THE GENOMIC ORGANIZATION AND EXPRESSION OF THE *STRONGYLOCENTROTUS PURPURATUS* ACTIN GENE FAMILY

Thesis by

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In Fond Memory of My Beloved Wife, JoAnne

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ABSTRACT

The actin gene family of the sea urchin *Strongylocentrotus purpuratus* was studied in detail, using subcloned probes specific to the 3'-terminal nontranslated actin gene sequences. By determining the often polymorphic restriction fragment band pattern displayed in genomic blots by each probe, the actin genes in this species could be classified. The evidence presented here shows that the *S. purpuratus* genome contains eight actin genes, and these can be assigned to four subtypes. Studies of the expression of the genes show that the actin genes of three of these subtypes code for cytoskeletal actins (Cy), while the fourth gives rise to a muscle-specific actin (M). There is a single CyI actin gene, three CyII genes (CyIIa, CyIIb, and CyIIc), three CyIII genes (CyIIIa, CyIIIb, and CyIIIc), and a single M actin gene. Primary sequence data shows that two of these genes, CyIIc and CyIIIc, have anomolous structures and are probably pseudogenes.

RNA gel blots, using the 3'-trailer probes, show that during embryogenesis, as well as in a number of adult tissues, the CyI, CyII, and M subtype genes are differentially expressed. Genes of the CyIII subtype are not active (i.e., transcripts do not accumulate) in any adult tissues. Moreover, molecular titration experiments, using Sp6 polymerase-generated RNA probes, measured the absolute prevalence of transcripts from five of the six active actin genes at different points in development. This analysis showed that actin transcripts begin to accumulate after seven hours postfertilization and are not prevalent in the maternal RNA pool. *In situ* hybridizations of sections through the embryo establish that the accumulation of actin transcripts occurs in a cell lineage specific manner. Together with the molecular titrations these analyses provide per-cell prevalence data for transcripts from each actin gene. *In vitro* nuclear run-off experiments show that actin transcripts accumulate as the result of the activation and continued transcription of each actin gene during embryogenesis.

Additional *in vivo* kinetic labeling experiments were used to measure the rates of nuclear synthesis and decay. This data shows that once activated each actin gene is transcribed at a constant rate and these rates are the prominent kinetic parameters determining actin transcript prevalence levels.

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INTRODUCTION

The sea urchin embryo has been one of the most widely studied experimental system used by developmental biologists. Investigations at the turn-of-the-century (Driesch, 1892, 1898; Boveri, 1901a) were drawn to this embryo because of fortuitous parameters such as the availability of large amounts of gametes (MacBride, 1906; Harvey, 1956), the transparency and size of the egg/embryo (Lönning and Wennerberg, 1963), and the simplicity in overall structure of the embryo (Hörstadius, 1939). These parameters made the embryo accessible to experimental manipulation and as a result the sea urchin quickly became the best known embryonic system. The wealth of information gleaned from these early studies provides a framework that has allowed the molecular biologist to exploit this embryonic system (Davidson *et al.*, 1982). Interestingly, molecular biologists are drawn to the sea urchin embryo for exactly the same parameters that attracted the turn-of-the-century cell biologists. Thus, studies of specific gene transcripts (Bruskin *et al.*, 1981; Cox *et al.*, 1984; reviewed by Angerer and Davidson, 1984) and their protein products (Cohen *et al.*, 1975; Bruskin *et al.*, 1982) has in many respects made the sea urchin embryo the system of choice (Davidson *et al.*, 1982).

Lineage-specific gene expression in the sea urchin embryo

An underlying feature of sea urchin development is the organization of blastomeres into discrete lineages. The morphogenetic values of cell lineages descending from specific blastomeres in this embryo was documented early in this century (reviewed by Hörstadius, 1939). This study showed that as development proceeds each cell lineage differentiates, acquiring the morphogenetic qualities of a particular part of the embryo. Furthermore, the origins of these different cell lineages can be traced back to progenitor cells of the cleavage stage embryo. This suggests that early cleavage blastomeres are determined by their position in the embryo with respect to the distribution of maternal components. Experiments

which removed specific cleavage stage blastomeres and/or groups of blastomeres, however, have shown that inductive effects between neighboring cells are also an essential part of the embryo's ontogenic program (Hörstadius, 1939). These seemingly contradictory results indicate that sea urchin embryogenesis is the result of a complex interplay of maternal components and information derived from the nuclei of the embryonic blastomeres.

The application of *in situ* technologies has provided a link between molecular events occurring in specific blastomeres and the morphogenesis of different embryonic cell lineages. McClay *et al.*, 1983, using various monoclonal antibodies, showed by fluorescent staining that the ingression of primary mesenchymic cells into the blastocoel cavity is accompanied by the appearance of a new cell surface antigen. The antigen was shown to be expressed only by this cell lineage and once activated, its expression continues throughout the rest of embryogenesis. Additional cell lineage specific markers have been characterized (McClay and Chambers, 1978; McClay and Fink, 1982) and their expression delineates cell lineages that had been designated earlier based on morphological criteria. The expression of a number of these lineage markers have in fact been shown to precede the actual morphogenesis of the cell lineage. Studies of transcript accumulation by Cox *et al.*, 1984 showed, for example, that transcripts from the gene Spec 1 (*Strongylocentrotus purpuratus* ectoderm; Carpenter *et al.*, 1984; Bruskin *et al.*, 1981; 1982) accumulate in the presumptive cells of the aboral ectoderm. The point of interest is that these transcripts accumulate before the aboral ectoderm cells exhibit any characteristics associated with this cell lineage. Similar experiments with a primary mesenchyme-specific marker, the 50 kd spicule matrix protein (Sucov *et al.*, unpublished observations), showed that transcripts coding for this protein are also expressed before this lineage shows evidence of characteristics associated with this lineage (e.g., deposition of larval

skeleton). These results suggest that morphological differentiation of a cell lineage causes and/or is a consequence of the expression of specific genes in the cells of that lineage.

The actin gene family

The use of actin protein as a structural component of the cell's cytoskeleton (Buckingham and Minty, 1983) is a feature common to all eukaryotic organisms (Pollard and Weihing, 1974). In addition, actin protein serves as the major contractile protein in the muscle tissue of higher animals (Potter, 1974). Evidence from studies of actin genes, from a wide phylogenetic spectrum of organisms (Gallwitz and Sures, 1980; Ng and Abelson, 1980; Fyrberg *et al.*, 1980; Cleveland *et al.*, 1980; Minty *et al.*, 1983; Scheller *et al.*, 1981; Kaine and Spear, 1980; McKeown *et al.*, 1978; reviewed by Firtel, 1981) has shown a strong conservation of the actin protein coding information. The homology is strongest at the amino acid level (Vandekerckhove and Weber, 1978; 1980), and the phenotypic pressure maintaining the amino acid homology has been sufficient as to allow nucleic acid probes of an actin gene from one species to crossreact with corresponding sequences in nearly any other organisms. Thus, a DNA probe of a Drosophila actin has been used to isolate actin sequences from such diverse organisms as Dictyostelium (Kindle and Firtel, 1978; McKeown *et al.*, 1978), the sea urchin (Durica *et al.*, 1980). With the exception of yeast, actin proteins are encoded by multiple genes in eukaryotes. In general, the number of actin genes is proportional with genome size and/or evolutionary complexity; however, exceptions do exist (e.g., Dictyostelium has ~17 actin genes (Kindle and Firtel, 1978; McKeown and Firtel, 1981a; McKeown *et al.*, 1982) while the chicken possesses only 5-10 actin genes (Cleveland *et al.*, 1980; 1981a). While these multiple actin genes code for proteins that perform diverse functions (i.e., cytoskeletal vs. muscle), members of any one gene family exhibit a great deal of

sequence homology. The presence of this high degree of sequence conservation poses an interesting problem for higher multicellular organisms. *Viz.*, how can essentially homologous actin genes be independently regulated during the life cycle of the animal? Not only must the cells of a higher animal choose between functionally different actin proteins but it also must express a given actin gene (or group of genes) at an appropriate level(s) in order to meet the needs of that cell.

The regulation of actin gene expression during sea urchin development was initially shown by screening embryo cDNA libraries for sequences expressed during embryogenesis (Laskey *et al.*, 1980). A number of actin clones was isolated using this assay. The work presented in this thesis examines the details of actin gene expression in the sea urchin ***Stronglyocentrotus purpuratus***. An analysis of the genomic organization of the actin genes in this organism demonstrates the size and complexity of this multi-gene family. Moreover detailed kinetic analysis of actin gene transcription during embryonic development provides evidence that the different cell lineages of the *S. purpuratus* embryo independently regulate the levels of actin transcript prevalence and therefore the degree of actin gene expression.

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CHAPTER 1

Sea Urchin Actin Gene Subtypes

Gene Number, Linkage and Evolution

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The actin gene family of the sea urchin *Strongylocentrotus purpuratus* was analyzed by the genome blot method, using subcloned probes specific to the 3′ terminal non-translated actin gene sequence, intervening sequence and coding region probes. We define an actin gene subtype as that gene or set of genes displaying homology with a given 3′ terminal sequence probe, when hybridized at 55°C, 0·75 M-Na^+. By determining the often polymorphic restriction fragment band pattern displayed in genome blots by each probe, all, or almost all of the actin genes in this species could be classified. Our evidence shows that the *S. purpuratus* genome probably contains seven to eight actin genes, and these can be assigned to four subtypes. Studies of the expression of the genes (Shott *et al.*, 1983) show that the actin genes of three of these subtypes code for cytoskeletal actins (Cy), while the fourth gives rise to a muscle-specific actin (M). We denote the array of *S. purpuratus* actin genes indicated by our data as follows. There is a single CyI actin gene, two or possibly three CyII genes (CyIIa, CyIIb, and possibly CyIIc), three CyIII actin genes (CyIIIa, CyIIIb, CyIIIc), and a single M actin gene. Comparative studies were carried out on the actin gene families of five other sea urchin species. At least the CyIIa and CyIIb genes are also linked in the *Strongylocentrotus franciscanus* genome, and this species also has a CyI gene, an M actin gene and at least two CyIII actin genes. It is not clear whether it also possesses a CyIIc actin gene, or a CyIIIc actin gene. The genome of a more closely related congener, *Strongylocentrotus dröbachiensis*, includes 3′ terminal sequences suggesting the presence of a CyIIc gene. In *S. franciscanus* and *S. dröbachiensis* the first intron of the CyI gene has remained homologous with intron sequences of both the CyIIa and CyIIb genes, indicating a common origin of these three linked cytoskeletal actin genes. Of the four *S. purpuratus* 3′ terminal subtype probe sequences only the CyI 3′ terminal sequence has been conserved sufficiently during evolution to permit detection outside of the genus *Strongylocentrotus*. An unexpected observation was that a sequence found only in the 3′ untranslated region of the CyII actin gene in the DNA of *S. dröbachiensis* and *S. purpuratus* is represented as a large family of interspersed repeat sequences in the genome of *S. franciscanus*.

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1. Introduction

Previous studies have shown that the sea urchin genome contains multiple actin genes, and that these genes are diverse in both structural and functional respects (Durica *et al.*, 1980; Scheller *et al.*, 1981; Overbeek *et al.*, 1981; Cooper & Crain, 1982; Schuler *et al.*, 1983). Durica *et al.* (1980) estimated that in *Strongylocentrotus purpuratus* the number of actin genes per haploid genome lies between 5 and 20. Scheller *et al.* (1981) suggested that there are at least 11 actin genes in this species and Overbeek *et al.* (1981) proposed at least five different actin genes. Complete nucleotide sequences have now been reported for two of the actin genes (Cooper & Crain, 1982; Schuler *et al.*, 1983). These two genes are linked at distances of a few thousand nt† (Schuler & Keller, 1981; Scheller *et al.*, 1981), and both code for cytoskeletal actins (Cooper & Crain, 1982; Schuler *et al.*, 1983; Shott *et al.*, 1983). These genes each possess two intervening sequences, between codons 121 and 122, and codons 203 and 204. Although the protein sequences determined by these two genes are extremely similar, their intervening sequences differ at 40% of nucleotide positions. A third actin gene for which unpublished sequence information exists is expressed predominantly in muscle (cited by Cooper & Crain, 1982). This gene possesses four intervening sequences, located at codon positions 41/42, 121/122, 203/204 and 267/268. Several additional sea urchin actin genes have been cloned (Scheller *et al.*, 1981), but their structure is less well known. However, it was recognized by Scheller *et al.* (1981) that all the cloned sea urchin actin genes possess distinctive transcribed but non-translated 3′ terminal sequences, by which they can be classified into several subtypes. In the study presented here we constructed subclones of the 3′ terminal regions, using the genomic recombinants described earlier, and have utilized these probes to identify the actin genes of the *S. purpuratus* genome. We conclude that there are probably seven or eight actin genes in the genome of this echinoderm, and by the criterion of 3′ terminal sequence homology, these fall into four actin gene subtypes. In a separate study presented elsewhere in which the same 3′ terminal actin gene probes were utilized, we report that at least six of the actin genes produce cellular messenger RNAs, and that each displays a unique pattern of expression during the life cycle (Shott *et al.*, 1983).

Sea urchins are particularly favorable material for the study of both actin gene regulation and evolution. This follows from the depth of molecular knowledge regarding gene expression in this organism, and also from the availability of a range of reasonably well-studied species of known phylogenetic relationship. In this paper we consider the evolution of the 3′ non-translated actin gene region during sea urchin phylogeny, and we provide an initial comparison of this gene family among three species of the genus *Strongylocentrotus*.

2. Materials and Methods

(a) *Preparation of sea urchin sperm DNA*

Sperm of *S. purpuratus* and *Strongylocentrotus franciscanus* were collected by intracoelomic injection of 0·5 M-KCl. The dry sperm (i.e. undiluted semen) was kept on ice until used.

† Abbreviations used: nt, nucleotides; kb, 10^3 bases; SDS, sodium dodecyl sulfate.

High molecular weight genomic DNA was isolated from the sperm of individual animals using a modified version of the procedure described by Blin & Stafford (1976). Dry sperm was diluted 100-fold by dropwise addition at room temperature to rapidly stirred sperm DNA isolation buffer (SDIB), which is 0·2 M-Tris·HCl, 0·2 M-EDTA (pH 8). Pronase (Calbiochem) suspended in 0·1 × SDIB and preincubated at 37°C for 2 h) was added to a final concentration of 500 μg/ml. The solution was then brought to 0·1% (w/v) sodium dodecyl sulfate, and incubated at 37°C overnight (12 to 16 h). The digested sperm was extracted twice with equal volumes of phenol/chloroform (1 : 1), saturated with 100 mM-Tris·HCl (pH 8), and once with an equal volume of chloroform. The aqueous phase was then dialyzed at room temperature against 0·1 × SDIB with frequent changes for 2 to 3 days. The dialyzed DNA solution was brought to 0·1 M-NaCl and 100 μg heat treated RNase A/ml were added. This solution was then incubated at 37°C for 1 h. Pronase (preincubated at 37°C for 2 h) was added to 500 μg/ml and the solution was incubated an additional 1 h at 37°C. The DNA was again extracted twice with equal volumes of phenol/chloroform (1 : 1) and once with an equal volume of chloroform. The aqueous phase was dialyzed at room temperature against 0·1 × SDIB for 2 to 3 days. The DNA solution was then concentrated with a rotary evaporator to approximately 0·5 μg DNA/μl buffer.

(b) *Construction of 3′ untranslated sequence subclones, a 5′ coding region subclone, and an intron-specific subclone*

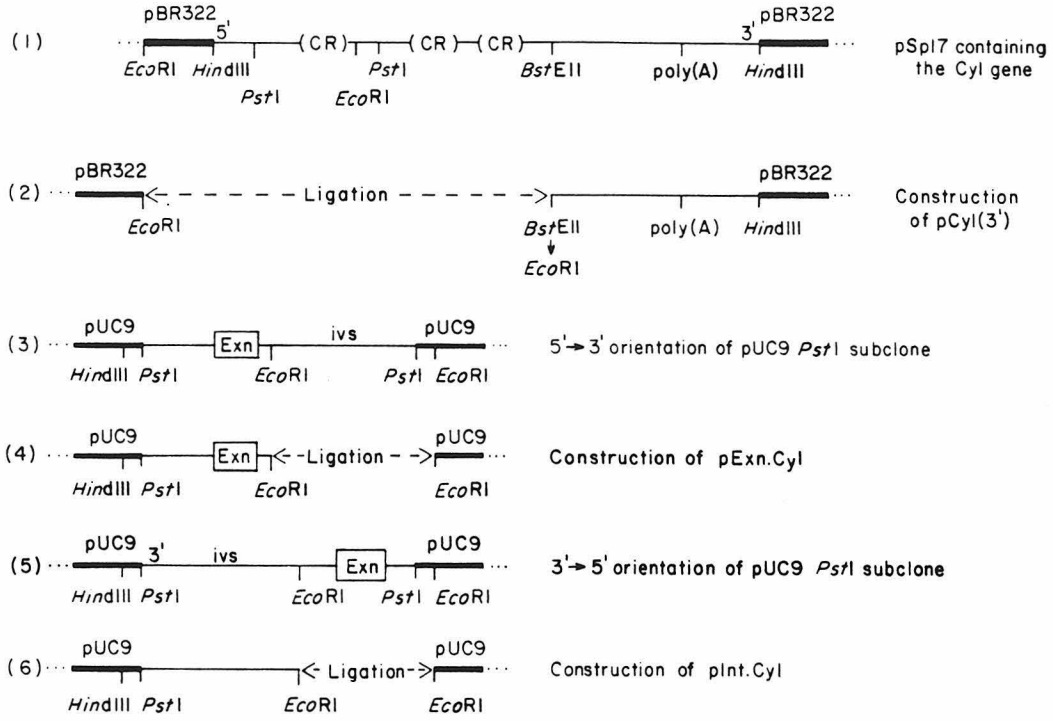
The actin genomic clones used as starting material for these constructions were as described by Scheller *et al.* (1981) and Durica *et al.* (1980). In this work bacteria containing recombinant plasmids were grown and the plasmids were amplified according to the method of Norgard *et al.* (1979). Plasmid DNAs were isolated as described (Scheller *et al.*, 1977).

(i) *Subclone pCyI(3′): the 3′ untranslated region of actin gene CyI*

Sequence analysis of the plasmid genomic isolate pSp17 (Cooper & Crain, 1982) positioned the actin protein-coding regions with respect to the restriction map (see diagram (1)). A *Bst*EII site, 203 nt 3′ to the stop codon, was utilized. After digestion with this enzyme the termini were filled in with cold α-deoxynucleotide triphosphates by repair synthesis using Klenow polymerase I. Ligation of *Eco*RI linkers and subsequent digestion of the plasmid with *Eco*RI yielded a 5·14 kb *Eco*RI fragment containing pBR322 (excepting the 20 nt separating the *Hin*dIII and *Eco*RI sites), the distal portion of the 3′ untranslated region of the CyI gene (i.e. the 517 nt separating the *Bst*EII site from the end of the transcript), plus 263 nt of flanking downstream sequence. The proximal 203 nt following the stop codon and all protein-coding sequences are excluded. The 5·14 kb fragment was cloned by self-ligation under conditions that promote cyclization. It contains a 780 nt sea urchin DNA insert that can be isolated by digestion with *Eco*RI and *Hin*dIII. This is seen schematically in diagrams (1) and (2). This and the following line diagrams in this section are for orientation only and are *not* drawn to scale.

(ii) *Subclones pInt.CyI, first intron probe and pExn.CyI, a first coding region probe for the CyI gene*

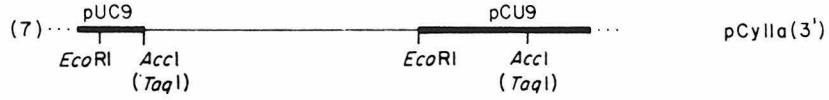
As can be seen in diagram (1) digestion of pSp17 with *Pst*I yields a fragment containing the 5′ flanking sequence, the first exon of the CyI gene, and its first intervening sequence. This fragment, which is about 900 nt in length, contains an *Eco*RI site 20 nt 3′ of the donor splice site in the intervening sequence. This *Pst*I fragment was cloned into the polylinker *Pst*I site of pUC9 (Vieira & Messing, 1982), in both orientations. As indicated in diagrams (3) to (6), digestion of these clones with *Eco*RI, and religation, yields the desired subclones. pInt.CyI contains a 200 nt insert consisting exclusively of intervening sequence. pExn.CyI contains a 700 nt insert which includes all of the CyI 5′ exon and flanking extragenic sequence.



(iii) *Subclone pCyIIa(3'): the 3' untranslated region of actin gene CyIIa*

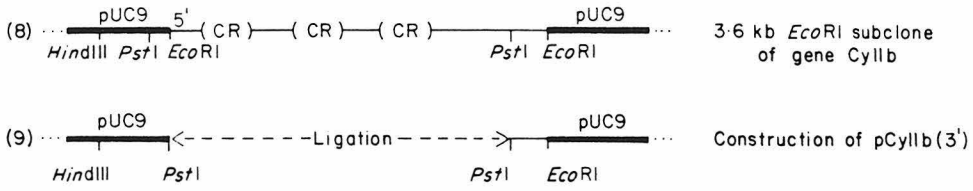
*Eco*RI digestion of the genomic isolate λ SpG2-8 generates two *Eco*RI fragments that contain actin genes (Scheller *et al.*, 1981). The CyIIa gene is contained on the larger of these (4·0 kb) and the CyIIb gene is located on the smaller (3·6 kb). These *Eco*RI fragments were subcloned into pUC9.

Scheller *et al.* (1981) sequenced a portion of the 3' untranslated region of gene CyIIa (referred to by them as gene "G") and discovered a *Taq*I site 80 nt following the stop codon. *Taq*I digestion of the 4·0 kb CyIIa *Eco*RI subclone yields a 1·4 kb fragment which contains the distal region of the 3' untranslated sequence (720 nt), as well as the region of the pUC9 sequence between the polylinker *Eco*RI site and the next *Taq*I site (see diagram (7)). This fragment was isolated and cloned into *Acc*I-digested pUC9.



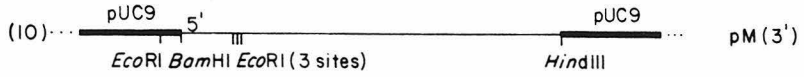
(iv) *Subclone pCyIIb(3'): the 3' untranslated region of actin gene CyIIb*

Construction of this subclone was based on a *Pst*I site downstream from the stop codon. An appropriately oriented pUC9 subclone of the 3·6 kb *Eco*RI fragment of λ SpG2-8 (Scheller *et al.*, 1981) was digested with *Pst*I and recircularized to generate the desired subclone, as indicated in diagrams (8) and (9). The insert is about 400 nt long.



(v) *Subclone pM(3'): the 3' untranslated region of actin gene M*

This gene was initially isolated from *S. purpuratus* genomic DNA as a *Hin*dIII fragment cloned in pBR322 by Crain *et al.* (1982), who designated this clone pSp28. Sequence data demonstrated a *Bam*HI site following the stop codon (W. R. Crain, personal communication). The desired subclone was constructed from the *Bam*HI-*Hin*dIII fragment of pSp28, by ligation into pUC9 DNA that had been cut with these enzymes. It contains a 1·3 kb insert, oriented with respect to the polylinker as indicated in diagram (10).



(vi) *Subclone pCyIIIb(3'): the 3' untranslated region of actin gene CyIIIb*

This subclone was generated from an 800 nt *Ava*II-*Pst*I fragment of λSpG2-51 (gene "K") of Scheller *et al.*, 1981). The *Ava*II site is 30 nt 5' to the stop codon and the following 500 nt are included in the 3' untranslated region of CyIIIb mRNAs. The *Ava*II site was filled in with the Klenow polymerase reaction, and *Pst*I linkers were added. The fragment was then cloned into the *Pst*I site of the polylinker of pUC9.

(c) *Preparation of terminally labeled DNA fragments for genomic blots and sequencing*

The insert DNA of each subclone is released by a *Hin*dIII/*Eco*RI double digest, except for subclone pCyIIa (3'), where *Eco*RI alone releases the insert. These enzymes leave 5' overhanging bases. Digests of the various plasmid DNAs were labeled by repair synthesis, utilizing the Klenow polymerase reaction. The labeled DNA fragments were separated on a 1% (w/v) agarose gel (containing 1 μg ethidium bromide/ml) run at 30 V/35 mA. Following electrophoresis, insert DNA bands were localized *via* u.v. illumination, and excised. The labeled DNA was recovered from the gel splice by electroelution and stored at −20°C. Specific activities of the labeled probes (size range: 200 to 1500 nt) ranged from about 10^7 to about 10^8 cts/min per μg. The labeled preparations were used without further purification for genome blots. For sequencing, the labeled fragments were purified additionally by DEAE-cellulose column chromatography (Posakony *et al.*, 1981).

(d) *Agarose gel electrophoresis and nitrocellulose blotting of genomic DNA*

Restriction enzyme digests of genomic DNA were resolved by electrophoresis on agarose gels in Tris-acetate/EDTA buffer (40 mM-Tris, 20 mM-sodium acetate, 1·3 mM-EDTA, pH 7·8). *Hin*dIII-digested wild-type λ DNA was used as a size marker (Murray & Murray, 1975). After staining with ethidium bromide (1 μg/ml) and gel photography (using a shortwave u.v. transilluminator), the DNA was transferred to nitrocellulose paper by the method of Southern (1975), using 10 × SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate) as the transfer medium. The filters were blotted for 8 to 12 h and then washed extensively in 3 × SSC. The filters were then air dried and baked for 2 to 3 h at 80°C in a vacuum oven.

(e) *Genome blot hybridizations*

The genome blots were prehybridized in 5× SET (SET is 0·15 M-NaCl, 0·03 M-Tris (pH 8), 2 mM-EDTA), Denhardt's solution (Denhardt, 1966), 20 mM-sodium phosphate (pH 6·8), and 50 μg sheared and denatured calf thymus DNA. After 2 to 4 h prehybridization heat-denatured radioactive probe DNA was added, and incubation was continued for 14 to 20 h. In this study blot hybridizations were carried out at 55°C (low criterion) or 68°C (high criterion), as indicated, in sealed plastic bags in a shaking waterbath. The hybridized filters were washed at the temperature of hybridization, as follows: two 30-min washes in 4× SET, 0·2% SDS; two 30-min washes in 2× SET, 0·2% SDS; two 60-min washes in 1× SET, 0·2% SDS (for low criterion hybridizations) or two 60-min washes in 0·2× SET, 0·2% SDS (for high criterion hybridizations).

(f) *DNA sequencing*

DNA sequencing was carried out by the method of Maxam & Gilbert (1977), using duplex restriction fragments asymmetrically labeled by repair synthesis with the Klenow polymerase.

