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FRACTIONATION OF mRNAs

FROM DICTYOSTELIUM DISCOIDEUM

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SUMMARY

We describe methods for fractionating mRNAs from Dictyostelium discoideum according to size, base composition, secondary structure and poly(A) content. RPC-5 chromatography appears to give the best resolution of a mixed population of mRNAs. Fractionation on RPC-5 columns does not correlate with mRNA size and may be useful in effecting a multi-dimensional fractionation of mRNA. By fractionating mRNAs on poly(U) Sepharose columns, we also show methods for separating mitochondrial mRNAs from the remaining poly(A)-containing RNAs and for isolating newly synthesized mRNAs. Furthermore, we show that not all mRNAs are metabolized in the same fashion as the "average" mRNA. These results serve to emphasize the importance of studying the transcription and organization of specific genes, rather than the behavior of "average" genes derived from studying mixed populations of mRNAs.

INTRODUCTION

The ability to clone eukaryotic DNAs in bacterial cells[1,2] should eventually make possible a detailed analysis of the transcription and organization of specific genes in higher organisms. In order to realize this goal, procedures for screening for recombinant DNAs have been recently developed.[3,4] These procedures are presently limited to a very small number of genes, however, because the specific homologous RNA or DNA sequences present in a probe must represent a significant percentage of the total nucleic acid in the probe. For example, if poly(A)-containing RNA from Dictyostelium discoideum is used as a probe, approximately 10 sequences of a possible 10,000 are effective screening agents. While study of DNA sequences homologous with these abundant mRNAs will undoubtedly provide a great deal of valuable information about mRNA metabolism in Dictyostelium, most mRNAs, including most of those which are presumed to be developmentally regulated, are not sufficiently abundant and require further fractionation and purification for gene isolation purposes.

Unlike fractionation procedures for proteins and tRNAs, both of which have

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reached a high level of sophistication, fractionation procedures for mRNAs are quite primitive. For the past few years, therefore, we have been developing methods for fractionating mRNAs. Since Dictyostelium cells may contain 10,000 or more mRNAs, our approach has been to develop methods which fractionate mRNAs on a multi-dimensional basis. The characteristics which we have most successfully exploited are size, base composition and secondary structure. We have also developed procedures for fractionating mRNAs according to poly(A) content. Using these procedures, we have been able to separate mitochondrial mRNAs from the remaining poly(A)-containing RNAs and to isolate newly synthesized mRNAs. Furthermore, we have shown that not all mRNAs are metabolized in the same fashion as the "average" mRNA. These results serve to emphasize the importance of studying the transcription and organization of specific genes, rather than the behavior of "average" genes derived from studying mixed populations of mRNAs.

RESULTS

Electrophoresis of RNA on polyacrylamide gels containing 99% formamide: The best results which we have been able to obtain for fractionation of mRNAs according to size have been by electrophoresis of RNA on polyacrylamide gels containing 99% formamide.[5] Figure 1 illustrates the extent of fractionation we have been able to obtain. In this experiment 100-200μg of mRNA isolated from vegetative cells was fractionated on a formamide gel, portions of the gel were excised and the RNA from each portion was eluted and repurified by oligo(dT) cellulose chromatography. Aliquots of 0.2-0.5μg of purified RNA were then translated in wheat germ extracts containing ^{35}S-methionine. The total in vitro product was layered onto a polyacrylamide slab gel containing 0.1% SDS and subjected to electrophoresis and autoradiography. The numbers above each slot are the mobilities of a given RNA fraction relative to that of 17S rRNA. The slots beneath these numbers correspond to the in vitro products whose synthesis was programmed by these RNAs.

Analysis of the translation products clearly indicates that a high degree of fractionation has been achieved. For example, the peak of translation activity for actin migrates about 20% faster than 17S rRNA. Furthermore, the fractions richest in actin translation activity are at least 50% pure with respect to translation activity.

