assuming asymmetric transcription, this equates to 3100 and 3300 average sized sequences. These values are somewhat higher than previous estimates (Timberlake et al., 1977) but are in good agreement with those derived from cDNA hybridization (Table III). The reason for the difference between these and earlier results is not presently understood. When equal amounts of the two types of RNA were mixed together and reacted with single copy DNA, 7.1% of the DNA hybridized equating to 3400 number average sequences. While the differences observed in the three experimental treatments may reflect actual differences in complexity, we could not reject the null hypothesis that the means are the same by one-way analysis of variance (F = 2.46).

Discussion

The evidence presented in this paper suggests that the majority of the poly(A$^+$) RNA of *Achlya* is mRNA active in translation and that most of the mRNA, both in terms of mass and complexity, contains sequences of poly(A). A small population of mRNA lacking poly(A), for example, histone mRNA, would not have been detected. The cellular content of poly(A$^+$) RNA (2.3%) and the length of the poly(A) isostichs (33 nucleotides) are similar to those reported for other fungi (Johnson et al., 1977; Groner et al., 1974; McLaughlin et al., 1973). In agreement with our previous results (Timberlake et al., 1977) we found that the number average size of the mRNA, isolated either by poly(U)-agarose or oligo(dT)-cellulose chromatography, is 1100–1200 nucleotides, and that 10% of the sequences are transcribed from reiterated DNA while the remainder are transcribed from single copy DNA.

The kinetics of hybridization of *Achlya* poly(A$^+$) RNA to cDNA are qualitatively similar to those observed with poly(A$^+$) mRNA derived from other eucaryotic cells (Bishop et al., 1974; Axel et al., 1976; Levy & Dixon, 1977; Monahan et al., 1976; Levy et al., 1976; Hereford & Rosbash, 1977a; also, see Lewin, 1975b). Hybridization proceeds over several log units of R_0t, indicating that the poly(A$^+$) RNA sequences are present at varying cellular concentrations. These data are accurately fit using three kinetic components. Thus, in *Achlya*, the mRNA is distributed into three abundance classes. Our data are strikingly similar to those recently obtained by Hereford & Rosbash (1977a) for yeast cells. We find that over 90% of the poly(A$^+$) RNA sequence complexity comprises 36% of the mass. The remainder of the mass contains only 250 different sequences. Yeast cells are also similar to *Achlya* in that the complexity of the polysomal poly(A$^+$) RNA is essentially the same as whole cellular poly(A$^+$) RNA (Hereford &

Rosbash, 1977a; Timberlake et al., 1977). The failure to identify putative nuclear precursor molecules which are much more complex than mRNA in two distantly related fungi suggests that this group of organisms may, in general, lack metazoan-like hnRNA.

We were unable to distinguish any statistically significant difference between the complexity or abundance of poly(A^+) RNA derived from cells cultured in defined or enriched media. It is interesting to consider this result in relationship to the concept of auxotrophic genes in *E. coli*. As discussed by Bukhari (1976) it can be estimated that there are in the range of 250 genes (of 3000–5000), mutations in which would induce auxotrophy in *E. coli*. If a similar number of genes are required for the growth of *Achlya* in basal medium as compared with enriched medium, and, if these are transcribed and accumulated during growth in the former but not in the latter, then we would have expected to observe only about an 8% difference in sequence complexity [(250/3200) × 100]. This degree of difference would be difficult to detect with either of the experimental procedures used in this study. Nevertheless, it is tempting to speculate that the differences observed in the data presented in Table IV may actually reflect changes in mRNA sequence complexity.

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