Volume 9 Number 12 1981 Nucleic Acids Research

Detection of poly A^+ RNA in sea urchin eggs and embryos by quantitative in situ hybridization

Lynne M. Angerer and Robert C. Angerer

Department of Biology, The University of Rochester, Rochester, NY 14627, USA

Received 17 February 1981

ABSTRACT

We present an improved procedure for detecting poly A tracts in situ by hybridization of ^3H poly U. Glutaraldehyde fixation achieves significantly higher retention of RNA and better morphologic preservation than does Carnoy's. A dramatic increase in signal to noise is obtained by prehybridization treatment of glutaraldehyde-fixed sections with proteinase K and acetic anhydride. Measurement of the increase in poly A concentration after fertilization by solution titration and by in situ hybridization are in excellent agreement indicating that in situ measurements yield accurate relative estimates of local RNA concentrations in sections. Examination of the grain density distribution in sections of sea urchin eggs and cleaving embryos reveals no major cytoplasmic localization of poly A+ RNA, although nuclei show much less labelling and micromeres of 16-cell embryos have a small, but significant, reduction in poly A concentration.

INTRODUCTION

The technique of in situ hybridization has become a powerful tool for mapping specific DNA sequences on chromosomes. Recently, this approach has been applied to the detection of RNA transcripts in individual cells (1-13). It offers the potential for comparing quantitatively the relative concentrations of specific RNA transcripts in different cells of a heterogeneous population. Such an approach is particularly valuable for systems in which quantities of material are limited or in which cell fractionation techniques are not feasible. The latter situation exists in the case of developing sea urchin embryos for which limited purification of different cell types is possible only at a few stages, and consequently almost all information on developmental changes in RNA transcript populations has been derived from whole-embryo measurements.

As a prerequisite for determining the distribution of individual RNA sequences in developing embryos we have used radioactively labelled poly U as a model probe to develop improved in situ hybridization methods

which optimize the signal and minimize nonspecific binding of heterologous sequences. We demonstrate that the hybridization is specific, and that grain densities determined from *in situ* hybridization measurements are proportional to the known poly A content of eggs and 4-cell embryos.

We have used these methods to examine the distribution of poly A tracts in eggs and cleaving embryos. The concentration of poly A is much lower in nuclei than in surrounding cytoplasm. No general localization of poly (A)+ RNA is evident in unfertilized eggs or 4-cell embryos. At 16-cell stage micromeres exhibit a slightly lower cytoplasmic concentration of poly A compared to the rest of the embryo.

MATERIALS AND METHODS

Embryo culture. *Strongylocentrotus purpuratus* were obtained from Pacific Biomarine Supply, Inc. Spawning was induced by intracoelomic injection of 0.5M KCl. Eggs were washed gently three times with Millipore-filtered artificial sea water and fertilized at a concentration of $1\text{-}2\times10^4$/ml with the lowest concentration of sperm yielding greater than 98% fertilization. Embryos developed in artificial sea water at 15°C at a density of $1\text{-}3\times10^3$/ml. Cultures were monitored for normal development as evidenced by normal gastrulation 36 hours after fertilization.

RNA Extraction. Total RNA was extracted and purified from unfertilized eggs and 4-cell embryos according to the method of Hough *et al.* (14) with minor modifications. Briefly, this procedure includes lysis in 7M urea, 50 mM sodium acetate, pH 5.1, 10 mM EDTA, 0.5% SDS, 10 µg/ml polyvinylsulphate and 200 µg/ml bentonite, and subsequent deproteinization with an equal volume of a 1:1 mixture of { phenol, 0.1% 8-hydroxyquinoline: chloroform, isoamyl alcohol (24:1)}. After extraction of the interface with 1M sodium perchlorate, 0.1M Tris, pH 8.0, 1% SDS, aqueous phases were pooled and extracted with chloroform:isoamyl alcohol (24:1). All extractions were carried out at room temperature. Nucleic acids were precipitated with ethanol at -20°C, dissolved in 50 mM Tris, pH 7.4, 5 mM $MgCl_2$ and incubated at 37°C for one hour with 100 µg DNAase I/ml (Worthington, DPFF) freed of contaminating RNAase according to the method of Maxwell *et al.* (15). Samples were then digested with 50 µg proteinase K/ml (EM Laboratories) in 0.1 M Tris, 0.2% SDS and 50 mM EDTA, pH 8.0, for one hour at 37°C. After extraction with phenol:chloroform and chloroform, RNA was precipitated with two volumes of ethanol at -20°C. Each preparation was dissolved in sterile water and stored frozen at -20°C.

Synthesis of ^{3}H polyuridylic acid (poly U) and ^{3}H polycytidylic acid (poly C). Tritiated poly U and poly C were synthesized by polynucleotide phosphorylase catalyzed polymerization of either UDP or CDP following modifications of a procedure described by Jones et al. (16). ^{3}H UDP or ^{3}H CDP (11.0 and 12.7 Ci/mmole, respectively; Amersham) were dried down and a 4 or 5 fold molar excess of cold UDP or CDP added to adjust the specific activity. The reaction was carried to completion at 37°C for one hour at final concentrations of 2.5 mM XDP in 50 mM Tris, adjusted to pH 8.5 (at 37°C), 10 mM KCl, 6 mM $MgCl_2$, 66 units polynucleotide phosphorylase/ml (Miles). Approximately 25-35% of the isotope was incorporated into TCA precipitable material. The reaction was terminated by addition of EDTA, SDS and sodium acetate, pH 6.0, to final concentrations of 10 mM, 0.1% and 0.2M, respectively. Samples were purified by successive extractions with 1 phenol:1 chloroform, and with chloroform, followed by gel filtration on 5 ml columns of G-100-120 Sephadex in 0.2M sodium acetate, pH 6.0, and 0.1% SDS. Excluded material was collected, concentrated approximately 10 fold by repeated extraction with 2-butanol and precipitated with 2 volumes of ethanol at -20°C. After a second ethanol precipitation, polymers were dissolved in sterile water and their specific activities determined. Several different preparations were used in the experiments reported here and their specific activities are noted in the footnotes to the Tables. Weight average lengths of initial products, determined by electrophoresis on appropriate acrylamide or agarose gels, were approximately 500 nucleotides with a broad distribution ranging from 50 to 1200 nucleotides. For most experiments, the mass average length was reduced by limited alkaline hydrolysis (3 hours, 0.1M NaOH at 0°C or 25°C for poly U or poly C, respectively). Lengths of hydrolyzed fragments were 50-100 nucleotides as determined by electrophoresis on 12% polyacrylamide, 10M urea gels run at 60°C at 8 volts per cm. For short fragment length markers, radioactive DNA derived from a DNAase I digest of Tetrahymena nuclei (a gift from Dr. C. Giri) was used.

