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Actin Gene Expression in Developing Sea Urchin Embryos

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We show that the synthesis of actin is regulated developmentally during early sea urchin embryogenesis and that the level of synthesis of this protein parallels the steady-state amounts of the actin messenger ribonucleic acids (RNA). An in vitro translation and RNA blotting analysis of embryo RNA from several stages of early development indicated that during the first 8 h after fertilization there was a low and relatively constant level of actin messenger RNA in the embryo. Between 8 and 13 h of development, the amount of actin messenger RNA began to increase both in the cytoplasm and on polysomes, and by 18 h the amounts of actin message per embryo had risen between approximately 10- and 25-fold in the cytoplasm and between 15- and 40-fold on polysomes. Two size classes of actin messenger RNA (2.2 and 1.8 kilobases) were identified in unfertilized eggs and in all of the developmental stages examined. The amount of each actin message class increased over a similar time interval during early development. However, the amounts of these size classes in the cytoplasm relative to each other shifted between the earliest stages examined (2 to 5 h) and the hatching blastula stage (18 h), with the ratio of the 1.8-kilobase actin messenger RNA to the 2.2-kilobase actin messenger RNA increasing almost threefold during this period.

Sea urchin eggs and early embryos contain large quantities of stored maternal messenger ribonucleic acid (mRNA), a fraction of which is mobilized at fertilization for translation on polysomes (6, 9). In addition, detectable levels of newly synthesized mRNA appear on polysomes early in development (4, 12–14, 22). Therefore, gene expression during early sea urchin development is probably regulated at both transcriptional and post-transcriptional levels.

Hough-Evans et al. (11) have demonstrated that a large fraction of the sequences in the complex class of sea urchin egg RNA are represented on polysomes throughout early development, beginning as high as 73% in 16-cell embryos and dropping only to 38% in pluteus stage embryos. These results suggest that many of the sequences in the complex class of egg RNA encode proteins which are synthesized throughout early development. The mechanism by which the concentrations of these mRNA sequences are regulated in the embryo is unclear. Experiments which measure the variation in prevalence during development of a large number of different RNA transcripts by using cloned deoxyribonucleic acid (DNA) sequences as probes have revealed several patterns of developmental expression. Transcripts complementary to two cloned sequences which are represented in the complex class of sea urchin egg RNA have been shown to disappear from the cytoplasm almost completely by the blastula stage (18). In this case it is possible that only maternal RNA molecules encoding these sequences are translated during early development. Lasky et al. (17) have assayed the prevalence, at several developmental stages, of a large number of specific RNA transcripts which are abundant in the cytoplasm of early embryos. They found that, in general, sequences which are prevalent in the cytoplasm of gastrula and pluteus stage embryos are also prevalent in egg RNA. However, they also reported examples of sequences which are present at high and moderate levels in later stages but are not detectable in eggs. Shepherd and Nemer (27) have found that of the mRNA species which are abundant on sea urchin blastula polysomes, 15% remain abundant in gastrula, 15% decrease to a somewhat rarer frequency, and 70% essentially disappear from polysomes. Thus, it is clear that the patterns of developmental expression of individual mRNA sequences differ considerably and may be determined by different mechanisms; that is, some embryonic mRNA's may be supplied entirely by the maternal mRNA pool, others may be supplied entirely by new transcription, and still others may be supplied by a combination of these mechanisms.

To examine the question of differential gene

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expression during development more closely, we studied the expression of the actin genes. We chose actin for the study of gene expression during sea urchin development for several reasons. The actin genes are members of a multigene family whose protein products appear to be involved in several essential cellular functions (8, 26). Also, these protein products are readily identifiable by one- and two-dimensional gel electrophoresis techniques. Thus, it is possible to determine the amount and location of the final gene product, as well as the RNA intermediates, and it may be possible to correlate developmental expression with protein function. The experiments described here demonstrate that the expression of actin genes is regulated developmentally in sea urchin embryos. We report that the synthesis of actin increases sharply between 8 and 18 h of development and that the steady-state amounts of two size classes of polysomal actin mRNA increase approximately 14- and 40-fold at this time during development. We also show that maternal actin mRNA is present in unfertilized eggs and that the amount of actin-encoding RNA per embryo remains similar to the amount in eggs through the first 8 h of development. It is possible that the maternal actin mRNA supports most actin synthesis through the first 8 h of development and that after this new actin messages begin to enter the cytoplasm, allowing a much higher rate of actin synthesis.

MATERIALS AND METHODS

Analysis of in vivo- and in vitro-labeled proteins. *Strongylocenteotus purpuratus* embryos were grown at a concentration of 10^4 embryos per ml in membrane-filtered (Millipore Corp.) seawater containing 10 U of penicillin per ml at 15°C. Embryos from selected stages of development were labeled for 1 h with [^{35}S]methionine (100 μCi/ml; Amersham). After labeling, the embryos were washed with seawater and lysed with lysis buffer (23). In vitro translation of purified embryo RNA in a message-dependent reticulocyte lysate and sodium dodecyl sulfate-polyacrylamide gel analyses of in vivo- and in vitro-labeled proteins were performed as previously described (7).