3. Results

(a) *Subcloned 3′ terminal probes specific to the actin gene subtypes*

Previous studies left problematical both the overall number of actin genes and the variety of actin gene subtypes in the *S. purpuratus* genome. To clarify these issues we constructed a set of five molecular probes capable of distinguishing one actin gene subtype from another. We show in the following section that these probes suffice to identify all of the actin genes in the DNA of this species with one possible exception. Strategy for the construction of the necessary 3′ terminal region subclones from previously described genomic recombinants containing

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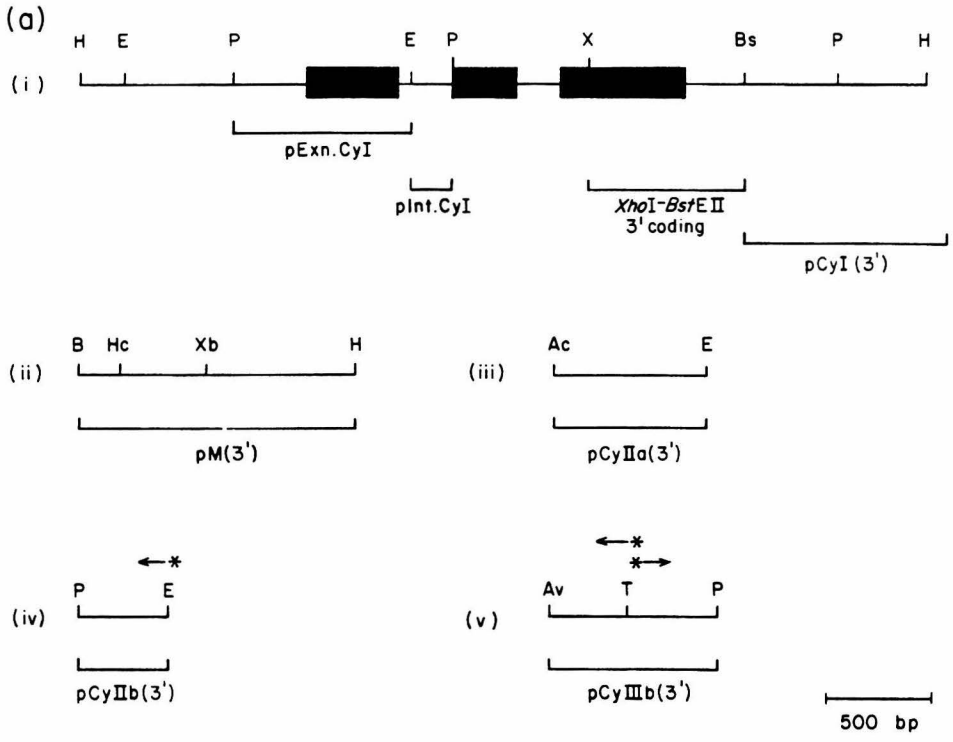
FIG. 1. Restriction maps and partial nucleotide sequences of subcloned 3′ terminal actin gene probes. Construction of the subclones referred to in this Figure is described in Materials and Methods, section (b). (a) Restriction maps. In (i) a map of the genomic clone pSp17 (Durica *et al.*, 1980) is presented. This clone contains the CyI gene, and from it 4 of the hybridization probes utilized in this paper were derived. The regions included in these probes are indicated by brackets. The indicated restriction sites are: H (*Hin*dIII), E (*Eco*RI), P (*Pst*I), X (*Xho*I), Bs (*Bst*EII). The protein coding regions of the CyI gene are represented by filled boxes. There may be an additional intron in the 5′ leader sequence of the gene (Schuler *et al.*, 1983). (ii) Restriction map of the insert in the 3′ terminal subclone specific for gene M (pM(3′)). In addition to the restriction sites indicated in (i), the sites for Hc (*Hin*cII), Xb (*Xba*I) are included. (iii) The 3′ terminal subclone specific for gene CyIIa (pCyIIa(3′)); Ac (*Acc*I). (iv) The 3′ terminal subclone specific for gene CyIIb (pCyIIb(3′)). In this diagram and in (v) the asterisk and arrow indicate the sites of labeling and the directions in which nucleotide sequences were obtained. (v) The CyIIIb 3′ terminal subclone (pCyIIIb(3′)); Av, *Ava*II. All maps shown are to the scale indicated. bp, base-pair. (b) Nucleotide sequence comparison of 3′ untranslated regions from the CyI, CyIII and CyII actin gene subtypes. The underlined TAA in each sequence represents the stop codon of the protein coding region. Boxed regions show the significant homology shared by all three 3′ untranslated sequences proximal to the stop codon. The underlined nucleotides in the CyI and CyIIa sequences represent a further region in which the two sequences are homologous in 53 of 55 nucleotides. The CyI sequence is from Cooper & Crain (1982) and the CyIIa sequence is from Scheller *et al.* (1981). (c) Sequence homology in the 3′ untranslated regions of two members of the CyII actin gene subtype, genes CyIIa and CyIIb. Maxam–Gilbert sequence analysis was performed on DNA fragments labeled at the *Eco*RI site in each gene as indicated in (a). Undetermined nucleotides are represented as X's. The dashes represent spaces introduced into the sequences in order to maximize homology. Homogeneity between the 2 sequences is indicated by dots. The CyIIa sequence was determined by Schuler *et al.* (1983). Sequence hyphens have been omitted for clarity.



(b)

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CyI      TAA  ACAACTCGCT'TTTGTTCTAC'ATCTGTTGAG'CACAATAACG'AACTCATGGG'
CyIIIb   TAA  ACAACTTGAT'TTTCTTCTAC'TTCTAATGAG'CAACCTGATT'TTTTTAATTC'
CyIIa    TAA  ACAAATCGCT'TTTGTTCTAC'ATCTGTTGAG'CACAATAACG'AACTCATGGG'

CyI           GTGGGTAAAT'GGGTCGCCTT'ACCGTGTGGC'TAACTCTTTG'GACAGTCGGG'
CyIIIb        TGTTTCACTC'CATGTTGCCA'CCTCTTGATA'GCCTTTGGTT'TCGGATGGTT'
CyIIa         GTTGGGGTTT'TTTGTGAACA'AACTCCCGAA'TATCAACTTT'GCTATACGAG'

CyI           TCTATTAGAA'ATTATCTAGC'TATCCATCTC'TGATAATA.....3'
CyIIIb        ACATGTTATT'AGATATTCAC'CACTGACTTT'ATCAAAAA.....3'
CyIIa         ATGATTCCTT'TTTGCCTCGT'CGATAAGGTG'ATTATAAC.....3'
```

(c)

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CyIIa    TTTTTGTTCT'TTGXACACGT'TTTGTAAGCA'ATGACGGGAT'GCCTAAATTG'CCACAAATCA'
CyIIb    TATTTGTTTT'CTGAACACGT'TTCGTAAACA'AATTTGGGAT'GCCACAATTG'CCACAAATCA'

CyIIa    TTTTCTTTTT'TTAAAGAAAT'ATTTGTTATT'GTTAATCTAA'ATACCAAGAA'ATCTTTTAAA'
CyIIb    T----ATTTT'TCAAAA----'--------TT'GTTAACCTGA'ATGCTAGGAA'ATCCTTCAAA'

CyIIa    AAA--TTCAA'AGATTATTAT'TT----ATAA'CATGAGAGCT'GCTACXXXXX'XXXXXXXXXX'
CyIIb    AAACCTTGAA'AGATTATTTT'TTCTTCATAA'CTTTAACGTT'CCTTAACATT'TATATGTTTG'

CyIIa    XXXXXXXXXX'XXXXXATTGT'ATACACTATT'TCATAGATAA'A..(X)n..GAATTC
CyIIb    AAGCTCTCAC'AAGGTATTGT'ATATACCATT'TCATATAGAA'A..(X)n..GAATTC
                                                      EcoRI
```

FIG. 1

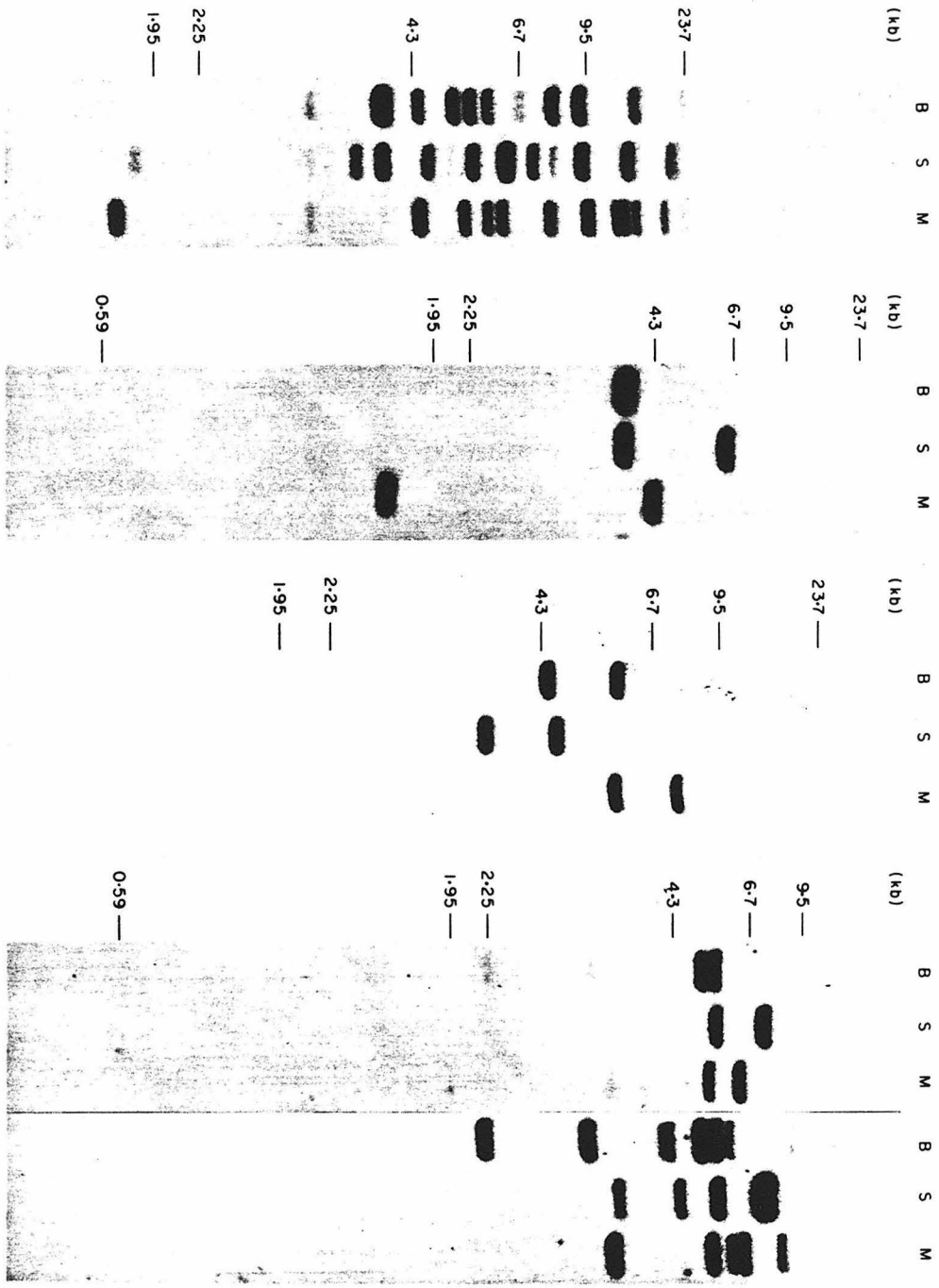

(kb)
B S M
23·7 —
9·5 —
6·7 —
4·3 —
2·25 —
1·95 —
0·59 —
Probe XhoI-BsrEII(CyI)
(a)
pCyI(3')
(b)
pCyIIa(3')
(c)
pCyIIb(3')
(d)
pCyIIb(3')Δ
(e)

FIG. 2

S. purpuratus actin genes (Scheller *et al.*, 1981; Durica *et al.*, 1980) is given in Materials and Methods, section (b). Restriction maps of the 3′ subcloned probe inserts are shown in Figure 1(a).

We define an *actin gene subtype* as that actin gene, or those actin genes, that react at a 55°C, 0·75 M-Na^+ hybridization condition with a specific 3′ terminal probe. Two of the five probes are themselves sufficiently homologous to cross react in blot hybridizations at this low criterion (see Materials and Methods, section (e)), though they do not react at the 68°C, 0·75 M-Na^+ criterion. No other cross reactions are observed among any of the five probes at either criterion. The five 3′ terminal probes thus represent four distinct actin gene subtypes.

The nomenclature we utilize in this paper for the *S. purpuratus* actin genes is based on the reaction of these subcloned 3′ terminal probes with RNAs of sea urchin embryos and adult tissues. These studies are described elsewhere (Shott *et al.*, 1983). It was found that four of the five subtype probes display prominent actin messages in *non*-muscle tissue (as well as in muscle); namely, in adult coelomocytes, or in eggs or early embryos. We conclude that these actin messages code for cytoskeletal actin proteins, and we designate the actin gene subtypes reacting with these probes as CyI, CyII and CyIII. The two actin genes represented by the two partially homologous CyII probes are called CyIIa and CyIIb. The fifth probe reacts only with transcripts found in muscle, or muscle-containing structures; namely, adult sea urchin tubefoot, lantern muscle and intestine. This actin gene subtype is denoted M.

In Figure 1(b) are shown nucleotide sequences immediately following the TAA stop codons of three cytoskeletal actin genes belonging to the CyI, CyII and CyIII subtypes. These sequences diverge sharply within 55 nt of the coding region termination. No significant homology can be discerned beyond this point in the sequences shown, nor in additional more distal sequences for which data are available (not shown). As demonstrated below in detail, the hybridization of each of the 3′ terminal probes is completely specific for genomic actin genes of its own subtype. Except for the CyIII probe, the subcloned sequences do not include the region of homology proximal to the stop codon.

Figure 1(c) illustrates the homology between the CyIIa and the CyIIb 3′ terminal sequences. In the alignment shown 48 out of the first 60 nt are

FIG. 2. Genome blots of the DNAs of 3 individual sea urchins were hybridized with actin gene coding regions and 3′ untranslated sequences. *Hin*dIII-digested sea urchin DNAs were extracted from sperm and are labeled B, S and M. Each panel represents DNAs run in parallel in contiguous tracks of the same gel. In the Figure are reproduced the autoradiographic images produced after hybridization of the labeled probes with the DNA blots. *Hin*dIII-digested λ DNA was used as a size marker (Murray & Murray, 1975). The coding region probe in (a) is a 680 nt *Xho*I-*Bst*EII fragment (see Fig. 1(a)) from the clone pSp17 (Durica *et al.*, 1980). The *Bst*EII site is located 203 nt beyond the translation stop codon (see Fig. 1(b)), and the *Xho*I site is located 477 nt prior to the stop codon. The probe thus includes about 2/3 of the coding region in the third exon of the CyI gene. It was labeled by the Klenow repair reaction as described. Probes utilized in (b) to (e) were as indicated: in (b) the probe represents the 3′ terminal sequence of gene CyI; in (c) the corresponding sequence of gene CyIIIa; and in (d) and (e) the corresponding sequence of gene CyIIb. Restriction maps and some primary sequence data for these probes are presented in Fig. 1. Each track contains 5 μg of DNA. The DNA fragments were resolved by electrophoresis and transferred to nitrocellulose. Hybridization was carried out at the 68°C, 0·75 M-Na^+ criterion described in Materials and Methods in (a) to (d), and at the 55°C, 0·75 M-Na^+ criterion in (e).

homologous. An approximate calculation suggests that the T_m value of this region would be ~62°C in 0·75 M-Na^+. This would account for the cross reaction of the CyIIa and CyIIb probes at 55°C, 0·75 M-Na^+, and probably for their failure to cross react at 68°C, 0·75 M-Na^+.

(b) *Number of* S. purpuratus *actin genes of each subtype*

S. purpuratus genome blots carried out with actin gene coding region probes typically reveal 10 to 20 bands (e.g. see Durica *et al.*, 1980; Scheller *et al.*, 1981). However, comparison of the genomes of different individuals by this method shows that the specific patterns of reaction are remarkably polymorphic. This is illustrated in Figure 2(a). An implication is that the number of actin genes in *S. purpuratus* DNA could have been overestimated in previous genome blot studies, since in many cases a single gene would be represented by two different restriction fragment alleles. A highly polymorphic genome blot pattern is expected for this species. Britten *et al.* (1978) showed that the average single copy sequence of any two *S. purpuratus* genomes differs at 4% of nucleotides, and the large amount of restriction fragment polymorphism predicted on this basis has already been observed in genome blot studies carried out with other cloned probes (Posakony *et al.*, 1983; Thomas *et al.*, 1982).

We took advantage of the restriction site polymorphism to distinguish allelic from non-allelic actin gene variants. Each of the 3′ terminal subtype probes was reacted with genome blots of the DNA of three individual sea urchins. The restriction fragment bands displayed by these probes were then matched with the bands displayed in the same DNAs by a probe deriving from the contiguous 3′ protein-coding regions. This coding region probe cross reacts even under elevated criterion conditions with all the actin genes in the genome, since, as is well known, the actin protein sequence is very highly conserved. The interspecific illustrations of this conservation are impressive. For example, Durica *et al.* (1980) found that sea urchin actin genes are detected by a coding region probe from *Drosophila,* and Fyrberg *et al.* (1980) used both *Dictyostelium* and chick actin probes to isolate the actin genes of *Drosophila.*

Reactions of individual 3′ terminal probes that demonstrate the high degree of restriction site polymorphism in the vicinity of specific *S. purpuratus* actin genes are shown in Figure 2(b) to (e). In the following we apply the term "allele" to denote a specific genomic restriction fragment containing a 3′ terminal actin gene sequence. In panel (b) it can be seen that there are four different CyI *Hin*dIII fragment alleles present in the six haploid genomes included in the experiment. Individual B appears homozygous for the CyI restriction fragment, while the other individuals are heterozygous. Panels (c) and (d) show that each of these individuals is heterozygous with respect to both the CyIIa and CyIIb actin gene restriction fragments. The main conclusion to be derived from the reaction patterns shown in Figure 2(b) to (d) is that each haploid *S. purpuratus* genome contains a single CyI gene, a single CyIIa gene, and a single CyIIb gene.

Panel (e) again shows reactions of the CyII(b) probe, but carried out at the 55°C rather than the 68°C criterion at which the remainder of the hybridizations were

conducted. The *Hin*dIII-digested DNAs in panels (d) and (e) were run on the same gel and were then blotted and separated for hybridization under the two criterion conditions. Band positions in (d) and (e) are thus directly comparable. In (e) the CyIIa genes are displayed by the CyIIb probe as light bands. However, the major result of lowering the criterion is to sharply increase the intensity of two *additional* bands that are only lightly labeled in each track in (d) and are not visible at all in (c). This can be seen most clearly in the DNAs of individuals B and M. It follows from the experiment in (e) that there must be three different sequences per haploid genome that react with the CyII subtype probes, those belonging to the actin genes CyIIa and CyIIb, and a third non-allelic sequence that is evidently more closely related to the CyIIb than to the CyIIa 3′ terminal gene region. This observation raises the possibility that there is another actin gene for the CyII subtype, which has not yet been cloned. We refer to this putative actin gene as CyIIc, though as discussed below it is not yet established that the sequence reacting with the CyIIb probe is actually associated with an actin-coding region.

In Figure 3(a) to (c) are shown sets of genome blots made with the same three individual DNAs as are represented in Figure 2. For each individual DNA the left-hand tracks display reactions with coding region probes, and the remaining tracks show reactions with the various 3′ terminal region subclones. We could thus directly align the reactions occurring with each of the subtype probes with those occurring with the 3′ coding region probe, as indicated. The results of this analysis are as follows. We found above that each haploid genome contains one gene of the CyI subtype, one CyIIa gene, and one CyIIb gene. Figure 3 shows that there is also a single M gene. In contrast, the CyIII probe displays four to six bands, depending on the genome tested. A gene of this subtype, now termed CyIIIb, was cloned by Scheller *et al.* (1981) (i.e. that from which the CyIII probe was derived) and a second, the CyIIIa gene, has recently been isolated from genomic and complementary DNA libraries (unpublished data). Figure 3 indicates that the *S. purpuratus* genome probably contains a third actin gene of the CyIII subtype as well.

The restriction fragments reacting in each of the three genomes with the 3′ terminal probes for genes CyI, CyIIa, CyIIb, CyIIIb and M correspond exactly with restriction fragments reacting with the 3′ coding region probes. This set of reactions accounts for all but two actin bands per haploid genome in the 3′ coding region probe blots. On the other hand, the single CyIIc sequence in each haploid genome also fails to line up with any of the bands displayed by the coding region probes. These unmatched bands are marked by dots in Figure 3. One possibility is that the two unmatched sequences per haploid genome belong to the same gene, i.e. CyIIc. In this case the CyIIc gene must have a *Hin*dIII site that separates the 3′ terminal sequence from the sequence reacting with the 3′ coding region probe.

These conclusions are partially at variance with the interpretation of Scheller *et al.* (1981), which was based on an analysis of a set of cloned actin gene isolates. Scheller *et al.* (1981) found several λ recombinants containing actin genes that reacted specifically with the CyI 3′ terminal sequence, and yet contained diverse flanking sequences. They inferred that the genome must include several genes of

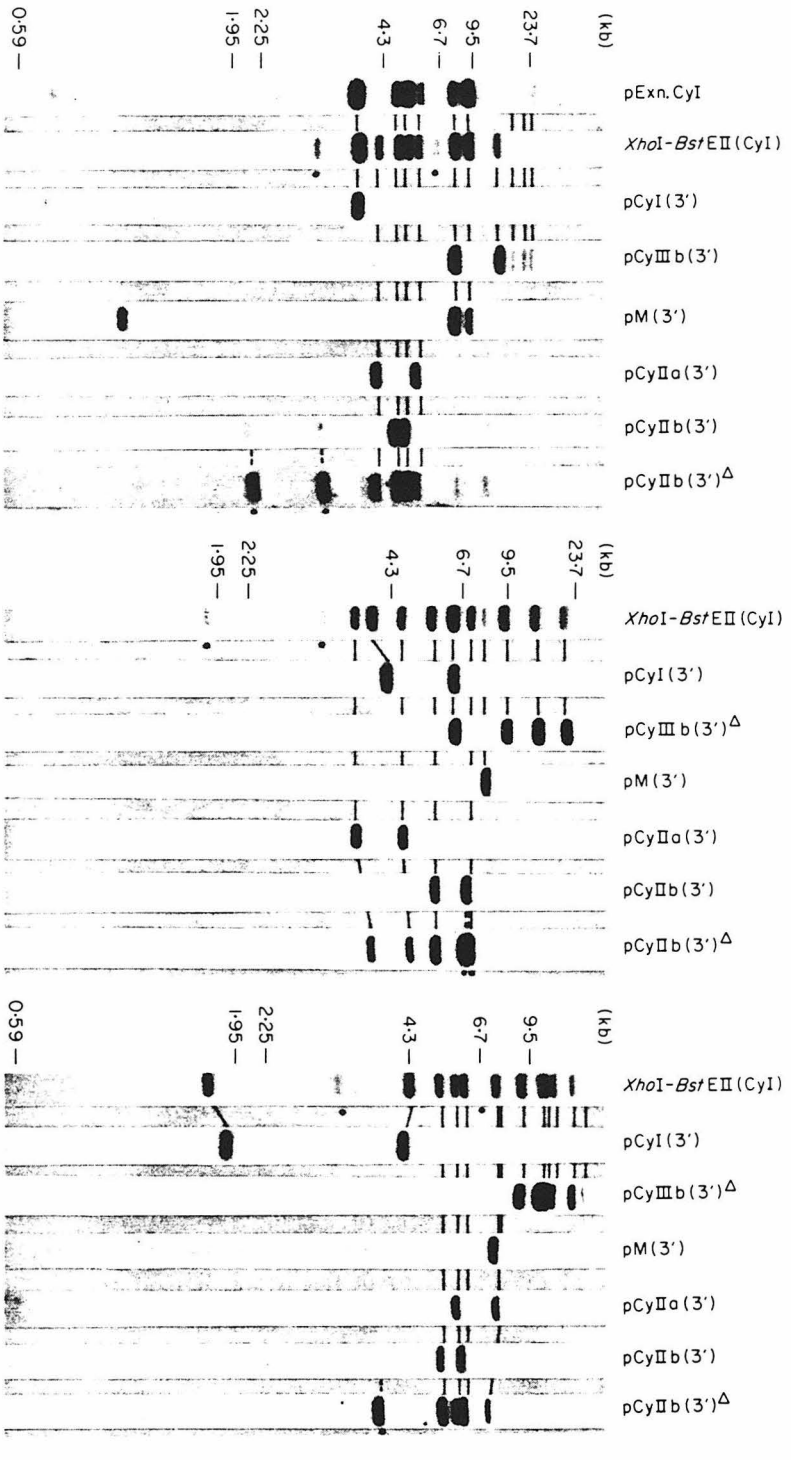


FIG. 3. Analysis of the *S. purpuratus* actin gene family utilizing the 3′ subtype probes. The genome blots were carried out with *Hin*dIII-digested DNA. Construction and labeling of the subcloned probes are described in Materials and Methods. The *Xho*I-*Bst*EII coding region probe is described in the legend to Fig. 2. Hybridizations were carried out at the 68°C, 0·75 M-Na^+ criterion, except where the track is labeled Δ. The lanes thus marked were hybridized at the 55°C, 0·75 M-Na^+ criterion. The transverse lines connect corresponding bands. Where these lines are not parallel, the gels were run for different lengths of time and the assignments are based on the calibration markers shown. Bands marked with a large dot in the left-hand coding region probe lanes represent actin genes for which directly corresponding 3′ terminal sequences are not observed. Bands marked with a large dot in the right-hand CyIIb probe lanes represent the bands assigned to the putative CyIIc gene. In (a), representing DNA of individual B, all the lanes were derived from a single gel, except the low criterion lane; (b) similar analysis representing the DNA of individual S; (c) same, with DNA of individual M.

the CyI subtype. Prompted by the results shown in Figure 2, we further examined these isolates, and have now concluded that they are cloning artifacts. In one experiment (not shown) a new set of CyI actin genes was isolated from another genome library and reacted with flanking sequence probes derived from several of the recombinants containing CyI actin genes reported by Scheller *et al.* (1981). In several of the original recombinants the sequences surrounding the CyI gene proved to be illegitimate. Possibly there are sequence features in the vicinity of this gene that promote deletion or recombination. It seems incontrovertible, from the data in Figure 2(b), that there is in fact only one CyI gene. Crain *et al.* (1982) have also arrived at this conclusion.

TABLE 1

S. purpuratus *actin gene subtypes*

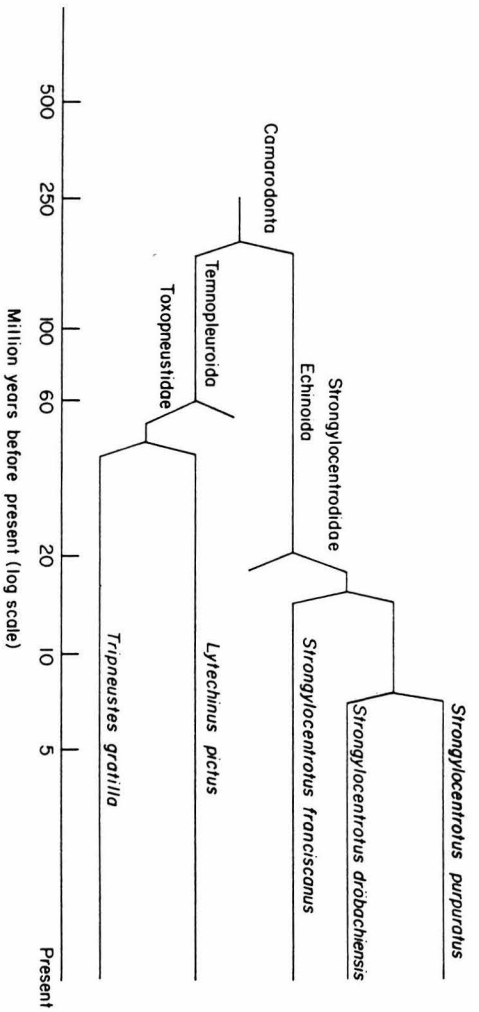
Actin gene type†	Subtype designations‡	Individual gene designations	Gene designations in previous studies‖
Cytoskeletal	CyI	CyI	Gene C[a], gene 2[b], pSp17[c], λSA16[c], λSA22[c]
	CyII	CyIIa	Gene G/F[a], gene 1[b]
		CyIIb	λSA11[c], gene I/H[a], pSA38[d], λSA11[c]
		(CyIIc)§	—
	CyIII	CyIIIa	—
		CyIIIb	Gene K[a]
		CyIIIc	—
Muscle	M	M	Gene J[a], pSp28[c]

† The actin genes are classified as cytoskeletal or muscle types on the basis of their representation in the poly(A) RNA of various adult and embryo sea urchin tissues (Shott *et al.*, 1983). The *cytoskeletal* actin genes are represented in the RNAs of eggs, or pregastrula embryos, or adult coelomocytes, none of which include muscle. *Muscle* actin gene transcripts are not detectable in any of these cell types, but are found in adult sea urchin lantern muscle and tubefeet.

‡ Subtype designation (Cy, cytoskeletal; M, muscle) refers to the transcribed but not-translated 3′ terminal sequence region of the gene, as described in the text. Genes of a given subtype are those that display reaction with the respective 3′ terminal subcloned probe at a hybridization criterion of 55°C, 0·75 M-Na^+.

§ The existence of this gene is only inferred, as discussed in the text, from genome blot reactions with the CyIIb 3′ terminal probe sequence. However, it is not yet demonstrated that this sequence is associated with sequences homologous to actin gene coding regions.

‖ [a] Scheller *et al.* (1981); [b] Schuler *et al.* (1983) isolated "gene 1" and "gene 2" from the same recombinant DNA library as utilized by Scheller *et al.* (1981). This library was described by Anderson *et al.* (1981). [c] Durica *et al.* (1980); [d] Merlino *et al.* (1980). This clone is a complementary DNA clone. Its identification as a cloned transcript of the CyIIb gene is based on 3′ terminal sequence data. The clone was sequenced by Schuler *et al.* (1983). Comparison with our data for the CyIIb gene shows the two sequences to be almost identical. [e] Overbeek *et al.* (1981). These identifications are tentative, as they are based only on restriction map homologies. λSA11 probably contains both the CyIIa and the CyIIb genes. It is probably identical to λSpG2-39 of Scheller *et al.* (1981). The isolates of Overbeek *et al.* (1981) are also derived from the genome library of Anderson *et al.* (1981).