Poly A Titrations. Increasing concentrations of egg or 4-cell total RNA were annealed with an excess of either ^{3}H poly U (1.4 Ci/mmole) or ^{3}H poly C (2.0 Ci/mmole) in 0.3M NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA at 37°C for one hour, using unhydrolyzed probes. After a 30 minute digestion with 20 µg RNAase A/ml at 37°C in 0.5 M NaCl, 2 mM Tris, 2 mM EDTA, pH 8.0, hybrids were precipitated with cetyltrimethylammonium bromide according to the method of Darnbrough and Ford (17), collected on Whatman GF/C filters and counted in Betafluor (National Diagnostics).

Labelling Embryo RNA in vivo. ^{3}H uridine (45 Ci/mmole; Amersham) was added to embryo cultures ($2x10^{4}$/ml) ten minutes after fertilization at a concentration of 10 μCi/ml. After two hours at 15°C, embryos were collected by low speed centrifugation and suspended in fresh sea water for one hour prior to fixation and embedding.

Fixation, and Paraffin Embedding

Carnoy's (3 ethanol: 1 acetic acid). Aliquots of eggs or embryos were collected by centrifugation at 1000 rpm in the Beckman JS13 rotor for two minutes. A thick slurry of eggs in sea water was added dropwise to an approximately 50 fold volume excess of fixative with gentle stirring in order to avoid aggregation. Embryos were suspended directly in fixative since, unlike eggs, they do not aggregate. Both eggs and embryos were centrifuged as above and suspended in fresh fixative for 30 minutes at room temperature. They were washed 30 min with 99% ethanol (2x), xylene (2x), 45 minutes in 1 xylene: 1 paraffin at 55°C and three times 5 min in melted paraffin (m.p. 55-57°C) at 58°C. Small drops of paraffin containing eggs or embryos were placed in embedding capsules, allowed to solidify, heated until they began to glisten and then covered with liquid paraffin. Paraffin blocks were stored at 4°C.

1% Glutaraldehyde. Eggs and embryos were fixed as described above in 1% glutaraldehyde (Ladd), 3% NaCl, 50 mM sodium phosphate, pH 7.4, at 0°C for 60 minutes. They were washed in two changes of buffer, each 30 minutes at 0°C and dehydrated by sequential 30 minute changes through increasing ethanol concentrations (50%, 70%, 85%, 95%, 99% and 99%) before embedding as described above.

Sections were cut nominally 5μ thick, placed on subbed slides (18) and dried for at least two days on a 40°C slide warming rack.

Tissue Pretreatments. Sections of eggs or embryos were deparaffinized by two 10 minute incubations in fresh xylene and hydrated by passage through descending ethanol concentrations to water. As noted in the text, some sections were incubated with proteinase K (EM Laboratories) or acetic anhydride prior to hybridization. Proteinase K digestions were at 37°C in 0.1 M Tris, 50 mM EDTA, pH 8.0, at an enzyme concentration of 1 μg/ml unless otherwise noted. Following digestion, slides were washed briefly in distilled water, and treated with acetic anhydride as described by Hayashi et al. (19). Acetylated slides were washed briefly in 2xSSC, water and dehydrated through increasing ethanol concentrations (30%, 50%, 70%, 85%, 95%, 99% and 99%).

In certain control experiments listed in Table 3, deparaffinized sections were digested with either DNAase I or RNAase T2 (Calbiochem). Slides were incubated at 37°C for 1 hour in a solution containing 50 mM Tris, pH 7.4, 5 mM $MgCl_2$ and 100 µg RNAase-free DNAase I/ml. Other slides were incubated at 37°C for 1 hour in 10 mM Tris, pH 7.6, 0.1 M KCl, 1 mM $MgCl_2$ containing 100 units RNAase T2/ml. In both cases, control slides were incubated in buffer only. Following RNAase T2 or DNAase I digestion, slides were washed with proteinase K digestion buffer and processed as described above.

Hybridization. Hybridization mixtures (100 µl) containing 0.1-1.0 nmoles of either ^3H poly U or ^3H poly C nucleotides in 0.3 M NaCl, 0.01 M Tris, pH 8, and 1 mM EDTA were applied to deparaffinized and dehydrated sections on dry slides and covered with siliconized coverslips (22x60mm). Slides were incubated in moist chambers at 37°C for one hour. We estimate that these conditions correspond to approximately Tm - 20°C for hybrids 60 nucleotides long. Coverslips were removed by soaking each slide individually in 50 ml of hybridization buffer. All slides in a group were then washed successively in 500 ml of 0.5M NaCl, 2 mM Tris, pH 8, 2 mM EDTA, containing 20 µg RNAase A/ml for 30 minutes at 37°C, with shaking; and for one hour in 4 l of hybridization buffer at room temperature. Slides were then immersed in 400 ml of cold 5% TCA after the method of Capco and Jeffrey (11) and dehydrated through increasing ethanol concentrations. Each ethanol solution, except 99%, contained 300 mM ammonium acetate as suggested by Brahic and Haase (7).

Autoradiography. Slides were dipped at 45°C in Kodak NTB-2 liquid nuclear track emulsion diluted with an equal volume of 600 mM ammonium acetate. They were dried vertically for 30 minutes at room temperature, incubated in a moist chamber for three hours at room temperature to reduce the number of latent grains, placed in light-tight slide boxes containing Drierite and exposed for 5.5-12.5 days at 4°C. In later experiments, we took the additional precaution of storing the slides in an evacuated dessicator. The emulsion was developed as described previously (18) and the sections were dehydrated through an ethanol series, stained with eosin Y and mounted in Permount.

Data Analysis. Random sections were photographed using a Zeiss Universal microscope equipped with a 40x planapochromat lens with iris at a numerical aperature of 0.6-0.8. Areas of sections were measured with a Tektronix digitizer (model 4956)-controller (model 4052) and grain densities computed

as number of grains/μ^2. Grains were counted in equivalent areas adjacent to sections to determine emulsion backgrounds which have been subtracted from the values presented here.

RESULTS

Optimization of *in situ* hybridization methods

We wished to determine fixation and *in situ* hybridization conditions which maximize RNA retention and hybridization efficiency, and yield quantitative estimates of poly A concentrations in embryo sections. For initial experiments, unfertilized eggs or 4-cell embryos were fixed either in Carnoy's or in 1% glutaraldehyde, and sections were used for *in situ* hybridization without further treatment. ^{3}H poly U and ^{3}H poly C of similar specific activity and fragment length (50 nucleotides weight average) were hybridized to separate sections of egg and 4-cell embryos. Specific hybridization (grain yield for poly U minus that for poly C) was low. Preliminary estimates of hybridization efficiency (pmoles poly U bound/pmole poly A present in sections) gave values of 0.5-1%, based on measure poly A contents of eggs and 2 or 4-cell embryos (20; see below also). (See Discussion for considerations involved in estimation of hybridization efficiencies). The signal/noise ratios were also low, in the range of 1.3-3.0 for different samples. However, specific hybridization was measurably higher for glutaraldehyde-fixed cells.

