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Polyadenylation of Maternal RNA of Sea Urchin Eggs After Fertilization

(oogenesis/parthenogenetic merogons)

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ABSTRACT **Between fertilization, or parthenogenetic activation, and the two-cell stage, the content of polyadenylic acid in sea urchin eggs doubles, and the increase occurs primarily in the ribosome–polyribosome fraction. The increase is due to polyadenylation of preexisting RNA molecules synthesized during oogenesis. The polyadenylation occurs in activated, enucleated merogons. It is argued that cytoplasmic polyadenylation may play a role in mobilization of maternal messenger RNA for translation and the polyadenylic acid does not subserve an exclusively nuclear function.**

Fertilization or parthenogenetic activation of sea urchin eggs entrains a profound series of changes in the egg, including dramatic increases in synthesis of nucleic acids and proteins, as well as complete reorganization of the structure and permeability of the cell surface (1). The increase in protein synthesis is not affected by enucleation (2) or inhibition of transcription (3). The increase in protein synthesis reflects the increased capacity of the eggs to synthesize protein, is accompanied by a gradual increase in the number (but not size) of polyribosomes (4), and occurs on mRNA templates synthesized during oogenesis. The mechanism(s) of sequestration or inactivity of untranslated maternal mRNA of unfertilized eggs and its mobilization into active polysomes after fertilization is unknown.

I will report experiments that are based on the assumption that most mRNA molecules of metazoa contain sequences of poly(A) (5). First, I will show that during the 2-hr time span from activation to first cleavage (the time during which protein synthesis attains its maximum) the content of poly(A) doubles, and that the increase occurs primarily in the ribosome–polyribosome fraction of the cell. While this work was in progress, papers reporting similar conclusions appeared (6, 7). Second, the increase in poly(A) is primarily due to a polyadenylation after fertilization of RNA molecules transcribed during oogenesis. Third, polyadenylation occurs in enucleated activated merogons; hence, the reaction is cytoplasmic.

MATERIALS AND METHODS

[8-^{3}H]Adenosine (25.3 Ci/mol), [^{3}H]polyuridylic acid (13.3 Ci/mol), and [8-^{14}C]polyadenylic acid (0.36 Ci/mol) were purchased from Schwarz/Mann. Unlabeled poly(U) and poly(A) were purchased from Miles Laboratory. Oligo(dT)–cellulose with deoxythymidilate chain lengths ≤ 10 was purchased from Collaborative Research, Inc., and used by the method of Aviv and Leder (8). *Lytechinus pictus* sea urchins were purchased from Pacific Biomarine, and *Strongylocentrotus purpuratus* were collected locally. Actinomycin D was supplied by Merck, Sharpe and Dohme. Pancreatic RNase A was obtained from Worthington Biochemicals, and T1 and T2 nuclease, ethidium bromide, and cordycepin were purchased from Calbiochem.

Sea urchin gametes were obtained, and embryos were raised in Millipore-filtered sea water containing 50 μg/ml of streptomycin by conventional techniques. Parthenogenetic activation was done by a modified butyric acid method (9) followed by exposure for 20 min to sea water containing an extra 30 g/liter of NaCl, after which the eggs were returned to normal sea water.

Merogons were obtained by layering 0.2–1.0 ml of a 20% suspension of eggs over a linear gradient constructed from 1.0 M sucrose and a mixture of equal parts of sea water and 1.0 M sucrose. Gradients were centrifuged in either an SW 39 or SW 25 rotor at 5000 $\times$ *g* (middle of tube) for 6–7 min, followed by centrifugation at 26,000 $\times$ *g* (middle of tube) for 6–10 min. The well-separated egg fragments were collected by dripping the gradients through a puncture made in the bottom of the tube and were immediately washed in normal sea water.