Strongylocentrotus purpuratus
Strongylocentrotus dröbachiensis
Strongylocentrotus franciscanus
Strongylocentrotidae
Echinoida
Lytechinus pictus
Temnopleuroida
Toxopneustidae
Tripneustes gratilla
Camarodonta
500
250
100
60
20
10
5
Present
Million years before present (log scale)

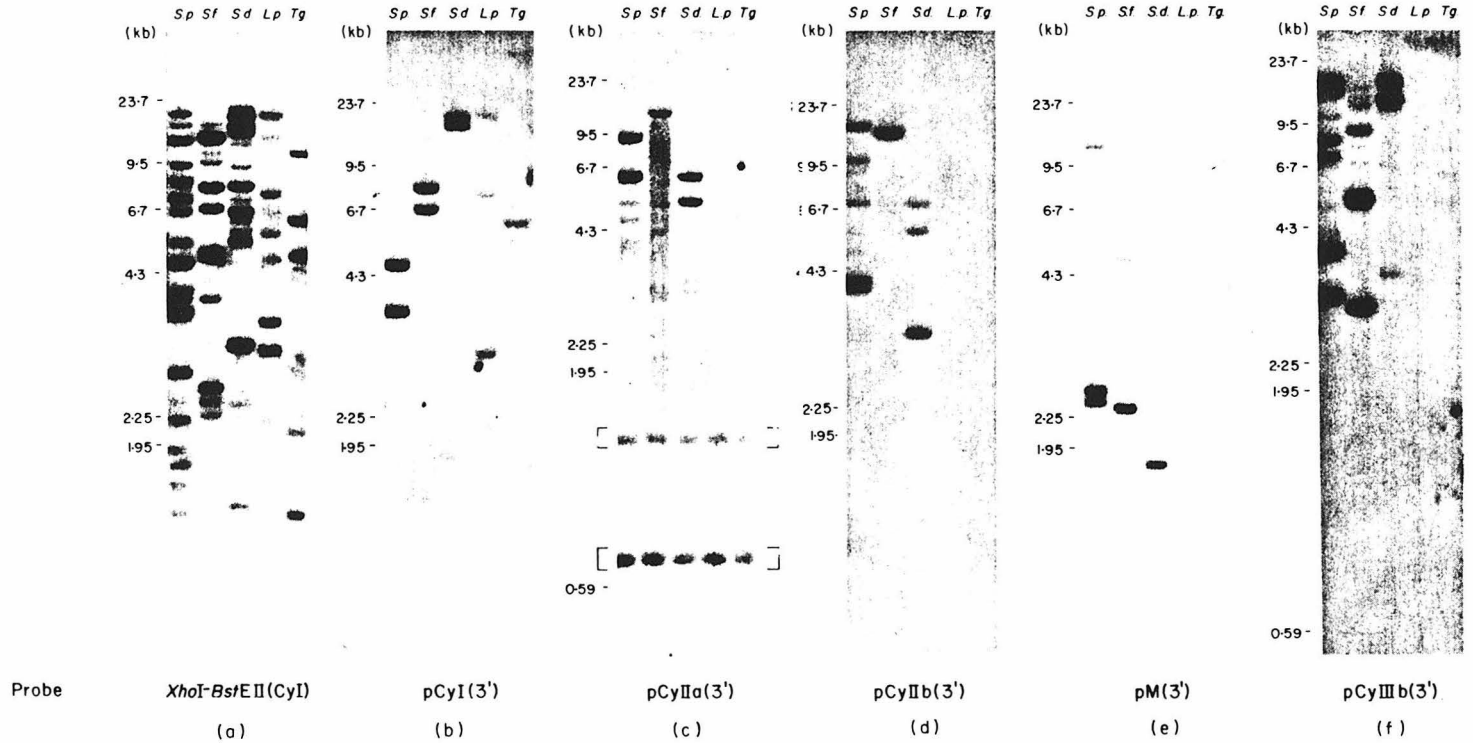


FIG. 4. Evolution of 3′ terminal actin gene sequences in 5 sea urchin species. Phylogenetic relation of the Toxopneustidae and the Strongylocentrotidae is indicated in the Figure at the top. The time scale is according to Durham (1966). It should be regarded as only approximate. Only the relevant branches are shown and the absence of forks is not meant to imply the absence of other members of the taxon. The bottom portion displays a series of genome blot analyses of the DNAs of 5 sea urchin species: Sp, *Strongylocentrotus purpuratus*; Sf, *S. franciscanus*; Sd, *S. dröbachiensis*; Lp, *Lytechinus pictus*; Tg, *Tripneustes gratilla*. The DNA preparations utilized were each obtained from an individual sea urchin. Each lane contained 5 μg of DNA that had been digested with *Eco*RI. The indicated probes (see Figs 1 and 3) were labeled and hybridized at the 55°C, 0·75 M-Na^+ criterion as described in Materials and Methods. The autoradiographs represent in each panel the reactions of DNAs blotted from a single gel. (a) Probe representing a portion of the 3′ coding region of the CyI gene; (b) probe representing the 3′ terminal untranslated region of the CyI gene; (c), (d), (e) and (f) probes representing the corresponding 3′ regions of the CyIIa gene, the CyIIb gene, the M gene, and the CyIIIb gene, respectively. The bands labeled with brackets in (c) are non-reproducible artifacts, apparently due to a contaminant introduced during the experiment.

(c) *The actin gene family of* S. purpuratus

The results so far considered can be summarized as follows. There are in the genome of *S. purpuratus* clearly seven actin genes; namely, CyI, CyIIa, CyIIb, CyIIIa, CyIIIb, CyIIIc and M, and if CyIIc is included, eight. It is important to note that all five of the 3′ subtype probes utilized in this analysis react with RNA transcripts (Shott *et al.*, 1983). An additional probe for gene CyIIIa, not included in this study, is also known to be represented in cellular RNAs. Thus, at least six of the seven to eight actin genes are expressed. The results of the experiments in Figures 2 and 3 are presented in Table 1. We also provide in Table 1 a cross reference to the various *S. purpuratus* actin clone nomenclatures utilized in previous studies, from our own and other laboratories.

Scheller *et al.* (1981) discovered that several of the actin genes are linked. This has been confirmed independently, in studies utilizing the same λ genomic recombinant libraries, by Schuler & Keller (1981) and G. P. Moore & L. J. Kleinsmith (personal communication). The linked genes, in terms of our present analysis, are the cytoskeletal actin genes CyI-CyIIa-CyIIb. These genes are all oriented in the same direction and are spaced at distances of about 10 kb (Scheller *et al.*, 1981). A remaining possibility, suggested by the large size of the fragments displayed by the CyIII probe in the experiments of Figure 3, is that these fragments could include more than one CyIII gene. However, in blots of genomic DNA cut with other restriction enzymes, e.g. *Eco*RI, the number of restriction fragments containing sequences homologous to CyIII genes is similar to the number present in genomic DNA cut with *Hin*dIII, and the restriction fragments are of sizes that rule out the possibility of more than one gene per fragment.

(d) *Evolutionary divergence of the 3′ terminal actin gene sequences*

Genome blot hybridizations with the *S. purpuratus* 3′ subtype actin gene probes were carried out on DNAs of five sea urchin species. The approximate evolutionary and taxonomic relation of these species, according to morphological and to paleontological data, is described diagrammatically at the top of Figure 4. As expected, the 3′ coding region probe displays a set of actin gene bands in the DNAs of all five species. This is shown in Figure 4(a). The major result of the experiment is that only the CyI 3′ terminal probe reacts outside of the genus *Strongylocentrotus,* though all five probes cross react completely within this genus. Restriction fragments containing the coding region of the actin genes are well-resolved in the *Tripneustes* and *Lytechinus* blots of Figure 4(a), and yet none of these genes includes sequences homologous to the *S. purpuratus* CyIIa, CyIIb, CyIII or M 3′ terminal regions, that is, at the 55°C, 0·75 M-Na^+ reaction criterion. On the other hand, the Strongylocentrotid CyI 3′ sequence is clearly evident in the genomes of the two Toxopneustidae, though the orders to which the Strongylocentrotidae and the Toxopneustidae belong apparently diverged over 150 million years ago. The average rate of change of single copy sequences during sea urchin evolution has been estimated at about 1% per million years (Grula *et al.*, 1982), and by either this measure or the standards provided by the CyIIa,

CyIIb, CyIII and M 3′ terminal sequence, the untranslated 3′ region of the CyI actin gene has evidently been specifically conserved.

Figure 4 provides two additional items of information, both with reference to the genome of *S. franciscanus*. In (a) it can be seen that the coding region probe reveals significantly fewer bands in the genome of a *S. franciscanus* individual than are obtained in individual *S. purpuratus* DNAs (compare Fig. 4 with Figs 2(a) and 3(a) to (c)). This is due partly to the failure of the *S. purpuratus* probe to reveal all of the *S. franciscanus* actin genes at the criterion of the experiment, and may also imply a greater degree of actin gene linkage in the latter species. An additional unexpected result seen in panel (c) is that the *S. purpuratus* CyIIa probe appears to react with a highly repetitive sequence in the *S. franciscanus* genome. These interesting evolutionary features of the actin gene family are examined in more detail in the following sections.

(e) *The actin gene family in* S. franciscanus

To further explore the differences between the actin gene sets of *S. purpuratus* and *S. franciscanus* we carried out a genome blot analysis on the DNAs of two individuals of the latter species similar to that shown in Figure 3 for *S. purpuratus*. In this experiment the *S. purpuratus* 3′ terminal probes were again utilized. The results are shown in Figure 5. It is apparent that in both individuals the actin gene regions are indeed significantly less polymorphic than in any *S. purpuratus* individual so far investigated. At the 68°C criterion the coding region probe reveals only four bands in the DNA of individual K (Fig. 5(a)) and six bands in the DNA of individual F (Fig. 5(b)) (at the 55°C criterion other bands appear indicating additional divergent sequences related to the actin gene coding regions). Only a single restriction fragment allele is observed for genes M and CyIIa at 55°C in both individuals K and F. In addition, individual K is homozygous with respect to the restriction fragments on which are located at least one of the CyIII genes, and the CyI gene, respectively. The gel blots in Figure 5 suggest that there are two (or more) genes of the CyIII subtype. Further analysis would be required to determine if in fact there exists a third (CyIIIc) gene, as in *S. purpuratus*. Though in both individuals examined one of the CyIII genes and the M actin gene appear at the same position, this must be the result of the co-migration of adventitiously similar sized fragments. These fragments are only about 2·4 kb in length, which is too small to accommodate two linked actin genes.

The conclusion that the actin genes of *S. franciscanus* reside in significantly less polymorphic regions of the genome than in *S. purpuratus* is not predicted by what is known of the overall sequence polymorphism of this species. Grula *et al.* (1982) found that the average intraspecific single copy sequence divergence in *S. franciscanus* is about 3·2% compared to about 4% for *S. purpuratus*.

It was not possible to determine the location in the *S. franciscanus* genome blots of the fragment or fragments containing the CyIIa gene. As initially noted in the experiment in Figure 4 the CyIIa probe reacts with a plethora of individual

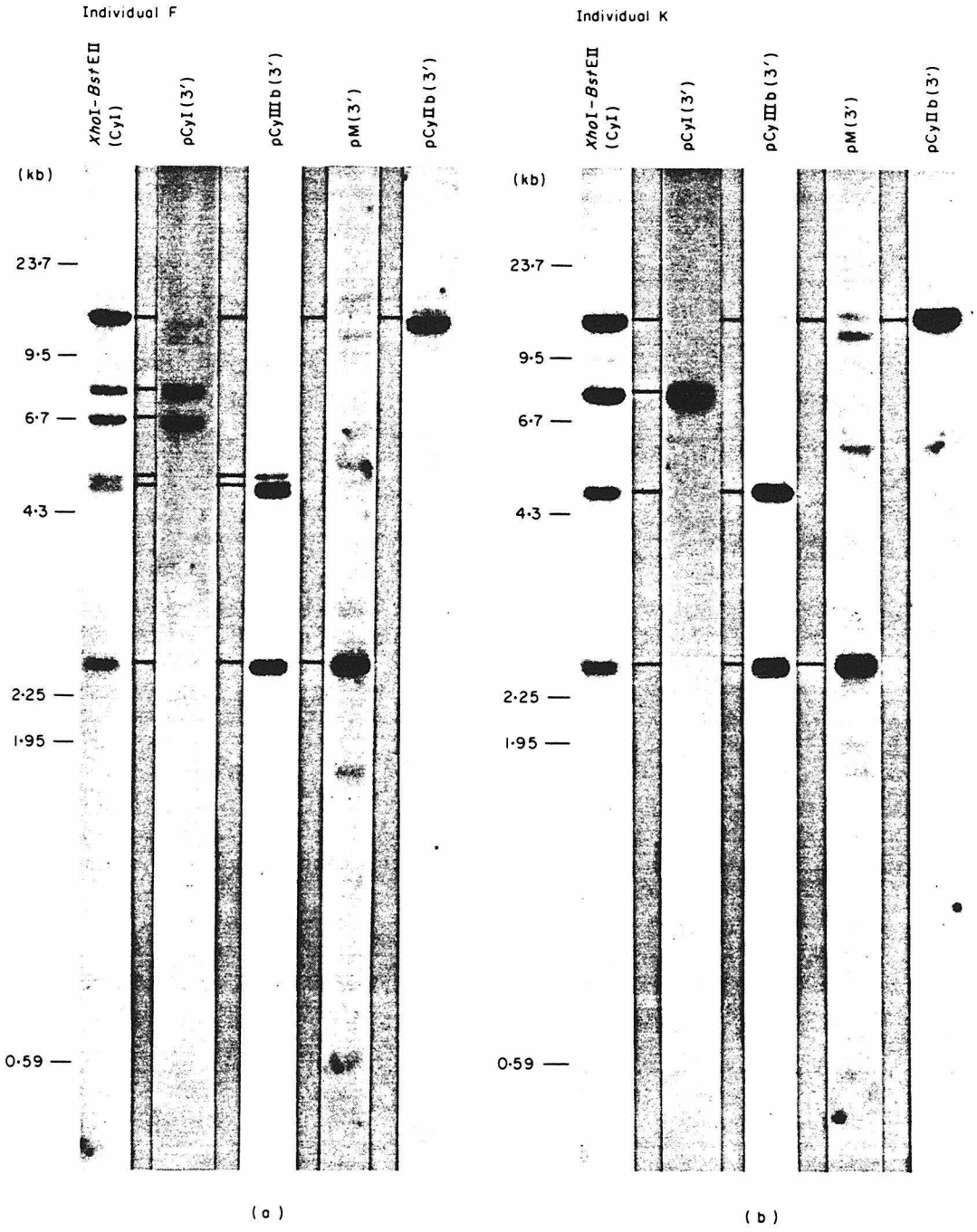

Individual F
XhoI-BstEII (Cγ1)
pCγ1(3′)
pCγIIIb(3′)
pM(3′)
pCγIIb(3′)
(kb)
23·7
9·5
6·7
4·3
2·25
1·95
0·59
(a)
Individual K
XhoI-BstEII (Cγ1)
pCγ1(3′)
pCγIIIb(3′)
pM(3′)
pCγIIb(3′)
(kb)
23·7
9·5
6·7
4·3
2·25
1·95
0·59
(b)

FIG. 5

fragments. This is demonstrated in Figure 6(a) for the DNAs of three *S. franciscanus* individuals, including the two utilized for the analysis presented in Figure 5. As a control, in Figure 6(b) the same probe is reacted at the same 55°C, 0·75 M-Na^+ criterion with the three *S. purpuratus* genomes investigated earlier. The difference in the pattern of these reactions provide dramatic evidence of the creation and expansion of an interspersed repetitive sequence family, an event that occurred in the relatively brief evolutionary time since the radiation of the genus *Strongylocentrotus*. In *S. purpuratus* the sequence carried in the CyIIa 3′ probe is represented exclusively in the 3′ non-coding or flanking sequences of the CyII actin gene subtype, though distantly related sequences not clearly detected in the genome blots shown could also be present. The same probe identifies in *S. franciscanus* DNA a middle repetitive sequence that has evidently been distributed so that it now inhabits a large number of diverse sequence environments.

(f) *Intrageneric evolution of the CyI-CyIIa-CyIIb actin gene linkage group*

Even though the location of the CyIIa gene cannot be directly identified, Figure 5 implies that, if it exists in the genome of *S. franciscanus*, it is linked to another actin gene. This follows from the fact that all the fragments containing actin genes revealed by the coding region probes are matched by corresponding fragments reacting with the subtype probes, *without* taking into account the CyIIa gene. In this section we show that the CyIIa gene is indeed present in the *S. franciscanus* genome, and that as in *S. purpuratus*, it is linked to the CyIIb gene. This demonstration requires the use of a different probe, and the DNA of a third species, *S. dröbachiensis* (see Fig. 4).

Figure 7(a) reproduces the familiar pattern of bands displayed in *S. purpuratus* genome blots by the CyI, CyIIa and CyIIb probes. The DNA is that of *S. purpuratus* individual B (see Fig. 2(e)). For convenience the particular bands containing the CyI, CyIIa, CyIIb and CyIIc sequences are identified throughout Figure 7. Figure 7(a) includes reactions of the CyI intron probe. In *S. purpuratus* DNA this probe apparently detects only the CyI gene, i.e. at the 55°C, 0·75 M-Na^+ criterion applied. This is expected from the sequence data of Schuler *et al.* (1983), which indicates that although related, the introns of the CyI gene and the CyIIa gene are too divergent for reaction to occur under our conditions. Scheller *et al.* (1981) demonstrated that reaction of both *S. purpuratus* CyI introns with the homologous introns of the CyIIa gene occurs at a lower criterion (50% (v/v) formamide, room temperature). On the other hand, as shown in Figure 7(b) in the

FIG. 5. Actin gene subtypes of the *S. franciscanus* genome. DNAs of two *S. franciscanus* individuals, denoted F (in (a)) and K (in (b)) were digested with *Eco*RI and subjected to a genome blot analysis with the 3′ subtype *S. purpuratus* probes indicated. The methods, the probes utilized, and the presentation of the data are as described for Fig. 3. Reactions with the coding region probes were carried out at the 68°C, 0·75 M-Na^+ criterion, and all other reactions shown were carried out at 55°C, 0·75 M-Na^+ criterion. The nature of the light bands on the CyIIIb and M probe lanes in (a) and (b) is unknown, since none of these bands corresponds to bands displayed by the 3′ coding region probe, and furthermore, all of the coding region bands can be accounted for. These light bands are unlikely to represent a partial digestion product, since all the DNAs in each panel of the Figure were digested together, and they are not cross reaction products, since the probes are totally non-homologous.

FIG. 6. Multiplication of the CyIIa 3′ teriminal sequence since evolutionary divergence of the *S. purpuratus* and *S. franciscanus* lineages. Each panel displays an autoradiograph of a blot taken from a single gel. (a) DNAs of *S. franciscanus* individuals K and F (see Fig. 5) and a third individual, P, were digested with *Eco*RI, blotted, and hybridized with the *S. purpuratus* CyIIa probe (i.e. pCyIIa(3′)) at the 55°C, 0·75 M-Na^+ criterion. (b) The same probe was reacted at the same criterion with genome blots of the *S. purpuratus* individuals B, S and M (see Figs 2 and 3). The restriction enzyme used in this case was *Hin*dIII. A similar number of discrete bands is observed in *Eco*RI-digested *S. purpuratus* DNA (not shown); the *Hin*dIII blot is presented to facilitate identification of the bands according to the analysis in Figs 2 and 3.

closely related species *S. dröbachiensis*, the *S. purpuratus* CyI intron probe clearly hybridizes to both the CyIIa and CyIIb genes at the 55°C criterion, as well as to the CyI gene, though with no other sequences in the genome. This interesting observation suggests that all three cytoskeletal actin genes that are known to be closely linked in *S. purpuratus*; namely, CyI, CyIIa and CyIIb, share a common ancestry. Note also that Figure 7(b) indicates the presence in *S. dröbachiensis* DNA of an additional actin gene of the CyII subtype that does *not* share homology with the CyI intron probe. This band is labeled (c) in Figure 7(b), since it could represent the third CyII subtype gene, CyIIc. The CyIIa probe displays no repetitive sequence reaction in the genome of *S. dröbachiensis*, so the evolutionary expansion of this sequence must have been confined to the lineage leading to *S. franciscanus* (see Fig. 4).

The *S. purpuratus* CyI intron probe also reacts with the CyIIa and CyIIb genes of *S. franciscanus*, as shown in Figure 7(c) to (e). This provides the key to an analysis of the CyII actin gene subtype in *S. franciscanus* DNA, since it is now possible to detect the CyIIa gene without encountering the 3′ terminal repeat sequence. In Figure 7(c) it is observed that the CyI intron displays a single dark band that migrates at the position of the fragment also containing the CyIIb gene. That this fragment, which is 12·5 kb long, contains both a CyIIa and a CyIIb gene is shown in Figure 7(d) and (e) (labeled a and b). Figure 7(d) displays reactions of the DNA of individual K with the CyI intron probe, and Figure 7(e) shows the reactions of this DNA with the CyIIb probe. The left-hand lanes of each Figure are blots of *Eco*RI digests, as in (c), and the subsequent lanes are blots of *Hin*dIII, and *Hin*dIII + *Eco*RI digests, as indicated. Bands present in Figure 7(d) and absent in (e) indicate fragments containing the CyI actin gene (labeled I in the Figure). Of the remaining bands, those that react more intensely with the CyI intron probe (in Fig. 7(d)) represent fragments containing the CyIIa gene, and those that react more intensely with the CyIIb probe (in Fig. 7(e)) indicate fragments containing the CyIIb gene. This experiment and others with different restriction enzymes (not shown) demonstrate that the *S. franciscanus* CyIIa and CyIIb genes are linked. We assume here that as in *S. purpuratus* and *S. dröbachiensis* the CyII subtype gene that is linked to CyIIb in *S. franciscanus* is indeed the CyIIa gene. These genes are separated and revealed individually in the double digestion experiment reproduced in the right-hand lanes of Figure 7(d) and (e).

The following statements summarize the differences and similarities in the actin gene families of the three *Strongylocentrotus* species investigated.

(1) *S. franciscanus* possesses at least a single M gene, a single CyI gene, a single CyIIa gene, and a single CyIIb gene. There are at least two CyIII genes in *S. franciscanus* and as we saw earlier, three CyIII genes in *S. purpuratus*. The closely related species *S. dröbachiensis* and *S. purpuratus* have three genes of the CyII subtype, while there is yet no evidence of a CyIIc sequence in *S. franciscanus*. However, since this gene is divergent in any case it is possible that it is present but was simply not detected at the reaction criterion utilized.

(2) The regions containing the actin genes of *S. franciscanus* are significantly less polymorphic than those containing the actin genes of *S. purpuratus*.

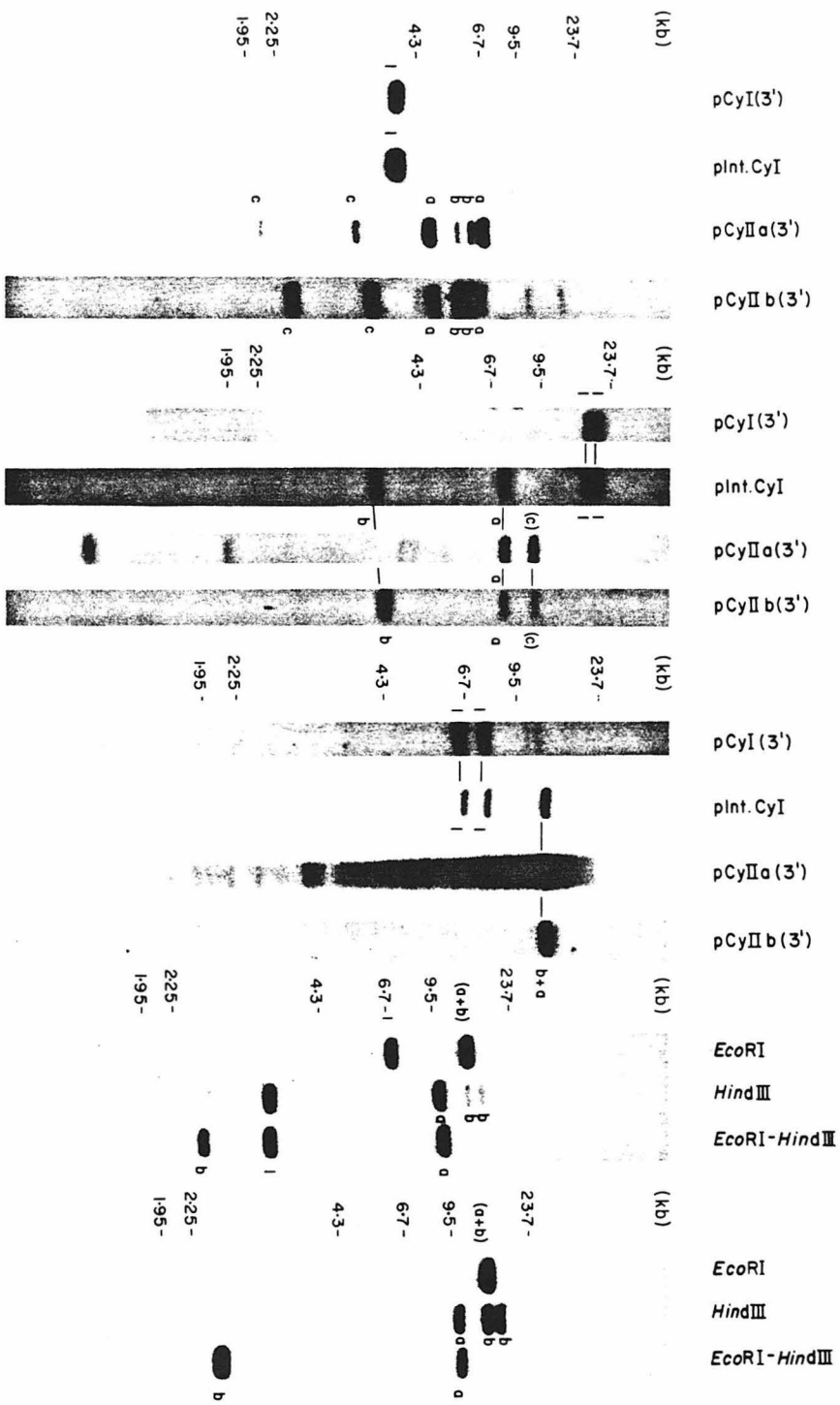

(a)
(b)
(c)
(d)
(e)
(kb)
23·7-
9·5-
6·7-
4·3-
2·25-
1·95-
pCyI(3')
pInt.CyI
pCyII a(3')
pCyII b(3')
EcoRI
HindIII
EcoRI-HindIII
Probe: pInt.CyI
pCyII b(3')

Fig. 7

(3) At least a portion of the CyI-CyIIa-CyIIb gene linkage discovered by Scheller *et al.* (1981) is evolutionarily old. Thus, the CyIIa and CyIIb genes must have been linked in the common ancestor of all existing *Strongylocentrotus* species. These genes and the CyI gene probably share a common origin, since in at least two of the three species studied homology with the CyI intron has been retained amongst all three genes (see also Schuler *et al.*, 1983).

(4) A sequence homologous to the CyIIa 3′ terminal sequence has given rise to an interspersed middle repetitive sequence family in the line leading to *S. franciscanus*.

4. Discussion

(a) *The actin gene family of* S. purpuratus

This work provides for the first time a relatively complete image of the actin gene family of *S. purpuratus*. There are seven or possibly eight actin genes in the genome of this species. The approach illustrated in Figures 2 and 3, the results of which are summarized in Table 1, bypasses a variety of difficulties that beset earlier studies. By making use of our cloned actin gene subtype probes we have been able to take into account, and indeed turn to our advantage, the high degree of sequence polymorphism characteristic of *S. purpuratus* DNA in general, and of the actin gene region in particular. Three individual genomes were analyzed, each displaying a unique set of polymorphic restriction fragments bearing actin genes. All three genomes yielded the same solution, with respect to the number of actin genes of each of the four subtypes.

Several specific uncertainties remain, pending isolation and examination of further genomic recombinants. There is yet no direct evidence that the CyIIc sequence detected by the CyIIb probe at the 55°C criterion is actually associated with an actin gene coding sequence. Figure 3 indicates one copy per haploid genome of a 3′ actin coding sequence that does not correspond to any of the fragments identified by the 3′ non-translated probes. On grounds of parsimony we tentatively assigned this actin gene to the CyII terminal sequence. Alternatively this actin gene could represent a fifth subtype. In this case the CyIIc sequence might instead be a disembodied copy of the CyII terminal region. The number of *S. purpuratus* actin genes would still total eight, though the number of subtypes would increase to five. An argument against the possibility of a fifth, yet unknown actin gene subtype derives from the *S. franciscanus* analysis in Figure 5. This shows that every one of the restriction fragments having actin gene coding regions

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FIG. 7. Linkage and evolutionary relationships amongst the CyI, CyIIa and CyIIb actin genes. The indicated probes were reacted with the genome blots at the 55°C, 0·75 M-Na^+ criterion. The *Xho*I-*Bst*EII fragment is the 3′ coding region probe of gene CyI; pInt.CyI is a probe for the first intron of the CyI gene (see Materials and Methods, section (b)); and pCyIIa(3′) and pCyIIb(3′) are the 3′ terminal non-translated subtype probes, as before. In this Figure the bands have been assigned to the different actin genes as described in the text: I, CyI; a CyIIa; b, CyIIb; c, CyIIc. Each panel displays an autoradiograph of genome blots prepared from a single gel. (a) *Hin*dIII-digested DNA of *S. purpuratus* individual B; (b) *Eco*RI-digested DNA of an *S. dröbachiensis* individual; (c) *Eco*RI-digested DNA of *S. franciscanus*, individual F; (d) and (e) DNA of *S. franciscanus* individual K digested with *Hin*dIII, *Eco*RI and *Eco*RI + *Hin*dIII, as indicated, and then reacted with the CyI intron probe in (d) and the CyIIb 3′ terminal probe in (e).

can be assigned to one of the four known *S. purpuratus* 3′ terminal actin gene subtypes. It seems unlikely that a fifth subtype is present in *S. purpuratus* but is missing from the genome of *S. franciscanus*. An element of doubt also persists regarding the CyIIIc gene, since characterization of genomic recombinants that may contain this gene has not yet been completed. Recombinant clones containing the CyI, CyIIa, CyIIb, CyIIIa or CyIIIb, and M actin genes are all available, and have been characterized to various extents (Scheller *et al.*, 1981; Durica *et al.*, 1980; Schuler & Keller, 1981; and unpublished data). Furthermore, each one of these six actin genes is known to be expressed in the *S. purpuratus* embryo or in adult tissues (Shott *et al.*, 1983).