A possible factor contributing to low absolute hybridization efficiency as well as to differences observed for the two fixatives is loss of poly A+ RNA from sections during *in situ* procedures. To assay relative retention of RNA, eggs were labelled beginning ten minutes after fertilization for two hours with ^{3}H uridine (10 μCi/ml), collected and washed at the 4-cell stage, and carried through fixation and *in situ* hybridization procedures, omitting RNAase A digestion. A comparison of resultant grain densities for embryos prepared in the two fixatives demonstrated that overall retention of RNA is 5.5 fold greater for glutaraldehyde- than for Carnoy's - fixed material. Examination of representative sections, shown in Figure 1, reveals an additional difference. While nuclear grain density is similar for the two fixatives, the cytoplasmic grain density is approximately 10-20 fold greater for glutaraldehyde-fixed embryos.

These considerations and the better histologic preservation afforded by glutaraldehyde led us to try several postfixation treatments on glutaraldehyde-fixed material in an effort to increase hybridization efficiency and

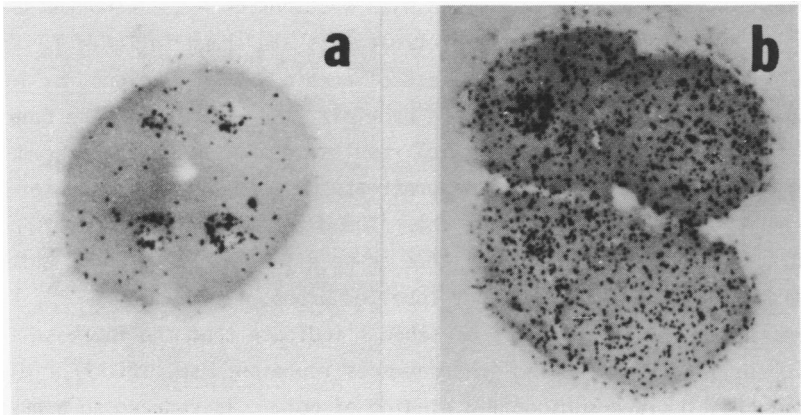

Figure 1. Retention of in vivo Labelled RNA in Sections of 4-cell Embryos. (a) Carnoy's fixation; (b) 1% glutaraldehyde fixation. Eggs were incubated in ^{3}H uridine for 2 hours as described in Materials and Methods, washed, fixed, embedded and sections were carried through the complete in situ hybridization protocol. Exposure time was 3.5 days.

decrease nonspecific binding of probe. Brahic and Haase (7) reported that procedures which partially remove cellular proteins (e.g., washes with 0.2N HCl, incubation at 70°C, and mild digestion with proteinase K) result in a 2 fold increase in hybridization efficiency of cDNA probes. However, others have shown that treatment with HCl disrupts cellular morphology with resultant loss of cytoplasmic organelles, and that exposure to high temperatures can lead to artifactual grain production (21). We therefore tested the effect of prehybridization treatment with proteinase K on in situ hybridization efficiency and cellular morphology. Examination of 4-cell embryo sections incubated with 1, 10 and 100 µg proteinase K/ml at 37°C for 30 minutes gave evidence of extensive damage to tissue at the two higher enzyme concentrations as well as loss of a large fraction of sections from the slides, especially at the highest enzyme concentration. We therefore chose pretreatment at 1 µg/ml for further study. To help decrease background binding of probe we employed prehybridization treatment of sections with acetic anhydride after proteinase K digestion. This procedure has been shown to be effective in reducing backgrounds for in situ hybridizations utilizing ^{125}I-labelled probes (19), presumably by acetylating positively charged groups and thereby decreasing nonspecific electrostatic binding of nucleic acids.

The results of three experiments with sections of 4-cell embryos employing combinations of these two pretreatments are shown in Table 1. In experiment 1, we examined the effect of acetic anhydride alone, or in combination with proteinase K, on the hybridization of poly U. The combination of treatments (experiment 1c) results in an 8 to 10 fold increase in poly U binding as compared to no pretreatment (experiment 1a) or to acetic anhydride alone (experiment 1b). The data of experiment 1 d-f show that the pretreatments have little or no effect on backgrounds determined from slides incubated without labelled probe.

The results of experiment 2 of Table 1 indicate that the increase in signal observed after combined pretreatments represents specific hybridization of poly U since nonspecific binding of poly C is reduced to background levels, (compare experiments 2b and 1f in Table 1). The combination of increased signal and decreased nonspecific background gained by these procedures results in a signal/noise ratio of at least 40 (Table 1, experiment 2). Representative sections of eggs and 4-cell embryos hybridized under

TABLE 1

Effect of Prehybridization Treatments on Hybridization *in situ* of Poly U to 4-cell Embryos

Expt.	Pretreatment	nmoles/slide[γ]	#μ² (x10⁻⁴)	Total grains counted	RELATIVE GRAIN YIELD[α] (x10³)
1	a) None	0.1(U)	2.3	522	1.9±0.7 (12)[β]
	b) Acetic anhy.	0.1(U)	2.0	605	2.4±1.2 (20)
	c) Prot. K + Acetic anhy.	0.1(U)	1.6	3356	17.2±2.3 (17)
	d) None	0	2.3	117	0.4±0.3 (15)
	e) Acetic anhy.	0	3.3	121	0.3±0.2 (15)
	f) Prot. K + Acetic anhy.	0	2.4	83	0.3±0.2 (11)
2	a) Prot. K + Acetic anhy.	0.1(U)	2.9	1590	5.6±1.4 (26)
	b) "	0.1(C)	1.4	19	0.1±0.0 (12)
3	a) Prot. K(1μg/ml) + Acetic anhy.	0.5(U)	1.6	1795	5.9±1.9 (14)
	b) Prot. K(3μg/ml) + Acetic anhy.	0.5(U)	1.0	3361	17.8±3.0 (13)

α: grains/μ²/day of exposure/Ci/mmole U or C
β: Values in ( ) refer to number of sections analyzed.
γ: Poly U and poly C specific activites were 1.4 and 2.0 Ci/mmole, respectively; exposure time was 5.5 days.

these conditions are presented in Figure 2.