Preparation of RNA. RNAs were isolated from embryos at selected stages of development as follows. The embryos were collected by centrifugation in a Sorvall GSA rotor for 5 min at 4,000 rpm, suspended at a concentration of 4×10^5 embryos per ml in a solution containing 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.5), 500 mM KCl, 12 mM $MgCl_2$, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid, 250 mM sucrose, 50 μg of polyvinyl sulfate per ml, and 0.5% diethylpyrocarbonate, and lysed in a glass homogenizer. To isolate RNA for in vitro translation, diethylpyrocarbonate was omitted, and 1 to 10 mM vanadyl adenosine (3) was added as an ribonuclease inhibitor. The homogenate was centrifuged at 12,000 rpm and 4°C for 15 min in a Sorvall HB-4 rotor to remove nuclei and mitochondria.

Polysomes were prepared by diluting each supernatant 5:8 and layering the resulting sample onto a 5 to 40% isokinetic sucrose gradient containing 10 mM PIPES (pH 6.5), 200 mM KCl, and 5 mM $MgCl_2$; these gradients were then centrifuged for 3.5 h at 27,000 rpm in an SW27 rotor. The >100S regions of the gradients were pooled as polysomes, and the <100S regions were pooled as nonpolysomal. When the polysomal material was to be ethylenediaminetetraacetic acid (EDTA) released (25), the polysomes were pelleted by centrifugation in an SW27 rotor at 27,000 rpm for 18 h. The pellets were suspended in 10 mM PIPES (pH 6.5)–50 mM KCl–1 mM EDTA, incubated for 1 to 2 h on ice, and then centrifuged on 5 to 40% sucrose gradients. The >100S regions of the gradients were pooled as nonpolysomal, and the <100S material was pooled as released polysome-associated material. The RNA fractions isolated from the sucrose gradients were extracted with phenol-IAC (IAC is 24 parts of chloroform plus 1 part of isoamyl alcohol).

To prepare total cytoplasmic RNA, the 12,000-rpm supernatant was brought to 4% (wt/vol) sodium lauroyl-*n*-sarcosinate and 0.5 g of CsCl per ml. This solution was heated to 65°C for 5 min, layered onto a 1.5-ml cushion of 5.7 M CsCl, and centrifuged at 36,000 rpm for 13.5 h in an SW50.1 rotor. The RNA pellets were suspended in guanidine hydrochloride and precipitated as described by Chirgwin et al. (5).

Polyadenylic acid-containing [poly(A)$^+$] RNA was prepared from total polysomal or cytoplasmic RNA by oligodeoxythymidylic acid cellulose chromatography (2).

RNA blotting experiments. RNAs isolated from selected developmental stages were blotted by two separate procedures. In some experiments the RNA was denatured, electrophoresed, and transferred to diazobenzyloxymethyl paper as described by Alwine et al. (1). In other experiments the RNA was denatured, electrophoresed, and transferred to nitrocellulose paper as follows (B. Seed and D. Goldberg, unpublished data). The RNA samples were made 50% formamide, 6% formaldehyde, 20 mM morpholinepropanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0). These samples were incubated at 60°C for 5 min and then separated on 1% agarose gels in 20 mM morpholinepropanesulfonic acid–5 mM sodium acetate–1 mM EDTA (pH 7.0). Each gel was washed several times with water and then washed for 45 min in 50 mM NaOH–100 mM NaCl, for 30 min in 0.5 M tris(hydroxymethyl)aminomethane (pH 7.6)–1.5 M NaCl, and for 30 min in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The RNA was transferred to nitrocellulose paper in 20× SSC. The nitrocellulose filter was washed in 3× SSC and baked at 80°C for 2 to 3 h. The filters containing the transferred RNAs from both procedures were prehybridized at 42°C for 8 h in a solution containing 50% formamide, 0.75 M NaCl, 0.075 M sodium citrate, 50 mM phosphate buffer (pH 6.5), 0.02% (wt/vol) Ficoll, 0.02% (wt/vol) polyvinylpyrrolidone, 20 μg of nuclease-free bovine serum albumin per ml, 1 mg of sonicated salmon sperm DNA per ml, and 1% (wt/vol) glycine. The ^{32}P-labeled probe was hybridized at 42°C for 18 h

in the same buffer minus glycine but containing 10% dextran sulfate. After hybridization, the filters were washed six to eight times over a period of 7 h with 30 ml of 50% formamide–5× SSC–50 mM phosphate buffer at 42°C. The filters were dried and exposed to prefogged Kodak XR-5 film at −70°C (16). The sizes of the actin-encoding RNA molecules were determined by comparing them with 18S and 26S sea urchin ribosomal RNA and four pBR322 restriction fragment markers.

Preparation of ^{32}P-labeled hybridization probe. In all of the experiments described here, the hybridization probe was derived from the *S. purpuratus* actin-encoding plasmid pSpG17. This plasmid contains a fragment of sea urchin genomic DNA which codes for actin (7) and no repetitive sequences other than actin-encoding sequences (unpublished data). In some experiments the entire plasmid was nick-translated and used as a hybridization probe. In other experiments a 0.525-kilobase (kb) *Xho-Pst* subfragment was prepared by cleaving 10 μg of pSpG17 DNA with *Xho* and *Pst* and separating the desired fragment on a 7% polyacrylamide gel. The fragment was eluted from the gel as described by Maxam and Gilbert (20) and nick-translated. This fragment has been shown to contain approximately 0.365 kb of actin-encoding sequence and 0.160 kb of intron-specific sequence (unpublished data). The DNA probes were nick-translated to high specific activities with three α-^{32}P-labeled nucleotides, as described by Maniatis et al. (19).

