POLY A METABOLISM IN SEA URCHIN EMBRYOS*

Roy S. Wu and Fred H. Wilt
Department of Zoology
University of California
Berkeley, California 94720

Received August 6, 1973

SUMMARY

Three different methods for the detection and isolation of RNA that contains poly A sequences are compared. All three methods selectively bind poly A containing sequences. However only the poly dT cellulose technique gave reproducible results and did not modify the binding characteristics of the RNA initially bound to the poly dT cellulose. The proportion of the total cellular RNA that contains poly A is about the same in the blastula and gastrula stages and is lower at cleavage. The kinetics of accumulation of RNA containing poly A for the blastula stage indicates extensive turnover of poly A containing material. RNA containing poly A is characterized by its heterogeneous sedimentation. Some of the RNA containing poly A is as large as 0.5 x 10^6 Daltons.

INTRODUCTION

Recent experiments have demonstrated the presence in eucaryotic organisms of polyriboadenylic acid (poly A) sequences which are between 150-200 nucleotides in length and are covalently linked to the 3' termini of heterogeneous nuclear RNA (HnRNA) and messenger RNA (mRNA) (1-7). The RNA that contains poly A can be isolated by a variety of methods, including: binding to millipore filters in 0.5 M KCl (2, 8); hybridization of poly A sequences to poly dT oligomers covalently bound to cellulose (3, 6, 7); binding of poly A sequences to poly U immobilized on glass fiber filters, cellulose powder (1, 9); or sepharose beads (11); hybridization of unlabeled RNA with radioactive poly U (12) and binding of poly A to cellulose (untreated) in high salt solutions or in low salt solutions at pH values of 5.5 or less (13). All the methods appear to have good specificity for the selective binding of poly A sequences. Since the chemical bases of separation on glass fiber filters and cellulose powder of RNA contain-

*This investigation was supported in part by an N.I.H. postdoctoral fellowship (GM 52558 to R.S.W.) and grant GM 13882.

ing poly A from the cellular RNA not containing poly A are not completely understood, it is important to test whether these methods will give differential binding when whole cell RNA from different developmental stages is used. The RNA obtained from the different embryonic stages may have different secondary or tertiary structure, which may influence the relative retention capacities of these methods.

We will discuss experiments that compare the binding specificities of three methods, poly U glass fiber filters, poly U cellulose and poly dT cellulose, using RNA extracted from cleavage, mesenchyme blastula and mid-gastrula stages of sea urchin embryos and will utilize the results to characterize RNA that contains poly A during sea urchin embryogeny.

MATERIALS AND METHODS

Embryos of Lytechinus pictus were cultured at 15°C in millipore filtered sea water containing 50 μg/ml of streptomycin and 2-^{14}C-thymidine (55 mc/mmole). Embryos were labeled with ^{3}H-adenosine (18 c/mmole) at 8 hr (cleavage), 16 hr (mesenchyme blastula) and 36 hr (mid-gastrula) post fertilization. The exact labeling conditions of these cultures are given in the Figure and Table Legends. RNA from the embryos was extracted with hot phenol-chloroform (14) and further purified by digestion with DNAse and pronase (12, 15, 16). The purified RNA was then analyzed by SDS-sucrose gradient sedimentation (14) or DMSO-sucrose gradient sedimentation (17).

RNA containing poly A was assayed on the basis of binding to poly U glass fiber filters or poly U cellulose (9) and poly dT cellulose (Collaborative Research Inc., Waltham, Mass.). The conditions for chromatography are described in the Figure and Table legends. 8-^{14}C-poly A (0.36 mc/mmole), poly U and poly A were purchased from Miles.

RESULTS

The binding of poly A or RNA that contains poly A to poly U glass fiber filters, poly U cellulose and poly dT cellulose.