A further attempt to improve hybridization efficiency is shown in experiment 3 of Table 1. For this experiment probe input was increased 5 fold and proteinase K concentration was raised from 1 to 3 μg/ml. Although the higher enzyme concentration yielded a 3 fold increase in grain density, there was noticeable damage to sections. We have therefore chosen 1 μg proteinase K/ml as our standard pretreatment.

To test whether these pretreatments have an effect on retention of RNA in sections, we subjected 4-cell embryos labelled in vivo to the complete optimized in situ hybridization protocol. Relative retention of RNA as indicated by autoradiographic grain densities is shown in Table 2. The pretreatment causes no observable decrease in grain yields; the relatively small increase in grain density over proteinase K-digested sections may result from lower self-absorption of ^{3}H disintegrations in partially deproteinized sections. We conclude that the pretreatments do not cause a significant decrease in RNA retention by sections.

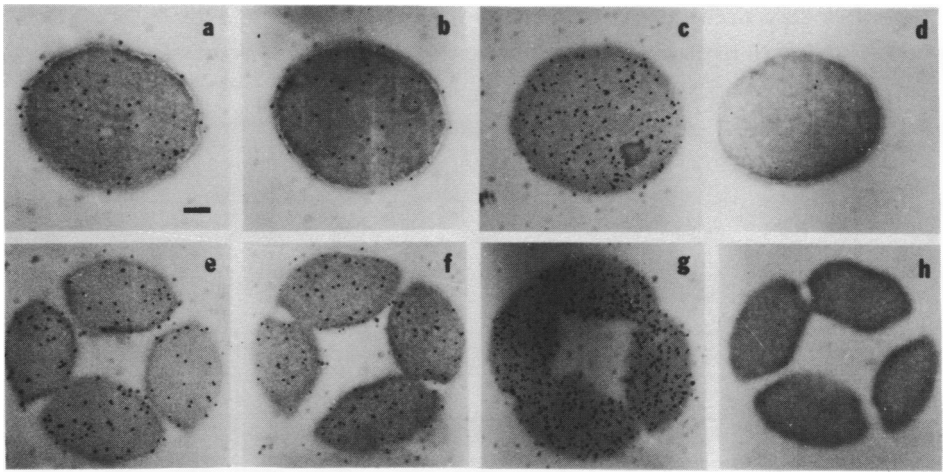

Figure 2. Effect of Pretreatments with Proteinase K and/or Acetic Anhydride on Signal (poly U hybridization)/Noise(poly C binding). (A-C) Sections of eggs fixed in 1% glutaraldehyde were subjected to (A) no pretreatment, (B) acetic anhydride, or (C) proteinase K + acetic anhydride, and hybridized with ^{3}H poly U (1.8 Ci/mmole) as described in Materials and Methods; (D), same as (C), except the sections were hybridized with ^{3}H poly C (2.0 Ci/mmole). (E-H) Same as (A-D), except the sections are from 4-cell embryos. The probes were approximately 50 nucleotides long and were hybridized at a concentration of 0.1 nmole CMP or UMP/ml. Exposure time was 6.8 days.

TABLE 2

Effect of Prehybridization Treatments on Retention of Labelled RNA in 4-cell Embryos

Pretreatment	# μ^2 ($x10^{-4}$)	Total grains counted	GRAINS/ μ^2 ($x10^2$)
a) None	5.4	3568	18.9±5.8 (19)[α]
b) Acetic anhy.	4.1	2419	16.7±7.1 (22)
c) Prot. K + Acetic anhy.	5.4	5195	27.6±13 (25)

α: Values in ( ) refer to number of sections analyzed.

It should be noted that no specific in situ hybridization of poly U was observed to sections fixed in Carnoy's and subjected to proteinase K and acetic anhydride pretreatments. We will consider the large differences in in situ hybridization signals observed for material fixed by these two methods in the Discussion.

Additional tests on specificity of hybridization are presented in Table 3. To control for the possibility that poly U hybridizes to AT-rich regions of mitochondrial DNA, sections of eggs and 4-cell embryos were incubated with RNAase-free DNAase I as described in Materials and Methods. After proteinase K pretreatment, the sections were hybridized with ^3H poly U (0.5 nmoles/slide). No decrease in grain density resulted for

TABLE 3

Prehybridization Treatments with DNAase I or RNAase T2

Expt.[γ]	Stage	Enzyme	# μ^2 ($x10^{-4}$)	Total Grains Counted	RELATIVE GRAIN[α] YIELD ($x10^3$)
1	Egg	0	2.4	1080	2.3±0.7 (12)[β]
	Egg	DNAase I	3.1	1643	2.7±0.7 (15)
	4-cell	0	2.0	3000	7.6±1.2 (14)
	4-cell	DNAase I	1.5	3300	11.3±3.4 (11)
2	4-cell	0	3.1	4588	10.0±2.6 (26)
	4-cell	RNAase T2	3.5	663	1.3±0.7 (26)

α: Grains/μ^2/day of exposure/Ci/mmole U.
β: Values in ( ) refer to number of sections analyzed.
γ: Poly U specific activity was 2.3 Ci/mmole; exposure time was 6.8 days.

either egg or 4-cell embryos relative to controls incubated in buffer. While hybridization signals are, in fact, somewhat higher for samples digested with DNAase I, they fall within the range of experimental variability.

In experiment 2 of Table 3, sections of 4-cell embryos were treated with RNAase T2 or buffer alone prior to hybridization (0.5 nmoles ^{3}H poly U/slide). Greater than 85% of the signal was eliminated by this treatment which indicates that target sequences are polyribonucleotides. The effect of varying RNAase T2 concentration on the reduction in signal (data not shown) implies that the signal remaining in this experiment represents poly U hybridized to residual poly A tracts. It is important to show that reduction in hybridization to RNAase T2-treated sections is not due to probe degradation *via* remaining enzyme activity. Excess ^{3}H poly U was recovered from both experimental and control slides after *in situ* hybridization. Analysis of the size of these recovered probes by gel electrophoresis and measurements of their hybridizability with excess poly A revealed no detectable differences.

Finally, we have examined the effect of variations in fragment length on specific hybridization. In two such experiments, probes of approximate weight average lengths of 50 and 500 nucleotides were separately hybridized to sections of eggs or 4-cell embryos. A summary of these experiments, presented in Table 4 indicates that the hybridization signal is 2-3 fold higher for the shorter probes. Qualitatively, these results agree with those of Brahic and Haase (7), who described a 10 fold increase in signal when cDNA probes were reduced from 500 to 50 nucleotides.