It should be pointed out that some bands which appear on the gels are present in all of the fractions and are either coded for by mRNAs which are not fractionated by the gel system or, more likely, by endogenous mRNAs present in the wheat germ extracts. Nevertheless, it is clear that translation activities can be fractionated extremely well on this gel system. Two disadvantages of this system are its limited capacity and the difficulty of extracting intact mRNA in good yield from individual gel fractions. While we are continuing to pursue this system because of its excellent resolving abilities, we are presently

exploring other size fractionation methods such as continuous elution from high capacity preparative gels.[6]

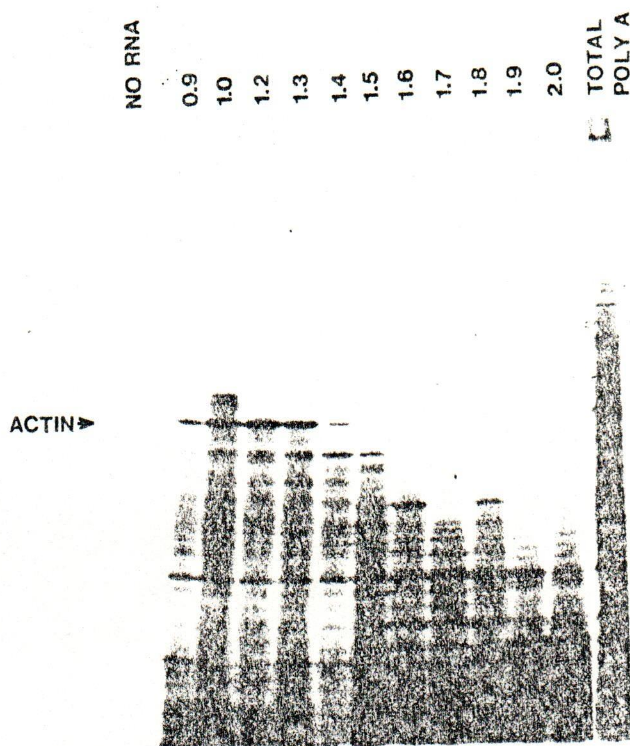

Fig. 1. Translation products of RNA fractionated on polyacrylamide gels containing 99% formamide. Whole cell RNA from vegetative cells was electrophoresed on a 3.75% polyacrylamide gel containing 99% formamide. Successive 1 cm fractions of the gel were excised, RNA was eluted and repurified by oligo(dT) cellulose chromatography and subsequently translated in cell-free extracts prepared from wheat germ.

RPC-5 Chromatography as an mRNA fractionation method: Reverse phase partition chromatography, particularly RPC-5 chromatography, has been used extensively for fractionation of tRNAs and has been shown to give resolution far superior to other existing methods.[8,9] In reverse phase chromatography the RNA is partitioned between a stationary hydrophobic amine and a mobile aqueous phase. As salt concentrations are increased, some RNAs become more soluble in the aqueous phase and elute from the resin. Because of hydrophobic interactions between the RNA and the resin, reverse phase supports are extremely sensitive to differences in base composition and fractionation does not correlate with RNA size. Furthermore, it has been shown that conditions which affect secondary structure or net charge on individual bases can alter the order of elution of individual tRNAs[10] (and presumably other RNAs as well).

Figure 2 shows the results when this technology is applied to Dictyostelium mRNAs. For the run illustrated, poly(U) Sepharose-purified total vegetative RNA was applied to an RPC-5 column and eluted with a salt gradient. At least 50 peaks are apparent in Figure 2. If these peaks represent discrete mRNA fractions,