Table I

Retention of ^{14}C-poly A by poly U or poly dT

		CPM Input	CPM bound	% bound
poly U filter		548	545	100
poly U cellulose		2,600	2,680	100
poly dT cellulose	(0.5 M KCl)	10,000	10,000	100
"	(0.1 M NaCl)	10,000	7,956	80
"	(0.5 M NaCl)	10,000	10,015	100

Various amounts of ^{14}C-poly A (47.6 CPM/μg) were bound to poly U filters, poly U cellulose and poly dT cellulose. Conditions for binding poly A to poly U filters and poly U cellulose were the same as those reported by Sheldon et al. (9) except buffers contained 0.05% SDS. Conditions used for poly dT cellulose chromatography were the same as those reported by Aviv and Leder (18) except the type and the concentration of the cation was varied and all buffers contained 0.05% SDS.

The three methods examined were tested for their ability to bind radioactive poly A. Table I indicates that all three methods will bind authentic poly A. Three different conditions were used to bind poly A to poly dT cellulose, the high KCl method reported by Aviv and Leder (18), a low NaCl method and a high NaCl method. It is clear that not all the radioactive poly A was bound to poly dT cellulose at 0.1 M NaCl, a result in agreement with those showing radioactive poly A is eluted at 0.1 M KCl (12). Thus, maximum retention of RNA containing poly A on poly dT cellulose dictates the use of 0.5 M salt but either Na^+ or K^+ may be used and the presence of 0.05% SDS in the binding buffer does not seem to affect the retention capacities of these methods.

Table II shows the fractionation of RNA from different stages of development using the three methods. The different techniques gave comparable binding regardless of the source of the RNA. Although the retention of RNA by poly U filters seems to agree with that obtained by poly U or poly dT cellulose, a

Table II

Retention of labeled sea urchin RNA by poly U and poly dT

Source of RNA	poly U filter* CPM Input	poly U filter* CPM bound	poly U filter* % bound	poly U cellulose CPM Input	poly U cellulose CPM bound	poly U cellulose % bound	poly dT cellulose (0.5 M KCl or NaCl) CPM Input	poly dT cellulose (0.5 M KCl or NaCl) CPM bound	poly dT cellulose (0.5 M KCl or NaCl) % bound
Cleavage	---	---	---	41,484	8,997	21.6%	29,998	6,412	21.3%
blastula	26,781	8,766	32.8%	27,114	8,963	33.0%	17,701	5,741	32.5%
mid-gastrula	---	---	---	181,747	30,250	16.5%	25,238	4,360	17.2%

*Number for percent bound varied between duplicate samples; chosen to show the number that agreed with those obtained by poly U and poly dT cellulose.

RNA from cleavage, mesenchyme blastula and mid-gastrula were isolated as described in Methods and assayed for RNA that contains poly A on poly U glass fiber filters, poly U cellulose and poly dT cellulose as described in the legend of Table I. The respective RNA's represented different fractions of SDS-sucrose gradients (Figure 1); cleavage RNA (fractions 0-4, Fig. 1A); blastula RNA (fractions 0-21, Fig. 1B); mid-gastrula (fractions 0-33, Fig. 1C). Labeling conditions are described in the legend of Figure 1.

word of caution is in order. The reproducibility of the poly U filter assay was very poor. The numbers on occasions can vary by an order of magnitude between duplicate samples. The fluctuation in the poly U filter assay may be due to inability to control the rates of flow through the filter. The poly U or dT cellulose columns consistently gave reproducible results.

The use of poly U or poly dT cellulose to isolate and to identify RNA that contains poly A may be used to isolate mRNA. It is therefore important to demonstrate that the binding techniques have not damaged or modified the RNA in any manner. One approach to monitor this issue is to determine if a fraction that is retained by poly U or poly dT cellulose is also bound when subjected to a second chromatographic fractionation on poly U or poly dT cellulose. Table III shows the results of such an experiment. Although a given RNA will bind equally well to poly U or poly dT cellulose on the first passage (line 1 and 8; Table III) up to 50% of the material that binds originally to poly U

cellulose will not bind upon a second chromatography to either poly U or poly dT cellulose (line 4, 5). Concentration of RNA by ethanol precipitation and resuspension of the RNA in fresh binding buffer does not eliminate this problem (line 6, 7). On the other hand all the RNA that binds to poly dT in a first chromatographic fractionation will bind to either poly U or poly dT upon rechromatography (line 11, 12). A possible explanation of this phenomenon is that poly U cellulose is unstable and poly U tends to be eluted along with the RNA containing poly A. Thus poly dT cellulose is the method of choice for preparing RNA containing poly A to be used for subsequent characterization.