Quantitative estimation of poly A concentrations by *in situ* hybridization

---

Demonstration that the quantity of poly U hybridized *in situ* is proportional to section poly A concentration is a prerequisite for examining the distribution of poly A in eggs and embryos. To demonstrate this proportionality we made use of the 2-3 fold increase in poly A content which occurs after fertilization (20,22,23). For these measurements total RNA was extracted from unfertilized eggs and 4-cell embryos. Aliquots of the same cultures were fixed in 1% glutaraldehyde and used for *in situ* hybridization experiments. The fraction of poly A in each RNA sample was determined by solution titration (Figure 3A) with excess ^{3}H poly U. The slope of the line for each RNA sample yields the fraction of that RNA which is poly A. These values are 0.032% and 0.087% for egg and 4-cell RNA, respectively, and agree well with literature values (20,22,23). The ratio of 4-cell/egg poly A content is 2.8.

TABLE 4

Effect of Fragment Length on Relative Grain Yield for poly U *in situ* Hybridization[α].

Expt.	Stage	Probe Length[β] (NT)	Relative Grain Yield[γ] ($\times 10^3$)	SHORT / LONG
1b	Egg	50	2.2	2.8
1c	Egg	500	0.8	
1b	4-cell	50	6.3	3.0
1c	4-cell	500	2.1	
2f	Egg	50	6.1	1.6
2b	Egg	500	3.7	
2f	4-cell	50	15.2	2.1
2b	4-cell	500	7.2	
2c	Egg	50	3.1	2.5
2a	Egg	500	1.2	
2c	4-cell	50	7.8	1.9
2a	4-cell	500	4.1	

α: For clarity of presentation, these data have been extracted from the complete data set shown in Table 5. Experiment numbers refer to experiments listed in Table 5.
β: Experiments 1 and 2 were done with different preparations of poly U.
γ: Grains/μ^2/day of exposure/Ci/mmole.

Quantitative *in situ* measurements of relative poly A concentrations in eggs and 4-cell embryos are presented in Table 5 and Figure 3B. These measurements were made using probes of two different average fragment lengths, each derived from 2 separate preparations of ^{3}H poly U, and hybridized at a variety of probe concentrations. Two important conclusions can be drawn from these data. First, measurements of relative concentrations of poly A in eggs and 4-cell embryos by *in situ* hybridization are completely independent of any factors leading to variation in the extent of hybridization. This is graphically illustrated in Figure 3B, where relative grain yields for 4-cell embryos are plotted as a function of relative grain yields for eggs. These data, spanning a range of almost 10 fold in extent of hybridization, accurately define a single straight line passing through the origin. Second, the slope of this line indicates the poly A content of 4-cell embryos is 2.7±0.4 times greater than that of eggs, which is not significantly different from the value of 2.8 obtained by solution hybridizations. We conclude that these *in situ* methods give accurate estimates of relative

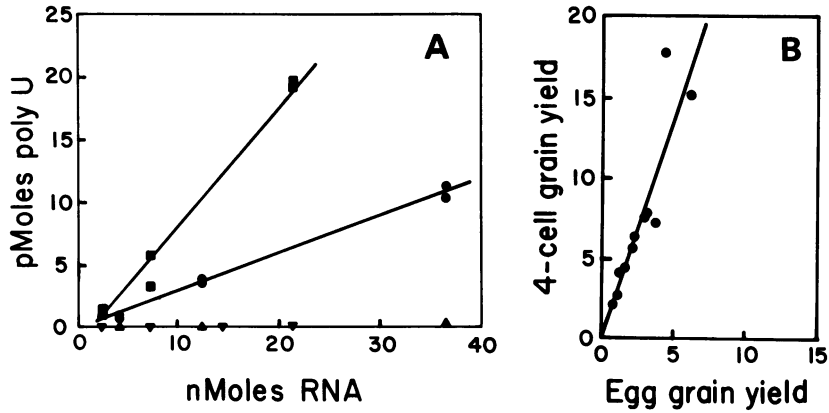

Figure 3. Measurement of Poly A Content of Eggs and 4-cell Embryos by Solution Titration and In Situ Hybridization. (a) Titration of poly A with poly U. Increasing amounts of either egg RNA (●) or 4-cell RNA (■) were added to a constant excess of ^{3}H poly U, annealed, and RNAase A-resistant hybrid measured as described in Materials and Methods. Identical reactions were carried out with ^{3}H poly C as controls; (▲) egg RNA, (▼) 4-cell RNA. Background counts obtained from control reactions containing no egg or 4-cell RNA were subtracted. The lines drawn represent least squares fits to the data. (b) In situ determination of the ratio of poly A contents in egg and 4-cell embryos. Using the data from Table 5, the relative grain yield obtained for 4-cell embryos is plotted as a function of the relative grain yield for eggs. The line represents a least squares fit to the data. Each point represents mean grain yields obtained in different experiments or from different slides from the same experiment. The slope of the line is 2.7 and the y-intercept is 0.

poly A concentration in sections.

Distribution of poly A+ RNA in eggs and early embryos

Available estimates of the total mRNA (24) and of the polyadenylated mRNA content (25) of unfertilized eggs indicate that a major fraction (50% or more) of maternal mRNA is polyadenylated (discussed in Costantini et al., 26). We have used in situ hybridization of ^{3}H-poly U to determine the general distribution of maternal mRNA in unfertilized eggs and early cleavage stage embryos. For this analysis we visually examined a large number of random sections of eggs and 4- and 16-cell embryos. In addition, for eggs and 16-cell embryos we analyzed 20 series of serial sections; a representative series of each is shown in Figure 4. In eggs and 4-cell embryos we found no evidence for enrichment of poly A+ RNA in any specific region of the cytoplasm. Visual examination of 16-cell embryos, however, implied a slightly lower grain density over the cytoplasm of micro-

TABLE 5

Measurements of Relative poly A Concentration in Eggs and 4-cell Embryos by *in situ* Hybridization.