Eggs and embryos were fractionated by washing twice in 1.5 M dextrose, suspension in 2 mM Mg acetate for 10 min, and then mixing them with an equal volume of cold 0.3 M sucrose–15 mM Mg acetate–0.48 M NH_4Cl–12 mM dithiothreitol–50 mM Tris·HCl (pH 7.8). The suspension was gently homogenized in a stainless steel Dounce homogenizer, and the homogenate was successively centrifuged at 10,000 $\times$ *g* for 10 min and 100,000 $\times$ *g* for 90 min, yielding a low-speed sediment, a high-speed pellet, and cell sap.

RNA was extracted by suspension of the cells or cell fractions in 20 volumes of 0.05 N Na acetate (pH 5), 10 mM EDTA, 0.5% Na dodecyl sulfate, 1 mg/ml of bentonite, and extraction once at room temperature and twice at 50° with 50% phenol–48% $CHCl_3$–2% isoamyl alcohol. The aqueous phase was again extracted at 50° with $CHCl_3$ (4% in isoamyl alcohol). The interface between the two phases was then extracted with 1 M $NaClO_4$ (pH 8) at 50°; the latter aqueous phase was mixed with the first extensively extracted aqueous phase. The combined aqueous phases were precipitated at −20° with two volumes of ethanol. The precipitate was subsequently purified by digestion with DNase and Pronase, followed by two extractions with phenol–$CHCl_3$–isoamyl alcohol and reprecipitation from ethanol three times (10). This procedure resulted in yields of $\geq$ 95% of the total RNA (as measured by absorbance in the Schmidt–Thannhauser procedure), as well as $\geq$ 95% of the radioactive label in cellular RNA or added ^{14}C-labeled polyadenylate. Sucrose density gradients were done by the usual techniques (10), and radioactivity was determined after

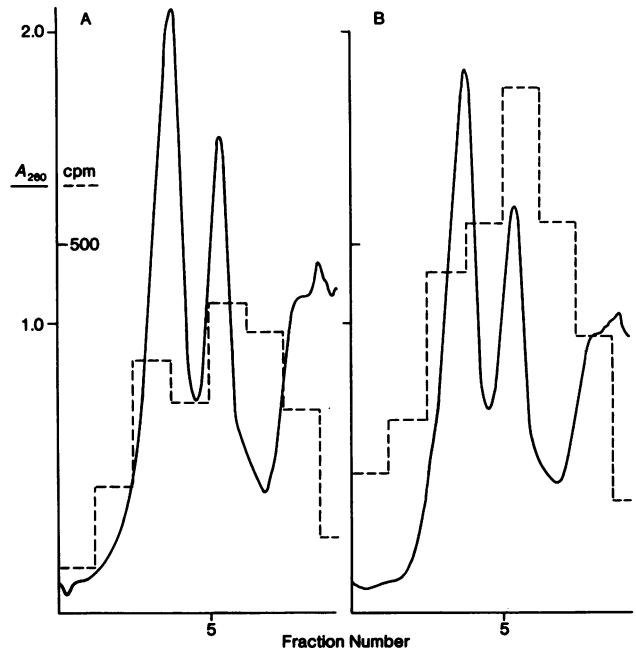

FIG. 1. Total RNA from unfertilized eggs and two-cell embryos was fractionated on 5-20% sucrose gradients made in 0.1 Na acetate, (pH 5)-10 mM EDTA. Fractions of the gradient were collected after passage through an ultraviolet absorbance detector, and assayed for poly(A) content by the radioactive poly(U) method. (*A*) unfertilized RNA; (*B*) RNA from 2-hr embryos.

acid precipitation. Poly(A) was isolated from enzymatic (20 units of T2 nuclease per ml in 300 mM NaCl-30 mM Na citrate at 37° for 3 hr) digests or acid hydrolysates (6% $HClO_4$ at 20° for 16 hr) by the ion exchange method of Katz and Comb (11). Oligonucleotides were separated from RNA on a 6-cm column of G-25 Sephadex in 300 mM NaCl-30 mM Na citrate.