RNA containing poly A in sea urchin embryos

The amount of RNA containing poly A was determined at 3 different embryonic stages. The embryos of each stage were labeled with ^{3}H-adenosine for 0.5, 2 and 4 hr and the total cellular RNA was extracted and centrifuged on SDS-sucrose gradients. Figure 1 shows the sucrose gradient patterns of labeled RNA's after a 4 hr labeling period. Fractions were pooled as indicated and assayed for their content of RNA containing poly A by chromatography on poly U cellulose. The proportion of the total cellular RNA that contains poly A is about the same in the blastula and gastrula stages, and is lower at cleavage, probably due to the large contribution to radioactivity by histone mRNA, 4S RNA and small pieces of DNA that were not eliminated by DNAse treatment (Fig. 1A). This suggestion is based on the facts that no ^{14}C-thymidine containing material bound to poly U cellulose and that 4S RNA and histone mRNA also do not bind to poly U cellulose (10, 19, unpublished results). The RNA containing poly A is characterized by its polydispersity at all 3 stages at all labeling periods (not shown). The bound fraction centering on 28S rRNA shows the highest percentage of binding to the cellulose columns (i.e. 38% for the blastula stage, Fig. 1B). However, if one expresses the bound material as percent of total RNA that contains poly A (numbers in parenthesis) the fraction larger than 28S rRNA has the largest amount of RNA containing poly A. Approximately 80% of the RNA containing poly A is located in the fractions containing RNA larger than 7S. The blastula

Table III

Rechromatography of Fractions from poly U and poly dT cellulose

	Cellulose polynuleotide	% bound $\frac{\text{CPM bound}}{\text{CPM Input}}$
Original chromatography of RNA from cleavage embryos	poly U	21.6%, 31%
rechromatography of front peak	poly U	<1%, <1%
rechromatography of front peak	poly dT	<1%, <1%
rechromatography of back peak	poly dU	50%, 50%
rechromatography of back peak	poly dT	50%, 50%
rechromatography of back peak (EtOH ppt.)	poly U	50%, ---
rechromatography of back peak (EtOH ppt.)	poly dT	50%, ---
Original chromatography of RNA from cleavage embryos	poly dT	21.3%, 33%
rechromatography of front peak	poly U	<1%, <1%
rechromatography of front peak	poly dT	<1%, <1%
rechromatography of back peak	poly U	100%, 100%
rechromatography of back peak	poly dT	100%, 100%
rechromatography of back peak (EtOH ppt.)	poly U	100%, ---
rechromatography of back peak (EtOH ppt.)	poly dT	100%, ---

stage RNA has a somewhat higher proportion of its radioactive RNA (> 7S) polyadenylated; probably the low value obtained for cleavage is due to histone mRNA, as indicated previously. The accumulation of rRNA (which does not bind to poly U cellulose) at gastrulation may proportionally reduce the binding at this stage. The kinetics of accumulation of material containing poly A shows that a maximum level is reached by 2 hr of labeling for the blastula stage embryos (Figure 2). Furthermore, labeling of total RNA also displays similar kinetics (20). Thus, there is probably an extensive turnover of poly A containing material at this stage of development. Kinetics of poly A accumulation at the other stages are currently under investigation.