Expt.	Probe[β]	nmoles/slide	Stage	$\#\mu^2$ ($\times 10^{-4}$)	Total grains counted	Relative Grain[α] Yield ($\times 10^3$)	4-CELL/EGG
1 a	U50	0.25	Egg	3.0	732	1.6±0.6 (18)[γ]	2.8
	U50	0.25	4-cell	1.9	1284	4.4±1.9 (16)	
b	U50	0.49	Egg	2.5	832	2.2±0.6 (16)	2.9
	U50	0.49	4-cell	1.5	1422	6.3±2.1 (10)	
c	U500	0.49	Egg	1.8	210	0.8±0.3 (12)	2.7
	U500	0.49	4-cell	1.6	510	2.1±0.9 (12)	
d	U500	0.98	Egg	1.8	291	1.1±0.3 (12)	2.5
	U500	0.98	4-cell	1.7	678	2.7±1.1 (11)	
e	C50	0.84	4-cell	1.2	-11	-0.3±0.1 (12)	-
	C500	0.84	4-cell	1.3	-34	-0.3±0.3 (8)	
2 a	U500	0.1	Egg	2.9	687	1.2±0.5 (15)	3.4
	U500	0.1	4-cell	2.1	1642	4.1±2.3 (15)	
b	U500	1.0	Egg	2.7	1936	3.8±0.5 (15)	1.9
	U500	1.0	4-cell	1.8	2504	7.2±2.6 (15)	
c	U50	0.1	Egg	2.9	1713	3.1±0.7 (16)	2.5
	U50	0.1	4-cell	2.1	3236	7.8±5.6 (13)	
d	U50	0.25	Egg	3.0	1707	2.9±0.6 (15)	2.6
	U50	0.25	4-cell	2.4	3517	7.5±1.3 (15)	
e	U50	0.5	Egg	2.7	1139	2.1±0.7 (16)	2.8
	U50	0.5	4-cell	1.6	1795	5.9±1.9 (14)	
f	U50	1.0	Egg	0.8	950	6.1±1.2 (15)	2.5
	U50	1.0	4-cell	0.8	2316	15.2±6.1 (14)	
g[δ]	U50	0.5	Egg	3.8	3226	4.3±0.9 (18)	4.0[δ]
	U50	0.5	4-cell	1.0	3361	17.8±3.0 (13)	

α: Grains/μ^2/day of exposure/Ci/mmole.
β: The specific activities of poly U and poly C in Experiment 1 were 1.4 and 2.0, respectively. The value for poly U in experiment 2 was 2.3 Ci/mmole. Exposure times were 10.5 and 8.5 days in experiments 1 and 2, respectively.
γ: Values in ( ) refer to number of sections analyzed.
δ: In experiments 3g, the sections were treated with 3 μg/ml proteinase K/ml; all other experiments listed in this table were carried out on sections pretreated with 1 μg proteinase K/ml.

meres. We have quantitated this difference as shown in Figure 5. Each point represents the cytoplasmic grain density (grains/μ^2) for a micromere or pair of micromeres *vs.* that for one or two adjacent macromeres.

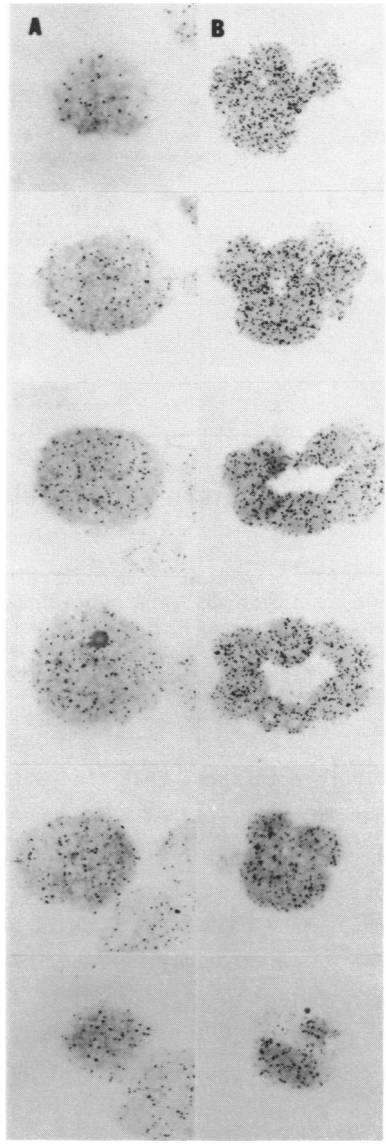

Figure 4. Serial Sections of an Egg and 16-cell Embryo Hybridized In Situ with ^{3}H poly U. (A) Egg or (B) 16-cell embryo serial sections were hybridized with ^{3}H poly U (0.5 nmoles/slide; 2.3 Ci/mmole UMP). Sections 1, 3, 4, 6, 8 and 10 and 1, 2, 3, 6, 7 and 8 are shown for egg and 16-cell, respectively. A complete egg or embryo requires 9-11 5 micron thick sections, all of which could not be obtained due to loss of some sections from the slide. In the 16-cell series, the fourth section contains 2 of the 4 micromeres which lie at the vegetal pole of the embryo. Exposure time was 5.5 days.

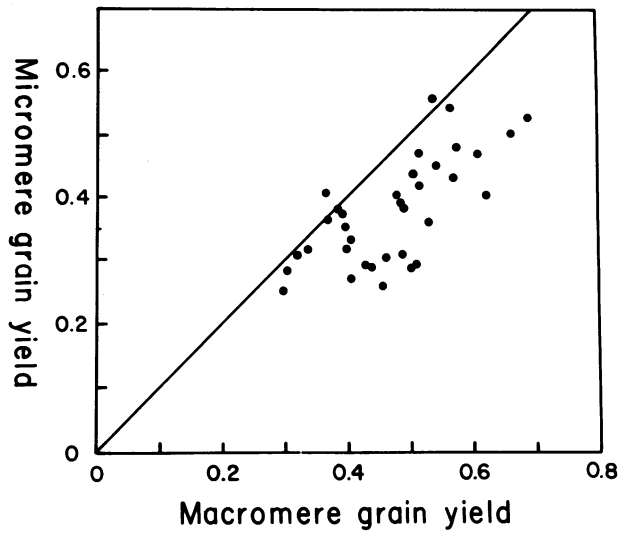

Figure 5. Relative Poly A Concentration in Micromere and Adjacent Macromere Cytoplasm. Sections of 16-cell embryos were hybridized as described in the legend to Figure 4. Exposure time was 12.5 days, resulting in grain densities 2-3 times greater than those shown in Figure 4. For comparison, the solid line represents the expected fit to the data for the case of equal poly A concentrations in micromere and macromere cytoplasm.

Statistical analysis of these data shows that the ratio of grain densities is significantly different from 1.0 (paired-difference test, $t_{34} = 7.78$, $p < 0.001$). The average grain density over micromere cytoplasm is 0.81 that over macromere cytoplasm. We know of no systematic errors which might cause this difference since each comparison is made for adjacent cells which have been treated identically. We conclude that the concentration of poly A in the cytoplasm of micromeres is slightly, but significantly lower than that in the cytoplasm of macromeres.

We also observed a much lower hybridization of poly U to nuclei than to surrounding cytoplasm. Measurements were made of grain density over egg nuclei and cytoplasm for 26 sections after an exposure sufficient to produce heavy cytoplasmic labelling (0.28 grains/μ^2, 2 to 3 fold higher than that in the egg sections illustrated in Fig.4). The nuclear grain density was 0.075 grains/μ^2, a factor of 3.7 lower. This is an overestimate of the true nuclear grain density for two reasons. First, grains over nuclei may actually be derived from poly U bound to cytoplasmic poly A in cases where the nucleus lies in the section below a layer of cytoplasm.