RESULTS

Differences in actin synthesis during early sea urchin development are reflected in actin mRNA levels. We studied actin synthesis during early sea urchin development by labeling embryos at selected stages for 1 h with [^{35}S]methionine. The proteins were extracted and separated on one-dimensional sodium dodecyl sulfate-polyacrylamide gels. Figure 1A shows that the amounts of actin synthesized relative to other proteins were much greater at the 23-h (blastula), 46-h (gastrula), 70-h (prism), and 100-h (pluteus) stages than in 8-h embryos, indicating a dramatic increase in actin synthesis between 8 and 23 h.

Since early sea urchin embryos contain large amounts of maternal mRNA, the increase in actin synthesis might have been due either to a differentially regulated "unmasking" of maternal actin mRNA's or to the appearance of new actin messages in the cytoplasm. To distinguish between these two possibilities, polysomal and cytoplasmic RNAs from several stages of development were translated in vitro in a rabbit reticulocyte lysate, and the ^{35}S-labeled products were separated on sodium dodecyl sulfate-polyacrylamide gels (Fig. 1B). Translation of polysomal RNAs from 5-h (16-cell stage) (Fig. 1B, lane 5) and 18-h (hatching blastula) (lane 6) embryos reflected the in vivo labeling results; that is, at the 16-cell stage very little translatable actin mRNA was present on polysomes, whereas at the blastula stage the actin mRNA was a major, abundant message. Translation of total egg RNA (Fig. 1B, lane 1), total 2-cell RNA (lane 2), 16-cell cytoplasmic RNA (lane 3), and hatching blastula cytoplasmic RNA (lane 4) also demonstrated increasing amounts of actin-encoding RNA during development. The low amounts of translatable actin message in the cytoplasm of the egg and early developmental stages (2- and 5-h embryos) suggested that the levels of maternal actin mRNA in the egg and early embryo were not sufficient to account for the dramatic increase in actin synthesis which occurred after 8 h. Therefore, this increase must have resulted either from the appearance of new actin mRNA molecules in the cytoplasm during development or from a modification of the structure of actin-encoding RNAs so that they produced the 42,000-dalton actin proteins in an in vitro translation system (e.g., RNA processing).

The 42,000-dalton in vivo- and in vitro-synthesized proteins observed in these experiments bound specifically to deoxyribonuclease I (unpublished data). In addition, a two-dimensional gel analysis of these proteins indicated that they resolved into at least three isoelectric variants, which had isoelectric points similar to those reported for actins from other species (7; unpublished data).

Two size classes of polysome-associated actin mRNA's are regulated developmentally. The sizes of the actin mRNA's and their relative amounts on polysomes during early development were examined by RNA blotting analyses of polysomal RNAs isolated from five developmental stages (2, 5, 8, 13, and 18 h after fertilization), using as a probe the sea urchin actin-encoding recombinant plasmid pSpG17 (7). Figure 2 shows the results of such an analysis of polysomal poly(A)$^+$ RNA (Fig. 2A) and total polysomal RNA (Fig. 2B). In the experiment shown in Fig. 2A, the poly(A)$^+$ RNA from similar amounts of total polysomal RNA was loaded in each lane, and in the experiment shown in Fig. 2B each lane contained a similar amount of total polysomal RNA. Therefore, the relative intensities of the actin-encoding bands in lanes a through e of Fig. 2A and B are direct indications of the relative amounts of these molecules in the total polysomal and poly(A)$^+$ polysomal RNAs at the different developmental stages; lane f of each panel is a lighter exposure of lane e. It is apparent that the amount of actin mRNA increased markedly during the first 18 h of development, with the most dramatic changes occurring between 8 and 18 h after fertilization