The covalent nature of attachment of poly A to RNA of high molecular weight was tested by centrifugation through DMSO-sucrose gradients. First RNA >7S from the blastula stage (fractions 1-14 Fig. 1B) was centrifuged through DMSO-sucrose gradients under conditions in which the RNA is denatured (Figure 3A). Fractions were collected from the gradient, pooled and bound to poly U cellulose. The denatured RNA sedimented heterogeneously and remained large in size. The material containing poly A also distributed throughout the gradient similar to the distribution of total radioactivity. Furthermore, the total number of counts bound to poly U cellulose after DMSO-sucrose gradient centrifugation is the same as that obtained for the RNA after SDS-sucrose gradient centrifugation. The comparability of binding of RNA from both DSMO and SDS-sucrose

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Legend for Table III

RNA from the pooled fractions of the cleavage stage SDS-sucrose gradient (Fig. 1A) were used for the original fractionation either on poly U or poly dT cellulose. The numbers in the "% bound" column represent respectively fractions 0-4, Fig. 1A and fractions 5-11, Fig. 1A. The columns were washed with the respective binding buffers: for poly U cellulose (0.01 M Tris pH 7.5-0.1 M NaCl-0.05% SDS); for poly dT cellulose (0.01 M Tris, pH 7.5-0.5 M NaCl-0.05% SDS) and eluted with elution buffer (0.01 M Tris, pH 7.5-0.5% SDS). The RNA that eluted with the binding buffer was designated the front peak and the RNA that eluted with the elution buffer was designated the back peak. The respective front and back peaks from the poly U and poly dT cellulose columns were rechromatographed on poly U or poly dT cellulose. RNA from certain samples of the back peak were first ethanol precipitated, resuspended in fresh binding buffer and then subjected to rechromatography on poly U or dT cellulose.