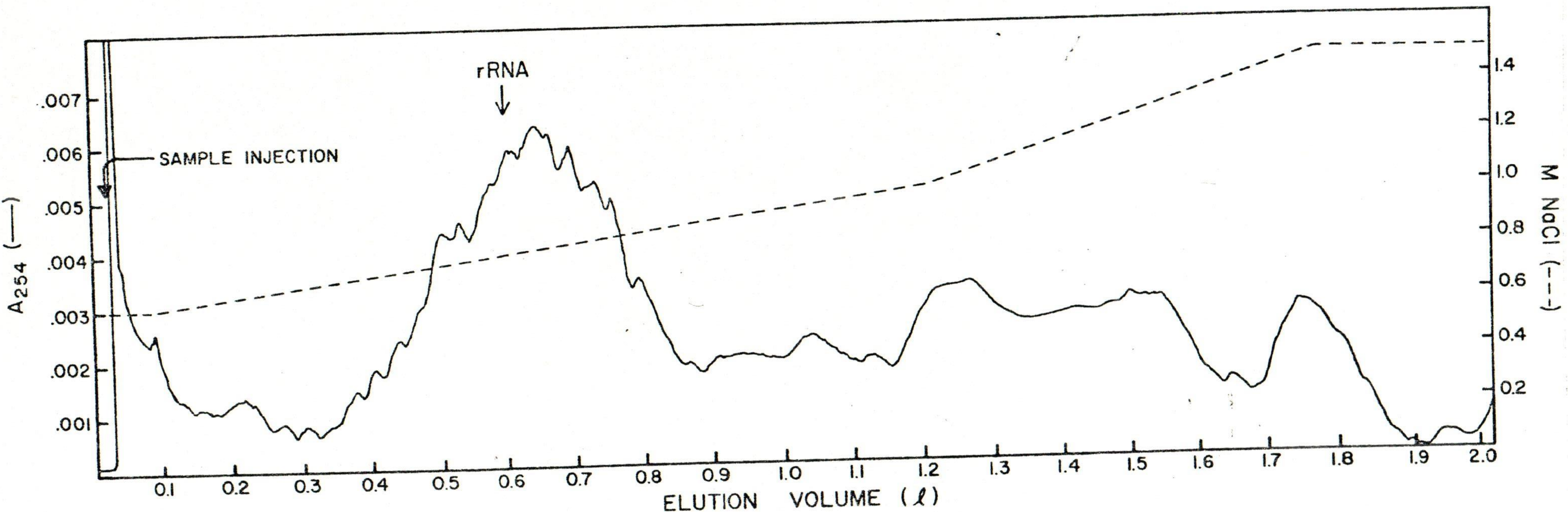

Fig. 2. RPC-5 chromatographic profile of poly(U) Sepharose-purified vegetative RNA (3 A_{260} units) from *Dictyostelium*. Chromatographic conditions were as previously described.[7] The column was monitored continuously at A_{254}. The region marked rRNA is estimated from chromatographic runs of material which did not bind to poly(U) Sepharose.

this is the best resolution of a mixed population of mRNAs which has been obtained by any fractionation method. This is probably not the best resolution obtainable on these columns, however. For example, every time we have made the salt gradient more shallow we have increased the resolution. Furthermore, alteration of elution conditions so as to affect secondary structure or net change on individual bases alters the elution profile. In theory, therefore, it may be possible to effect a multi-dimensional fractionation on RPC-5 columns alone.

Figure 3 illustrates <u>in vitro</u> translation products obtained from some fractions of an RPC-5 column which was run under conditions to the one illustrated in Figure 2. In this case, however, the column was run with total unfractionated vegetative RNA and also with a steeper salt gradient. Unlike some of the trans-

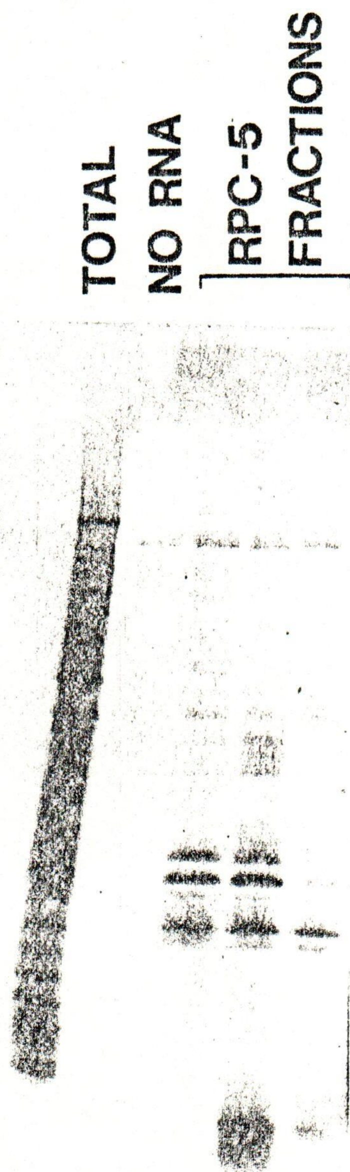

Fig. 3. Translation products of vegetative whole cell RNA fractionated by RPC-5 chromatography. Samples were translated in micrococcal nuclease-treated wheat germ extracts,[11] displayed on 6-15% linear gradient polyacrylamide gels and analyzed by fluorography. The RPC-5 translation products are from fractions analagous to the peak eluting between 200 and 250 ml, illustrated in Figure 2.