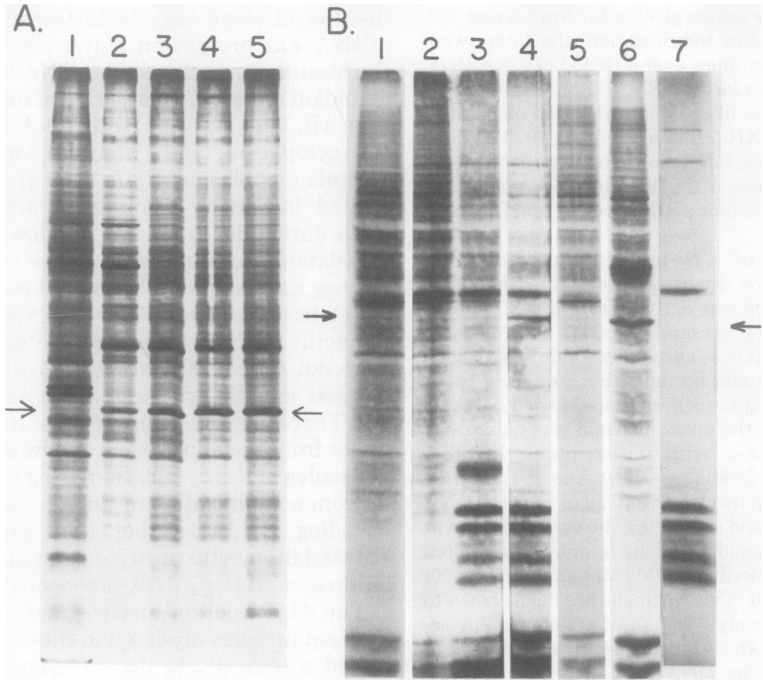

FIG. 1. *Actin synthesis during early sea urchin development. (A) In vivo-labeled proteins synthesized during a 1-h pulse with [^{35}S]methionine. Embryos from different stages of development were labeled for 1 h with [^{35}S]methionine, the proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15), and the gels were autoradiographed. Lanes 1 through 5, Embryos labeled at 8, 23, 46, 70, and 100 h, respectively. The arrows indicate the position of actin. (B) In vitro-labeled proteins synthesized from embryo RNA. Total, cytoplasmic, or polysomal RNA from eggs or from 2-, 5-, or 18-h embryos was isolated and purified. The RNA was translated in a message-dependent reticulocyte lysate (24), the products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the gels were autoradiographed. Lanes 1 and 2, Proteins synthesized from total RNA isolated from eggs and 2-h embryos, respectively; lanes 3 and 4, proteins synthesized from total cytoplasmic RNA from 5- and 18-h embryos, respectively; lanes 5 and 6, proteins synthesized from polysomal RNA from 5- and 18-h embryos, respectively; lane 7, no added RNA. Lanes 2, 3, 4, and 7 were exposed for 10 days, and lanes 1, 5, and 6 were exposed for 12 h. The arrows indicate the position of actin.*

(Fig. 2A and B). This result correlated directly with the synthesis of actin protein and with the amount of translatable actin mRNA (Fig. 1).

Two different size classes of actin mRNA which bound to oligodeoxythymidylic acid cellulose were present on polysomes (Fig. 2A). The sizes of these mRNA's were determined to be 2.2 and 1.8 kb by comparison with denatured RNA and DNA markers. The larger message class (2.2 kb) was detectable as early as 2 h after fertilization and increased in amount significantly between 8 and 18 h. In contrast, the smaller message class (1.8 kb) was not detectable under these conditions until after 8 h. We did not observe this 1.8-kb band at early stages in two separate experiments or with exposures up to six times longer than that shown here. However, by 13 h the 1.8-kb message was clearly evident, and at 18 h it appeared to be slightly more intense than the 2.2-kb band. Therefore, the relative amounts of these two different poly(A)$^+$ mRNA classes on polysomes changed noticeably during development. In addition, a similar blotting analysis of polysomal RNAs from 5-, 8-, and 13-h embryos which did not bind to an oligodeoxythymidylic acid cellulose column indicated that both the 2.2- and 1.8-kb bands were present in the poly(A)$^-$ fraction at these stages (data not shown).

Figure 2B shows that the total polysomal RNA in developing embryos contained the same two size classes of actin-encoding RNAs observed in polysomal poly(A)$^+$ RNA and that the amounts of these RNAs increased in a similar manner during development. However, in contrast to poly(A)$^+$ RNA, the 1.8-kb mRNA was detectable at the earlier stages (2, 5, and 8 h), indicating that a 1.8-kb message was present on polysomes which did not bind to oligodeoxythymidylic acid cellulose. In addition, the inten-

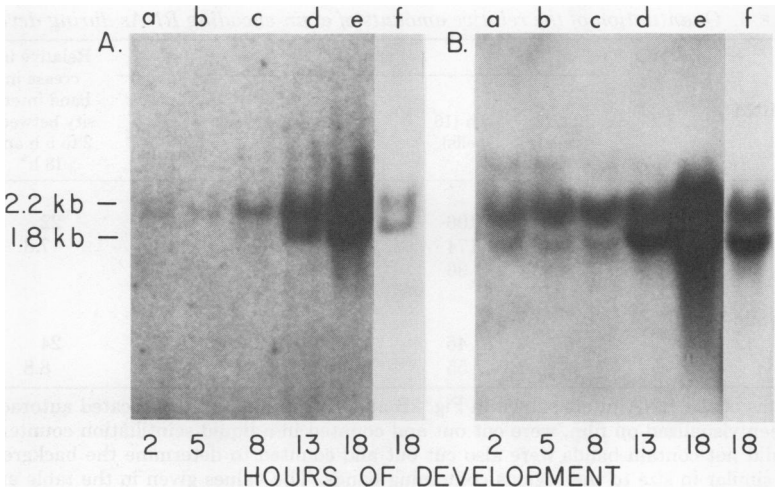

FIG. 2. *RNA blot analysis of actin-encoding polysomal RNAs at selected developmental stages. Polysomal RNA from 2-, 5-, 8-, 13-, and 18-h embryos was denatured in glyoxal and formamide, electrophoresed on 1% agarose gels, and transferred to diazobenzyloxymethyl paper; 0.5 μg of ^{32}P-labeled pSpG17 DNA containing an actin-encoding sequence was hybridized to the RNA on each filter. (A) A 30-μg sample of total polysomal RNA from each developmental stage was passed once over an oligodeoxythymidylic acid cellulose column. All of the RNA which bound to the column [poly(A)$^+$ enriched] was then loaded into one lane on the gel. The time of isolation is indicated on the figure. The specific activity of the probe was 5.2 × 10^7 cmp/μg. Lanes a through e were exposed for 8 days with preflashed Kodak XR5 film with an intensifying screen. Lane f shows a 20-h exposure of lane e under similar conditions. (B) A 30-μg sample of total polysomal RNA from each developmental stage was loaded into one lane of the gel. The time of isolation is indicated on the figure. The specific activity of the probe was 3.8 × 10^8 cpm/μg. Lanes a through e were exposed for 44 h as described above. Lane f shows a 40-h exposure of lane e in which Kodak no-screen film was used without an intensifying screen.*

sities of the 2.2- and 1.8-kb bands relative to each other shifted during development, with the 2.2-kb band being more intense at 2 and 5 h and the 1.8-kb band becoming more intense at 13 and 18 h (Fig. 2 and Table 1). Thus, it appears that although the overall amounts of these two actin message size classes increased during development, their levels relative to each other also changed in both the total polysomal and poly(A)$^+$ polysomal RNA populations.