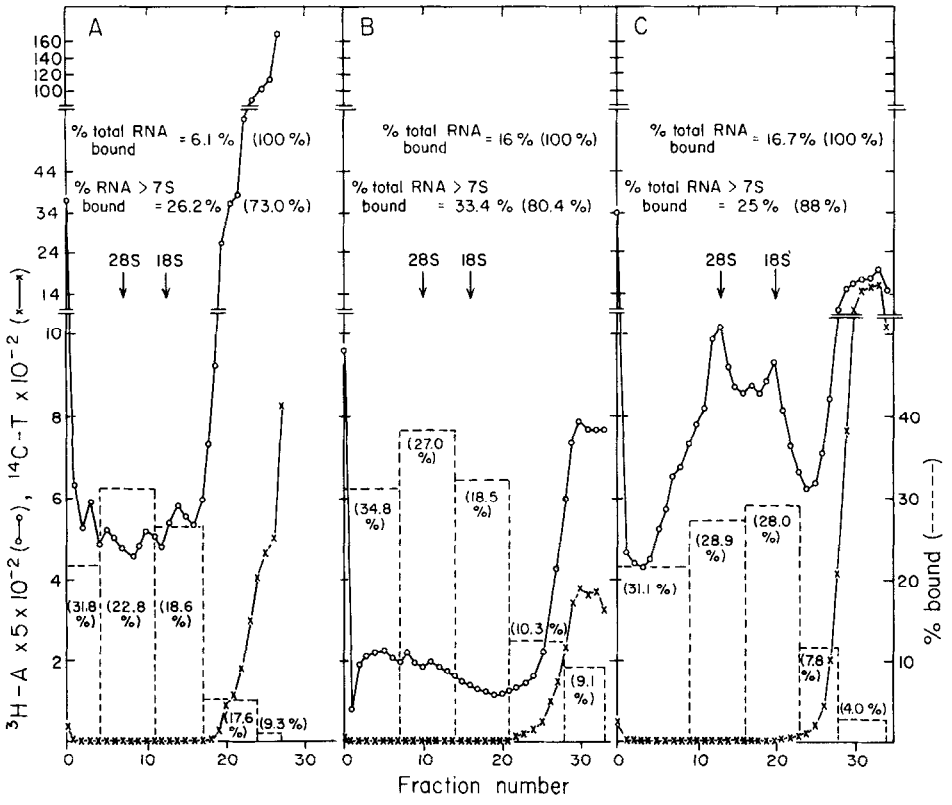

Figure 1. Eggs of Lytechinus pictus were fertilized and cultured in sea water containing 0.05 μc/ml of ^{14}C-thymidine. At the desired times the embryos were labeled for 0.5, 2 and 4 hr with ^{3}H-adenosine at 5 μc/ml. 50 ml cultures were removed at the end of each labeling period and the embryos were processed as described in Methods. The RNA was analyzed on 15-30% SDS-sucrose gradients. The gradients were centrifuged at 21,000 rpm for 20 hr in a Spinco SW 25.1 rotor at 25°C. Only the gradients for the 4 hr labeling time point are shown. (A) cleavage (8 hr post fertilization); (B) blastula (16 hr post fertilization) and (C) mid-gastrula (36 hr post fertilization). Fractions from the respective gradients were pooled and assayed for RNA containing poly A on poly U cellulose. Details of chromatography are given in the legends of Table I, II, and III. The amount of RNA containing poly A was expressed (1) as the percentage of total counts in each group (bar graphs) and (2) as the percentage of the total counts in RNA containing poly A from the whole gradient (numbers parenthesized). o—o ^{3}H-A; x—x ^{14}C-T ---- % bound.

gradients, as well as the large size of the RNA that contains poly A indicates that few internal nicks were produced in the RNA during the isolation and purification. Since the poly A sequence does not exceed 200 nucleotides (21, 22; unpublished results) we conclude that the poly A is attached covalently to large RNA transcripts. Second, RNA obtained from gastrula stage embryos were fractionated on poly dT cellulose and the bound fraction was analyzed on DMSO-

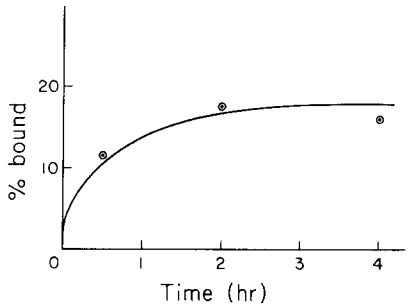

Figure 2. The kinetics of accumulation of RNA containing poly A extracted from the blastula stage embryos was obtained from the experiments described in Figure 1. The percentage of radioactivity in the RNA that binds to poly U columns is shown. Incorporation of ^{3}H-adenosine into RNA shows "saturation" kinetics at this stage (20).

sucrose gradients. Figure 3B shows the DMSO-sucrose gradient pattern of the RNA containing poly A. A preponderance of the material containing poly A sedimented close to 16S rRNA from Escherichia coli, which has a molecular weight around 0.5 x 10^{6} Daltons (17). This result is similar to that obtained for mRNA from sea urchin mesenchyme blastula (15).

DISCUSSION

The first result we wish to underline is that while three different methods of detection of poly A give comparable results, binding to poly U filters is rather unreliable in our hands. Fractionation in either 0.5 M NaCl or KCl on poly U or poly dT cellulose is very reproducible and useful, though the latter is preferable if further experimental work is to be done with RNA that contains poly A.

Second, it is illuminating to apply these methods to developing organisms in which relative rates of transcription of different RNA classes vary at different stages. RNA molecules that contain poly A have been reported for rabbit eggs (23), Dictyostelium (24), and eggs and zygotes of sea urchins (12, 25, 26), but no careful comparison of poly A metabolism at different developmental stages has yet been reported. At all stages examined during sea urchin embryogenesis poly A is synthesized and is found attached to large molecules of RNA, some exceeding 0.5 x 10^{6} Daltons, though the fraction of RNA transcripts that