The poly(A) content of RNA was determined by hybridization of RNA with radioactive poly(U) and then determination

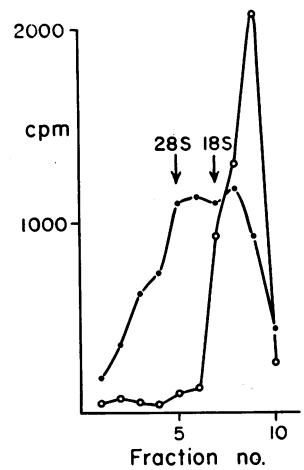

FIG. 2. Eggs were fertilized and cultured for 2 hr in sea water containing 5 μCi/ml of [^{3}H]adenosine. The extracted RNA was sedimented at 16° through a 5-20% sucrose gradient containing 0.1 M NaCl-10 mM EDTA-10 mM Tris·HCl (pH 7.6)-0.2% Na dodecyl sulfate. Untreated RNA (●) and RNA digested in 300 mM NaCl-30 mM Na citrate for 15 min at 37° with 5 μg/ml of RNase and 10 units/ml of T1 nuclease (○) are both shown.

TABLE 1. *Poly(A) content of sea urchin eggs and embryos*

Cells	nmol of poly(U) hybridized per A_{260} unit of RNA	Poly(A) (% total RNA)
Unfertilized	0.096 ± 0.007 (6)	0.048
20 min after fertilization	0.114 ± 0.014 (6)	0.057
2 hr after fertilization (2 cells)	0.159 ± 0.013 (6)	0.079
2 hr after fertilization (2 cells) + actinomycin D	0.179 ± 0.084 (5)	0.089

RNA was prepared from eggs and embryos of *Lytechinus pictus*, and the poly(A) content was assayed. The number of replicate determinations used for calculation of standard deviation is shown in parentheses. Treatment with actinomycin D began 20 min before fertilization with 35 μg/ml of sea water. Values in other experiments with the same species and with *S. purpuratus* were very similar. Percentage of poly(A) is mol (nucleotide) of poly(U) hybridized per mol (nucleotide) of RNA × 100 divided by 2.

of the ribonuclease resistance of the poly(U). Up to 1 A_{260} unit of RNA was mixed with 0.01-0.04 A_{260} unit of radioactive poly(U) in 0.5 ml of 300 mM NaCl-30 mM Na citrate and incubated at 45° for 30 min. The tubes were placed on an ice bath, and 1.5 ml of cold 300 mM NaCl-30 mM Na citrate containing 40 μg of pancreatic ribonuclease and 20 units of T1 ribonuclease were added; digestion proceeded at 4° for 30 min. Then 0.2 A_{260} units of nonradioactive carrier poly(A) [this step was essential to ensure recovery of undigested poly(U)] and 200 μg of yeast RNA were added. Trichloroacetic acid was added to a final concentration of 10%, and the precipitates were collected on glass-fiber filters and washed with more trichloroacetic acid, water, and methanol. Values of 9200 at 260 nm were used for the molar extinction coefficient of polyuridylate (12) (on a nucleotide basis), and 10,000 was the value used for RNA. The assay gave a linear response with addition of increasing amounts of RNA, and did not detect poly(A) in soluble RNA (Calbiochem) from *E. coli*. Prior digestion of sea urchin RNA with DNase or T1 ribonuclease did not lower the values obtained, but prior digestion with T2 ribonuclease abolished any detectable "poly(A)." The product formed during the assay is a triple-stranded molecule composed of two strands of polyuridylate and 1 strand of RNA rich in adenylate (12), and the hybrid is resistant to pancreatic, T1, and T2 ribonuclease. Controls with poly(C) were not able to detect "poly(A)."

Poly(A) was also detected by digestion of RNA labeled with [^{3}H]adenosine with 5 μg of pancreatic ribonuclease and 10 units of T1 nuclease per ml at 37° for 30 min in 300 mM NaCl-300 mM Na citrate. Controls in which 2 units of T2 ribonuclease were added were always used in conjunction with these assays.