The hybridization probe used in these experiments was the *S. purpuratus* actin-encoding genomic plasmid pSpG17 (7), which contains both actin-encoding and non-actin-encoding sequences. Since this plasmid does not contain repetitive sequences other than the sequence which encodes actin (unpublished data), it seemed unlikely that any non-actin-encoding RNA molecule would hybridize with the probe. To verify this assumption, we used a 0.525-kb *Pst-Xho* subfragment of the cloned sea urchin sequence which contained approximately 0.365 kb of actin-encoding sequence and 0.160 kb of intron as a hybridization probe on RNA blots of cytoplasmic poly(A)$^+$ RNA from 18-h embryos (hatching blastula). Figure 3A shows that both the 2.2-kb band and the 1.8-kb band hybridized with this probe, indicating that both contained an actin-encoding sequence.

In these experiments the RNA which sedimented at >100S on a polysome gradient was considered to be polysomal. To verify that the actin-encoding RNA which sedimented at >100S in a polysome gradient was polysome associated, the >100S portion of a polysome gradient from 40-h embryos (gastrula) was treated with EDTA and resedimented on a second gradient containing EDTA (25). The RNAs from the >100S and <100S portions of this second gradient were then purified and subjected to RNA blot analysis. Figure 3B, which shows the 2.2- and 1.8-kb actin bands from 18-h embryos (blastula) (Fig. 3B, lane 1) and 40-h embryos (gastrula) (lane 2), demonstrates that both RNA bands were released from polysomes by EDTA (lane 3) and that there were essentially no actin-encoding RNAs which still sedimented at >100S after EDTA release (lane 4). Thus, the two actin-encoding bands (2.2 and 1.8 kb) which sedimented at >100S before EDTA release were both polysome associated.

Cytopasmic actin-encoding RNA molecules are similar to polysomal actin mRNA. The in vitro translation of total and cytoplasmic RNAs from early developmental stages shown in Fig. 1B indicated that there were not high

TABLE 1. *Quantitation of the relative amounts of actin-encoding RNAs during development*

RNA	cpm at:[a]					Relative increase in band intensity between 2 to 5 h and 18 h[b]	Relative increase in amount per embryo between 2 to 5 h and 18 h[c]
	2 h (2 cells)	5 h (16 cells)	8 h	13 h	18 h (hatching blastula)		
Polysomal (total)							
1.8 kb	83	106	116	334	2,087	22	40
2.2 kb	144	174	152	237	1,242	7.8	14
~4 kb	38	36	11	28			
Cytoplasmic (total)							
1.8 kb		46	47	109	1,106	24	24
2.2 kb		55	50	68	482	8.8	8.8

[a] The regions of the RNA filters shown in Fig. 2B and 4B containing the indicated autoradiographic bands, which had been visualized on film, were cut out and counted in a liquid scintillation counter. Three separate areas which did not contain bands were also cut out and counted to determine the background level. These regions were similar in size to the regions containing bands. The values given in the table are the counts per minute above the background level. The background level was 90 cpm.

[b] Ratio of counts per minute at 18 h to counts per minute at 2 to 5 h. The counts per minute for 2 and 5 h were averaged when both values were available. If only the 5-h value was available, it was used.

[c] In the case of polysomal RNA, values were obtained by multiplying the relative increase in band intensity between 2 to 5 h and 18 h by the relative increase in polysome content between these two stages, which was determined by Infante and Nemer (13) to be 1.8. For the cytoplasmic RNAs no correction was made since the amount of cytoplasmic RNA remains relatively constant during early development.

levels of translatable actin maternal mRNA in the early embryos. However, we wanted to determine whether there were additional actin-encoding RNAs in the cytoplasm which did not correspond in size and relative amount to the RNAs on polysomes. To analyze the cytoplasmic actin-encoding RNA molecules, cytoplasmic poly(A)$^+$ RNA and total cytoplasmic RNA were examined in RNA blot experiments (Fig. 4). As with the polysomal RNAs, the amounts of the actin-encoding RNAs increased strikingly during development, with a detectable increase apparent by 13 h. The same two size classes (2.2 and 1.8 kb) were also present. Figure 4A shows that the accumulation of poly(A)$^+$ actin-encoding RNA was quite similar to that observed on polysomes; that is, at the earlier stages (5 and 8 h) only a 2.2-kb band was observed, whereas at the later stages (13 and 18 h) both a 2.2-kb band and a 1.8-kb band were observed in increasing amounts. Figure 4B shows the accumulation of total cytoplasmic actin-encoding RNA. As with total polysomal RNA, both mRNA size classes were evident in all stages, and the ratio of the 1.8-kb band to the 2.2-kb band increased during development (Table 1). Thus, it is clear that there were no size classes of nonpolysomal actin-encoding RNAs which were detectably different from those on polysomes and that the ratio between the 2.2-kb RNA and the 1.8-kb RNA in the cytoplasm at any given stage was similar to the ratio of these two actin RNA size classes on polysomes.