An isolate containing the CyIIIb gene was initially described by Scheller *et al.* (1981) while the CyIIIa gene is discussed here for the first time. The CyIII actin gene subtype is biologically both interesting and important. Shott *et al.* (1983) discovered that the CyIII genes are expressed exclusively in the embryonic and larval stages of life, and unpublished work of K. Cox, J. Lee, E. Davidson & R. Angerer demonstrates with the same probes used in the present work that CyIII transcripts are confined to ectoderm cells. This was also observed by Bruskin *et al.* (1981), whose "Spec 4" sequence is a CyIII actin gene (unpublished data). Furthermore, the CyIIIa gene contributes a larger amount of actin mRNA during embryogenesis than does any other of the actin genes (Shott *et al.*, 1983).

(b) *Evolution of the non-translated 3′ terminal actin gene sequences*

Unlike the 3′ terminal sequence of the CyII, CyIII or M actin gene subtypes, at least some elements of the 3′ non-translated CyI gene sequence have been conserved over immense periods of evolutionary time (Fig. 4). It is germane to compare the remarkable example of evolutionary conservation in a 3′ non-translated actin gene sequence recently discovered by Ordahl & Cooper (1983). They showed that the rat and chick skeletal muscle α-actin genes are highly homologous in the 3′ terminal region just proximal to the poly(A) addition site, including a perfectly matched sequence of 19 nt in identical positions in the two genes. The lineages giving rise to the Temnopleuroidan and Echinoidan orders of the echinoderms have been evolving separately for over 150 million years, and the last common ancestors of the birds and mammals existed over 250 million years ago. In both cases the actin genes displaying these highly conserved 3′ terminal sequences are single copy, and the sequence elements in question appear nowhere else in the genome. It seems inescapable that accumulation of random mutations in the 3′ terminal sequences of these actin genes has been prohibited by natural selection, and therefore that these conserved sequences must function in some way that is important for the expression of these genes. This is also the conclusion reached by Ordahl & Cooper (1983). Furthermore, if these particular actin gene 3′ terminal sequences are functionally significant, so might be others, even if they are less extensively conserved. It is a general and intriguing feature of the actin genes in several organisms that the specific genes utilized in different tissues or at different times in the life cycle are distinguished by their specific 3′ terminal sequences. This has now been shown for the rat (Shani *et al.*, 1981), the mouse

(Minty *et al.*, 1982), *Drosophila* (Fyrberg *et al.*, 1981), the chick (Ordahl & Cooper, 1983), *Dictyostelium* (McKeown & Firtel, 1981) and the sea urchin.

The most striking example of rapid evolutionary *change* observed in the present work also concerns a 3′ terminal actin gene sequence. As illustrated in Figure 6, sequence elements of the *S. purpuratus* CyIIa 3′ terminal probe are represented in a very large number of dispersed copies in the *S. franciscanus* genome. The CyIIb 3′ terminal probe sequence does not reveal this new middle repeat family, though it cross reacts with the CyIIa gene. This is because the CyIIb probe represents a more confined region of the terminal sequence than is included in the CyIIa probe, and this region was evidently not involved in the sequence multiplication phenomenon. Though it appears likely that the sequence that has undergone replication lies within the 3′ untranslated region of the message (unpublished data), its exact location and size are not yet determined. The explosive expansion of the CyIIa 3′ probe sequence must have occurred in the last 15 million years or so, since it is confined to the *S. franciscanus* branch of the genus (Fig. 7). Perhaps it is still in process.

Earlier studies from this laboratory have indicated that many of the large interspersed repeat sequence families in the *S. purpuratus* genome are the product of sequence multiplication events occurring since the radiation of the genus. These have resulted in some cases in more than a tenfold increase in the number of repeat sequence family members, compared to the homologous *S. franciscanus* sequence family (Moore *et al.*, 1978,1981). However, the present observation is unique in two respects. First, it provides a complementary example in which an *S. franciscanus* repeat family has expanded. More importantly, it seems likely that in this instance a middle repetitive sequence family has been created from a sequence found in congeners as a part of a very low copy number structural gene. The genome blot displayed in Figure 6(a) is indistinguishable from that obtained with known interspersed sea urchin repetitive sequence probes (e.g. see Moore *et al.*, 1981; Posakony *et al.*, 1983). The mechanism underlying this dramatic evolutionary change in genomic organization may be accessible through studies of the structure of the multiplied repeat sequence elements.

(c) *Organization and regulation in the actin gene family*

In a recent discussion of the evolution of the actin gene family we proposed that new actin genes may have arisen at frequent intervals through duplication, and that transposition of such genes into ontogenic genomic "regulatory modules" may be among the evolutionary processes leading to the phylogenetic appearance of novel forms (Davidson *et al.*, 1982). Our current results are useful in considering further this speculation.

Figure 3 shows that in *S. purpuratus* there is a single muscle-specific actin gene. Shott *et al.* (1983) demonstrated copious transcripts of the M actin gene in adult lantern muscle, tubefoot and intestine. Vertebrate genomes also appear to contain a single copy skeletal muscle α-actin gene (Nudel *et al.*, 1982; Ordahl *et al.*, 1980; Fornwald *et al.*, 1982) that must be utilized in a variety of different morphological structures. On the other hand the genomes of all animals so far studied include

multiple cytoskeletal actin genes, though in most cases it is not known how many are actually utilized (reviewed by Buckingham & Minty, 1983). Cytoskeletal actins appear in virtually every cell type, where they perform a variety of functions, both structural and contractile. Though it cannot be excluded, recent evidence does not argue strongly for the proposition that the cytoskeletal actin genes are multiple because their diverse functions require a variety of specific protein sequences. For example, several differentially expressed actin genes of *Dictyostelium* (McKeown & Firtel, 1981) code for an identical cytoskeletal actin (Vandekerckhove & Weber, 1980); the six actin genes of *Drosophila* (including those utilized specifically in muscle) all code for extremely similar proteins differing mainly by a few conservative amino acid substitutions and resembling mammalian cytoskeletal actins in sequence (Fyrberg *et al.*, 1981); and the two sequenced cytoskeletal actin genes of the sea urchin (i.e. CyI and CyIIa), which are also expressed differentially (Shott *et al.*, 1983) differ by only 1·3% of their amino acids (Schuler *et al.*, 1983). The data now emerging suggest that at least some of the multiple, differentially expressed cytoskeletal actin genes in the genome of a given organism may code for insignificantly diverse proteins.

An hypothesis along the lines of that discussed earlier (Davidson *et al.*, 1982) is that each of the cytoskeletal actin genes belongs to a regulatory module that determines an aspect of subcellular structure, for instance microvilli, or microfilaments. Depending on the specific differentiated state to which a given cell is assigned, it may require the expression of one or several such regulatory modules, each of which controls a set of individual genes, including an actin gene. Another such module, that is called upon in muscle cells, would control the set of genes required for myofibril construction, including in *S. purpuratus* the M actin gene, and in vertebrate skeletal muscle, the α-actin gene. This hierarchial view of the genomic basis of cell differentiation could ultimately be tested by gene transfer experiments in which given actin genes are associated with the regulatory sequence of other actin genes.

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Note added in proof: The existence of the actin gene CyIIIc, inferred from the genome blots, has now been confirmed by the isolation and characterization of λ genomic recombinants that contain this gene.

CHAPTER 2

Differential Expression of the Actin Gene Family of *Strongylocentrotus purpuratus*

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Differential Expression of the Actin Gene Family of *Strongylocentrotus purpuratus*

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Molecular probes that individually recognize the 3′ nontranslated regions of six actin genes were utilized in RNA gel blot hybridizations to detect RNAs complementary to each gene in embryonic and adult tissues of *Stronglyocentrotus purpuratus*. In addition the probes were used in DNA excess filter hybridizations to estimate the relative contribution of the different actin genes. All six genes produce relatively stable mRNAs, and each displays a characteristic and distinct pattern of expression. On the basis of their expression in the egg, early embryos, or in adult coelomocytes, it is concluded that genes termed CyI, CyIIa, CyIIb, CyIIIa, and CyIIIb encode cytoskeletal actin proteins. Actin gene M gives rise to mRNAs that are found only in tissues containing muscle. Actin genes CyI, CyIIa, CyIIb, and M are expressed in both adult and embryonic tissues, giving rise to transcripts 2.1-2.2 kb in length. Expression of genes CyIIIa and CyIIIb is confined to the embryo. Gene CyIIIa provides the major embryonic actin mRNA, which is 1.8 kb in length. Three of the cytoskeletal actin genes are linked over a 30-kb distance in the *S. purpuratus* genome. We show that the actin genes included in this linkage group are not coordinately expressed.

INTRODUCTION

The genome of the sea urchin *Strongylocentrotus purpuratus* contains eight nonallelic actin genes (Lee *et al.*, 1983). It was previously shown by Crain *et al.* (1981), Merlino *et al.* (1980), and Scheller *et al.* (1981) that at least two different actin genes are expressed differentially during early embryogenesis. Our object in the investigation described in this paper was to determine the patterns of expression, in embryos and in adult tissues, of the members of this complex gene family. We made use of a set of five subcloned probes prepared as described by Lee *et al.* (1983), each of which recognizes the nontranslated 3′ terminal region of a specific actin gene, and of an additional probe that was constructed for the purposes of this study. These probes were reacted with RNAs extracted from a variety of embryonic stages and adult sea urchin tissues. We show that at least six of the eight actin genes are expressed at some stage in the development of the sea urchin. Five of these genes can be classified as cytoskeletal (Cy) actin genes, according to the criterion that their expression is detectable in embryos prior to the differentiation of any muscle-containing tissues, or in adult sea urchin coelomocytes. The latter are free wandering nonmuscle cells, which have been implicated in a variety of functions, including formation of clots in response to injury, graft rejection, phagocytosis, and ingestion of unused ovarian oocytes (Hyman, 1955; Endean, 1966; Coffaro and Hinegardner, 1977). We found that the cytoskeletal actin genes are activated to different extents during embryogenesis, and that each displays a distinct pattern of expression during the life cycle, even though some of them are linked in the genome. A single muscle-specific actin gene, M, was found that produces high levels of mRNA in adult muscle tissues. Transcripts of this gene are not observed until the pluteus stage of development.

MATERIALS AND METHODS

Growth of sea urchin embryos and larvae. Eggs of *S. purpuratus* were collected, fertilized, and the embryos were cultured at 15°C with stirring as described previously (Smith *et al.*, 1974; Hough-Evans *et al.*, 1977). Larvae (one larva per 3 ml) were grown at 15°C with stirring and fed daily with *Rhodomonas* (3000 ml^{-1}). One week prior to metamorphosis (for these cultures, 6 weeks postfertilization), 500 premetamorphosis stage larvae were harvested by filtration through a Nitex filter. Metamorphosis was initiated by allowing 6-week-old embryos to settle on the bottom of the culture vessel following removal of the stirring apparatus. Juvenile sea urchins were harvested 1 week postmetamorphosis by washing the culture vessel in Millipore-filtered seawater to remove any cellular debris and unattached larvae. The animals were examined microscopically to ensure removal of all larval material. For RNA extraction 500 juvenile sea urchins were individually transferred to a Nitex filter, and rinsed in Millipore-filtered seawater.

RNA extractions. Embryos were harvested by centrifugation. Coelomocytes were harvested as previously

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described (Wold *et al.*, 1978). Ovary, testis, tubefoot, lantern, and intestine were removed by dissection. RNA was extracted by the method of Chirgwin *et al.* (1979). Approximately 10^7 eggs or embryos were homogenized in 20–40 ml (depending on developmental stage) of guanidinium thiocyanate reagent (5 *M* guanidinium thiocyanate, 50 m*M* EDTA, 50 m*M* Tris-HCl, pH 7.0, 5% β-mercaptoethanol) using a hand-held homogenizer. Similarly, coelomocytes from 50 sea urchins, tubefeet from 200 sea urchins, gonads from 5 sea urchins, or lantern structures from 10 sea urchins were homogenized in 30 ml of guanidinium thiocyanate reagent. The homogenate was sonicated until the solution lost its viscosity. Sarcosine was added to a final concentration of 2% and the homogenates heated at 60°C for 5 min. Cellular debris and test fragments were removed by centrifugation at 4000 rpm for 30 min. The supernatants were layered over a 10-ml cushion of 5.7 *M* CsCl, 50 m*M* EDTA (pH 7.0), and centrifuged for 40 hr at 39,000 rpm, 15°C (Glišin *et al.*, 1974) in a Beckman 70 Ti fixed-angle rotor. Supernatants were removed by aspiration and the RNA pellets were retrieved and dissolved in water. Five hundred larvae or juvenile sea urchins were extracted in 2 ml of guanidinium thiocyanate reagent as described above, and the extract layered over a 1.2-ml cesium chloride cushion. RNA was pelleted by centrifugation for 24 hr at 39,000 rpm, 15°C in a Beckman SW55 rotor. The RNA was phenol:chloroform-extracted, chloroform isoamyl alcohol-extracted, and ethanol-precipitated. Poly(A) RNA was isolated by affinity chromatography as described by Aviv and Leder (1972).

Preparation of ^{32}P-end-labeled probes. Probes used for RNA blot analysis are listed in Table 1. Ten micrograms of each plasmid were digested with the appropriate restriction enzymes. One hundred microcuries each of [α-^{32}P]dATP, [α-^{32}P]dCTP, [α-^{32}P]dGTP, and [α-^{32}P]dTTP (Amersham; specific activity >3000 Ci/mmole) were added and the reaction diluted to 80 μl with 40 m*M* Tris-HCl, pH 7.4. Five units of DNA polymerase Klenow fragment were added (New England Biolabs) and incubated for 10 min at room temperature. Following electrophoretic separation appropriate end-labeled fragments were electroeluted from 2% agarose gels and ethanol precipitated. The specific activity for 400–800 nucleotide (nt) fragments was 2-5 × 10^7 cpm/μg.

RNA gel blot analysis. RNA gel blots were performed

TABLE 1
CHARACTERISTICS OF GENE-SPECIFIC PROBES

Probe	Subclone[a]	Hybridization specificity at 55°C[b]	Hybridization specificity at 65°C[b]	Length of probe complementary to mRNA[c] (nt)	Probe length (nt)
Actin[d] coding region	pExn.CyI	All actin genes	All actin genes	480	700
CyI	pCyI (3′)	CyI	CyI	300	780
CyIIa	pCyIIa (3′)	CyIIa CyIIb	CyIIa	720	720
CyIIb	pCyIIb (3′)	CyIIa CyIIb	CyIIb	400	400
CyIIIa	pCyIIIa (3′)	CyIIIa CyIIIb CyIIIc	CyIIIa CyIIIb CyIIIc	400	400
CyIIIb	pCyIIIb (3′)	CyIIIa CyIIIb CyIIIc	CyIIIa CyIIIb CyIIIc	550	800
M	pM (3′)	M	M	227	1300

[a] Restriction maps and details on the construction of these subclones are presented in Lee *et al.* (1983), except for pCyIIIa (3′), for which see Materials and Methods.

[b] Hybridizations performed at 55 and 65°C in 5× SET, 0.025 *M* phosphate buffer (pH 6.8). The 65°C hybridization conditions are equivalent to those used in the RNA gel blot experiments presented here.

[c] Inferred from location of putative poly(A) addition sites revealed by DNA sequencing (see Lee *et al.*, 1983), except CyIIIa which as described, is a cDNA probe.

[d] A 480-nt section of this probe is occupied by the sequence of the coding region of the first exon of gene CyI, and 200 nt by the flanking 5′ noncoding region (Lee *et al.*, 1983).

as described by Scheller *et al.* (1981) using either 10 μg of total RNA or 1 μg of poly(A) RNA. RNA size markers were sea urchin mRNAs. Radioactive bands were cut from nitrocellulose filters and counted by scintillation in Ready Solv (Beckman).

DNA dot analysis. DNAs were bound to nitrocellulose essentially as described by Kafatos *et al.* (1979). Whole-plasmid DNA (32 μg/ml) was nicked and denatured by boiling for 1 min in 0.4 *M* NaOH. Samples were chilled on ice and neutralized with ice-cold 2 *M* ammonium acetate. Equimolar quantities of approximately 1 μg were spotted onto 0.22-μm nitrocellulose filters (Millipore) under suction. Filters were baked at 80°C *in vacuo* for 2 hr.

Preparation of [^{32}P]cDNA. One microgram of poly(A) RNA was incubated for 2 hr at 42°C in a 50-μl reaction mix containing 100 m*M* Tris-HCl (pH 8.7), 100 m*M* KCl, 10 m*M* $MgCl_2$, 30 m*M* β-mercaptoethanol, 1 m*M* dATP, 1 m*M* dGTP, 1 m*M* dTTP, 1 μg oligo$(dT)_{17}$, 50 μCi [α-^{32}P]dCTP (Amersham; specific activity 800 Ci/mmole), and 1 μl reverse transcriptase (Life Sciences Corp.). Unincorporated triphosphates were removed by Sepharose G-75 chromatography. Specific activity of cDNA was 1.3×10^9 cpm/μg.

Hybridization. All hybridizations were performed overnight at 43°C in 5× SET, 1× Denhardt's solution (Denhardt, 1966), 0.025 *M* phosphate buffer (pH 6.8), 10% dextran sulfate, 50% formamide, 50 μg/ml calf thymus DNA, 10 μg/ml poly(rA), and 10^6 cpm/ml ^{32}P-labeled DNA probe. SET is 0.15 *M* NaCl, 0.02 *M* EDTA, 0.01 *M* Tris-HCl (pH 7.4). Filters were washed for 1 hr in 1× SET and 1 hr in 0.3× SET at 65°C.

Preparation of subclone pCyIIIa (3'). This probe was isolated from a gastrula-stage cDNA library constructed by dG-dC tailing of double-stranded cDNA, and ligation into the *Pst*I site of pBR322 (Sutcliffe, 1978) (library provided by W. Klein). The probe sequence was defined initially by virtue of cross-reaction with a characterized 3′ terminal sequence belonging to the CyIII actin gene subtype (Lee *et al.*, 1983). The cDNA insert was excised with *Pst*I and subcloned into the corresponding site of pUC9 (Vieira and Messing, 1982) to facilitate insert excision and end-labeling. This probe extends 400 nucleotides (nt) in a 3′ direction from an *Ava*II site located 30 nt 5′ to the translation stop codon. The probe also contains approximately 50 nt of the poly(A) tail of the mRNA. For labeling by the Klenow repair reaction the insert was excised by an *Ava*II-*Eco*RI digest.

RESULTS

Probes Specific to Individual Actin Genes

In order to analyze the expression of the various actin genes during development and differentiation subcloned probes specific to each individual gene were required. These probes were prepared by subcloning the 3′ noncoding actin gene regions of appropriate genomic DNA isolates into the plasmid vectors pUC9 or pBR322. Construction of probes specific for the four cytoskeletal actin genes CyI, CyIIa, CyIIb, CyIIIb, and the single muscle actin gene, M, was described by Lee *et al.* (1983). An additional probe for the cytoskeletal actin gene CyIIIa was obtained in this work from a cDNA clone (see Materials and Methods). We did not investigate the seventh and eighth actin genes, CyIIc and CyIIIc, since cloned genomic isolates of these genes have not as yet been fully characterized.

Even under the stringent conditions of hybridization used in this work, the DNA probe for gene CyIIIb showed some cross-reaction to the transcript of gene CyIIIa. However, since gene CyIIIa gives rise to a 1.8-kilobase (kb) transcript while gene CyIIIb produces a 2.1-kb transcript their expression can be distinguished. There remains a possibility that the 3′ CyIIIa or CyIIIb gene probes cross-react with transcripts of gene CyIIIc. Thus mRNAs detected by the CyIIIa or CyIIIb probe sequences may also represent the transcription products of gene CyIIIc. Under the reaction conditions used the other four 3′ probes were completely specific to the actin genes from which they were derived (Lee *et al.*, 1983). Characteristics of all of the subcloned probes that we utilized are summarized in Table 1.

Expression of Actin Genes in Adult Sea Urchin Tissues

To investigate variations in the levels of actin transcripts in different adult tissues, we performed a series of RNA gel blot hybridizations. The six specific 3′ probes and the actin coding region probe indicated in Table 1 were labeled with ^{32}P by the Klenow repair reaction, and used in RNA gel blot hybridization experiments with either poly(A) RNA or total RNA isolated from ovary, testis, coelomocytes, intestine, tubefoot, or lantern muscle. Autoradiographs of some of these blots are shown in Fig. 1 and the results are summarized in Table 2.

Only four of the six actin genes represented by our probes are expressed in the adult somatic tissues or in testis, at least at the level of detection afforded by the gel blot method. Genes CyI, CyIIa, and M produce 2.2-kb mRNA species, whereas gene CyIIb gives rise to a 2.1-kb actin mRNA (Fig. 1). Among the adult RNAs only that from ovary contained detectable levels of either of the CyIII gene transcripts. As indicated in Table 2 the 1.8-kb CyIIIa actin mRNA is a major embryonic species. The presence of this transcript in ovary RNA is most

TABLE 2
QUALITATIVE ESTIMATES OF EXPRESSION OF ACTIN GENES IN SEA URCHIN EMBRYO AND ADULT TISSUES

Gene	Transcript length (kb)	First appearance in embryogenesis	Expression in adult tissues relative to pluteus embryos						
			Testis	Ovary	Coelomocytes	Intestine	Tubefoot	Lantern muscle	Pluteus
CyI	2.2	Maternal mRNA	+++++	+++++	+++++	+++++	+++++	+++++	+++++
CyIIa	2.2	Gastrula (40 hr)	−	−	+/−	+	+/−	+/−	+
CyIIb	2.1	Early blastula (14 hr)	+/−	+/−	+	+	+	+	+
CyIIIa	1.8	Maternal mRNA	−	+	−	−	−	−	+++++
CyIIIb	2.1	Early blastula (14 hr)	−	+	−	−	−	−	+
M	2.2	Early pluteus (62 hr)	+/−	+/−	−	+	+++	+++++	+

Note. Crosses indicate relative abundance of each gene transcript in different tissues. (−) Denotes undetectable (we believe this means less than two transcripts per average cell at the pluteus stage). +/− indicates detectable transcript but only on long exposure.

likely due to expression of the CyIIIa gene in the developing oocytes.

In order to determine the approximate relative concentrations of different actin transcripts within each tissue, a DNA excess "dot" blot analysis was performed (Kafatos *et al.*, 1979). DNAs of the specific actin gene probes were bound to nitrocellulose filters and hybridized with oligo(dT)-primed [^{32}P]cDNA transcribed from the various poly(A) RNAs. Examples of these experiments are shown in Fig. 2. It can be seen qualitatively that in the three adult somatic tissues for which results are illustrated (Figs. 2a–c), the majority of actin mRNA derives from the CyI gene. In Fig. 2b gene M is again seen to be expressed in intestine (cf. Fig. 1d and Table 2), which contains among other cell types, a thin circular muscle. Gene M contributes a more prominent actin species in the RNA population of adult tubefoot, and in lantern muscle gene M provides an even larger portion of the actin message. Figure 2 also demonstrates that neither in the adult tissues nor in embryo cells (Fig. 2d) do the two CyII genes contribute more than a minor fraction of the actin mRNA. Furthermore, genes CyIIa and CyIIb are expressed differently. Gene CyIIa is most highly represented in the intestine cDNA (Fig. 2b; see also Fig. 1b). The pattern of expression of gene CyIIb in adult tissues seems more closely to resemble that of gene CyI, though at a much reduced level. In addition, as shown below, gene CyIIb is expressed at low levels in both gonadal tissues, while gene CyIIa is not.

A summary of the tubefoot dot blot experiments is presented in Table 3. Data from two different experiments agree satisfactorily, though of course any systematic errors that might increase or decrease hybridization with a given probe could be shared. The dot blot experiments are insufficiently sensitive to detect expression of gene CyIIa, though as shown in Fig. 1b some CyIIa transcripts are in fact present in tubefoot RNA. Three-fourths of the actin mRNA in this contractile organ system is produced by gene CyI, and about 20% by gene M. The relatively low apparent expression of the CyII actin genes in tubefoot cells is not due to lack of polyadenylation of their transcripts, since similar results were obtained in RNA gel blot experiments carried out on total RNA (not shown).

The experiments so far considered yield two main conclusions. First, they provide part of the basis for the classification of the *S. purpuratus* actin genes. The CyI and CyII probes clearly react with actin mRNAs in adult *nonmuscle* cell-types, viz coelomocytes, and in gastrula stage or earlier embryos (Table 2). It follows that these

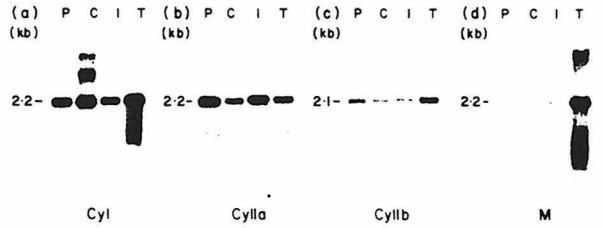

FIG. 1. Expression of actin genes in sea urchin adult tissues. Each gel blot (a–d) contains in order (left to right) 1 μg of poly(A) RNA from pluteus stage embryos (P), adult coelomocytes (C), adult intestine (I), and adult tubefoot (T). The four blots were hybridized with [^{32}P]DNA of the subcloned probes described in Table 1 for (a) gene CyI, (b) gene CyIIa, (c) gene CyIIb, (d) gene M.

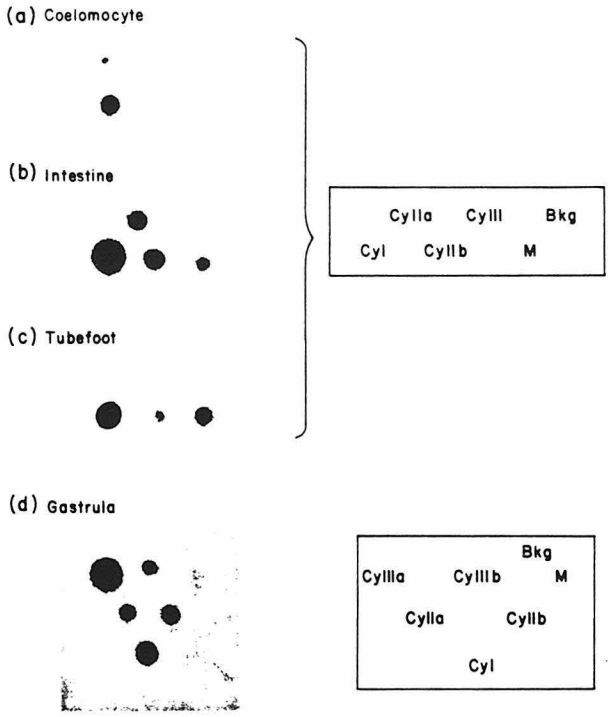

FIG. 2. Relative prevalence of actin transcripts within the embryo and in adult tissues. [^{32}P]cDNAs transcribed from poly(A) RNA from (a) adult coelomocytes, (b) adult intestine, (c) adult tubefoot, and (d) gastrula-stage embryos were hybridized with DNA dots (1 μg) of the probes for the actin genes indicated in the figure (Bkg, background dot containing pBR322 DNA). The filter-bound DNA was present in enormous sequence excess, and when 10 ng rather than 1000 ng of DNA was loaded per dot no more than a threefold difference in signal intensity was detected by scintillation counting.

genes code for forms of cytoskeletal actin proteins, while gene M can be regarded as a muscle-specific actin gene. Second, the three cytoskeletal actin genes that react with adult somatic cell transcripts, genes CyI, CyIIa, and CyIIb, are expressed in unique patterns with respect to one another. The activities of each of these genes, and of the M actin gene, are thus regulated differentially.