RESULTS

The amount of poly(A) in RNA from sea urchin eggs and embryos

The assay based on protection of [^{3}H]poly(U) from digestion with ribonuclease after hybridization with RNA was able to detect putative poly(A); the product had the properties of authentic hybrid between poly(U) and fragments composed

TABLE 2. *Subcellular distribution of poly(A)*

	% Total cellular RNA	nmol of poly(U) hybridized per A_{260} unit of RNA	% Distribution of poly(A)
Unfertilized eggs			
10,000 × *g* pellet	8.6	0.173	25
1000,000 × *g* pellet	81.5	0.042	58
Cell sap	9.9	0.102	17
2-Hr embryos (2 cell)			
10,000 × *g* pellet	13.0	0.245	25
100,000 × *g* pellet	73.0	0.111	62
Cell sap	14.0	0.123	13

Eggs and embryos of *Lytechinus pictus* were fractionated as described, the RNA was extracted, and the poly(A) content was assayed. % Total RNA is simply the percentage of the cell's RNA found in the different cell fractions. The percent distribution of poly(A) was calculated by multiplication of the content of poly(A) per A_{260} unit of RNA by the total amount of RNA in a given cell fraction (expressed in A_{260} units) and division of this value by the total poly(A) recovered in all three cell fractions × 100. The two-cell embryos in this experiment were raised in the presence of 35 μg/ml of actinomycin D. The results presented are the averages of two experiments.

predominantly of poly(A) (*Methods* and unpublished). Model experiments with authentic poly(A) and the melting behavior of the hybrids indicated that the hybrids were triple-stranded structures composed of 2 mol of U for every mol of A, and hence, the amount of any RNA preparation that is "poly(A)" is simply the mol of poly(U) protected against nuclease digestion divided by 2.

Table 1 describes the results obtained with various stages of *Lytechinus pictus*. Poly(A) represents about 0.045% of the total RNA of unfertilized eggs, and increases to twice that value by 2 hr after fertilization, when protein synthesis has reached a maximum and first cleavage is completed. The increase occurs in the presence of actinomycin D.

The subcellular distribution of poly(A) was determined by fractionation of homogenates (prepared in the absence of detergents) by differential centrifugation. As shown in Table 2, the low-speed pellet containing nuclei, yolk, mitochondria, and cell debris was richest in poly(A), but the largest total amount of poly(A) is found in a high-speed pellet composed of ribosomes and polyribosomes. There is a dramatic increase in the poly(A) content of the small particulate fraction when one compares unfertilized eggs with 2-hr embryos, and this increase is so extensive it cannot have occurred by translocation from one cell fraction to another. The increase in poly(A) in the ribosome–polyribosome fraction is unaffected by inclusion of high doses of actinomycin D (Table 2).

The size distribution of the molecules containing poly(A) can be determined by applying the poly(A) assay to fractions of a sucrose gradient. As shown in Fig. 1, the molecules containing poly(A) in both fertilized eggs and 2-hr embryos show a similar broad distribution with a predominant type sedimenting about 18 S. Some of the poly(A)-containing molecules sediment more rapidly than the larger ribosomal RNA under these conditions.

TABLE 3. *Fractionation of RNA on oligo(dT)–cellulose*

	A_{260}	cpm ^3H	cpm ^{14}C	Nuclease resistance (% precipitable)
Total	84.0	374,000	67,350	63
Front peak	80.5	166,800	0	3
Mid peak	1.02	111,700	13,000	97
Back peak	1.62	91,200	54,000	102

Recoveries of labeled RNA from an oligo(dT)–cellulose column (Fig. 3) are shown. RNA was prepared from *Lytechinus* embryos labeled with 5 μCi/ml of [^{3}H]adenosine for 2 hr from the time of fertilization. Authentic [^{14}C]polyadenylate was added to the cells at the time of RNA extraction (94% recovered). The oligo(dT)-cellulose column was eluted with 0.5 M KCl-0.01 M Tris·HCl (pH 7.6) (front peak), 0.1 M KCl-0.01 M Tris·HCl (pH 7.6) (mid-peak), and 0.01 M Tris·HCl pH 7.6 (back peak). Nuclease resistance indicates the retention of acid precipitability of radioactivity after treatment with pancreatic ribonuclease and T1 nuclease.