Actin mRNA is present in unfertilized eggs and shows no detectable change at fertilization. Figures 2 and 4 show that the actin mRNA's were present at relatively low levels in the cytoplasm and on polysomes at early developmental stages (i.e., 2 and 5 h). We next tried to determine whether these actin messages were present in eggs at fertilization (i.e., maternal actin mRNA) or whether they resulted entirely from the synthesis of new actin-encoding molecules during the first few hours of development. To distinguish between these possibilities, we fractionated RNAs from unfertilized eggs and 1- and 2-h embryos into material which sedimented at >100S (polysomal) and material which sedimented at <100S (nonpolysomal) on a sucrose gradient. RNAs from these fractions were then analyzed by RNA blotting to detect any actin-encoding molecules (Fig. 5). The two known actin-encoding RNAs (2.2 and 1.8 kb) and a larger RNA (~4 kb) (see below) were present in both the >100S fractions and the <100S fractions of egg RNA and 1- and 2-h embryo RNA and were present in similar amounts in each stage. The material in the >100S fraction from the 2-h embryos was EDTA released and run on a second sucrose gradient to verify that the bands in the >100S fraction were polysome associated and not rapidly sedimenting nonpolysomal ribonucleoprotein particles. The RNAs from the >100S and <100S fractions on the second gradient were blotted and probed for actin-encoding sequences. Figure 5 shows

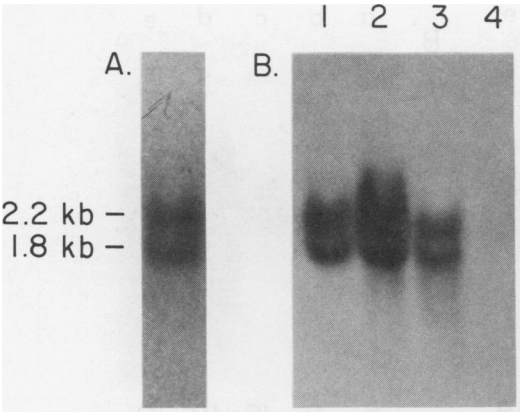

FIG. 3. *RNA blot analysis of cytoplasmic poly(A)$^+$ RNA, in which an actin-encoding specific probe was used, and EDTA release of actin-encoding RNA from polysomes. (A) A 33-μg sample of cytoplasmic RNA from 18-h embryos was passed once over oligodeoxythymidylic acid cellulose. The RNA that bound to the column [poly(A)$^+$ enriched] was recovered, denatured in glyoxal and formamide, electrophoresed on a 1% agarose gel, and transferred to diazobenzyloxymethyl paper. The RNA on the filter was then hybridized with 2 × 10^7 cpm of a ^{32}P-labeled Xho-Pst fragment from the actin-encoding plasmid pSpG17. This fragment contains approximately 0.365 kb of actin-encoding sequence and 0.160 kb of intron-specific sequence (unpublished data). (B) RNA was denatured in glyoxal and formamide, electrophoresed on a 1% agarose gel, transferred to diazobenzyloxymethyl paper, and hybridized with 0.5 μg of ^{32}P-labeled pSpG17 DNA (specific activity, 1.4 × 10^9 cpm/μg). Lane 1, 30 μg of RNA from the >100S fraction of a polysomal gradient from 18-h embryos; lane 2, 30 μg of RNA from the >100S fraction of a polysomal gradient from 40-h embryos; lane 3, 30 μg of RNA from the <100S fraction of an EDTA release gradient of material from 40-h embryos; lane 4, 30 μg of RNA from the >100S fraction of an EDTA release gradient of material from 40-h embryos.*

that most of the actin-encoding RNA was in the released (<100S) fraction (Fig. 5, lane 7), with smaller amounts appearing in the >100S fraction (lane 8). Since virtually all of the ribosomal RNA was released into the <100S fraction and equal amounts of RNA from the >100S and <100S fractions were loaded onto the gel, significantly more >100S RNA was loaded on a per embryo basis. Therefore, we concluded that essentially all of the >100S actin-encoding RNA molecules in the initial polysome gradient were released by the EDTA treatment and were polysome associated.

The egg preparation and the 1- and 2-h embryos each contained three hybridization bands in the >100S and <100S fractions, indicating that these sequences were both on and off polysomes. In each of the three cases shown in Fig. 5, approximately one-half as much >100S RNA was loaded per lane as <100S. The yields of RNA from the >100S and <100S fractions indicated that only 10, 17.5, and 19% of the RNA were in polysomes in egg and 1- and 2-h embryos, respectively. Therefore, the amount of RNA loaded on a per embryo basis was between two and five times lower in the <100S fraction than in the >100S fraction. It follows from an inspection of the band intensities, where the <100S band was more intense in every case, that most of these actin-encoding RNA molecules were off polysomes in eggs and through 2 h of development.

The approximately 4-kb hybridization band which was evident in eggs and in 1- and 2-h embryos (Fig. 5) was also present in total polysomal RNA at 2, 5, 8, and 13 h of development (Fig. 2B) and in poly(A)$^-$ RNA at 8 and 13 h of development (data not shown). This band did not increase in intensity during development as the 2.2- and 1.8-kb bands did. In fact, its intensity may have decreased slightly between 2 and 13 h (Fig. 2B and Table 1). This RNA is of considerable potential interest since it may represent a larger actin mRNA which is not developmentally regulated. However, our data here did not demonstrate that it contains an actin-encoding sequence. It is possible that it is an RNA which contains a sequence complementary to a non-actin-encoding sequence on pSpG17.

DISCUSSION

From the data in Fig. 1, 2, and 4, we concluded that the amount of actin mRNA increases strikingly during early sea urchin development and that the rate of synthesis of actin relative to other proteins increases similarly during the same developmental period. An increase in the level of actin-encoding RNA has been noted previously by Merlino et al. (21). An inspection of the RNA blot experiments indicates that the two size classes of actin mRNA are present in relatively low and constant amounts during the first 8 h of development and that the amount of each of these mRNA's begins to increase by 13 h after fertilization. To quantitate these results, the hybridization bands in the RNA blots of total polysomal RNA (Fig. 2B) and total cytoplasmic RNA (Fig. 4B) were cut from the diazobenzyloxymethyl paper and counted in a liquid scintillation counter (Table 1).