Actin Genes Utilized Only in Embryo and Larval Stages

We have already seen that transcripts of the CyIII genes are not detectable in any of the adult tissues examined, with the exception of the ovary. This suggested that the expression of these genes might be limited to the embryonic and larval stages of the life cycle, though it remained possible that they are also expressed in a specific adult tissue not examined in our study. To investigate this possibility further, RNA extracted from whole juvenile sea urchins 1 week postmetamorphosis was probed for CyIII actin gene transcripts. These animals are about 2 mm in test diameter and they contain the organ systems of an adult sea urchin, except for the oral apparatus, the gonads and associated sexual structures. RNA was also extracted from advanced larvae about 1 week premetamorphosis, and reacted with the CyIII probes in the same series of gel blots. The results of this experiment are shown in Fig. 3. Both the CyIIIa and CyIIIb transcripts are prevalent in the premetamorphosis larval RNA, though significantly less so than in pluteus-stage embryo RNA (Fig. 3a, c). As indicated in Table 2 and Figs. 1 and 2, the CyIIIa transcript is extremely abundant in the embryo. Figure 3 demonstrates that, in contrast, neither of the CyIII mRNAs can be detected at all in the postmetamorphosis larvae. The quality of the juvenile sea urchin RNA used in these experiments was verified by washing away the hybrid-

TABLE 3
RELATIVE ABUNDANCE OF ACTIN TRANSCRIPTS IN ADULT TUBEFOOT POLY(A) RNA

Gene	Counts per minute A	Counts per minute B	Average counts per minute above background	Total hybridized counts per minute (%)
CyI	188.0	238.9	152.6	74.6
CyIIa	44.1	65.2	0.0	0
CyIIb	75.1	78.4	15.9	7.8
CyIIIa	75.9	71.9	0.0	0
CyIIIb	56.7	54.1	0.0	0
M	96.7	97.4	36.2	17.6
Background pBR322	60.7	61.0		

Note. DNA dots autoradiographed in Fig. 2 were cut from the nitrocellulose filters, and the radioactivity was measured by scintillation counting.

ized ^{32}P-CyIII DNA probes and rehybridizing with [^{32}P]DNAs representing the actin coding region (Fig. 3b) or gene CyIIb (Fig. 3d). Comparing juvenile to larval RNAs, no decreases in transcript prevalence were seen with the latter two probes.

Thomas *et al.* (1982) showed that RNA gel blot hybridizations performed as described can display comfortably transcripts present at only $\sim 10^3$ molecules per embryo, or approximately a molecule per average cell in the gastrula or pluteus stage. It cannot be excluded that CyIII transcripts occur in the postmetamorphosis somatic cells of some minor structures that do not contribute significantly to the overall RNA. However, considering the high sensitivity of the experiment shown in Fig. 3 the most likely conclusion is that the CyIII genes are expressed exclusively in embryo and larval cells.

The juvenile sea urchin includes only a fraction of the cells present in the larva at metamorphosis. It derives from imaginal structures that develop from outpocketings of mesoderm and from portions of the gut (Hyman, 1955; Czihak, 1971). Thus the disappearance of the CyIII transcripts is probably due to their presence in larval cells that are among those discarded at metamorphosis. Almost certainly these cells are the aboral ectoderm cells of the larva, since in pluteus-stage embryos CyIII transcripts are confined to the cells of this portion of the ectodermal wall (Cox, Lee, Britten, Davidson, Angerer, and Angerer, in preparation). This group

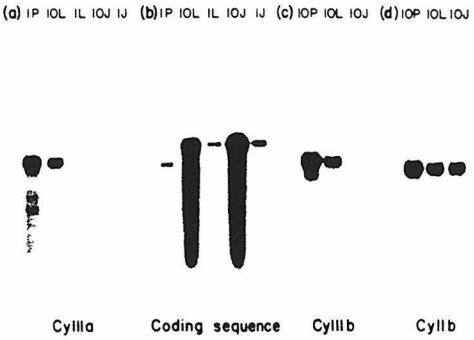

FIG. 3. Expression of CyIII actin genes is specific to embryonic and larval tissues. Autoradiographs are shown of RNA gel blot hybridizations carried out with subcloned probes recognizing the four actin genes indicated (see Table 1). Lane 1 of (a) contained 1 μg of total pluteus RNA (1P); lane 2, 10 μg of total advanced larva RNA (10L); lane 3, 1 μg of total advanced larva RNA (1L); lane 4, 10 μg of total RNA extracted from whole juvenile sea urchins 1 week postmetamorphosis (10J); lane 5, 1 μg of total juvenile RNA. Nomenclature in the remaining portions of the figure is the same.

of ectoderm cells makes no contribution whatsoever to the rudimentary structures that ultimately give rise to the juvenile sea urchin (see Czihak, 1971).

Regulation of Actin Genes during Embryogenesis

To investigate the developmental expression of the six actin genes represented by our subcloned probes, we carried out a series of RNA gel blot hybridizations with total RNAs extracted at intervals between fertilization and the late pluteus stage of embryogenesis. Since the quantity of rRNA in the embryo does not change significantly during this period (Davidson, 1976) the observed changes in relative abundance of actin transcripts reflect absolute changes in mRNA concentrations. Autoradiographs of some of the gel blots are shown in Fig. 4. These data were further analyzed by measuring the amount of radioactive probe hybridized either by scintillation counting of the gel transfer filters or by densitometry carried out on the autoradiographs. Data from two or three independent experiments using two different RNA preparations are summarized in Fig. 5. We estimate that the relative transcript accumulations shown in Fig. 5 are accurate at each point within a twofold range (see legend to Fig. 5).

FIG. 4. Developmental patterns of actin transcript accumulation in *S. purpuratus* embryos. RNA gel blots were reacted with the subcloned probes that recognize the indicated actin genes (see Table 1), as in Fig. 1 and 3. The RNAs loaded in each slot were as follows: (a) (left to right) 10 μg of total RNA from eggs (E), from embryos 6, 10, 14, 18, 41, 62, and 74 hr postfertilization, and from adult lantern muscle (L), and ovary (O), in (b-g), each gel blot shown contained (left to right) 5 μg of total RNA from testis (T) and 10 μg of total RNA from ovary (O), eggs (E) and embryos at 6, 10, 14, 18, 20, 41, 62, and 74 hr postfertilization. Note that in (g) the CyIIIb probe crossreacts with the 1.8-kb CyIIIa transcript as well as with the 2.1-kb CyIIIb transcript.

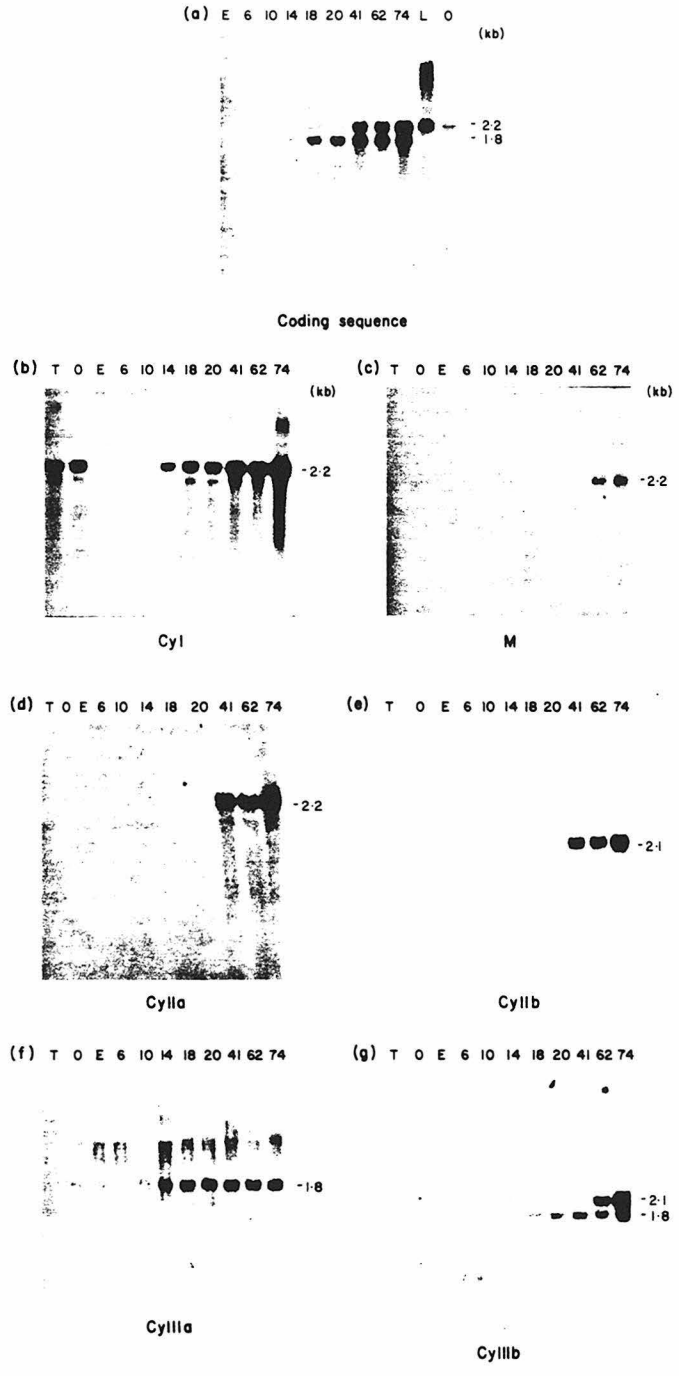
(a) E 6 10 14 18 20 41 62 74 L 0
(kb)
2·2
1·8
Coding sequence
(b) T 0 E 6 10 14 18 20 41 62 74
(kb)
2·2
CyI
(c) T 0 E 6 10 14 18 20 41 62 74
(kb)
2·2
M
(d) T 0 E 6 10 14 18 20 41 62 74
2·2
CyIIa
(e) T 0 E 6 10 14 18 20 41 62 74
2·1
CyIIb
(f) T 0 E 6 10 14 18 20 41 62 74
1·8
CyIIIa
(g) T 0 E 6 10 14 18 20 41 62 74
2·1
1·8
CyIIIb

Figure 4 shows that there is no large store of actin mRNA in the egg. The only detectable actin transcripts derive from genes CyI (2.2 kb) and CyIIIa (1.8 kb). Though appropriate direct measurements of the prevalence of actin message in the egg have not yet been carried out, an estimate that is probably reliable within a factor of several fold has been made. A λgt10 cDNA clone library constructed from gastrula poly(A) RNA by Dr. T. Thomas of this laboratory was found to contain 0.36% CyIIIa clones (36/10,000). This implies about 1.5×10^5 CyIIIa transcripts per embryo, assuming that each embryo has about 5×10^7 poly(A) RNA molecules (Lasky *et al.*, 1980). Since CyIIIa transcript prevalence appears to increase 50-fold from egg to gastrula (Fig. 5), there would be about 3000 CyIIIa transcripts per egg, and about an equal number of CyI transcripts. Even taking into account the inaccuracies of this estimation, actin must thus be classified as a rare maternal mRNA as was also concluded from colony clone screens by Flytzanis *et al.* (1982). This provides the first identification of a message belonging to the low-prevalence class of sea urchin maternal mRNA. It should also be noted that the prevalence of CyIIIa transcripts is higher in ovarian RNA than in maternal RNA (Fig. 4). As CyIIIa is evidently expressed only in embryos and larvae, the implication is that the concentration of CyIIIa transcripts is greater in developing oocytes than in mature eggs.

All six of the actin genes examined produce messages at some point during the first 80 hr of development. In all six cases there is an accumulation of RNA transcripts per embryo as development proceeds. However, the time of onset of the first detectable gene expression and the kinetics of accumulation of the transcripts for each gene differ significantly (Figs. 4 and 5). Genes CyI and CyIIb display similarities in their temporal patterns of transcript accumulation (Fig. 5), the major differences being that CyIIb transcripts are present at a much lower absolute concentration than are those of the CyI gene (Fig. 2d). Other than this these two genes could be coordinately expressed. However, gene CyIIa is utilized differently. Its transcripts are not detectable until gastrulation (40 hr) even after long autoradiographic exposure times, while there is a sharp early increase in the concentrations of CyI and CyIIb transcripts between blastula (18 hr) and gastrula (40 hr) stages. In conjunction with the expression data from adult tissues it can be concluded that the CyIIa gene is subject to a different regulatory regime from that controlling either gene CyI or gene CyIIb.

Transcripts of the single muscle specific actin gene, M, are not detectable until the early pluteus stage (62 hr) (Figs. 4 and 5). Even in late pluteus-stage embryos, the M gene transcript concentrations are low, compared to the level of expression seen in differentiated adult muscle tissue (see Figs. 1 and 2). This is probably due to limitation of the expression of this gene to muscle cells, which are a relatively minor component of the pluteus, confined to regions of the digestive tract.

The prevalence of gene CyIIIa transcripts rises rapidly, beginning early in development. Our estimate of a 30- to 36-fold increase in concentration from egg to blastula (18 hr) agrees well with the 10- to 25-fold estimate made by Crain *et al.* (1981) for the 1.8-kb transcript. We see a further increase of only 1.3- to 2-fold in the next 20 hr. In terms of transcripts per whole organism the concentration of CyIIIa mRNA falls by an order of magnitude during the several weeks leading to metamorphosis, as the number of cells other than aboral ectoderm increases. In embryos the CyIIIa transcript accumulation remains relatively constant after the early gastrula stage, which may reflect the early cessation of cell division in the ectodermal wall of the embryo (Lynn *et al.*, 1983).

It can be concluded from Fig. 5 that genes CyIIa, CyIIb, CyIIIb, and M are activated during development, probably at the transcriptional level, since there could not be more than 10^3 mRNA molecules of these species in the egg or the cleavage stage embryo, and since the developmental periods at which their transcripts first appear occur many hours following the termination of the phase of rapid cell division. It will require further measurements to determine whether genes CyI and CyIIIa undergo sharp increases in the rates of their transcription. Though this may be likely, it is not demonstrated by the data we present. Were these actin genes transcribed continuously at a rate that yields two molecules of mRNA per cell/min, a rate equal to that measured for a different moderately prevalent cloned sequence by Cabrera *et al.* (1983a), *constitutive* expression of the CyIIIa or CyI genes could account for the appearance of $>10^6$ new transcripts by 10 hr of development. This requires that the transcripts are at least moderately stable, and takes into account the exponential increase in cell number (i.e., number of sites of synthesis) during cleavage. On the other hand, the CyIIIa gene is certainly not expressed constitutively in all cells, since its transcripts are found only in the aboral ectoderm.

In Table 4 we show the results of a dot blot analysis carried out on gastrula stage embryo poly(A) RNA. The purpose of this experiment was to determine the relative contributions of the six actin genes. This cannot be extracted from the RNA gel blots discussed above, because of differing probe specific activities and other factors that render individual experiments difficult to compare. It can be seen that the majority of the actin mRNA indeed originates from genes CyI and CyIIIa. These genes contribute approximately 25 and 65% of total gas-

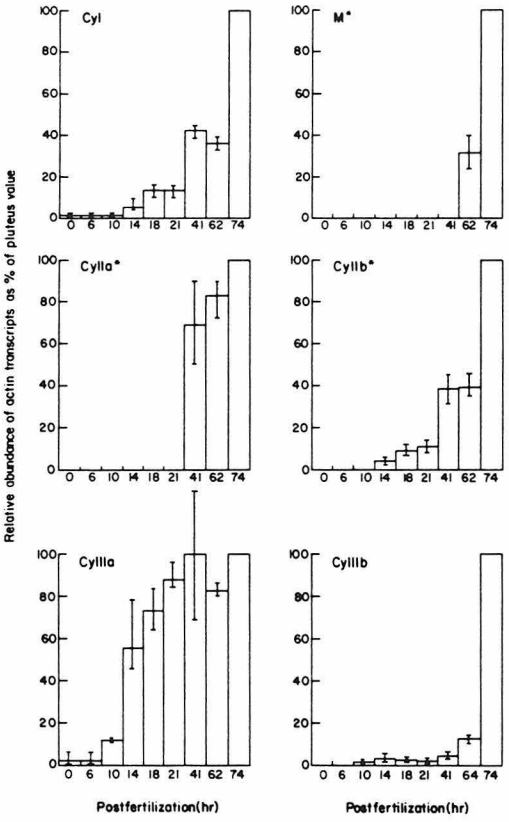

FIG. 5. Diagrammatic representation of actin transcript accumulation during embryogenesis. The radioactive bands in the autoradiographs shown in Fig. 4 were cut from the nitrocellulose filters and counted in scintillation fluid. In the cases of probes for genes CyIIa, CyIIIb, and M, the number of cpm was insufficient, and the autoradiographs were instead analyzed by densitometry to obtain estimates of relative transcript abundance (asterisk). When cross-checked with more radioactive samples, the densitometry measurements were found to be in good agreement with those obtained by scintillation counting. As a control, to ensure equal loading of RNAs and transfer to nitrocellulose, the filters were subsequently hybridized with a cloned probe for 16 S mitochondrial rRNA, which is represented as a highly prevalent transcript in egg and embryo poly(A) RNA (Cabrera *et al.*, 1983b). The amount of hybridization obtained varied from lane to lane by less than a factor of two. To confirm linear proportionality between signal intensity and relative RNA concentration, a titration series, covering a 100-fold range of RNA concentrations was performed by diluting the gastrula poly(A) RNA with chicken rRNA. These mixtures were then used for RNA blots which were reacted with the CyI gene probe. No systematic deviation from proportionality was detected, though individual duplicate points varied by a factor of two. Data are presented in the figure as the average percentage of the transcript prevalence observed for each probe at the pluteus stage of development. Error bars denote the range of the values measured from the two or three independent experiments from which the averages were obtained. In each panel 0 hr indicates unfertilized egg RNA.

TABLE 4
RELATIVE ABUNDANCE OF ACTIN TRANSCRIPTS IN GASTRULA POLY(A) RNA

Gene	Counts per minute A	Counts per minute B	Average counts per minute above background	Total transcripts (%)[c]
CyI	102.4	128.1	67.1	26.1
CyIIa	63.7	61.8	14.6	4.3
CyIIb	64.0	68.9	18.35	5.3
CyIIIa[a]	363.3	197.4	212.2	61.7
CyIIIb[b]	61.2	60.0	12.45	2.6
M	47.8	43.1	0.0	0.0
Background pBR322	45.8	50.5		

Note. DNA dots from Fig. 2d were cut from the nitrocellulose filters and counts per minute measured by scintillation counting.

[a] The value for CyIIIa includes an approximately 10% higher background, due to a slight reaction of the probe poly(dA:dT) sequence with other molecules present in the cDNA. This has been taken into account in the calculation of relative transcript prevalence in the final column of the table.

[b] The amount of CyIIIb transcript may also be overestimated since this probe cross-reacts with the homologous CyIIIa sequence. However, the quantity of CyIIIb RNA is so low (see Fig. 4) that the effect of such cross reaction on the overall distribution of actin transcripts is negligible (see Table 1).

[c] Corrected for length of probe complementary to mRNA and for background.

trula stage actin poly(A) RNA, respectively. Genes CyIIa, CyIIb, and CyIIIb each contributes, at most, 5% of the actin transcripts at this stage. Qualitatively, the RNA gel blot data of Fig. 4 reinforce the conclusion that the CyIIIa gene is the major source of embryo actin mRNA, in that the 1.8-kb mRNA appears to be the major actin mRNA species at the 40-hr gastrula stage.

DISCUSSION

Regulated Expression of Six Sea Urchin Actin Genes

The major finding of this study is that at least six of the eight actin genes in the genome of *S. purpuratus* are expressed at some stage in the life cycle, and that each of these genes is regulated differently. Thus the genes that we investigated all appear to possess their own control mechanisms, which determine the level and the timing of their expression, and the cell types in which they are active. Five of the six genes evidently code for cytoskeletal actins. Though each of these genes is distinguished by its own pattern of regulation, it is not known whether the proteins they encode are significantly different. Primary sequence data indicate that

the cytoskeletal actin genes CyI and CyIIa code for proteins that differ by only 1.3% (Cooper and Crain, 1982; Schuler *et al.*, 1983). Extensive coding region sequences are not yet available for the other three cytoskeletal actin genes included in this analysis, viz genes CyIIb, CyIIIa, and CyIIIb. It will be important to determine whether the individual Cy actin genes produce actins confined to specific cytoskeletal elements, e.g., structural components such as microfilaments, or the contractile components of ameboid processes. The alternative is that a given cytoskeletal actin gene would contribute to a variety of diverse structures. We have argued that the actin genes may be multiple because different copies are inserted in regulatory gene sets, each of which determines a specific multicellular or subcellular structure (Davidson *et al.*, 1982a; Lee *et al.*, 1983). Were this the case the major functional differences among cytoskeletal actin genes might reside in their regulatory rather than their protein coding sequences. This study shows that gene CyI produces prevalent transcripts in every cell type, and gene CyIIb also appears to be expressed in all the tissues studied. These genes might be required in the formation of ubiquitous cytoskeletal components, while other actin genes are utilized only in certain cells.

The discovery that genes CyIIIa and CyIIIb (and possibly CyIIIc if transcripts of this gene are detected by the CyIII probes) are expressed exclusively in embryonic and larval stages is particularly interesting. Cox *et al.* (in preparation; op cit) have shown that gene CyIIIa is active only in the aboral ectodermal wall. It cannot be excluded that this structure has a functional requirement for a special actin protein not shared by the remainder of the ectodermal wall, or for that matter by the external ectoderm of the postmetamorphosis juvenile. The alternative that the example suggests, however, is that the CyIIIa gene is one component of a set of genes responsible for construction and maintenance of a specific portion of the larva in this holometabolous animal. The physical and chemical properties of the protein this gene encodes could be very similar to those of cytoskeletal actins in other ectoderm cells, that belong to different regulatory gene sets.

The muscle actin gene, M, also displays a sharp cell type specificity. This single actin gene is utilized in all types of muscle so far investigated, viz adult tubefoot, lantern muscle, and intestine. There are also minor quantities of M gene transcript in whole ovary and testis which contain thin muscle layers utilized at least in the extrusion of gametes (Palmer, 1937). It is possible that the protein sequence encoded by the M actin gene differs functionally from the sequence(s) of the cytoskeletal actins. Vertebrate skeletal muscle α-actin, for example, displays an average of 6.5% amino acid sequence divergence from the cytoplasmic isoforms (Vandekerckhove and Weber, 1979). Likewise, the sea urchin M actin gene shows 12–15% nucleotide sequence divergence from gene CyI within the coding regions (D. Durica, personal communication). Whether this distinction reflects a necessary functional specialization remains unresolved.

Actin Gene Expression in the Embyro

Flytzanis *et al.* (1982) found that the large majority of transcripts that are abundant in the pluteus are equally prevalent in the maternal mRNA of the egg. A striking observation here, also noted by Crain *et al.* (1981) and Scheller *et al.* (1981) is that the sea urchin egg has no large store of maternal actin mRNA. We estimated that there may be about 6000 actin transcripts per egg prior to fertilization, thus placing actin message in the rare class of egg mRNAs. On the other hand the egg contains a large store of actin proteins (Mabuchi and Spudich, 1980) which must be synthesized earlier in oogenesis. In this context it is to be recalled (Table 2) that both CyI and CyIIIa transcripts are more abundant in ovary, which contains immature oocytes, than in the RNA of the mature egg. It might be supposed that the maternal cytoskeletal actin derives mainly from one or both of these genes. All the other actin genes included in this study fall in the class of genes expressed during development, but not represented detectably in maternal RNA. We have termed such genes, which contribute only about 10% of all actin transcripts expressed during embryogenesis "late genes" (Davidson *et al.*, 1982b; 1983). Perhaps the clearest example is afforded by the M actin gene, activation of which does not occur until pluteus stage (Fig. 5), when the first muscle cells appear in the digestive apparatus. In vertebrates as well, activation of muscle-specific actin genes occurs only at cell commitment during myoblast fusion (Shani *et al.*, 1981; Caravatti *et al.*, 1982; Garfinkel *et al.*, 1982; Schwartz and Rothblum, 1981). Similarly, in *Drosophila* the muscle actin genes are activated relatively late in development, as myogenesis takes place (Fyrberg *et al.*, 1983).

The fundamental problem in understanding late gene regulation is identification of the genomic sequence information specifying the ontogenic activation of these genes. The sea urchin actin genes, each displaying its individual regulatory pattern, are now sufficiently well characterized that they can serve as subjects for the investigation of this basic developmental process.

Actin Gene Linkage and Expression

It has been proposed that linkage of genes provides a mechanism for coordinated expression by localizing them within particular "regulatory domains" of the genome (Fritsch *et al.*, 1980; Stalder *et al.*, 1980; Stumph *et al.*, 1983). Correlative evidence for this hypothesis is

supplied, among other examples, by the organization of the histone genes in the sea urchin (Cohn *et al.*, 1976) and human (Plumb *et al.*, 1983); in *Drosophila* by the linkage of cuticle genes (Snyder *et al.*, 1981), some of the heat shock genes (Corces *et al.*, 1980) and yolk protein genes (Barnett *et al.*, 1980), and in the silk moth by the existence of a large coordinately utilized chorion gene domain (Eickbush and Kafatos, 1982). On the other hand the members of some linked gene families are not coordinately activated in development, for example the chorion genes of *Drosophila* (Griffen-Shea *et al.*, 1982) and the β-globin genes of mammals (Fritsch *et al.*, 1980) and birds (Stalder *et al.*, 1980).

Scheller *et al.* (1981) found earlier that the sea urchin actin genes CyI, CyIIa, and CyIIb are linked in that order, at intervals of 10 and 6 kb, respectively, and that these genes are all oriented in the same direction (5′ → 3′, as shown). The present study demonstrates that these three linked genes are neither coordinately nor sequentially regulated during development. The CyI gene is the only one of the group represented in the maternal mRNA. Gene CyIIa differs from genes CyI and CyIIb both in the developmental profile of its transcript accumulation and in its pattern of expression among adult tissues. Furthermore, the tissue distribution of CyIIa transcripts within the embryo differs from that of CyI and CyIIb transcripts (Cox *et al.*, in preparation; op cit). These three cytoskeletal actin genes thus belong to the class of linked, evolutionarily related genes that are *not* expressed in any common regulatory mode.

The linked arrangement of CyI and CyII actin genes is probably a relic of their evolution by duplication. Homologies within the intron sequences of the CyI, CyIIa, and CyIIb genes in *S. franciscanus* and *S. drobachiensis* (Lee *et al.*, 1983) as well as in *S. purpuratus* (Schuler *et al.*, 1983) suggest a single origin for these three genes. This example illustrates the difficulty of disentangling functional from evolutionary structural features. The same form of genomic organization that in some of the cases cited above may provide the phsyical basis for regulatory coordination, in these actin genes could have persisted by default. The functional distinction among the duplicated genes must have arisen subsequently, as a result of their acquisition of independent and individual regulatory information.

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CHAPTER 3

Cell Lineage-Specific Programs of Expression of Multiple Actin Genes During Sea Urchin Embryogenesis

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Cell Lineage-specific Programs of Expression of Multiple Actin Genes during Sea Urchin Embryogenesis

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We have determined spatial patterns of expression of individual actin genes in embryos of the sea urchin *Strongylocentrotus purpuratus*. Radioactively labeled probes specific for each of five cytoplasmic-type (Cy) and the single muscle-type (M) mRNAs were hybridized *in situ* to sections of fixed embryos. M actin mRNA appears only late in development and is confined to a few cells associated with the coelomic rudiments. The five Cy mRNAs fall into three sets, whose times and sites of expression during development are highly distinctive. Different cell lineages express messages of one or more of these sets, but never all three. Although all Cy actin mRNAs exhibit monophasic accumulation in the RNA of whole embryos during the course of development, such accumulation in many cases results from the summation of both increases and decreases in abundance within individual sets of cells. Within the genomic linkage group CyI-CyIIa-CyIIb, expression of CyI and CyIIb appears to be co-ordinate, and quite distinct from that of CyIIa. CyI and CyIIb are expressed in all lineages at some point in embryogenesis, but confined mainly to oral ectoderm and portions of the gut of the pluteus larva. CyIIa mRNAs are restricted to mesenchyme lineages throughout late gastrula stage, and subsequently accumulate in parts of the gut. The CyIIIa and CyIIIb genes, which form a separate linkage group, are expressed only in aboral ectoderm and its precursors. Furthermore, CyIII messages are the only detectable actin mRNAs in this cell lineage after late blastula stage.

1. Introduction

The sea urchin embryo presents a particularly advantageous system for analyzing the diversity of patterns of expression of individual actin genes because the set of actin genes has been well-characterized (Crain *et al.*, 1981, 1982; Overbeek *et al.*, 1981; Schuler *et al.*, 1983; Lee *et al.*, 1984), and probes specific for each gene have been identified (Lee *et al.*, 1984); the embryo is small and contains a limited number of cell types; and sensitive methods of hybridization *in situ* have been developed for determining which cells express individual mRNAs (Cox *et al.*, 1984). The sea urchin genome contains a single muscle actin gene and five cytoskeletal actin genes shown to be transcriptionally active (Shott *et al.*, 1984). Early studies identified two size classes of actin mRNAs whose abundance is differently regulated (Crain *et al.*, 1981; Merlino *et al.*, 1981). We have shown that mRNAs encoded by the individual genes accumulate differentially in the mRNA of whole embryos during development (Shott *et al.*, 1984; Lee *et al.*, 1986). We have now determined the spatial patterns of expression of each of these genes during embryogenesis and have interpreted these patterns in terms of the cell lineages of which the embryo is composed.

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2. Materials and Methods

(a) *Embryo culture*

Strongylocentrotus purpuratus were obtained from Pacific Biomarine Inc., Venice, California and from Patrick Leahy, Kerckhoff Marine Laboratory, California Institute of Technology, Corona del Mar, California. Embryos were cultured as described (Angerer & Angerer, 1981). All embryos used in these studies were harvested from the same culture, except where noted in the Figure legends.