Poly(A) is added after fertilization to RNA made during oogenesis

The probable reasons for the increase in poly(A) are either a differential extractability of poly(A) from the two different kinds of material or addition of poly(A) after fertilization to RNA made during oogenesis. If polyadenylation occurs after fertilization, one would predict that in the presence of radioactive adenosine, labeled poly(A) would be formed, and furthermore, that the labeled poly(A) would be attached to nonradioactive RNA molecules. This contention has been advanced on the basis of indirect evidence (6, 7). In order to test the hypothesis directly, eggs were fertilized and cultured in the presence of 5 μCi/ml of [^{3}H]adenosine. The RNA from 2-hr embryos was radioactive, the labeling of the RNA was unaffected by doses of actinomycin D that decreased uridine

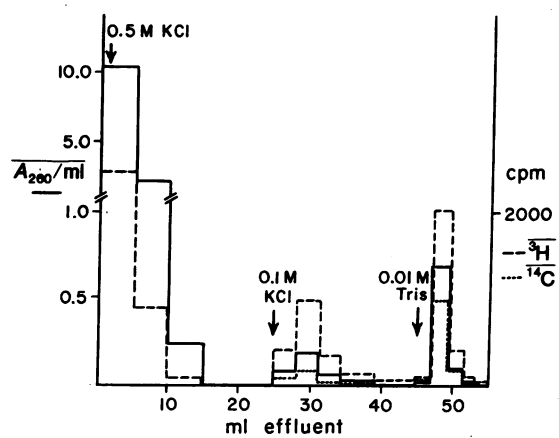

FIG. 3. Eggs of *Lytechinus pictus* were fertilized and cultured for 2 hr in 5 μCi/ml of [^{3}H]adenosine. The RNA was extracted and fractionated on 0.5 g of oligo(dT)–cellulose (8). Further details are given in the legend to Table 3.

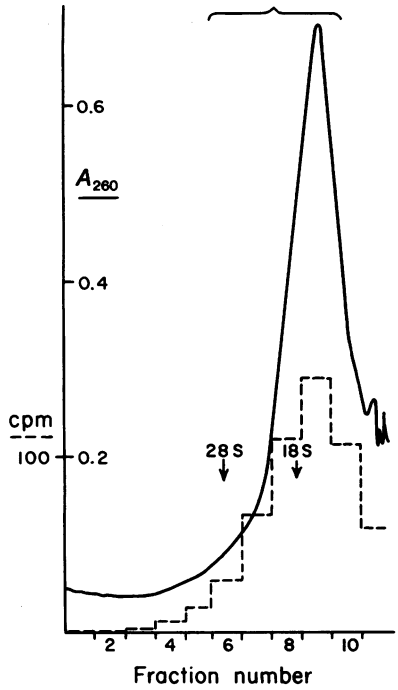

FIG. 4. RNA eluted from oligo(dT)-cellulose by 0.01 M Tris·HCl (see Fig. 3) was precipitated by K acetate and ethanol (8), dissolved in water, and sedimented through a 5–20% sucrose gradient containing 0.1 N Na acetate–(pH 5)–0.01 M EDTA. After the absorbance at 260 nm of the effluent from the centrifuge tube was monitored, aliquots were precipitated for radioactivity determination. The fractions included in the *bracket* were used for digestion with nuclease (Table 4).

labeling by 80%, and the labeling was also unaffected by ethidium bromide (10 μg/ml) or cordycepin (25 μg/ml).