lation products illustrated in Figure 1, the bands visible in these fractions are probably not wheat germ polypeptides because, for the translation of these fractions, the wheat germ extract was treated with micrococcal nuclease which destroys a considerable amount of endogenous mRNA activity.[11] In the slot from which no RNA was added to the wheat germ extract, no bands are visible. In addition, none of the bands of translation products from various fractions co-migrates with wheat germ proteins visible in extracts not treated with micrococcal nuclease. The translation products illustrated are from fractions analogous to the peak eluting between 200 and 250 ml in Figure 2. Two points can be made from examination of these translation products: (1) These fractions contain translation activities for a very small number of polypeptides and hence represent a considerable degree of purification and (2) the pattern is unlike any pattern obtained by other fractionation methods which we have tried. A comparison of Figures 1 and 3 clearly indicates that fractionation of translation activities on RPC-5 columns does not correlate with mRNA size.

Fractionation of mRNA by thermal elution from poly(U) Sepharose: In order to fractionate mRNA according to its poly(A) content, we developed the following

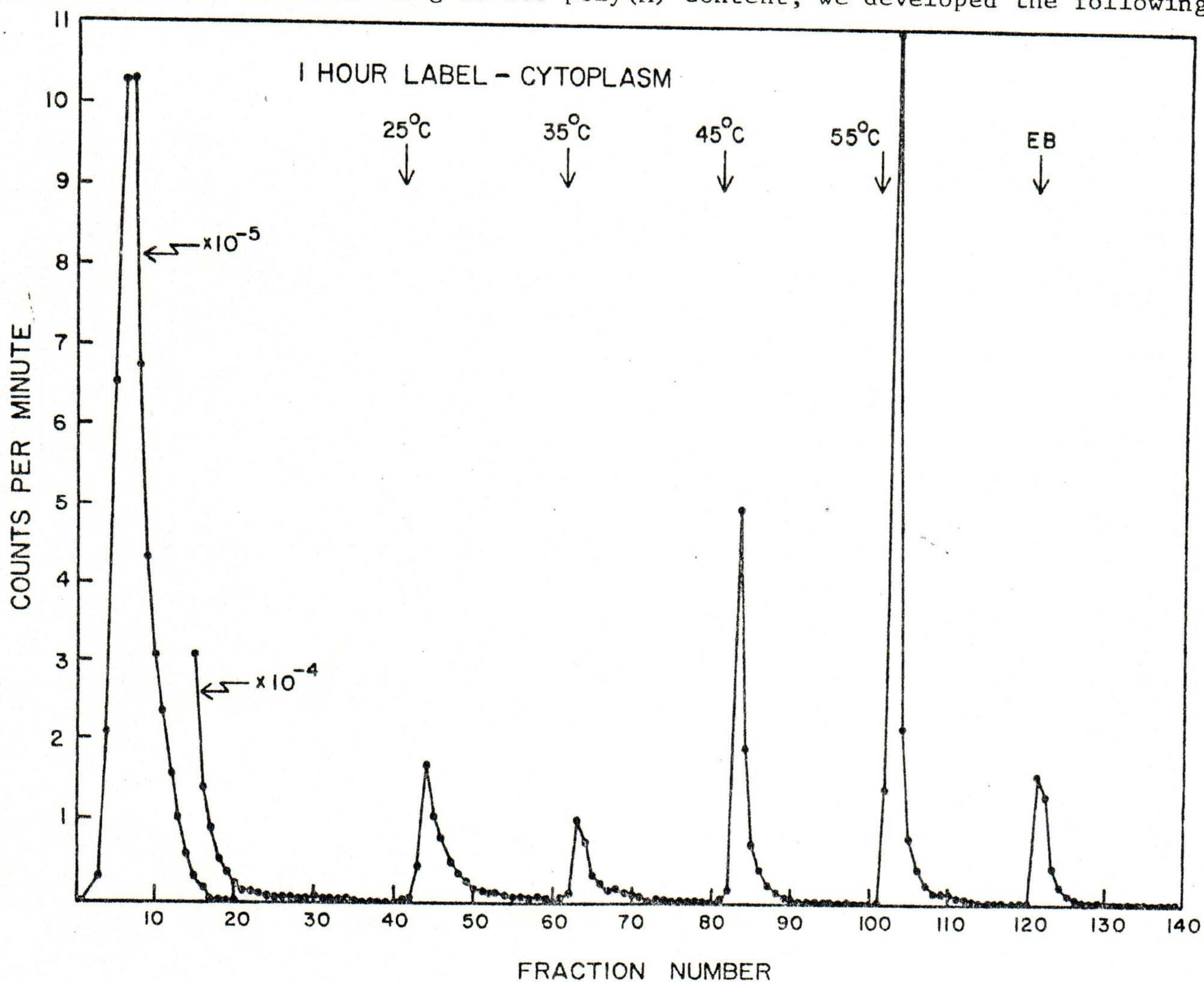

Fig. 4. Poly(U) Sepharose thermal elution profile of vegetative cytoplasmic RNA labeled for 1 hour with $^{32}PO_4$.

method. Using water jacketed columns, we bound mRNA to poly(U) Sepharose at 25°C in the presence of 0.7M NaCl and 25% formamide. To elute the bound mRNA, the salt concentration was reduced to 0.1M NaCl. We then washed the column with the same buffer at 25°C, 35°C, 45°C and 55°C. In a final wash at 55°C, we raised the formamide concentration to 90%. Figure 4 shows an elution profile from such a column which was run with vegetative cytoplasmic RNA labeled for 1 h with $^{32}PO_4$. Analysis of poly(A) tracts on polyacrylamide gels has shown that the mean poly(A) size of the different thermal eluates, in order of elution, is 25, 35, 90, 100 and 110 nucleotides, respectively.