The validity of this procedure as a method to quantitate the relative increases in the levels of the actin-encoding RNAs was tested by a reconstruction RNA blot experiment. Varying quantities of 18-h embryo cytoplasmic RNA (ranging

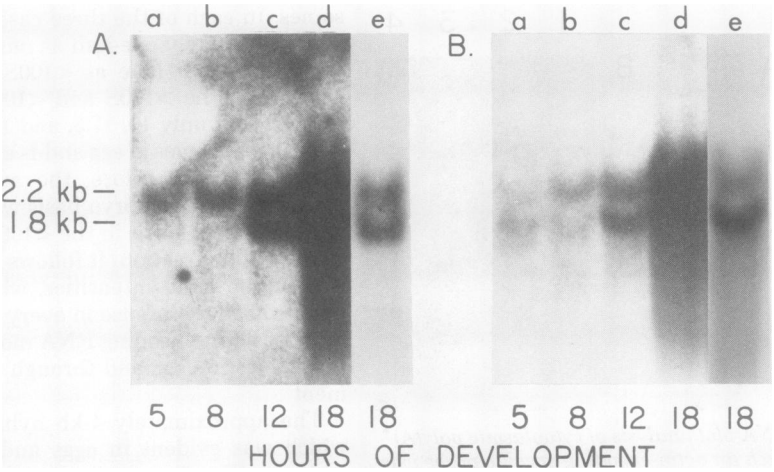

FIG. 4. *RNA blot analysis of actin-encoding cytoplasmic RNAs at selected developmental stages. Cytoplasmic RNAs from different developmental stages were denatured in glyoxal and formamide, electrophoresed on 1% agarose gels, and transferred to diazobenzyloxymethyl paper; 0.5 μg of ^{32}P-labeled pSpG17 DNA (specific activity, 4.4 × 10^8 cpm/μg) was hybridized to the RNA on each filter. (A) A 30-μg sample of cytoplasmic RNA from each stage was passed once over an oligodeoxythymidylic acid cellulose column. All of the RNA from each stage which bound to the column [poly(A)$^+$ enriched] was loaded into a single lane on the gel. Lanes a through d were exposed for 5 days witb preflashed Kodak XR5 film and an intensifying screen. Lane e shows a 40-h exposure of lane d on Kodak no-screen film without an intensifying screen. (B) A 30-μg sample of total cytoplasmic RNA from each developmental stage was loaded into one lane of the gel. The time of isolation is indicated on the figure. The exposure of lanes a through e was as described above.*

from 0.5 to 20.0 μg) were blotted and hybridized with the actin-encoding probe pSpG17. After autoradiography, the 2.2- and 1.8-kb actin-encoding bands were cut from the paper and counted. Figure 6 shows that the relationship between the amount of RNA loaded into a lane and the counts in a band is approximately linear. Therefore, a comparison of the amounts of radioactivity in the actin-encoding RNA bands, as measured by liquid scintillation counting, yields a reasonably accurate estimate of the relative changes in amounts of these sequences at different stages.

Table 1 shows the relative increases in the amounts of the 1.8- and 2.2-kb actin mRNA's from shortly after fertilization (an average of the values at 2 and 5 h) to 18 h of development (hatching blastula) for total polysomal and cytoplasmic RNAs. In the case of polysomal RNA, the increase was further corrected for the differences in the amounts of RNA in the polysomes at these different stages (13), so that the changes in the relative amounts of these polysomal mRNA species per embryo could be considered. It is clear that the relative increase in amount per embryo is significant, ranging from approximately 9- to 40-fold. The relative increases in the total polysomal and cytoplasmic messages during the period of greatest increase (13 to 18 h) were 5.2-fold (1,242 cpm/237 cpm) and 6.2-fold (2,087 cpm/334 cpm) for polysomal 2.2- and 1.8-kb mRNA's, respectively and 7.1-fold (482 cpm/68 cpm) and 10.1-fold (1,106 cpm/109 cpm) for cytoplasmic 2.2- and 1.8-kb mRNA's, respectively. Since the number of cells per embryo during this time increased only about twofold (10), this represented an increase of between 2.6- and 5-fold in the average number of actin messages per cell within each embryo between 13 and 18 h. It is clear that the increase in the amount of actin mRNA in developing embryos is not due simply to an increasing number of cells, each containing a constant amount of actin messenger. In fact, until 13 h the average number of actin messages in the cytoplasm of each cell must be decreasing significantly. This follows since the increases in the amounts of total embryo cytoplasmic actin mRNA's are 1.2-fold (68 cpm/55 cpm) and 2.4-fold (109 cpm/46 cpm) for 2.2- and 1.8-kb mRNA's, respectively (Table 1) during this time period, whereas the number of cells per embryo is increasing approximately 100-fold (10). Therefore, it is possible that no new actin mRNA appears in the cytoplasm until sometime between 8 and 13 h after fertilization. Clearly, no significant increases in the steady-state levels of these RNAs are detectable until 13 h.