Fig. 2 shows the distribution of the adenosine label in sucrose gradients; the label obviously cosediments with molecules that are very large and heterodisperse. After ribonuclease digestion under conditions that do not affect the acid precipitability of poly(A), 50–60% of the [^{3}H]adenosine is seen as a peak in the gradients at about 4 S. One may tentatively conclude that radioactive poly(A) of about 100 nucleotides in length is attached to RNA molecules of much greater size. Sedimentation of adenosine-labeled RNA is similar to the sedimentation distribution of poly(A) sequences determined by a completely different method (Fig. 1).

TABLE 4. *Adenylic acid-specific radioactivity (cpm/10 nmol)*

RNA from oligo(dT)-cellulose	Sephadex fraction	
	Excluded	Retarded
Front peak (0.5 M KCl)	None; none	163; 225
Back peak (0.01 M Tris·HCl)	3700; 3100	90; 110

RNA labeled with [^{3}H]adenosine was isolated and fractionated on oligo(dT)-cellulose. The fractions passing through the column with 0.5 M KCl–0.01 M Tris·HCl (pH 7.6) (front peak) and 0.01 M Tris·HCl (pH 7.6) (back peak) were collected and digested with RNase and T1 nuclease in 300 mM NaCl–30 mM Na citrate. The digests were passed through Sephadex G-25, and the excluded and retarded fractions were collected. The Sephadex fractions were then hydrolyzed to produce mononucleotides; adenylic acid was isolated and its specific activity was determined. Two different experiments on the same RNA preparation are shown. Other RNA preparations gave the same kinds of results.

TABLE 5. *Polyadenylate in merogons*

Source of RNA	Not activated		Parthenogenetically activated	
	nmol of poly(U) hybridized per A_{260} unit of RNA	% Total RNA	nmol of poly(U) hybridized per A_{260} unit of RNA	% Total RNA
Whole eggs	0.0845	0.042	0.163	0.081
Nucleated merogons	0.0847	0.043	0.161	0.081
Enucleated merogons	0.119	0.059	0.227	0.138

RNA was extracted from eggs and merogons of *S. purpuratus*, both with and without parthenogenetic activation, and the content of poly(A) was assayed. The numbers shown are the average of four determinations. Similar results have been obtained with *Lytechinus pictus*.

A similar labeled RNA preparation was then subjected to chromatography on oligo(dT)-cellulose in the presence of added authentic [^{14}C]poly(A). Although the chemical basis of fractionation on this column is not completely understood, the column can separate the preponderance of cellular RNA molecules from authentic poly(A) (Fig. 3). The radioactive sea urchin RNA eluted at low salt from the column is completely ribonuclease resistant (Table 3). Hence, in the 2 hr after fertilization, poly(A) is synthesized.

Can we be certain the poly(A) is attached to nonradioactive RNA transcribed in oogenesis? The question can be answered by isolating adenylic acid from the nuclease-sensitive and -insensitive portion of the RNA containing polyadenylate. Radioactive RNA labeled with adenosine was again prepared and fractionated on oligo(dT)-cellulose. The sedimentation of the RNA containing poly(A) is shown in Fig. 4. After fractionation most of the RNA sediments somewhat more slowly than 18 S, but still considerably faster than 4 S. The fraction of labeled RNA eluted at 0.5 M salt, and the fraction eluted at 0.01 M Tris·HCl were completely excluded from Sephadex G-25. These same fractions were next subjected to ribonuclease digestion, followed by passage through Sephadex; the small (retarded on G-25) and the larger nuclease-insensitive portions (excluded on G-25) of the enzymatic digests were separated. There were no large RNA fragments surviving nuclease digestion in RNA eluted with 0.5 M KCl, but some large fragments (about 25% of the UV-absorbing material) containing most of the radioactivity were found in digested RNA eluted with Tris·HCl. Both the large (excluded) and small (retarded) RNA fragments were degraded to ribonucleotides under conditions in which the 8-hydrogen isotope of purines does not exchange with water, e.g., by T2 nuclease digestion at neutral pH or mild acid hydrolysis. The adenylic acid produced was isolated on ion-exchange columns and its specific activity determined. Results of two experiments are