Translation products of vegetative mRNAs containing different size poly(A) tracts: Figure 5 shows *in vitro* protein synthetic patterns of RNAs isolated from various poly(U) Sepharose thermal eluates. Although this analysis has been initially made on a one-dimensional gel system, it can readily be seen that most

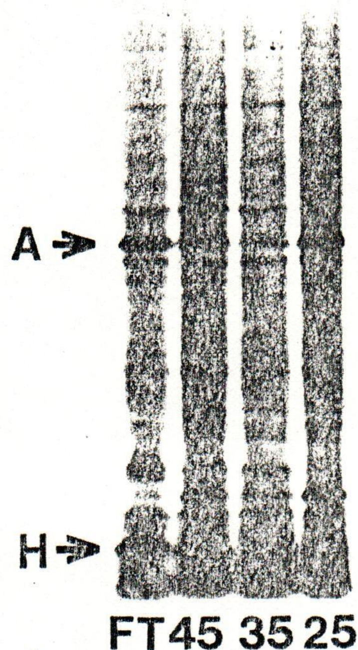

Fig. 5. Translation products of vegetative whole cell RNA fractionated by thermal elution from poly(U) Sepharose. Individual eluates were translated in wheat germ extracts, displayed on 6-15% linear gradient polyacrylamide gels and analyzed by fluorography. The actin and histone bands are labeled "A" and "H" respectively.

translatable mRNAs (approximately 90%) are distributed throughout the various eluates. We have, however, been able to detect at least 10 reproducible differences between the various eluates. For example, although it has been previously reported that poly(A) deficient mRNA is representative of all cellular mRNAs,[12] examination of the translation products of the poly(U)-Sepharose flow through, indicates that translation activities for two proteins, actin and histone, predominate. In addition, translation activities for two proteins also predominate in the 25°C eluate, actin and an unidentified protein of about 20,000 molecular weight. Furthermore, although it cannot be clearly seen from Figure 5, we have been able to detect translation activities in the 25°C and 35°C eluates which are absent from the 45°C and 55°C eluates. Preliminary experiments with inhibitors of RNA synthesis suggest that these RNAs are much less stable than the majority of cellular mRNAs. Since translation activities for these less stable mRNAs are not apparent in higher thermal eluates, they probably represent a second class of poly(A)-containing RNA which is synthesized and metabolized in a unique fashion.