(b) *Recombinant DNA templates*

Probes for CyI, CyIIa, CyIIb, CyIIIb and M actin mRNAs consisted of segments of genomic DNA clones encoding 3′ untranslated regions of the mRNAs; a cross-reacting exon sequence derived from the CyI gene was used to detect total actin mRNA. The construction of the original recombinants has been described (Lee *et al.*, 1984). The CyIIIa-specific probe was derived from a cDNA clone (Lee *et al.*, 1986). To prepare templates for transcription *in vitro* of labeled RNA probes with Sp6 RNA polymerase, sea urchin DNA sequences were excised from parental recombinants by restricting with 2 different enzymes that cut at flanking positions in the multiple cloning site. The resulting fragments were inserted by cohesive end ligation into either pSp6Z1 or pSp6Z2 (vectors described by Angerer *et al.*, 1985*a*), which had been digested with the same pair of restriction endonucleases. The 131 nt† CyIIIa-specific sequence initially inserted into pUC9 by blunt end ligation was transferred to pSp64 (a gift from Dr D. Melton) in similar fashion for this study. The characteristics of these probes are described in the accompanying paper (Lee *et al.*, 1986). All experiments were carried out in accordance with the N.I.H. Guidelines for Recombinant DNA Research.

(c) *Transcription* in vitro *of radioactively labeled RNA probes*

Plasmid DNAs were isolated and purified as described (Cox *et al.*, 1984). DNA templates in pSp6Z1 were truncated with *Hin*dIII just downstream from the actin inserts. Those templates in pSp6Z2 and pSp64 were truncated with *Eco*RI (Cy inserts) or *Bam*HI (M insert). [^{3}H]RNA probes were synthesized as described (Cox *et al.*, 1984) using [^{3}H]ATP and [^{3}H]UTP (58 and 45 Ci/mmol, respectively; Amersham) at concentrations resulting in the specific activities indicated in the Figure legends. Two alterations were made in this synthesis protocol. First, placental RNase inhibitor (BRL), or RNasin (Promega Biotec) was added at 1000 units/ml to prevent RNA degradation. Second, the transcription reaction (10 μl) was allowed to continue for up to 2 h and, in some cases, a second addition of 12 to 18 units of Sp6 RNA polymerase was made after the first hour. We have observed that some batches of [^{3}H]NTPs inhibit the enzyme when used in reaction mixtures at high concentration and have been able to overcome this inhibition only by addition of a large excess of enzyme. After synthesis, RNA products were purified as described (Cox *et al.*, 1984), with the exception that extraction with phenol/chloroform was omitted between synthesis and DNase steps. The fragment length of probes was reduced to approximately 150 nt by limited hydrolysis in carbonate buffer (pH 10·2) for times dependent on initial fragment length (Cox *et al.*, 1984).

† Abbreviation used: nt, nucleotide(s).

(d) *Hybridization* in situ

Fixation of embryos in 1% (v/v) glutaraldehyde and embedding were carried out as described (Angerer & Angerer, 1981). Sections of paraffin-embedded embryos were cut nominally 5 μm thick. We have found that retention of sections is much improved on poly-L-lysine-coated slides prepared by a modification of the procedure described by McClay *et al.* (1981). Acid-cleaned slides are soaked in 50 μg poly-L-lysine/ml, 10 mM-Tris·HCl (pH 8·0) at room temperature for 10 min and air-dried. Treated slides may be stored for at least 1 month. Slides are dipped in distilled water, sections are mounted, and the slides are dried on a slide-warmer at 40°C for at least 2 h. After deparaffinization and hydration, slides were incubated in 1% (w/v) bovine serum albumin, 10 mM-Tris·HCl (pH 8·0) for 10 min at room temperature, washed twice with distilled water and treated with proteinase K and acetic anhydride as described (Angerer & Angerer, 1981). However, we have not detected any increase in background binding of probes when the wash with albumin is omitted.

Hybridization of [^{3}H]RNA probes was carried out as described (Cox *et al.*, 1984) at 45°C in 50% (v/v) formamide, 0·3 M-NaCl, 20 mM-Tris·HCl (pH 8·0), 5 mM-EDTA. 1 × Denhardt's (0·02% each bovine serum albumin, polyvinyl pyrollidone and Ficoll), 500 μg tRNA/ml and 10% (w/v) dextran sulfate for 16 h. Non-specific background binding of probe was determined in identical parallel hybridizations with transcripts of the vector R7Δ7 (Lynn *et al.*, 1983). Post-hybridization washes were carried out as described by Cox *et al.* (1984), with the addition of a 10 min wash at 50°C in 4 l of 0·1 × SSC immediately following the 30 min wash in 2 × SSC (SSC is 0·15 M-NaCl, 0·015 M-trisodium citrate). Autoradiography and data analysis were carried out as described (Angerer & Angerer, 1981).

Experiments presented here indicate that individual actin mRNAs are present at widely different concentrations in different regions of the embryo. Because the maximal signal to noise ratio is achieved at probe concentrations at or below saturation (Cox *et al.*, 1984), we used probe concentrations 50% to 100% of those estimated to saturate target RNAs. To demonstrate that relative signals over areas of very different target density do not depend on probe concentration, we compared the saturation curve for the hybridization of the actin CyIIIb probe to aboral ectoderm in sections of plutei (data points) to that for a histone probe hybridized to 12 h embryos (line) as shown in Fig. 1. Although the concentration of sequence complementary to these 2 probes (i.e. nucleotides/μm^3) differs by a factor of 40 to 80 (Mauron *et al.*, 1982; Lee *et al.*, 1986), the saturation curves are virtually congruent. Thus, relative signals for target RNAs present at very different concentrations are essentially independent of probe concentration.

3. Results

(a) *Probes for mRNAs encoded by individual actin genes*

Although the protein-coding regions of actin genes are highly conserved, the greater divergence in 3′ untranslated mRNA sequences allows their use

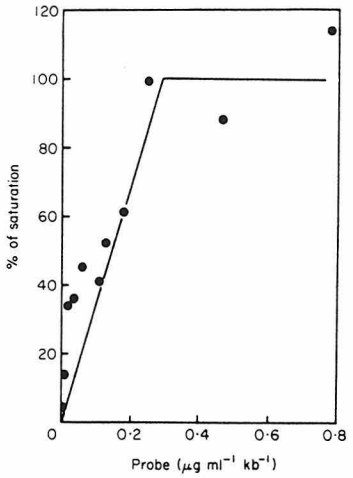

Figure 1. Relative signals over areas of different mRNA target density are independent of probe concentration. The percentage of available target mRNA hybridized is shown as a function of probe concentration for 2 mRNAs differing in concentration by at least 40-fold. Data points represent signals for the hybridization of the CyIIIb probe to aboral ectoderm of 82 h plutei. Probe specific activity was $1{\cdot}3 \times 10^8$ disints/min per μg, exposure time was 31 days, and the grain density at saturation was 15 grains/100 μm^2. The values shown were corrected for non-specific background binding of probe, which ranged from 5% of signal at the highest probe concentrations to 36% of the signal at the lowest probe concentrations. The line shown for comparison is the best fit to comparable data for the hybridization of a probe sequence complementary to early histone mRNAs in sections of 12 h embryos (Cox *et al.*, 1984). Probe concentrations have been normalized for the difference in complexity of the histone (6·8 kb) and actin (0·55 kb) sequences; kb, 10^3 bases.

as highly gene-specific hybridization probes. Lee *et al.* (1984) and Shott *et al.* (1984) have developed such probes for the actin genes of *S. purpuratus*, and classified the genes into related subfamilies. These analyses identified a single muscle-type (M) actin gene, and five genes encoding cytoplasmic (Cy) actins. The Cy actin genes comprise three subfamilies on the basis of homology within 3′ untranslated sequences. RNA blot hybridizations demonstrated that the single CyI, two CyII (a and b) and two CyIII (a and b) genes are transcriptionally active (Shott *et al.*, 1984). We have used probes for all six mRNAs and for an exon that cross-reacts with all actin mRNAs for analysis of the expression of individual actin genes in different cell lineages of developing *S. purpuratus* embryos. Because we had observed that much higher signals can be obtained using single-stranded RNA probes (Cox *et al.*, 1984), the actin sequences were transferred to transcription vectors and asymmetric RNA probes were synthesized *in vitro* with Sp6 RNA polymerase. Hybridizations *in situ* were carried out under conditions calculated to be slightly more stringent than the "high criterion" conditions used by Lee *et al.* (1984), taking into account the higher stability of RNA–RNA duplexes compared to that of DNA–RNA, and the slight reduction in t_m observed for duplexes formed *in situ* (Cox *et al.*, 1984). Except in one case, these conditions provide gene-specific hybridization (Lee *et al.*, 1984). In agreement with this, in most cases we observe distinct hybridization patterns *in situ* for each probe, indicating that each reacts with a unique target mRNA. The abundance of different actin transcripts in whole embryo RNA throughout development is known both in relative (Shott *et al.*, 1984) and absolute terms (see the accompanying paper, Lee *et al.*, 1986). In the present studies, exposure times (indicated in the Figure legends) were adjusted to yield adequate grain densities to determine spatial patterns of hybridization, rather than to provide comparisons of signals among different stages. Thus, relative grain densities over different areas of a given section indicate relative mRNA abundance, but grain densities over different sections are not always directly comparable.

Hybridization *in situ* provides a measure of local mRNA concentration in different regions of the embryo. While it is convenient to describe spatial patterns of actin mRNA distribution and developmental changes in these patterns in terms of actin gene expression, this does not imply any specific mechanism except control at the level of mRNA abundance. In describing distributions of signals, we will refer to areas of the embryo as unlabeled if grain densities were not above those observed in identical hybridizations with a control probe. Of course, failure to detect an mRNA in any cell type does not exclude its expression at a level below the sensitivity of our assay. This sensitivity varies with probe concentration, exposure time, probe sequence complexity and actual background observed in any one experiment. The average abundance for "labeled" cells ranges from about 50 to 200 molecules per typical pluteus cell (Lee *et al.*, 1986), and in many cases messages were detectable at earlier stages where they are considerably less abundant. We estimate that in most cases unlabeled regions contain at least five- to tenfold fewer transcripts. In some experiments (such as those shown in Fig. 9), the sensitivity approaches one mRNA molecule per pluteus cell volume using a probe complementary to 6% (131 nt) of the mRNA sequence.

(b) *Distribution of total actin mRNA*

We first illustrate the distribution of total actin mRNA in the embryo, detected with an exon sequence probe that cross-reacts with all actin mRNAs at a stringency similar to that used here (Lee *et al.*, 1984). While it was anticipated that all cells would contain actin mRNA, this analysis

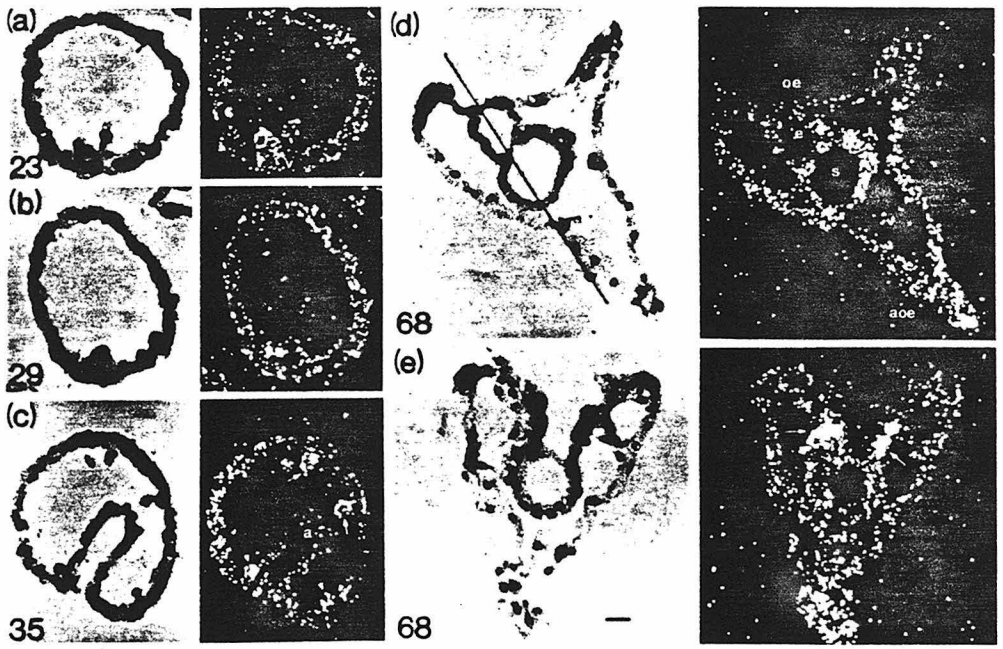

Figure 2. Distribution of total actin mRNA. Total actin mRNA was detected with a cross-reacting exon sequence from the CyI gene. Probe specific activity was $1{\cdot}1 \times 10^8$ disints/min per μg, and exposure time was 8 days. The probe contained 720 nt complementary to actin mRNA and was hybridized at a concentration 60% of that calculated to achieve saturation. Hours of development are indicated by the numbers in each panel; the stages illustrated are (a) middle and (b) late mesenchyme blastula, (c) gastrula, and (d) and (e) pluteus. (a) and (b) Sections are cut approximately through the animal–vegetal axis, the vegetal pole (v) is down and the presumptive aboral side is to the right. (c) The presumptive aboral side lies to the left, arrows point to secondary and arrowheads to primary mesenchyme cells. Sections in (d) and (e) pass approximately through the oral–aboral axis and are perpendicular to each other; the approximate plane of the section in (e) is indicated by the line in (d). In (d) the oral lobe lies to the left and anal arms to the right. In (e) arrows point to paired heavily labeled regions lying laterally on either side of the esophagus. oe, oral ectoderm; aoe, aboral ectoderm; e, esophagus; s, stomach; a, archenteron. All sections are illustrated at the same magnification, and the bar in (e) represents 10 μm. In this and subsequent Figures, pairs of micrographs of the same sections are shown, photographed under phase contrast (left) and darkfield (right) illumination.

emphasizes the fact that the distribution of total actin mRNA is the summation of discrete patterns demonstrated for individual subtypes in the following sections. Representative sections of embryos at several developmental stages are shown in Figure 2. Analysis of a large number of sections indicated that no major region of the pluteus larva is devoid of actin mRNA (Fig. 2(d) and (e)). Signals are significantly higher in all regions than non-specific background binding of probe measured by identical reactions with transcripts of vector sequence (data not shown). However, reproducible differences in actin mRNA concentration are observed among various cell types. In the ectoderm, the aboral region (aoe, the epithelial sheet of cells forming a cone whose vertex is opposite the mouth) is more heavily labeled than the oral region (oe). At least some mesenchyme cells lying along the inner surface of the ectoderm are labeled. Actin mRNA concentration varies in different portions of the gut at this stage, with the esophagus (e) showing consistently lower grain densities than those observed over stomach (s) and intestine (Fig. 2(d)). The concentration of actin mRNA in the late pluteus is highest in two small groups of cells on either side of the esophagus, including parts of the coelomic rudiments (arrows, Fig. 2(e)).

At gastrula stage (Fig. 2(c)), labeling over presumptive aboral ectoderm is also higher than over presumptive oral ectoderm. Signals over most of the archenteron (a) are relatively low, except for secondary mesenchyme cells. At early gastrula stage (not shown), these cells form a heavily labeled mass at the tip of the archenteron; they subsequently migrate from this region along the ectodermal wall (see arrows in Fig. 2(c)). Some primary mesenchyme cells, identifiable by their position around the base of the archenteron, are also labeled (arrowhead). At blastula stage, all regions of the embryo contain detectable actin mRNA (Fig. 2(a) and (b)). Labeling is heaviest over the presumptive aboral ectoderm and over vegetal pole blastomeres, especially those giving rise to mesenchyme cells.

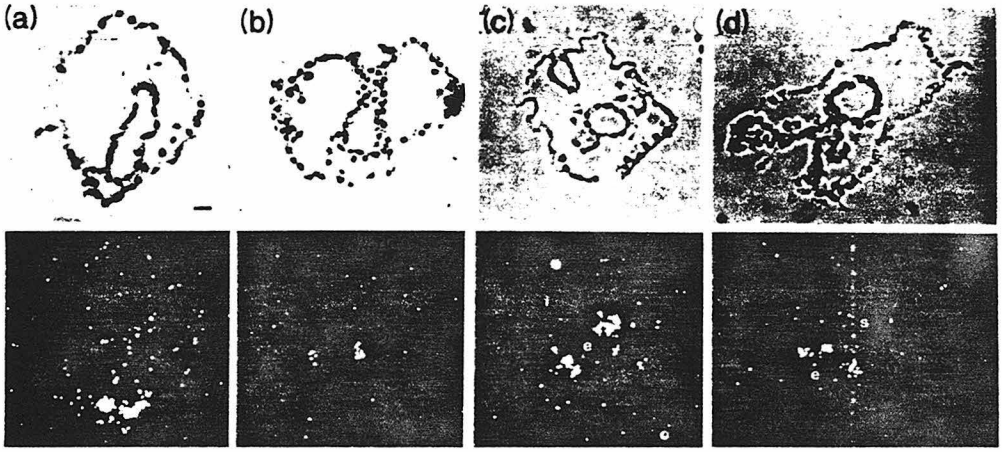

Figure 3. Localization of muscle-type actin mRNA. Sections were hybridized with the M probe ($1{\cdot}7 \times 10^8$ disints/min per μg), which contains 300 nt of sequence complementary to mRNA. Probe concentration was estimated to achieve saturation, and autoradiographs were exposed for 10 days ((a) and (b)) or 15 days ((c)). (a) 48 h late gastrula, showing labeling over cells at the region of fusion of the tip of the archenteron with the ectodermal wall. (b) 52 h late gastrula, showing a few labeled cells on either side of the archenteron. The sections in (a) and (b) are taken from different embryo cultures, which developed at slightly different rates. The developmental stage of the 52 h embryo is actually slightly earlier than that of the 48 h embryo. (c) and (d) 82 h plutei showing regions of labeling on either side of the esophagus. The section in (c) was 1 μm thick. Arrowheads in (c) indicate labeling over hollow vesicles of the coelomic rudiments. e, esophagus; s, stomach; i, intestine. All sections are shown at the same magnification, and the bar in (a) represents 10 μm.

(c) *Muscle actin mRNA*

In blot analyses of total RNA from embryos, M actin mRNA is not detectable until late embryogenesis (Shott *et al.*, 1984; Garcia *et al.*, 1984). Data presented in the accompanying paper (Lee *et al.*, 1986) show that transcripts of this actin gene are not detectable (i.e. <100 transcripts/embryo or 0·1/cell) until mid-gastrula stage, when they begin to accumulate rapidly. In the pluteus (65 h) there are over 25,000 molecules of M actin mRNA per embryo.

Hybridization *in situ* of the M probe to sections of late (82 h) plutei (Fig. 3(c) and (d)) indicates that these transcripts are abundant messages in the two small clusters of cells labeled most highly by the exon probe (Fig. 2(e)). Only the M probe gives high signals over this region of the embryo. Figure 3(c) shows that at least some of these cells are part of the hollow vesicles of the paired coelomic rudiments (arrows). In some sections, labeling is observed also over strands of several cells extending from the esophagus to the ectodermal wall, and some cells or cell processes extending around the outside of the esophageal wall (not shown). Based on time-lapse cinematographic analyses, Gustafson & Wolpert (1967) suggested that pseudopodia of cells derived from the coelomic sacs form the first embryonic muscle elements. This origin of the esophageal contractile bands has been confirmed in two urchin species by Ishimoda-Takagi *et al.* (1984), who showed by immunocytochemistry that these cells begin to accumulate muscle-specific tropomyosin at early pluteus stage when they first form pseudopodia. The distributions of muscle-type actin mRNA and muscle tropomyosin protein are consistent.

We detected M actin transcripts at late gastrula stage (48 to 52 h). As shown in Figure 3(a) and (b), a few cells in the wall of the archenteron are distinctly labeled. The position of these cells is consistent with their being precursors to the cells labeled at later stages. The number of cells labeled by the M probe increases between late gastrula and pluteus stages, which suggests that active cell division occurs after they begin expressing M actin mRNA. After departure of the secondary mesenchyme cells from the tip of the archenteron, the muscle cells form small clusters on either side of the archenteron adjacent to the area where it fuses with the ectoderm during formation of the mouth (Fig. 3(a)).

(d) *CyI and CyIIb mRNAs*

The CyI and CyIIb genes are found in the same 5′ to 3′ orientation separated by about 10,000 bases, which contains the CyIIa gene. Distributions of CyI and CyIIb mRNAs will be described together because they are not detectably different, either temporally or spatially. The concentration of both mRNAs begins to increase around mid-cleavage (before 12 h of development in the case of CyI, see below). CyI mRNA is about 1·5-fold more abundant than CyIIb mRNA in every adult tissue examined

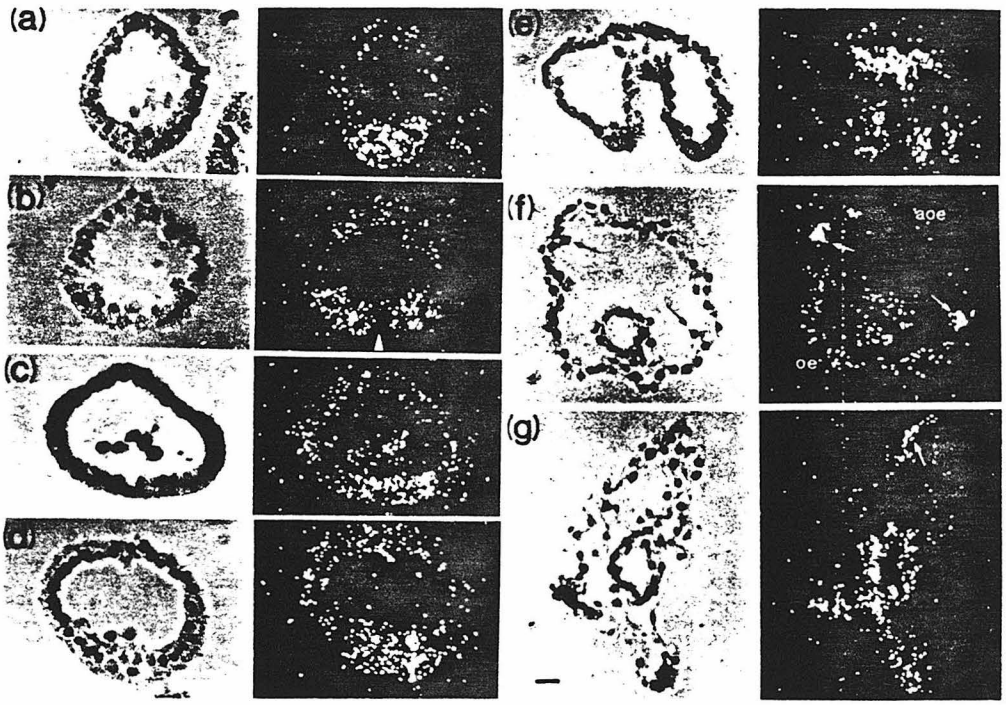

Figure 4. Distribution of CyI mRNA. The CyI probe ($1{\cdot}3 \times 10^8$ disints/min per μg) containing 780 nt of sequence complementary to the mRNA was hybridized at a concentration estimated to be 85% that required for saturation. Autoradiographic exposure was for 68 days (a) to (e) or for 42 days ((f) and (g)). (a) to (c) Mesenchyme blastula, 23 h; (d) late mesenchyme blastula, 29 h; (e) gastrula, 35 h; (f) late gastrula/early prism, 48 h; (g) pluteus, 82 h. Sections in (a), (b), (d) and (e) pass approximately through the animal–vegetal axis, and the vegetal pole is at the bottom; the section in (c) is probably slightly oblique and in (f) is approximately perpendicular to this axis. The arrows in different panels indicate: (b) the small gap in labeling at the vegetal pole; (c) 1 heavily labeled primary mesenchyme cell in a cluster of 5 of these cells; (e) 3 mesenchyme cells, that on the left unlabeled and the 2 on the right moderately labeled; (f) 2 highly labeled mesenchyme cells, probably secondary; and (g) labeled mesenchyme cells, which characteristically appear at the vertex of the embryo closely applied to the aboral ectoderm. aoe, aboral ectoderm; oe, oral ectoderm. All sections are shown at the same magnification, and the bar in (g) represents 10 μm.

and in whole embryos at different developmental stages (Lee *et al.*, 1986). Throughout most of embryogenesis it is the second most abundant actin message, comprising about 30% of total actin mRNA in whole embryos at gastrula stage.

We describe mRNA distributions proceeding backwards in developmental time because patterns are most easily elucidated in the differentiated pluteus larva in which the major cell lineages are easily identified. This final distribution often aids in understanding the patterns at progressively earlier stages. Analysis by hybridization *in situ* shows that CyI mRNA is widely distributed in the pluteus larva (Fig. 4(g)). Highest grain densities are observed over the gut. In most sections, we observed higher labeling over stomach and intestine than over esophagus. The differences among embryos in relative labeling could not be localized to a specific region of esophagus; possibly this variability is due to differences in timing of CyI mRNA accumulation in different regions of the gut, coupled with heterogeneity in developmental age of different embryos in the culture. Distinct labeling, but at slightly lower levels, is observed over oral ectoderm and at least some mesenchyme cells (arrows, Fig. 4(g)), which are probably secondary (see below). CyI mRNA is undetectable in aboral ectoderm, which comprises at least one-third of the embryo. No labeling is observed over the coelomic rudiments in late plutei.

The temporal pattern of CyI mRNA expression varies in different cell lineages. Clearly defined changes occur between gastrula and pluteus stages (Fig. 4(e) to (g)). Labeling is not uniform in the archenteron of gastrula stage (Fig. 4(e)). Heavily labeled cells at the tip are mainly secondary mesenchyme that have not migrated from this region. Labeling within the gut lineage proper is usually restricted to approximately the one-third of the archenteron nearest the blastopore. Examination of stages between gastrula (35 h) and pluteus (72 to 84 h) indicated that CyI mRNA accumulates

in the remainder of the gut by about 48 hours (data not shown). In ectoderm, labeling is confined to the presumptive oral region during this period (oe, Fig. 4(f)). The identity of these cells can be inferred from the CyI labeling pattern observed for plutei. It was demonstrated directly by experiments in which adjacent 1 μm thick sections were separately hybridized with probes for CyI and for Spec 1 mRNA, which identifies aboral ectoderm (Lynn *et al.*, 1983). These two probes label reciprocal and non-overlapping portions of ectoderm at gastrula stage (Angerer *et al.*, 1985*b*). Isolated mesenchyme cells lying along the ectodermal wall show signals ranging from no labeling to rather high grain densities (arrowheads, Fig. 4(e) and (f); Table 1). Although we cannot distinguish all primary from secondary mesenchyme cells that have migrated along the ectodermal wall, patterns of labeling over mesenchyme at earlier stages (see below) suggest that the most heavily labeled mesenchyme cells at gastrula are secondary. The absence of heavily labeled mesenchyme cells at pluteus stage suggest that CyI mRNA may decrease in abundance in many of these cells after they have migrated from the tip of the archenteron.

There is a major transition in the pattern of CyI mRNA distribution between about 18 hours (early blastula) and 29 hours (late blastula). At and before 18 hours, CyI mRNA is uniformly distributed in embryo blastomeres (see below and Fig. 9), while at stages later than late blastula, expression in the ectoderm and its precursors is restricted to the oral region (Fig. 4(d) to (g)). Examination of individual and serial sections showed that the distribution of this message is variable among embryos around the midpoint of this transition, i.e. in the 23 hour blastula (Fig. 4(a) to (c)). Labeling is also heterogeneous over ingressed primary mesenchyme at 23 and 29 hours (e.g. Fig. 4(c)). Our observations are consistent with the idea that most or all of these cells express CyI mRNA before ingression, because there is no large region of unlabeled cells in the vegetal pole at 23 hours or earlier stages. The labeling heterogeneity over internal primary mesenchyme implies that CyI mRNA disappears from individual cells with a variable time-course, beginning at about the time they ingress.

Throughout the blastula stage, cells of the vegetal pole form the major and most highly labeled region of the embryo (Fig. 4(a) to (d)). The pattern observed at slightly later stages and the size of this area indicate that it includes the presumptive archenteron (including secondary mesenchyme) and, at 23 hours, perhaps some primary mesenchyme that has not ingressed. A small gap in labeling at the center of the vegetal pole is consistently observed in those sections of 23 hour blastulae cut in the plane of the animal–vegetal axis (Fig. 4(b)). In any such section, this gap typically includes only one or two cells, so their total number in the embryo is small. The consistent position of these few cells exactly at the vegetal pole, and the fact that they are distinct from their neighbors with regard to expression of CyI mRNA, suggest that they correspond to the small daughter cells of the micromeres. In *S. purpuratus* blastulae, these include eight cells derived from the four smaller first division products of the micromeres, destined to participate in formation of the coelomic rudiments, and whose fate is thus distinct from that of the larger micromere derivatives that form primary mesenchyme cells (Boveri, 1901; Hörstadius, 1939; Pehrson & Cohen, personal communication). This unlabeled region is not consistently observed at 29 hours, suggesting that CyI mRNA accumulates in these cells by that time. Because we cannot identify these unlabeled cells after ingression of primary mesenchyme is complete (29 h), we cannot exclude the possibility that they represent the last primary mesenchyme to depart from the blastocoel wall.

The spatial distribution of CyIIb mRNA in embryos at stages of development from blastula (23 h) through pluteus is not detectably different from that described for CyI (Fig. 5). While we have not carried out quantitative analyses of grain densities, there are no obvious differences in relative levels of labeling of different embryo regions for the two mRNAs at any stage. We conclude that, according to all available data, CyI and CyIIb actin mRNAs are co-ordinately expressed, although quantitatively at slightly different levels.