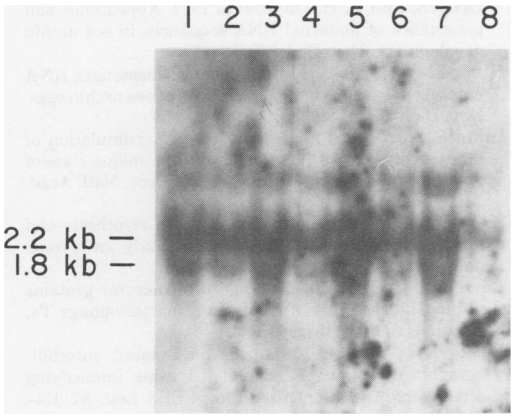

FIG. 5. *RNA blot of >100S and <100S fractions from polysome gradients of eggs and 1- and 2-h embryos. The post-mitochondrial supernatants from eggs and 1- and 2-h embryos were centrifuged on 5 to 40% sucrose gradients, and the RNAs from the >100S and <100S fractions were recovered. A portion of the >100S fraction from 2-h embryos was EDTA released and sedimented on a second gradient, from which the >100S and <100S fractions were recovered and the RNA was purified. The RNA from each fraction was denatured by heating in formamide and formaldehyde, electrophoresed on a 1% agarose gel, and transferred to a nitrocellulose filter. The RNA on each filter was hybridized with 0.5 μg of ^{32}P-labeled pSpG17 DNA (specific activity, 2 × 10^8 cpm/μg). Lane 1, Egg, <100S (16 μg); lane 2, egg, >100S (9 μg); lane 3, 1-h embryo, < 100S (16 μg); lane 4, 1-h embryo, >100S (7 μg); lane 5, 2-h embryo, <100S (16 μg); lane 6, 2-h embryo, >100S (9 μg); lane 7, 2-h embryo, <100S (9 μg; from EDTA release gradient); lane 8, 2-h embryo, >100S (9 μg; from EDTA release gradient).*

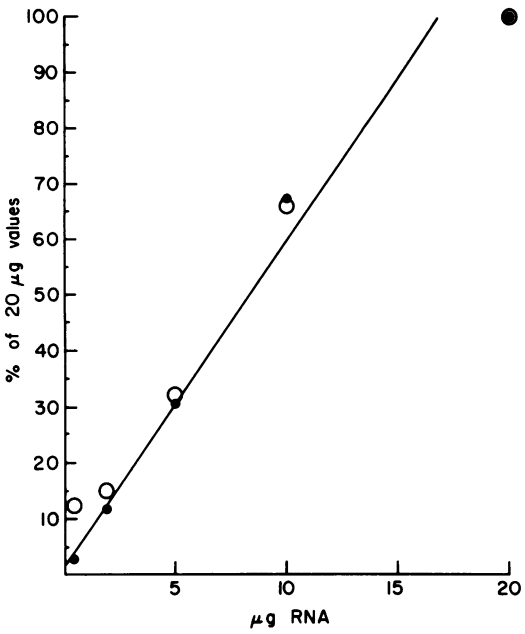

FIG. 6. *Reconstruction RNA blot. Varying amounts of total RNA from 18-h embryos were mixed with transfer RNA to a total of 30 μg of RNA, denatured by heating in formamide and formaldehyde, electrophoresed on 1% agarose, and transferred to a nitrocellulose filter; 0.1 μg of ^{32}P-labeled pSpG17 DNA (specific activity, 3.4 × 10^8 cpm/μg) was hybridized to the RNA on the filter. The regions of the paper containing the autoradiographic bands representing the 2.2- and 1.8-kb actin RNAs were cut out and counted in a liquid scintillation counter. Six additional regions of similar size and shape which contained no bands were also cut out and counted as background controls. In order to plot the counts from each band on the same scale, the counts were normalized (the number of counts in the bands in the 20-μg lane was defined as 100%). The background was determined to be 26 cpm, and the 100% values for the 2.2-kb RNA (●) and 1.8-kb RNA (○) were 90 and 139 cpm, respectively.*

The data described here demonstrate that there are at least two size classes of actin mRNA in developing sea urchin embryos (2.2 and 1.8 kb) and that there may be a third class (~4 kb). Although we cannot exclude the possibility that both known actin-encoding RNAs are the products of one gene whose precursor has been processed differentially, the simplest interpretation is that there are at least two distinct actin mRNA's being expressed. Of course, the issue of whether the actin messages are expressed differentially is dependent on the analysis of the number of different messages. If we consider each size class as a single message, certain patterns are found. In every case examined [i.e., polysomal total and poly(A)$^+$ RNAs and cytoplasmic total and poly(A)$^+$ RNAs], the amounts of the 2.2- and 1.8-kb messages change relative to each other; that is, the 1.8-kb band is always less intense than the 2.2-kb band at the early stages and reaches an intensity greater than or equal to that of the 2.2-kb band by 18 h (Fig. 2 and 4 and Table 1). In the total cytoplasmic actin RNAs, where the ratio of the counts in the 1.8-kb band to the counts in the 2.2-kb band is 0.84 (46 cpm/55 cpm) at 2 to 5 h and reaches 2.3 (1,106 cpm/482 cpm) by 18 h, this represents a change in the relative intensities of the two bands of 2.7-fold (2.3/0.84). Thus, the evidence demonstrates that the accumulation of these two RNA species is significantly different, suggesting the possibility of differential activation of two genes. However, this is a somewhat different issue than whether the messages appear in the cytoplasm or on polysomes or at both places at the same time during development. The data in Table 1 do not show any large

differences in the kinetics of appearance between the two message size classes. However, if low levels of the two actin message classes exist within eggs and no new message appears during the first 8 h of development, a difference of a few hours in the time of appearance of different new actin messages would be difficult to detect in these experiments.

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