<u>Separation of mitochondrial poly(A)-containing RNA from cytoplasmic poly(A)-containing RNA</u>: Figure 6 illustrates thermal elution profiles of RNA isolated

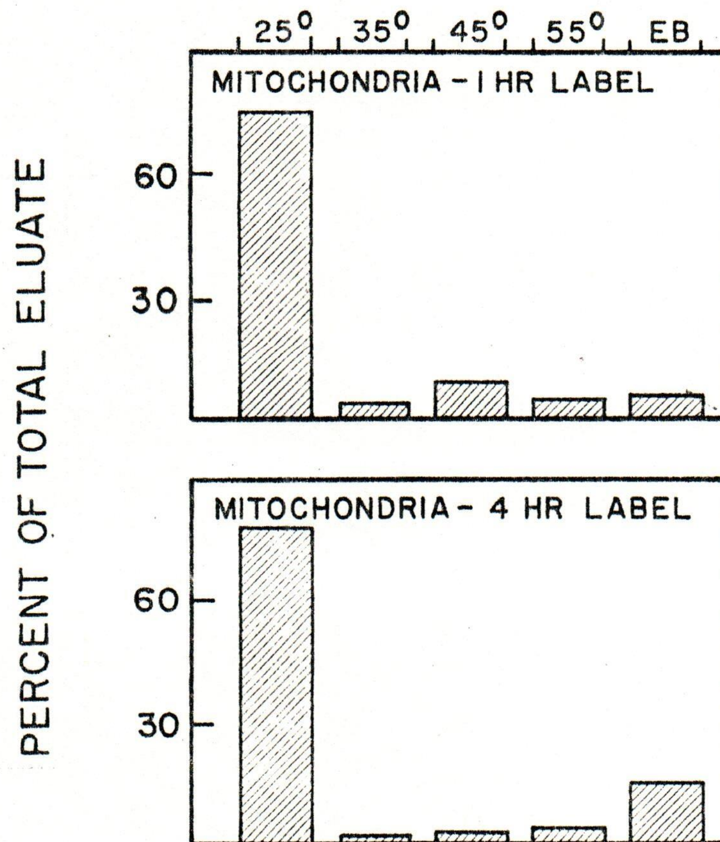

Fig. 6. Poly(U) Sepharose thermal elution profile of RNA isolated from mitochondria after labeling vegetative cells with $^{32}PO_4$ for 1 and 4 hours, respectively.

from mitochondria after labeling cells with $^{32}PO_4$ for 1 and 4 hours, respectively. In both cases, most of the RNA elutes from the column at 25°C. The small amount of material which elutes at higher temperatures probably represents contamination of the mitochondrial preparations with cytoplasmic RNA.

Isolation of newly synthesized mRNA: Figure 7 compares the elution profiles of three RNA samples isolated from vegetative cells. Figures 7A and 7B are profiles of cytoplasmic RNA extracted from cells labeled with $^{32}PO_4$ for 1 and 4 hours, respectively. Figure 7C is the "steady state" elution profile of whole