(e) *CyIIa mRNA*

The CyIIa gene encodes a relatively rare message in whole embryo RNA. Shott *et al.* (1984) showed that this message begins to accumulate after 21 hours of development (around hatching blastula), and Lee *et al.* (1986) demonstrated a very low level of this mRNA (about 1400 molecules/embryo) in the 20 h blastula. By 36 hours (mid-gastrula), CyIIa mRNA comprises about 6% of total actin message, or about 9000 molecules per embryo, and the abundance of this message increases only slightly by the pluteus stage (Lee *et al.*, 1986).

The pattern of expression of the CyIIa actin gene is distinct from that of the CyI and CyIIb genes that flank it, and is divisible into three phases during which the message is differently distributed in the embryo. In the pluteus (Fig. 6(f) and (g)), the major labeled region is the gut, in which the mRNA is differentially expressed. Sections through the stomach usually show high signals, while those through intestine are less frequently labeled, implying that CyIIa mRNA begins to accumulate in stomach before intestine. Within both regions, patches of cells often show relatively large differences in grain density, although no consistent pattern was evident. Esophagus is unlabeled, but in several sections labeling could be identified over the adjacent coelomic rudiments. Labeling is frequently observed over mesenchyme cells many of which, as discussed below, are probably secondary mesenchyme. The entire ectoderm of the pluteus

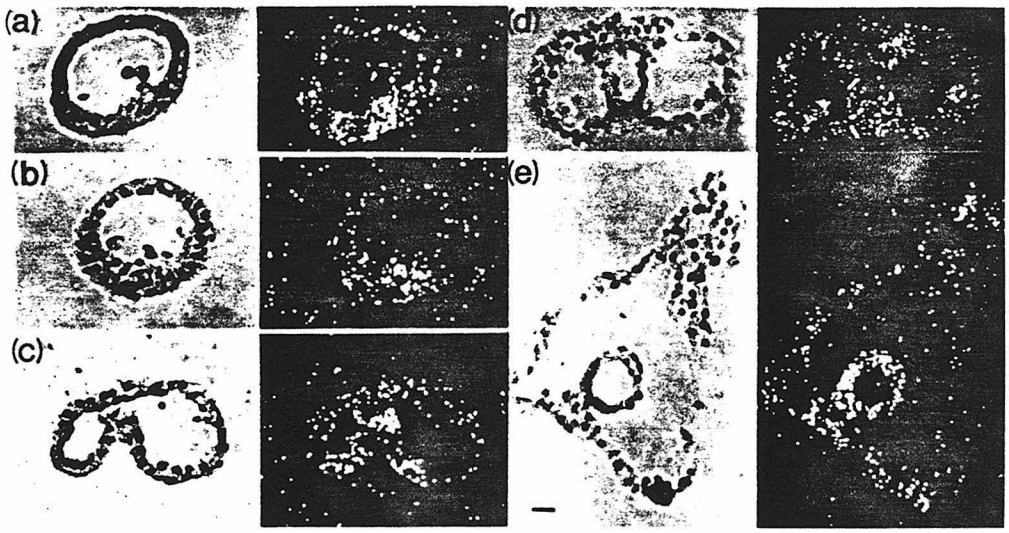

Figure 5. Distribution of CyIIb mRNA. The CyIIb probe ($1{\cdot}3 \times 10^8$ disints/min per μg) containing 400 nt of sequence complementary to mRNA was hybridized to sections at a concentration estimated to be 70% of that required to achieve saturation. Exposure time was 85 days for (a) to (c) and 64 days for (d) and (e). (a) Mesenchyme blastula, 23 h; (b) late mesenchyme blastula, 29 h; (c) gastrula, 35 h; (d) late gastrula/early prism, 48 h; (e) pluteus, 82 h. The sections in (a) to (d) are all cut approximately parallel to the animal–vegetal axis, and the vegetal pole is at the bottom. In (d) the presumptive oral ectoderm (oe) is at the left. All sections are shown at the same magnification, and the bar in (e) represents 10 μm.

appears to be largely devoid of CyIIa mRNA, since grain densities are similar to background non-specific binding, which is slightly higher over oral than over aboral ectoderm.

Between mid-blastula and late gastrula (Fig. 6(b) to (e)), CyIIa mRNA is detectable only in a single set of cells, the secondary mesenchyme, in which it increases in concentration at least fivefold (Lee *et al.*, 1986). These cells are identifiable as secondary mesenchyme at gastrula stage by their initial confinement to the tip of the archenteron (Fig. 6(d)) and their subsequent migration along the wall of the blastocoel (Fig. 6(e)) as the tip of the archenteron fuses with the ectodermal wall to form the mouth. No labeled cells remain in the esophagus after this process is complete. CyIIa mRNA decays rather gradually from these cells after they begin migration. At blastula stage (Fig. 6(b) and (c)), labeling is confined to the vegetal pole. This labeled area is noticeably smaller than that delineated by the CyI probe which marks both presumptive gut and secondary mesenchyme (compare Fig. 4(a) to (d)). Together with the absence of labeling in the archenteron proper at gastrula stage (Fig. 6(d)), the blastula pattern implies that CyIIa mRNA is not expressed in cells of the gut lineage at these stages. Examination of the archenteron/gut at stages between 48 hours (late gastrula) and 72 hours (pluteus) indicates that CyIIa transcripts are not detectable in this structure until about 60 hours, at least 1·5 days after they appear in secondary mesenchyme. Thus, while the content of CyIIa transcripts changes by only about 50% between gastrula and pluteus stages (Lee *et al.*, 1986), the predominant site of expression of this gene shifts from mesenchyme to gut.

The first phase of CyIIa mRNA expression differs from the second in that labeling is observed over primary as well as presumptive secondary mesenchyme cells (Fig. 6(a) and (b)). Other regions of the blastula are unlabeled. At 23 hours, labeling of primary mesenchyme cells within the blastocoel is quite heterogeneous, while at 29 hours, when all primary mesenchyme have ingressed, only a few cells show possible low levels of labeling. The labeled region at the vegetal pole becomes smaller between 23 and 29 hours, presumably reflecting the departure of the last primary mesenchyme cells into the blastocoel in this interval. These observations suggest that primary mesenchyme cells express CyIIa message in the early blastula before ingression, but discontinue expression around the time they depart from the blastocoel wall and turn over this message with a timing or rate that may differ between individual cells. We cannot always distinguish primary from secondary mesenchyme cells at later stages. It is clear that the primary mesenchyme cells do not reinitiate expression of CyIIa mRNA through gastrula stage, because no labeled cells are observed in the blastocoel until the time of emigration of secondary mesenchyme from the archenteron tip. However, we cannot exclude the possibility that these cells again express this message after the gastrula stage, perhaps when they

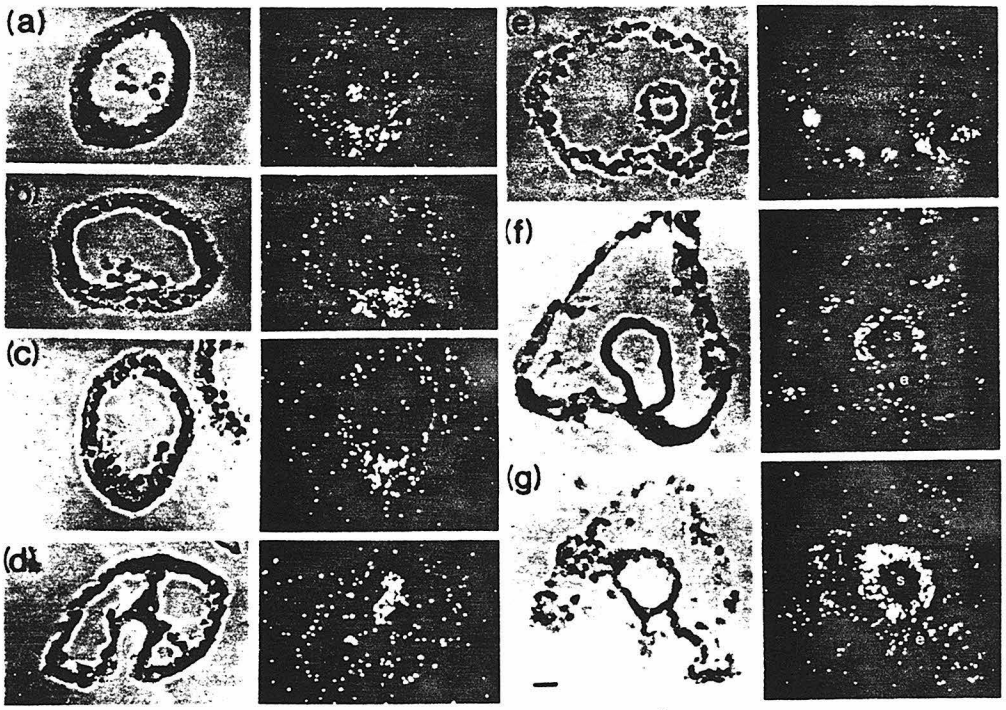

Figure 6. Pattern of expression of CyIIa mRNA. The CyIIa probe ($1{\cdot}3 \times 10^8$ disints/min per μg) containing 700 nt of sequence complementary to mRNA was hybridized at a concentration calculated to be 70% of that required to achieve saturation. Autoradiographic exposures were 85 days for sections shown in (a) to (d), 64 days in (e) and (g), and 26 days in (f). (a) and (b) Mesenchyme blastula, 23 h; (c) late mesenchyme blastula, 29 h; (d) gastrula, 35 h; (e) late gastrula/early prism, 48 h; (f) pluteus, 68 h; and (g) pluteus, 82 h. The section shown in (f) is taken from a separate experiment using embryos from a different culture. Sections in (a) to (d) pass approximately through the animal–vegetal axis, with the vegetal pole at the bottom. The section in (e) is perpendicular to this axis. The heterogeneity of labeling of ingressed primary mesenchyme cells is illustrated in (a) to (c). The gap in labeling at the vegetal pole is indicated by the arrowhead in (b). Heterogeneity in labeling of the gut at pluteus stage is shown in (f) and (g); arrowheads in (f) indicate several labeled mesenchyme cells (probably secondary). Because of the relatively low abundance of CyIIa transcripts, signal/noise is somewhat lower in these examples; labeling over ectoderm and its precursors is not consistently above background at any stage examined. e, esophagus; s, stomach; i, intestine. All sections are shown at the same magnification, and the bar in (g) represents 10 μm.

migrate from the base of the archenteron (see Discussion). The same small unlabeled region, as observed for the CyI probe, is found at the vegetal pole of 23 hour embryos (Fig. 6(b)), but not consistently in 29 hour embryos, suggesting that this mRNA also is initially absent from the small micromere derivatives and subsequently accumulates at late blastula stage.

(f) *CyIIIa and CyIIIb*

The CyIIIa and CyIIIb genes are linked in the same transcriptional orientation and are separated by about 6000 bases (Shott-Akhurst *et al.*, 1984). The data presented by Shott *et al.* (1984) and Lee *et al.* (1986) show that transcripts of these two genes accumulate during very different periods of development. CyIIIa mRNA levels rise rapidly between 15 and 18 hours and subsequently increase by a factor of only about 1·5. In contrast, there is little CyIIIb mRNA in the embryo before late gastrula stage, and it accumulates significantly only after 64 hours of development. Throughout most of early embryogenesis, CyIIIa mRNA is the major actin message in the embryo, alone accounting for about 44% of total actin mRNA at gastrula stage. However, calculations from the data presented by Shott *et al.* (1984) indicate that in the late pluteus, CyIIIb mRNA may achieve a concentration similar to that of CyIIIa message. We have used probes derived from both CyIII sequences. The CyIIIa probe is gene-specific when hybridized at a stringency similar to that used here (Lee *et al.*, 1986), while the CyIIIb probe does cross-react (Shott *et al.*, 1984).

We described hybridizations *in situ* with the cross-reacting CyIIIb probe showing that CyIII actin mRNAs are confined to aboral ectoderm of

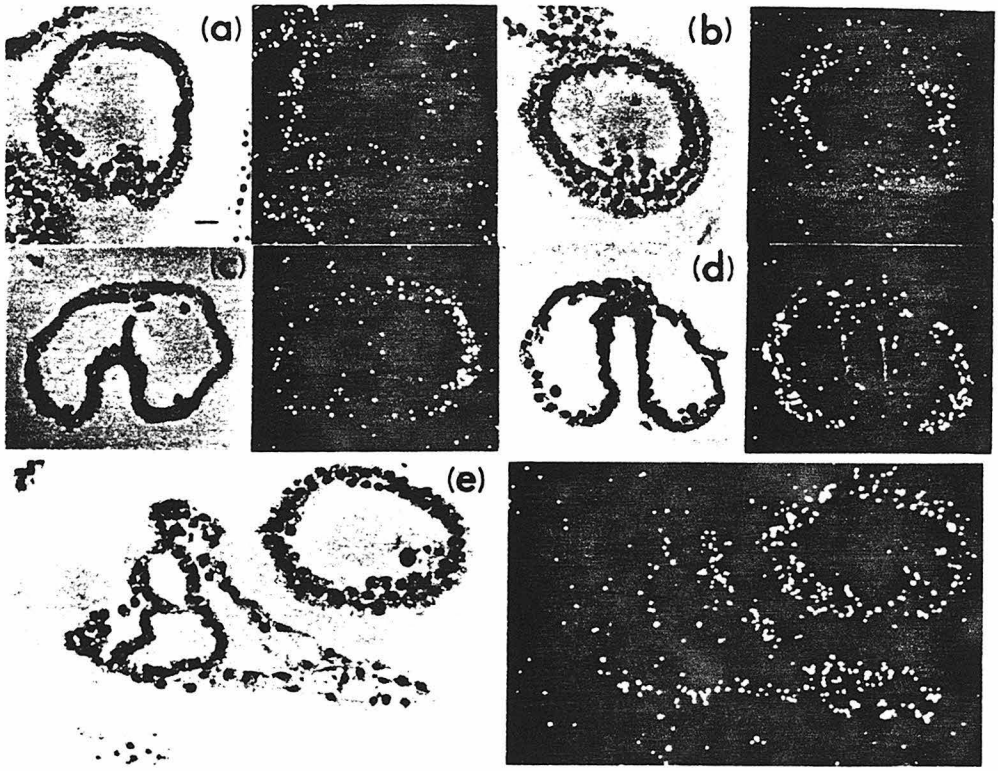

Figure 7. Restriction of CyIIIa transcripts to the aboral ectoderm lineage. The CyIIIa probe ($1{\cdot}5 \times 10^8$ disints/min per μg) containing 131 nt of sequence complementary to mRNA was hybridized at a concentration calculated to be slightly above that required to saturate complementary transcripts. Exposure time was 60 days. (a) Mesenchyme blastula, 23 h; (b) late mesenchyme blastula, 29 h; (c) and (d) gastrula, 35 h; (e) pluteus, 82 h. Labeling at all stages is restricted to aboral ectoderm and its precursors. This identification was confirmed at earlier stages (23 and 35 h), in which aboral and oral ectoderm cannot be distinguished readily by morphology, by comparing hybridization of the CyIIIb probe with that of a probe for Spec 1 mRNA, which is a marker for the aboral ectoderm lineage (Lynn *et al.*, 1983; Carpenter *et al.*, 1984) CyIII and Spec 1 mRNAs were found to be present in the same cells when these probes were hybridized separately to adjacent 1 μm thick sections (data not shown). At stages through gastrula this hybridization presents 2 characteristic patterns in sections passing through the animal–vegetal axis and either in the plane of bilateral symmetry ((a) and (c)) or perpendicular to it ((b) and (d)). In (a) the presumptive aboral region is on the left, while in (b) it is on the right. The labeled regions in (b) and (d) pass through the 2 lateral projections of presumptive aboral ectoderm described by Lynn *et al.* (1983). All sections are shown at the same magnification, and the bar in (a) represents 10 μm.

the late pluteus and to presumptive aboral ectoderm at blastula stage (Angerer & Davidson, 1984). Hybridizations with the probe specific for CyIIIa message show that it is confined throughout development to the aboral ectoderm lineage (Fig. 7), which at pluteus stage appears to include a single morphological cell type (Fig. 7(e)). At gastrula stage, the aboral surface includes approximately 60% of the actoderm in one contiguous sheet of cells (Lynn *et al.*, 1983; Lee *et al.*, 1986). Sections through the animal-vegetal axis cut either in (Fig. 7(c)) or perpendicular to (Fig. 7(d)) the plane of bilateral symmetry revealed by this mRNA marker show characteristic labeling patterns. (A diagram of the ectoderm fate map showing presumptive oral and aboral regions is given by Lynn *et al.*, 1983). A quite similar pattern of labeling is observed at blastula stages (23 and 29 h, Fig. 7(a) and (b)), except that the unlabeled region at the vegetal pole is larger because of inclusion of cells of the archenteron and primary mesenchyme lineages.

Hybridizations with the CyIIIb probe reveal exactly the same pattern as is observed for CyIIIa at stages from 23 hour blastula to late pluteus. The distribution at pluteus is illustrated in Figure 8(e). Because CyIIIb transcripts are present in very low concentration at stages before pluteus (Shott *et al.*, 1984), the signal obtained with the CyIIIb probe must result from cross-reaction with CyIIIa mRNA. The relative concentration of CyIIIb message increases markedly in late pluteus, and it

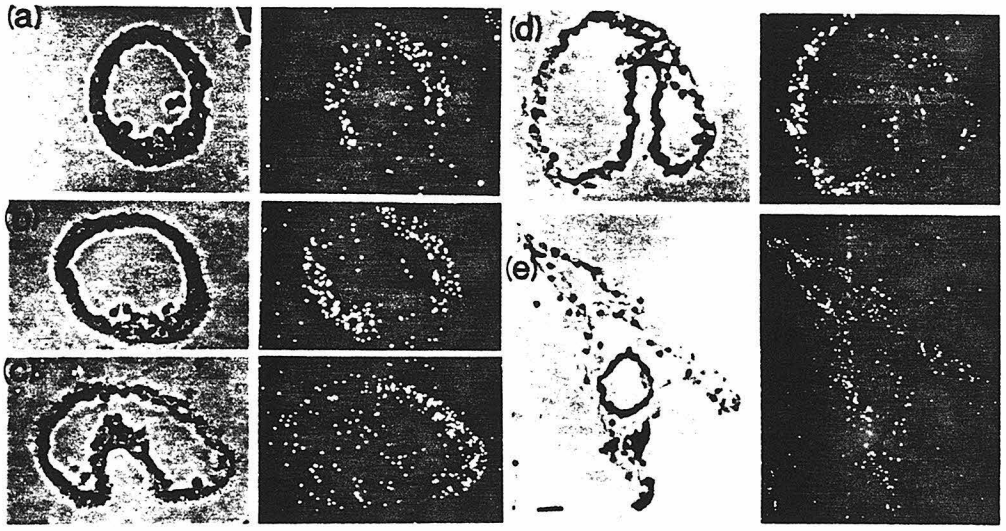

Figure 8. Hybridization pattern of the CyIIIb probe. The CyIIIb probe ($1{\cdot}3 \times 10^8$ disints/min per μg) containing 800 nt of sequence complementary to mRNA was hybridized at a concentration calculated to saturate 80% of available target mRNA. As discussed in the text, this probe cross-reacts with CyIIIa mRNA. Autoradiographic exposure was (a) to (c) 68 days or (d) and (e) 42 days. (a) Mesenchyme blastula, 23 h; (b) late mesenchyme blastula, 29 h; (c) gastrula, 35 h; (d) late gastrula/early prism, 48 h; (e) pluteus, 82 h. Sections in (a) to (d) all pass approximately through the animal–vegetal axis. Labeling is restricted to aboral ectoderm and its precursors at all stages, resulting in characteristic patterns described in the legend to Fig. 7. All sections are shown at the same magnification and the bar in (e) represents 10 μm.

would have been detectable if expressed at significant concentration in cells other than aboral ectoderm. We therefore conclude that CyIIIb mRNA is restricted to some, and probably expressed in all, cells of the aboral ectoderm.

(g) *Is actin mRNA localized in the egg or early embryo?*

The observation that each Cy actin probe labels a subset of blastomeres at blastula and later stages raises the questions of how early in development this specificity is established and whether maternal transcripts of any actin genes are localized in the unfertilized egg. Only CyI and CyIIIa sequences are detectable in maternal RNA of the unfertilized egg by RNA blot analyses (Shott *et al.*, 1984), and they are present at the concentration typical of rare mRNAs (about 2000 and 1400 transcripts/egg, respectively; Lee *et al.*, 1986). We attempted to detect these mRNAs at stages before mesenchyme blastula.

We observe low signals with the CyI probe over 2-cell embryos. The magnitude of these signals is consistent with that expected from calculations based on the measured prevalence of this message in unfertilized eggs (Lee *et al.*, 1986) and previous estimates of efficiency of hybridization *in situ* (Cox *et al.*, 1984). Furthermore, grain densities over 2-cell embryos are higher than those over unlabeled ectoderm of gastrula stage embryos (Table 1) compared over sections on the same slide, suggesting both that the 2-cell signals are real and that maternal mRNA decays within the aboral ectoderm

Table 1
Quantitation of hybridization by the CyI probe

Target	n†	Signal‡	Noise	Signal:noise
2-Cell	17	4·2± 1·1	2·9±0·6	1·3
12 h	14	13·6± 2·9	3·7±1·0	9·9
35 h unlabeled ectoderm	32	2·9± 1·0	3·8±0·8§	0
35 h labeled ectoderm	33	11·3± 3·8	3·8±0·8§	8·0
35 h mesenchyme	35	32·6±13·8	3·8±0·8§	29·3

† n is the number of individual 2-cell or 12 h embryos, ectoderm regions or mesenchyme cells measured.

‡ Signal, noise and (signal:noise) are given in grains/100 μm^2.

§ For 35 h gastrula, the noise values were determined from measurements of entire ectoderm in sections, since regions corresponding to those labeled or unlabeled cannot be identified.

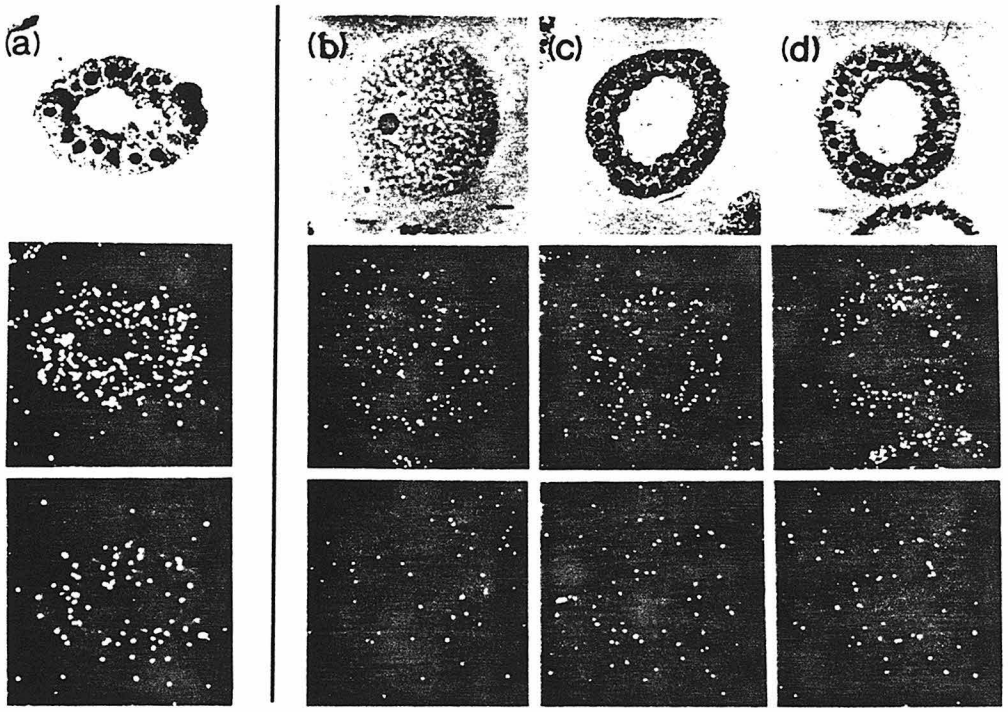

Figure 9. Distribution of CyI and CyIIIa transcripts at early developmental stages. The top and middle rows are phase-contrast and darkfield micrographs of the same section. The bottom row shows comparable sections hybridized under identical conditions with a control probe to measure non-specific background binding. (a) Hybridization of the CyI probe to a section of a 12 h embryo. Probe specific activity was $1{\cdot}3 \times 10^8$ disints/min per μg, probe concentration was estimated to be 85% of saturation, and exposure time was 117 days. The magnitude of the signal and homogeneity of labeling indicate that newly synthesized CyI mRNA is initially uniformly distributed in the embryo. (b) to (d) The CyIIIa probe ($1{\cdot}5 \times 10^8$ disints/min per μg) was hybridized at saturating concentration, and autoradiographs were exposed for 90 days.(b) Unfertilized egg exhibiting a low signal not detectably localized; (c) 12 h cleavage stage embryo in which the signal is equivalent to that observed over unfertilized eggs and is not detectably higher over any region of the embryo; (d) 18 h late cleavage stage. Such sections consistently exhibit inhomogeneities of labeling consistent with characteristic aboral ectoderm patterns. In the section shown, regions of lower signal lie on the right and left sides of the embryo. All sections are shown at the same magnification, and the bar in (b) represents 10 μm.

lineage. While such low signals must be interpreted with considerable caution, examination of a large number of sections gave no indication of non-uniform distribution of CyI maternal mRNA in the 2-cell embryo (data not shown).

Signals over sections of 12 hour embryos (about 180 cells in this culture) were approximately sevenfold higher than those over 2-cell embryos (Table 1). Thus at 12 hours, at least 80% to 90% of the hybridization signal must result from new transcription. At both 12 hours (Fig. 9(a)) and 18 hours (data not shown), these new CyI transcripts appear to be uniformly distributed in the embryo. We cannot exclude the possibility that a few cells are unlabeled, or that some lineages might have a slightly lower or higher content of CyI mRNA. However, blastomeres that give rise to aboral ectoderm, from which CyI mRNA is absent after blastula stages, occupy at least one-third of the embryo volume and would be detectable easily if unlabeled at 12 hours. The data in Table 1 show that the abundance of CyI mRNA characteristic of oral ectoderm at gastrula stage is already achieved by 12 hours. Thus the ultimate difference in abundance of this mRNA in oral and aboral regions reflects primarily decay of the mRNA in the aboral ectoderm lineage, presumably resulting from cessation of synthesis of this mRNA beginning between 18 and 23 hours.

A similar analysis for CyIIIa transcripts showed low and equivalent signals over both unfertilized eggs and 12 hour embryos, with no indication of a heterogeneous distribution (Fig. 9(b) and (c)). Signals were about twofold higher at 18 hours, reflecting the accumulation of newly synthesized mRNAs. In contrast to the uniform distribution observed for CyI mRNA when it begins to accumulate, examination of a large number of sections of 18 hour embryos hybridized with the CyIIIa probe showed regions of higher grain density

in patterns characteristic of presumptive aboral ectoderm (Fig. 9(d)). This implies that the initial expression of CyIIIa mRNA by embryonic nuclei is confined to this single lineage.

4. Discussion

Our previous analysis showed that the different actin mRNAs are, in most cases, separately regulated during development, in that they accumulate at different times and to different absolute levels in the whole embryo (Shott *et al.*, 1984). The results presented here amplify and interpret this conclusion by demonstrating that the individual mRNAs are differentially expressed in different cell lineages. The accumulation of three of the mRNAs (CyI, CyIIb and CyIIa) in the whole embryo is resolved to a complex set of changes in abundance in individual lineages, involving both increases and decreases. The other three messages (CyIIIa, CyIIIb and M) are shown to be restricted to single cell lineages.

The individual actin genes exhibit a surprising diversity of patterns of spatial/temporal expression during embryogenesis. The six mRNAs can be grouped into four sets according to spatial pattern (M, CyI/CyIIb, CyIIa and CyIIIa/CyIIIb) and accumulation of CyIIIa and CyIIIb may be separated into quite different temporal patterns (Shott *et al.*, 1984). Each spatial pattern shows some correlation with processes of cell division, motility or change in shape. Such correlations could indicate subtle functional distinctions among individual cytoplasmic actin proteins whose sequences as determined to date, however, differ by only 0·4% to 1% (Schuler *et al.*, 1983). Alternatively, as discussed (Davidson *et al.*, 1982; Crain *et al.*, 1982), the gene multiplicity might serve requirements for differentially regulating levels of actin protein in different cells and/or for coordinating expression of actin with that of other genes in regulatory sets.

Expression of muscle-type actin mRNA is confined to a single very small lineage, which is part of the coelomic rudiments (about 16 to 20 cells in the late unfed pluteus; Ishimoda-Takagi *et al.*, 1984). The precursors to these cells are situated at late gastrula stage on either side of the archenteron, distinctly below its tip. In contrast, the derivatives of the small micromeres, which also contribute to the coelom, are situated at the tip of the archenteron (Pehrson & Cohen, personal communication). Therefore, the muscle cells must derive from (probably a subset of) the "veg2" tier of eight macromere descendants in the 64-cell embryo, at the vegetal pole just above the micromeres.