shown in Table 4; there is a vast difference in the specific activity of the adenylic acid derived from the nuclease-sensitive and -insensitive portions of the poly(A)-containing RNA. I consider it likely the retarded portion of the RNA is not radioactive at all, and that the low radioactivity found in the nuclease-sensitive portion is due to use of a Sephadex column with poor resolution. The experiments provide strong and direct evidence that polyadenylation is an addition to adenylate to preexistent RNA after fertilization.

Polyadenylation occurs in the cytoplasm

The insensitivity of the increase in poly(A) to inhibitors of transcription suggests polyadenylation may occur in the cytoplasm. This hypothesis can be directly tested by preparation of enucleated merogons from the eggs by a centrifugation method (13). Centrifugation at low speed in a density gradient bands the eggs at their isodense position in the gradient and stratifies the cell organelles within the egg according to their relative densities. A subsequent acceleration of the centrifuge causes the eggs to separate into an upper, lighter fragment (merogon) that contains the nucleus, and a lower, dense fragment (merogon) lacking a nucleus. The enucleated merogons are smaller than their nucleated counterpart. A diffuse band sometimes forms below the enculeated merogons and is composed of products of secondary fragmentation of the enucleated merogons under the conditions used. The fragments obtained by this method were separated by 7–8 mm in the gradient; there was no detectable cross contamination of the enucleated layer, but some unfragmented eggs remain in the nucleated layer. After each band of merogons was washed with sea water, a portion of each was activated parthenogenetically and incubated in sea water for 2 hr. RNA was then extracted from the egg fragments and analyzed for its poly(A) content (Table 5). First, the content of poly(A) is somewhat higher in enucleated fragments than in nucleated fragments and gametes. Second, all fragments that are activated, regardless of whether they contain the oocyte nucleus, show a clear increase of about twofold in poly(A) after activation. Clearly, addition of poly(A) to RNA synthesized during oogenesis does not require a nucleus.

DISCUSSION

There is an increase in the polyadenylate content of RNA of sea urchin eggs in the 2 hr after fertilization. This is almost certainly due, as has been suggested (6, 7), to addition to poly(A) sequences to RNA molecules transcribed weeks or months before, during oogenesis. The experiments with merogons show that a nucleus is not required for polyadenylation. The results with inhibitors of mitochondrial RNA synthesis, as well as unpublished experiments in our laboratory on subcellular localization of polyadenylation, make mitochondria an unlikely candidate for the site of the reaction. It will be interesting to find out whether polyadenylation after fertilization occurs by lengthening of the poly(A) sequences present in RNA of unfertilized eggs. Since about half the adenosine incorporated during the first 2 hr after fertilization goes into polyadenylate, and much of the remaining adenosine incorporation is in mitochondrial RNA (14) and turnover of the 3′-OH terminus of transfer RNA (15), it is possible there is very little nuclear transcription during this period of development.

The main conclusions reached are similar to those of Slater *et al.* (6, 7). There are differences between the present data and those of Slater *et al.* (7) on the subcellular localization of RNA that contains poly(A). They contend a subribosomal fraction is rich in poly(A), while I find the preponderance of the poly(A) associated with a "ribosome" fraction. The differences found in the two sets of experiments are probably real and potentially interesting, for they used detergent while I did not, and centrifugation conditions were also different.

The conclusions reached bear on two important biological issues. First, the results argue against polyadenylation being simply a mechanism associated with transcription or export of RNA from the nucleus. The present data are more compatible with the idea that polyadenylation confers stability to RNA molecules while being translated and exposed to the vicissitudes of cytoplasmic nucleases, a view suggested by Perry and his collaborators (16). Second, the results suggest polyadenylation may be involved in regulation of protein synthesis after egg activation.

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