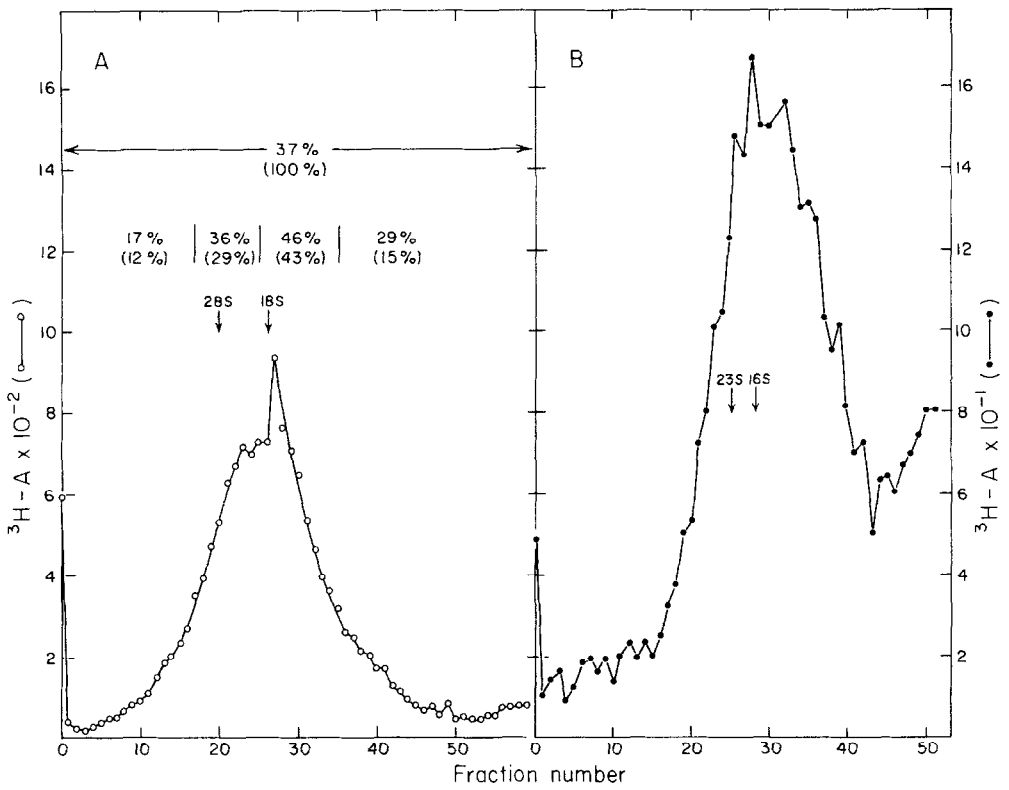

Figure 3. (A) RNA from fractions 0-19 of SDS-sucrose gradient (Fig. 1B) were precipitated in ethanol. The RNA was suspended in 10 microliters of RNA buffer (1 mM EDTA, 0.01 M Tris, pH 7.1), mixed with 10 microliters of dimethylformamide and 90 microliters of 99% DMSO. The mixture of denatured RNA was layered on a 5-20% (w/v) sucrose density gradient made in 99% DMSO (containing 1 mM EDTA, 0.01 M Tris, pH 7.1). Gradients were centrifuged in a SW 50.1 rotor at 47,000 rpm for 16 hr at 25°C. ^{14}C-28S and 18S rRNA were added as internal markers. The RNA from each group was chromatographed on poly U cellulose column after the concentration of DMSO in each group was diluted to 10% with the binding buffer. The amount of RNA containing poly A was expressed in 2 different ways as explained in legend of Figure 1 o——o ^{3}H-A.

(B) RNA from fractions 0-33 of SDS-sucrose gradient (Fig. 1C) were precipitated in ethanol. The RNA was subjected to fractionation on poly dT cellulose as described in legend of Table I. The RNA containing poly A (back peak) was subjected to DMSO-sucrose gradient centrifugation as described above. ^{14}C-23S and 16S rRNA were added as internal markers. ●——● ^{3}H-A.

contain poly A is somewhat higher at blastula than during cleavage or gastrulation. Poly A appears to have a limited half life at the blastula stage, a surprising finding in view of the proposition that poly A may be conserved during HnRNA degradation (27). We believe a detailed examination of poly A metabolism during sea urchin development may help understand just what the role(s) of poly A is in the cell.

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