Second, many grains counted as nuclear are just within the boundary of the nuclear membrane, and may also result from hybridization to the adjacent cytoplasm. While exact quantitation of these effects is not possible, it is likely that the true nuclear grain density is at least a factor of two lower than the value directly observed.

DISCUSSION

We have optimized the *in situ* hybridization of poly U to poly A-containing RNAs in sea urchin eggs and embryos. In developing this procedure, we have incorporated some parts of previously described methods (7,11,21). We established glutaraldehyde as the fixative because it affords superior morphological preservation, results in higher retention of RNA during *in situ* procedures and, in conjunction with mild proteinase K digestion prior to hybridization, yields much higher signal to noise ratios. This is true not only because of increased specific hybridization but also because nonspecific binding of ^{3}H poly C is undetectable. Our experiments show that accurate estimates of local RNA concentration can be obtained as demonstrated by the fact that the relative 4-cell and egg poly A contents are the same as determined by *in situ* and solution hybridization.

The specificity of poly U hybridization is demonstrated by 1) absence of labelling with poly C of similar specific activity and fragment length, 2) lack of sensitivity of poly U hybridization to pretreatment of sections with DNAase I or proteinase K, 3) high sensitivity of hybridization to prehybridization treatment with RNAase T2 and 4) the proportionality between known poly A content and *in situ* grain density. Thus, the signal we observe cannot be due to nonspecific electrostatic binding of nucleic acid polymers nor to hybridization to AT-rich segments of mitochondrial DNA. Since the signal increases with proteinase K digestion, it is unlikely that poly U binds to a sequence-specific binding protein in these cells. The reduction in grain density over sections digested with RNAase T2 indicates that the target sequences are RNA molecules.

The extent of hybridization of poly U to poly A in our optimized procedure is much greater for glutaraldehyde- than for Carnoy's-fixed sections. We believe this is primarily due to much higher retention either of poly A^{+} RNA during hybridization or of poly U:poly A duplexes during posthybridization washes. In support of this interpretation is our observation that RNA labelled during the first cleavages, which is largely end-labelled tRNA (27), is retained in 15-20 times higher concentration in glutaraldehyde-

fixed embryos (see Fig.1). Retention of tRNAs offers an appropriate model for that of RNAase-resistant poly A:poly U duplexes since the weight average poly A length is only 45-60 nucleotides (25). Furthermore, when recombinant DNA probes are hybridized in situ to longer RNAs without subsequent RNAase treatment, the signal obtained is only 2-4 times higher for glutaraldehyde-fixed material (our unpublished observations).

Among experiments we observe considerable variability in the extent of hybridization. We have identified several sources of this variation. First, the absolute signal is quite sensitive to extent of proteinase K digestion. When the enzyme concentration is increased 3 fold, the grain density after in situ hybridization at equivalent probe input increases proportionately (Table 1, experiment 3). There is a reasonably narrow optimum for proteinase K digestion which yields maximum hybridization without significant destruction of cytological structure. Second, the hybridization signal depends on the length and amount of hybridizable probe. We observed as much as a 3 fold increase in hybridization when the weight average fragment length was reduced from 500 to 50 nucleotides. However, a fraction of such short probes is probably not hybridizable under our conditions. In hybridizations at probe concentrations less than saturating we observe reduced signals as a function of age of probe, presumably due to an increase in this nonhybridizable fraction. However, we should note that our saturation measurements with probes 500 and 50 nucleotides long (data not shown) imply that fewer poly A sequences are detected by longer probes since apparent saturation values are lower.

Determinations of hybridization efficiency using ^{3}H probes are complicated and are only approximations owing to large and uncertain corrections for self-absorption. Our best estimates of the expected grains/μ^2/day in a 5 μ thick section are calculated as follows, assuming 100% hybridization efficiency and 100% retention of RNA:

$$\frac{8.7\times10^{-15}\ \text{mole poly A}^{(24)}}{\text{4-cell}} \times \frac{7.3\times10^{18}\ \text{disint.}}{\text{(day)(mole poly U)}} \times \frac{5\mu^3/\mu^2}{3.9\times10^{4}\ \mu^3/\text{4-cell}} \times \frac{0.02\ \text{grains}^{(28,29)}}{\text{disint.}} = 0.16$$

Using the highest probe concentration and optimized conditions (Table 5, expt. 2, line f), we obtain a grain density of 0.035 grains/μ^2/day (15.2×10^{-3}grains/μ^2/day/Ci/mmole x 2.3 Ci/mmole). These calculations imply that 22% of the total poly A in a 5 μ section is hybridized.

Despite variations in grain yield among experiments we wish to stress that within any such experiment, accurate estimates of the relative content of target poly A sequences in different sections are obtained when enough random sections are analyzed. This point is underscored by the data presented in Table 5 and Figure 3B which illustrate that the ratio of grain yields for 4-cell embryos and eggs is completely independent of variations in absolute extent of hybridization. Thus, the fraction of target sequences hybridized is independent of their local concentration in the section. This conclusion is consistent with the results of Szabo et al. (30) on the kinetics of in situ hybridization of labelled 5s RNA to Drosophila polytene chromosomes and with our observations using a recombinant DNA probe complementary to sea urchin early histone mRNA variants (31). In both of these cases, the reassociation is observed empirically to obey pseudo first order kinetics with respect to labelled probe. An important consequence of these findings is that local relative concentrations of target sequences in the tissue can be accurately quantitated even at subsaturating probe concentrations.

We have used these techniques to examine the distribution of poly A (and presumably of poly A+ RNA in sea urchin eggs and early embryos. Visual exmination of serial sections of eggs (Figure 4) revealed no major inhomogeneities in the distribution of polyadenylated maternal RNA, al--though grain density differences smaller than 2 fold could easily be detected. Based on these observations we can rule out the possibility of general gradients of poly A+ RNA concentration, for example along the animal-vegetal axis, or in the cortical vs. central cytoplasm. However, in the absence of morphological reference points, we cannot exclude the possibility that some restricted regions of egg cytoplasm may have a slightly different concentration of polyA+ RNA. The same analysis and arguments indicate that the distribution of poly A in 4-cell embryos is also largely homogeneous, although the concentration of poly A increases almost 3 fold between these two stages. These observations indicate that the increase in poly A content after fertilization must also occur relatively uniformly throughout the embryo cytoplasm.