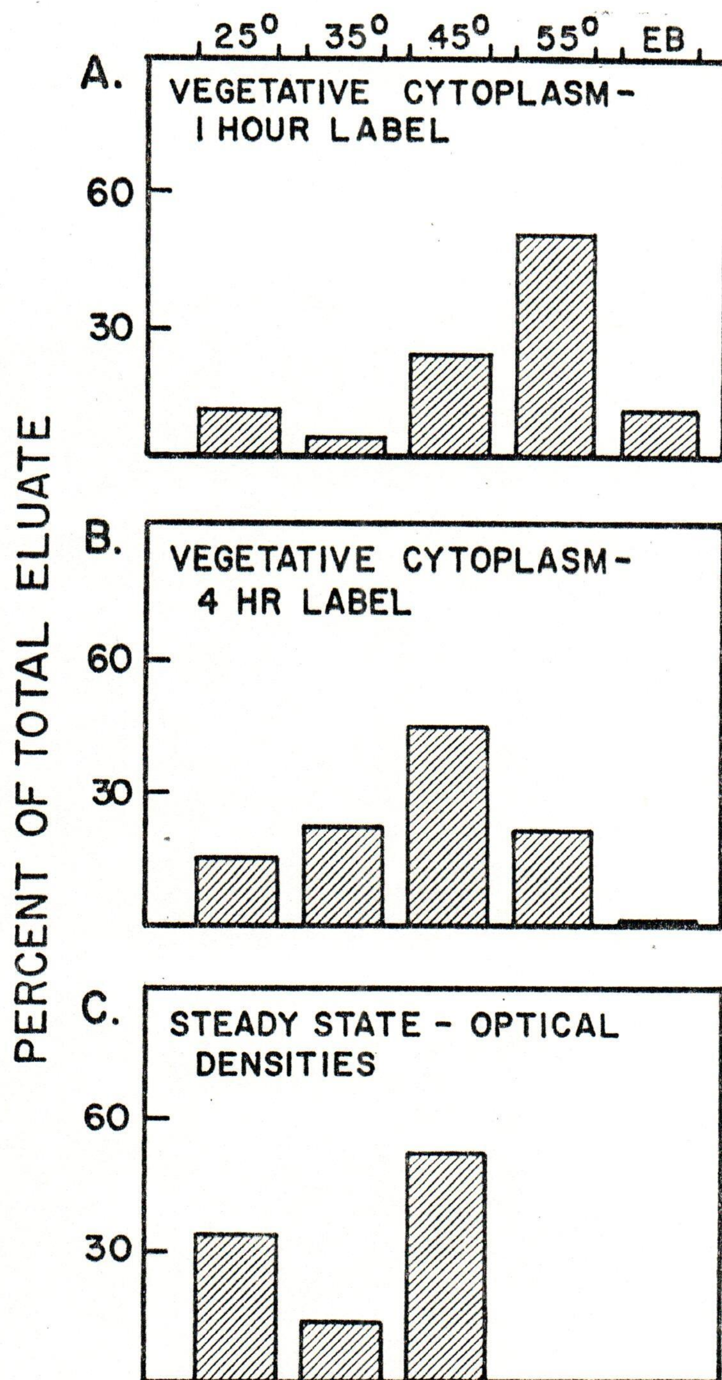

Fig. 7. Poly(U) Sepharose thermal elution profiles of vegetative RNA: (A) cytoplasmic RNA labeled with $^{32}PO_4$ for 1 hour, (B) cytoplasmic RNA labeled with $^{32}PO_4$ for 4 hours and (C) whole cell RNA monitored at A_{254}.

cell RNA, obtained by monitoring the optical densities of the various fractions. As shown, newly synthesized poly(A)-containing RNA contains predominantly long poly(A) tracts. By 4 hours of labeling, however, the size of the poly(A) tracts has shortened considerably and has begun to approach a steady state size distribution. We have taken advantage of this observation in order to isolate newly synthesized mRNA from various developmental stages and have been able to show (Palatnik, Wilkins and Jacobson, in preparation) that significant changes in transcription occur within the first 5 minutes of development.

DISCUSSION

We have shown that we can fractionate mRNA by size on polyacrylamide gels containing formamide and by criteria which do not correlate with size on RPC-5 columns. RPC-5 columns appear to have great potential as vehicles for multi-dimensional fractionation of mRNAs. In addition to their apparent unequaled resolution by conventional methods, they offer the advantages of high capacity (2mg RNA/g of resin), inexpensive cost (except for the initial high pressure liquid chromatography set-up) and short run time. If the peaks which we see represent discrete mRNA fractions, we believe that use of RPC-5 chromatography should rapidly yield at least 100-fold purification of individual mRNA species, enough for gene isolation of most developmentally regulated mRNAs in *Dictyostelium.*

The ability to fractionate mRNAs according to their poly(A) content has enabled us to gain insight into several important aspects of poly(A) metabolism in *Dictyostelium discoideum.*[13] (Palatnik, Mabie, Wilkins and Jacobson, in preparation). Using this technique, we have also shown that most of the newly synthesized mRNA in vegetative cells contains long poly(A) tracts which shorten with age. This, coupled with our previous work which showed that nuclear RNA contains longer poly(A) stretches than cytoplasmic RNA,[14] enables us to isolate newly synthesized RNA from various developmental stages without having to resort to procedures involving *in vivo* labeling of the RNA with Hg^{++}, which could potentially lead to artifacts.

We have also shown that we can separate $^{32}PO_4$-labeled mitochondrial RNA which binds to poly(U) Sepharose, from cytoplasmic poly(A)-containing RNA. This is significant in that we have been unable to isolate totally undegraded preparations of polysomal RNA from *Dictyostelium* (Palatnik, Wilkins and Jacobson, unpublished observations). On the other hand, we have been able to isolate totally undegraded whole cell RNA. Mitochondrial mRNA in these whole cell RNA preparations, however, is a contaminant which could interfere with nuclear gene isolation. Using thermal elution from poly(U) Sepharose, we can circumvent this problem.

Finally, the behavior of vegetative mRNAs on poly(U) Sepharose shows that there are at least two classes of poly(A)-containing RNA in *Dictyostelium.*

This serves to emphasize the importance of studying the transcription and organization of specific genes.

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