We observed that egg pronuclei and nuclei of 4 or 16-cell embryos have much lower grain densities than does the surrounding cytoplasm. We have shown previously that egg nuclei are not refractory to hybridization probes since we have measured high concentrations of pronuclear RNA transcripts complementary to an early histone gene repeat unit (31). Direct

measurements indicate that the grain density due to poly U hybridization is at least 3.7 fold lower over nuclei than over surrounding cytoplasm. We estimate the maximum extent of polyadenylation of egg pronuclear RNA as follows: Brandhorst has recently shown that the egg contains 11 pg of RNA which can be radioactively labelled; about 30% of this, or 3.3 pg, is located in the pronucleus at steady state (32). This corresponds to 5.4×10^9 NT of nuclear RNA, which is a minimum value since there may exist stable nuclear RNAs not labelled in Brandhorst's studies. Using the maximum estimate for nuclear grain density (0.27 that of cytoplasm), the total amount of poly A in the egg (1.9×10^9 NT) and the volume ratio of nucleus to cytoplasm (1:375; 31), we estimate that no more than 0.025% of the mass of egg pronuclear RNA is poly A. In contrast, Dubroff and Nemer (33) have determined the extent of polyadenylation of nuclear RNA labelled for 1 hr at various times between 4 and 20 hrs of development. Since the half life of nuclear RNA is approximately 20 min (24), this labelling should approach the steady state distribution. From the data of Dubroff and Nemer (33,34) we calculate that 0.5-0.8% of nuclear RNA is poly A during this period, a value which is 20 fold greater than our maximum estimates for egg pronuclear RNA. We conclude that the relatively large amount of RNA in egg pronuclei [at least 3 pg (32) vs. about 0.2 at later stages (24)] is markedly underpolyadenylated.

Quantitation of the poly A concentration in micromeres (Fig.5) indicates that it is about 20% lower than in macromeres. It is possible that the average poly A tract is 20% shorter in micromere RNA. However, it is interesting to compare this result to solution hybridization measurements which show that micromeres lack approximately 25% of the sequence complexity found in the rest of the embryo (35,36). Our results are consistent with the idea that the reduction of micromere RNA complexity is accompanied by a significant reduction in the mass of poly A+ RNA.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Joanna Olmsted for the use of her microscope; Dr. Martin Gorovsky for helpful comments on the manuscript; Dr. David Swinton for help in writing and adapting a computer program for digitizing section areas, and Dr. Thomas Caraco for assistance with statistical analyses. This work was supported by a grant from the National Institutes of General Medical Sciences.

REFERENCES

1. Harrison, P.R., Conkie, D., Affara, N., and Paul, J. (1974) J. Cell Biol. 165, 54-59.
2. Moar, M.H. and Jones, K.W. (1975) Int. J. Cancer 16, 998-1007.
3. Clissold, P.M., Arnstein, H.R.V. and Chesterton, C.J. (1977) Cell 11, 353-361.
4. John, H.A., Patrinou-Georgoulas, M. and Jones, K.W. (1977) Cell 12, 501-508.
5. Neer, A., Bara, N. and Manor, H. (1977) Cell 11, 65-71.
6. Harding, J.D., MacDonald, R.J., Przybyla, A.E., Chirgwin, J.M., Pietet, R.L. and Rutter, W.J. (1977) J. Biol. Chem. 252, 7391-7397.
7. Brahic, M. and Haase, A.T. (1978) Proc. Nat. Acad. Sci. USA 75, 6125-6129.
8. Minty, A.J., Birnie, G.D. and Paul, J. (1978) Exp. Cell Res. 115, 1-14.
9. Godard, C. and Jones, J.W. (1979) Nuc. Acids Res. 6, 2849-2861.
10. Kaufman, S.L., Gallo, R.C. and Miller, N.R. (1979) J. Virol. 30, 637-641.
11. Capco, D.G. and Jeffery, W.R. (1978) Dev. Biol. 67, 137-157.
12. Jeffery, W.R. and Capco, D.G. (1978) Dev. Biol. 67, 152-166.
13. Capco, D.G. and Jeffery, W.R. (1979) J. Cell Sci. 39, 63-76.
14. Hough-Evans, B.R., Wold, B.J., Ernst, S.G., Britten, R.J. and Davidson, E.H. (1977) Dev. Biol. 60, 152-166.
15. Maxwell, I.H., Maxwell, F. and Hahn, W.E. (1977) Nuc. Acids Res. 4, 241-246.
16. Jones, K.W., Bishop, J.O. and Brito-da-Cunha, A. (1973) Chromsoma 43, 375-390.
17. Darnbrough, C. and Ford, P.J. (1976) Dev. Biol. 50, 285-301.
18. Gall, J.G. and Pardue, M.L. (1971) In Methods in Enzymology 38, 470-480.
19. Hayashi, S., Gillam, I.C., Delaney, A.D. and Tener, G.M. (1978) J. Histochem. Cytochem. 26, 677-679.
20. Wilt, F.H. (1973) Proc. Nat. Acad. Sci. USA 70, 2345-2349.
21. Godard, C. and Jones, K.W. (1980) Histochemistry 65, 291-300.
22. Mescher, A. and Hymphreys, T. (1974) Nature 249, 138-139.
23. Slater, I. and Slater, D.W. (1972) Nature 240, 333-337.
24. Davidson, E.H. (1976) In Gene Activity in Early Development, Chapter 7, Academic Press; and our measurements of poly A content.
25. Wilt, F.H. (1977) Cell 11, 673-681.
26. Costantini, F.D., Britten, R.J. and Davidson, E.H. (1980) Nature 287, 111-117.
27. Gross, P.R., Malkin, L.I. and Moyer, W.A. (1964) Proc. Natl. Acad. Sci. USA 51, 407-414.

28. Pelc, S.R. and Welton, M.G.E. (1967) Nature 216, 925-927.

29. Ada, G.L., Humphrey, J.H., Askonas, B.A., McDevitt, H.O. and Nossal, G.V. (1966) Exp. Cell Res. 41, 557-572.

30. Szabo, P., Elder, R., Steffensen, D.M. and Uhlenbeck, O.C. (1977) J. Mol. Biol. 115, 539-561.

31. Venezky, D.V., Angerer, L.M. and Angerer, R.C. (1981) Cell 24, 385-391.

32. Brandhorst, B. (1980) Dev. Biol. 79, 139-148.

33. Dubroff, L.M. and Nemer, M. (1976) Nature 260, 120-124.

34. Dubroff, L.M. and Nemer, M. (1975) J. Mol. Biol. 95, 455-476.

35. Rodgers, W.H. and Gross, P.R. (1978) Cell 14, 279-288.

36. Ernst, S.G., Hough-Evans, B.R., Britten, R.J. and Davidson, E.H. (1980) Dev. Biol. 79, 119-127.