The major feature of expression of the CyI (and probably CyIIb) gene is its initial expression in all cells of the early blastula and its subsequent disappearance from several lineages at different times during embryogenesis. In general CyI and CyIIb mRNAs are found in embryonic lineages whose cells are dividing and disappear from lineages as they reach their final cell number in the unfed pluteus. CyI mRNA is one of two actin messages detectable in the unfertilized egg, implying that at least some of the maternal actin protein pool is of this subtype. New transcription appears to be initiated, and the messages accumulate in all blastomeres between 10 and 14 hours. Between 18 and 27 hours, CyI and CyIIb mRNAs decay from a major cell lineage, the aboral ectoderm, and this coincides with the time during which these cells withdraw from the division cycle (L. Cohen, personal communication; the analyses presented by Masuda (1979) and Masuda & Sato (1984) show, for several sea urchin species, that some ectodermal cells withdraw from rapid division around the 10th cleavage). These messages disappear from primary mesenchyme cells shortly after they ingress into the blastocoel (20 to 29 h), and it is tempting to speculate that the variability in signals over individual cells reflects the stage they have reached in the programmed number of cell divisions. Both mRNAs disappear gradually from secondary mesenchyme when these cells migrate from the tip of the archenteron. In these cases, withdrawal from expression of CyI and CyIIb appears to be permanent, at least in the absence of further development of the pluteus dependent on feeding. In the case of parts of the gut lineage, the withdrawal is temporary, and these mRNAs reaccumulate as the gut differentiates. We do not know if there is a corresponding pause and resumption of cell division in these regions.

Throughout all of early embryogenesis, CyIIa mRNA accumulates only in cells that utilize contractile pseudopodia for morphogenetic purposes. CyIIa mRNA is found in primary mesenchyme cells just before ingression and subsequently decays. These cells come to lie at the vegetal pole, forming a ring around the base of the archenteron at early gastrula stage. Subsequently, movement is reinitiated to establish the syncytium of cells that deposits the skeleton in a specific pattern, but we were unable to tell whether any of the labeled mesenchyme cells at this stage were primary, as might be predicted. Extension of contractile pseudopodia by secondary mesenchyme at the tip of the invaginating archenteron is instrumental in the culmination of gastrulation (Gustafson & Wolpert, 1967). Subsequently, most of these cells migrate from this region along the wall of the blastocoel. In a few sections of plutei where coelomic rudiments could be identified unambiguously, they also were labeled. Cells of the coelomic rudiments actively extend pseudopodia (Gustafson & Wolpert, 1967). Quite late in development, CyIIa mRNA accumulates to moderately high levels in stomach and intestine. While the precursors to these cells were noted by Gustafson & Wolpert (1967) to exhibit "pulsatile activity", cells of the differentiating gut are not motile and, to our knowledge, do not construct pseudopodia. Finally, we note that the distribution of CyIIa mRNA most strikingly reflects the animal–vegetal embryonic axis. Thus, this message

is restricted throughout pre-feeding embryogenesis to derivatives of vegetal pole blastomeres, primary mesenchyme and some cells derived from the "veg2" blastomeres, excluding the esophagus.

The most striking specificity of expression is displayed by the CyIII genes, whose transcripts appear to be expressed only in the embryo (Shott *et al.*, 1984; Garcia *et al.*, 1984), in which they are confined to the single cell type of the aboral ectoderm. The shift from division to differentiation of cells in this lineage is accompanied by a switch in expression of actin genes from CyI/CyIIb to CyIIIa/CyIIIb, and only CyIII mRNAs are detectable in aboral ectoderm at stages after late blastula. The exclusive relationship between aboral ectoderm and CyIII actins suggests a singular functional requirement either for co-ordinating the synthesis of large amounts of actin mRNA with that of other mRNAs or for actin proteins with distinct properties. Recent analyses suggest that CyIII actins and calcium-binding proteins encoded by the Spec 1 and/or Spec 2 gene families comprise part of the cytoskeletal mechanism for establishing and maintaining the flattened epithelial shape of aboral ectoderm and/or regulating the contraction of this tissue at the culmination of metamorphosis (Carpenter *et al.*, 1984; see also Angerer & Davidson, 1984).

Cell division, control of cell shape, pseudopodial activity and contractility, and motility are fundamental processes in the morphogenesis of the sea urchin embryo that must involve actin proteins. We have established some suggestive associations between these processes and expression of specific members of the actin gene family. Whether the multiplicity of these genes serves regulatory, biochemical or both kinds of purposes, it is clear that each major cell lineage of the embryo considered here utilizes a characteristic program of actin gene expression. Initiation of these programs at early blastula stage is one of the first molecular signals of cell differentiation in the embryo.

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CHAPTER 4

Activation of Sea Urchin Actin Genes During Embryogenesis:

Measurement of Transcript Accumulation from Five Different Genes

in *Strongylocentrotus purpuratus*

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Activation of Sea Urchin Actin Genes during Embryogenesis

Measurement of Transcript Accumulation from Five Different Genes in *Strongylocentrotus purpuratus*

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The number of molecules of mRNA transcribed from each of five different actin genes are reported for developing embryos of the sea urchin *Strongylocentrotus purpuratus*. Transcripts of the cytoskeletal actin genes CyI, CyIIa, CyIIb and CyIIIa, and of the muscle actin gene M, were measured in unfertilized egg and embryo RNAs of cleavage, blastula, gastrula and pluteus stages. The measurements were obtained by probe excess titrations of these RNAs, using a set of single-stranded RNA probes each identifying the mRNA transcripts of a specific actin gene. These mRNAs can be identified by their distinct 3′ non-translated trailer sequences. We confirm prior observations that the prevalence of actin mRNA in the unfertilized egg is low. Cytoskeletal actin genes CyI and CyIIIa each contribute 1×10^3 to 2×10^3 maternal mRNA molecules, and CyIIb contributes $< 2 \times 10^2$ mRNA molecules, while no detectable maternal mRNAs derive from cytoskeletal actin gene CyIIa or the muscle actin gene M. During certain periods of development, transcripts derived from the individual cytoskeletal actin genes accumulate rapidly, with kinetics specific to each mRNA. Transcripts of the muscle actin gene are absent until after gastrulation, when the initial muscle progenitor cells are formed. At late stages of development, each of the five genes studied is represented by 10^4 to 10^5 mRNA molecules per embryo. The present measurements permit calculation of the levels of each actin mRNA species in the particular cell types in which each gene functions in the fully differentiated embryo.

1. Introduction

The genome of the sea urchin *Strongylocentrotus purpuratus* contains eight different actin genes, of which six are known to be functional (Lee *et al.*, 1984; Shott *et al.*, 1984). Previous studies (Shott *et al.*, 1984; Garcia *et al.*, 1984; Angerer & Davidson, 1984) and the observations described in the accompanying paper (Cox *et al.*, 1986) demonstrate that each of the active genes of this family is regulated independently during development. These genes display distinct patterns of expression in adult sea urchin tissues and, while they are all activated to some extent during embryogenesis, they function in diverse cell lineages or sets of cell lineages at diverse stages of development. In the present study, we provide quantitative measurements of the accumulation of mRNA during embryogenesis from five of the six functional actin genes. Approximate estimates of the relative prevalence of transcripts of these actin genes had been obtained from gel blot and dot blot hybridization data (Shott *et al.*, 1984).

The patterns of expression and the linkage relationships among the six functional actin genes can be summarized as follows. As in the accompanying paper, we utilize the nomenclature of Lee *et al.* (1984), in which the actin genes are classified according to homologies in the 3′ non-translated trailer sequences of their messages. One gene, called M, which displays a unique 3′ sequence, is expressed only in muscle cells in adult sea urchins (Crain *et al.*, 1981; Shott *et al.*, 1984) and, as demonstrated by Cox *et al.* (1986), during embryogenesis transcripts of this gene appear exclusively in a bilateral set of muscle anlage cells that differentiate after gastrulation. The remaining five active genes are all expressed in non-muscle cell

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types, and presumably code for cytoskeletal actin proteins (Schuler & Keller, 1981; Shott *et al.*, 1984; Cox *et al.*, 1984). Three of these genes are linked at intervals of several thousand bases, in the order CyI-CyIIa-CyIIb (Scheller *et al.*, 1981; Schuler & Keller, 1981). CyI and CyIIb display very similar patterns of activity in the embryo. Both are represented at low levels in the maternal mRNA, and are activated in most or all regions of the early embryo. However, their transcripts ultimately disappear from cells of the aboral ectoderm. The CyIIa gene is activated later than are CyI and CyIIb; it is not detectably represented in maternal mRNA; and its transcripts are confined to mesenchyme cells and some regions of the gut and its precursors. CyIIIa and CyIIIb are also linked at a distance of several thousand bases. The 3′-terminal sequences of these genes are not homologous with those of other actin genes and are very similar to each other, though they can be distinguished at the primary sequence level (Shott *et al.*, unpublished results). CyIIIa and CyIIIb are both expressed specifically in the aboral ectoderm of embryos and larvae (Angerer & Davidson, 1984; Cox *et al.*, 1986). CyIIIa provides more embryonic actin message than does any other actin gene, but neither CyIIIa nor CyIIIb is utilized after metamorphosis (Shott *et al.*, 1984). A probe for CyIIIb that does not cross react with CyIIIa is difficult to generate, whereas a probe specific for CyIIIa has been constructed as described below. Shott *et al.* (1984) showed that CyIIIb produces less than 3% of the total actin mRNA synthesized before gastrulation, though it later becomes more active. The measurements described in this paper outline the development course of accumulation for the mRNAs produced by genes M, CyI, CyIIa, CyIIb and CyIIIa.

2. Materials and Methods

(a) *Growth of sea urchin embryos*

Gametes of *S. purpuratus* were collected by intracoelomic injection of 0·5 M-KCl. Eggs were washed by repeated settling in Millipore-filtered seawater. Embryos were fertilized and cultured at 15°C with constant stirring and aeration as described (Smith *et al.*, 1974; Hough-Evans *et al.*, 1977). The embryos were grown at a concentration of 10^4/ml in Millipore-filtered seawater containing 40 μg of gentamicin sulfate/ml (Schering Corp.).

The developmental stages relevant to the experiments in this paper were described briefly by Davidson *et al.* (1982*a*). At 7 h after fertilization, the embryos contain 32 to 64 cells. Hatching of early blastulae occurred at 18·5 h post-fertilization, and swimming blastula stage embryos were collected at 20 h. Gastrula RNA was prepared from embryos at 36 h post-fertilization. At this stage they have initiated archenteron invagination and skeletal formation has begun. Plutei were collected at 65 h post-fertilization.

(b) *Isolation of total egg and embryo RNA*

Total embryo RNA was prepared essentially as described by Posakony *et al.* (1983). Eggs or embryos were washed and pelleted through ice-cold Ca/Mg-free seawater brought to pH 3 with citric acid (Hynes & Gross, 1970). The pellets were suspended in a buffer containing 7 M-urea, 50 mM-sodium acetate (pH 5·5), 10 mM-EDTA, 15 mM-ethyleneglycol-bis(β-aminoethyl ether), 1% sarcosine and 10 μg polyvinyl sulfate/ml. Proteinase K (Merck) was added to a final concentration of 300 μg/ml of lysate and the homogenate was incubated at 25°C for 30 min. RNA was purified from this homogenate by the CsCl pelleting method of Glišin *et al.* (1974). The homogenate was diluted 1 : 1 with diethylpyrocarbonate-treated distilled water, and solid CsCl (1 g/ml) was added. The homogenate was transferred to Beckman Quick-Seal tubes and underlayered with a cushion of 5·7 M-CsCl in 10 mM-sodium acetate (pH 5·5). Centrifugation was carried out for 24 h at 39,000 revs/min at 26°C in a 60 Ti rotor. The RNA pellets were dissolved in diethylpyrocarbonate-treated distilled water at 4°C and precipitated from 0·2 M-sodium acetate (pH 5·5) with the addition of 2 vol. ethanol. The RNA was again dissolved in distilled water and precipitated overnight at −20°C with 2 vol. 4·5 M-sodium acetate (pH 5·5) (Childs *et al.*, 1979). These RNA pellets were redissolved in distilled water, precipitated a final time with 2 vol. ethanol at −20°C, and the resulting pellets were dissolved in distilled water and stored at −70°C.

(c) *Construction of 3′ transcribed but untranslated actin-gene-specific probes*

Bacteria containing recombinant plasmids were grown and the plasmids were amplified according to the method of Norgard *et al.* (1979). Plasmid DNAs were isolated as described by Scheller *et al.* (1977).

Actin-gene-specific probes pCyI, pCyIIa, pCyIIb and pM are subclones whose construction and specificity were described by Lee *et al.* (1984). An additional subclone was constructed for this work that is specific to the cytoskeletal actin gene CyIIIa. Briefly, a gel-purified *Ava*II–*Rsa*I fragment from the cDNA clone p9-KIA (Shott *et al.*, 1984; see Fig. 1(d)) was digested with *Mbo*I and the reaction incubated with Klenow polymerase I (Boehringer-Mannheim) in the absence and then presence of deoxynucleoside triphosphates. The resulting blunt-end fragments were cloned into the *Sma*I site of pUC9. Primary nucleotide sequences obtained by Shott *et al.* (unpublished results) for the 3′ region of the CyIII genes show that the 131 nucleotide (nt)† *Mbo*I–*Rsa*I fragment, located 30 nt 3′ from the translational stop codon, is homologous only to the CyIIIa gene.

Each of the five actin-gene-specific probes was recloned into M13 phage vectors Mp10 and Mp11 (Messing, 1983) and into plasmid vectors (pSpZ1, pSpZ2 or pSp62-PL) that have an SP6 RNA polymerase promoter adjacent to their polylinker region (Green *et al.*, 1983; Angerer *et al.*, 1985). M13 subclones were constructed by directionally cloning the double-stranded actin inserts in the replicative form DNA. The orientation of each clone was chosen such that primer extension reactions using the sequencing primer and single-stranded phage DNA would generate a probe that hybridizes to mRNA. The plasmid vector pSpZ1 was used for pCyIIa and pCyIIb, and pSpZ2 was used for pCyI and pM (Cox *et al.*, 1985). The vector pSp62-PL (Green *et al.*, 1983) was used for pCyIIIa.

† Abbreviation used: nt, nucleotides.

(d) *Determination of probe sequence length represented in mRNA by digestion with* Exo*VII and* S_1 *nucleases*

Uniformly labeled, single-stranded M13 probes of defined length were prepared as described by Hu & Messing (1982). Synthesis reactions were carried out in 40-μl reaction volumes under the following conditions. Reactions typically contained 0·5 to 1·5 μg of single-stranded DNA template and 5 ng of 15mer sequencing primer (New England Biolabs) in a buffer containing 50 mM-NaCl, 7 mM-Tris·HCl (pH 7·5), 10 mM-$MgCl_2$. Cold deoxynucleoside triphosphates (dGTP, dCTP, dTTP) were added to a final concentration of 100 μM and 50 μCi of [α-^{32}P]ATP (410 Ci/mmol; Amersham) was included as the label. Reactions were started with the addition of 10 units of Klenow polymerase (Boehringer-Mannheim) and incubated first at room temperature for 10 min followed by 30 min at 37°C. Reactions were chased with unlabeled dATP (50 μM final concn) for 30 min at 37°C. The synthesis reactions were stopped by incubation for 5 min at 68°C.

To generate a probe of defined length, the synthesis products were digested with an appropriate restriction enzyme that truncates the primer extension product immediately after the insert. The reactions were denatured with the addition of 5 M-NaOH and 0·5 M-EDTA to final concentrations of 0·2 M and 10 mM, respectively. The denatured synthesis products were separated on a 1·5% neutral agarose gel (running buffer: 40 mM-Tris·HCl (pH 7·8), 20 mM-sodium acetate, 2 mM-EDTA) and the labeled single-stranded DNA probe was gel-purified by electroelution onto DE-81 filter paper and eluted with 1·5 M-NaCl, 200 mM-NaOH, 1 mM-EDTA, 5 μg sheared calf thymus DNA/ml. The eluted probe was then brought to pH 7 with the addition of 3 M-sodium acetate (pH 5·5).

The single-stranded DNA probes (~0·3 ng) were hybridized overnight (~20 h) with 2 μg of poly(A)$^+$ RNA from a developmental stage or adult tissue shown to contain a significant amount of the mRNA of interest. Hybridizations were typically done at 37°C in 10 μl final volumes containing 80% (v/v) formamide, 0·4 M-NaCl, 25 mM-PIPES (pH 6·8), 200 μM-EDTA. The hybridization reactions were subsequently digested with either S_1 nuclease (Miles) or *Exo*VII (Bethesda Research Laboratories). S_1 nuclease assays were done essentially as described by Berk & Sharp (1977). Assays were performed by diluting the hybridization reactions with 300 μl of S_1 buffer (0·25 M-NaCl, 0·03 M-potassium acetate (pH 4·5), 1 mM-zinc sulfate, 5% (v/v) glycerol) containing 5 μg of double-stranded and 5 μg of single-stranded *Escherichia coli* chromosomal DNA. S_1 nuclease digestions were carried out at 15°C for 2 h with an amount of enzyme sufficient to digest 95% of the single-stranded DNA in the reactions. *Exo*VII assays were performed essentially as recommended by the manufacturer. Hybridization reactions were diluted to 300 μl with *Exo*VII digestion buffer (30 mM-KCl, 10 mM-Tris·HCl (pH 7·4), 10 mM-EDTA, 10 mM-β-mercaptoethanol), 5 μg of yeast tRNA and 6 units of *Exo*VII. Digestions were carried out at 37°C for 45 min. The nuclease reactions (both S_1 and *Exo*VII) were stopped with the addition of an equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1). Following this extraction, the hybridized molecules were precipitated overnight at −20°C with 2·5 vol. ethanol. The precipitates were resuspended in DNA sequencing buffer (90% formamide, 10 mM-NaOH, 0·25% (w/v) bromophenol blue, 0·25% (w/v) xylene cyanol), heat-denatured (95°C for 5 min) and a sample was loaded onto a denaturing 5% polyacrylamide gel.

(e) *Synthesis and isolation of RNA probes using Sp6 RNA polymerase*

Specific RNA probes of defined length were generated using the plasmid clones that contain the Sp6 RNA polymerase promoter (see section (c), above). The synthesis reactions were carried out with linearized plasmid as template. Run-off transcripts were synthesized as described by Green *et al.* (1983) but with the following modifications. Standard reactions (25 μl) contained 1 μg of linearized DNA template and 1 mCi of [α-^{32}P]UTP (410 Ci/mmol; Amersham) diluted to approx. 100 Ci/mmol with cold UTP. Synthesis reactions were initiated with the addition of 15 units of Sp6 RNA polymerase (Promega Biotec) and incubated at 37°C for 1 h. RNase-free DNase (Promega Biotec) was added to a final concentration of 20 μg/ml, and the reactions were incubated at 37°C for 10 min. Following extraction with phenol/Sevag, the [^{32}P]RNA was purified from unincorporated nucleotides by Sephadex G-100 chromatography. Typically, 2 to 3 μg of [^{32}P]RNA with a specific activity of $\sim 2 \times 10^8$ cts/min per μg was obtained.

(f) *Solution hybridizations and assay of RNase resistant hybrids*

Tracer excess hybridizations were carried out with the purified RNA probes and varying amounts of total RNA isolated from various developmental stages. The hybridizations were performed in 1·5-ml Eppendorf tubes in final volumes of 20 μl.

Reactions typically contained 0·1 to 1·5 ng of tracer (Sp6-derived RNA probe) and various amounts (5 to 100 μg) of input embryo RNA. These 2 components, plus added yeast RNA to maintain a constant amount of RNA in each set of reactions, were precipitated from 0·2 M-ammonium acetate overnight at −20°C. The final RNA content in each series was either 50 or 100 μg. The resulting pellets were freed from residual salt by drying under reduced pressure in a Savant Speed-vac concentrator. The dried pellets were taken up in 10 μl of 100% deionized formamide and 6 μl of water. Once dissolved, 4 μl of 5 × hybridization mix (2·0 M-NaCl, 125 mM-PIPES (pH 6·8), 1 mM-EDTA) were added, and the reactions were covered with 50 μl of mineral oil. The samples were heated to 85°C for 5 min and then incubated at 50°C for 20 h. The hybridization criterion condition, 0·4 M-Na^+, 50% formamide, 50°C, was sufficiently high to prevent any probe cross-reaction (Lee *et al.*, 1984).

After incubation for 20 h at 50°C, the reactions were quenched on ice until treatment with nuclease. Unhybridized tracer was removed from the reactions by diluting the samples to 300 μl with 2·5 × SET (SET is 0·15 M-NaCl, 0·03 M-Tris·HCl (pH 8·0), 2 mM-EDTA) and digesting with RNase A and RNase T_1. Reactions containing 50 μg of RNA (yeast and total sea urchin RNA) were digested with 65 μg of RNase A (Worthington) and 150 units of RNase T_1 (Worthington); reactions containing 100 μg of RNA were digested with 97·5 μg of RNase A and 225 units of RNase T_1. The amount of each RNase added was predetermined by titrating the activity of each enzyme preparation. The reactions were incubated at 37°C for 1 h. RNase-resistant hybrids were precipitated with ice-cold 5% (w/v) trichloroacetic acid. The precipitants were collected on

GF/C filters (Whatman), and their radioactivity was determined by scintillation counting in Ready-Solv EP (Beckman). The fraction of probe resistant to RNase after hybridization in the presence of heterologous RNA was approximately 0·5% of the input radioactivity.

3. Results

(a) *Determination of the length of mRNA transcript contained on each actin-gene-specific probe*

The construction of the gene-specific probes used in this work is described in Materials and Methods and by Lee *et al.* (1984). The exact length of sequence represented in the respective actin mRNAs was not known for three probes derived from genomic rather than cDNA clones; namely, pCyI, pCyIIa and pM. To obtain this necessary parameter, labeled single-stranded DNA transcripts were synthesized from M13 clones and reacted with embryo RNA. The length of sequence protected by the mRNA probe hybrid from digestion with S_1 nuclease or *Exo*VII was measured. These experiments are shown in Figure 1. The determination shown in Figure 1(a) demonstrates protection of a 517 nt fragment of the CyI gene after hybridization with embryo RNA. The 5′ terminus of this probe is located 203 nt beyond the translation stop codon, and thus the CyI gene possesses a 3′ transcribed but non-translated trailer 720 nt in length. We note that this result fails to confirm the poly(A) addition site proposed by Cooper & Crain (1982), based on the occurrence of the sequence A-A-U-U-A-A-A, which is a variant of

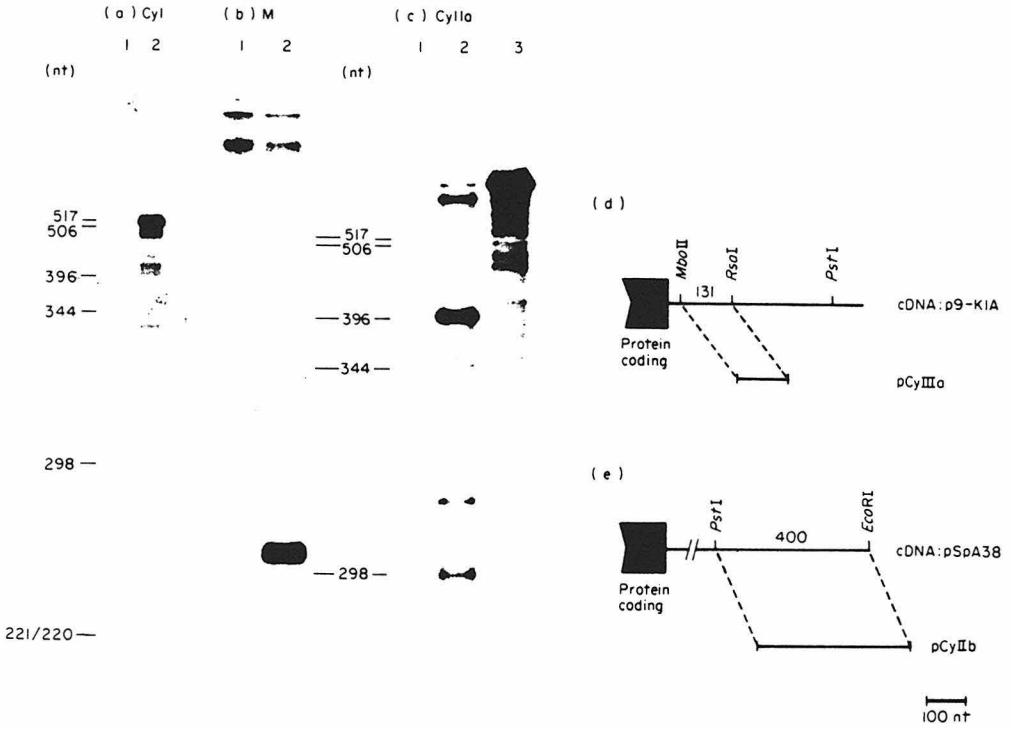

Figure 1. The length of actin mRNA transcript represented on each actin-gene-specific probe. Poly(A)$^+$ RNA (2 μg) was hybridized with ~0·3 ng of single-stranded labeled DNA probe synthesized from the respective M13 clones (see Materials and Methods). (a) CyI 3′-terminal probe; lane 1, yeast RNA; lane 2, 36 h poly(A)$^+$ embryo RNA. (b) M 3′-terminal probe; lane 1, yeast RNA; lane 2, poly(A)$^+$ tubefoot RNA. (c) CyIIa 3′-terminal probe; lane 1, yeast RNA; lanes 2–3, intestine poly(A)$^+$ RNA. RNA–DNA hybrids were digested for 2 h at 15°C with S_1 nuclease (lane 2). The CyIIa reaction (lane 3) was analyzed by digestion with *Exo*VII as well. Digestion was for 45 min at 37°C with 6 units of *Exo*VII. Nuclease digestion products were loaded on an 8 M-urea/5% polyacrylamide gel. Marker fragment lengths (nt) are the fragments resulting from *Hin*fI digestion of pBR322, labeled by means of the Klenow repair synthesis reaction. (d) Restriction map of the CyIIIa gene probe (see Materials and Methods for construction). (e) Restriction map of the CyIIb gene probe (Lee *et al.*, 1984).

Table 1
Actin gene probes and mRNA length

Actin gene probe	Length of probe complementary to mRNA (nt)	Length of mRNA† (nt)
CyI	517	2200
CyIIa	720	2200
CyIIb	400	2100
CyIIIa	131	1800
M	300	2200

Restriction maps and details of the construction of the subclones were presented by Lee *et al.* (1984), except for pCyIIIa, for which see Materials and Methods, and Fig. 1(d).

† The lengths of the mature mRNAs are derived from the RNA gel blots presented by Shott *et al.* (1984).

the canonical poly(A) addition signal that is located only 487 nt 3′ of the translational stop codon.

An S_1 nuclease experiment with the M gene probe was carried out with poly(A)$^+$ RNA from adult sea urchin tubefoot, which serves as a convenient source for muscle tissue. Figure 1(b) shows that 300 nt of the probe sequence is included in the mature mRNA. A similar experiment carried out with the CyIIa probe resulted in a series of S_1-nuclease-resistant fragments ranging from about 200 nt to the full length of the probe, about 720 nt (Fig. 1(c)). Since the CyIIa gene is represented by a single mRNA of discrete length (Shott *et al.*, 1984), the result obtained after digestion with S_1 nuclease implied the presence of multiple mismatched bases in the RNA–DNA hybrid, due to sequence polymorphisms. On the average, about 4% of nucleotides in any two genomes of *S. purpuratus* differ at any given location (Britten *et al.*, 1978). The regions surrounding the actin-gene-coding sequences are known specifically to exhibit high levels of sequence polymorphism and, in particular, the 3′-terminal sequence of gene CyIIa was shown by Lee *et al.* (1984) to have undergone very recent evolutionary changes in both copy number and sequence. To avoid strand scission at putative sites of polymorphic nucleotide mismatch, we digested CyIIa probe hybrid with *Exo*VII rather than S_1 nuclease. As shown in Figure 1(c), a fragment of 720 nt representing the full length of the probe insert now survives the digestion. A simplified restriction map for the CyIIIa gene probe constructed for this study is shown in Figure 1(d). This probe was derived from a cDNA clone described by Shott *et al.* (1984: and see Materials and Methods). The 3′ mRNA sequence included on this probe is 131 nt in length (Shott *et al.*, unpublished results). The probe representing the actin gene CyIIb was isolated from a genomic clone and, from the colinearity of this clone with a cDNA clone described by Merlino *et al.* (1980), it is known that it includes about 400 nt of 3′ mRNA trailer sequence (Fig. 1(e)). A summary of these data is given in Table 1, which includes the lengths of the various actin mRNAs as determined from the RNA gel blot hybridizations presented by Shott *et al.* (1984).

(b) *Prevalence of actin gene transcripts during embryogenesis*

Titrations of embryo RNAs were carried out using RNA probes derived from the actin-gene-specific clones. To obtain these, the recombinants listed in Table 1 were recloned into vectors containing the Sp6 RNA polymerase promoter (Green *et al.*, 1983). Embryo RNAs were prepared from eggs (0 h), midcleavage embryos (7 h), blastulae (20 h), gastrulae (36 h) and plutei (65 h). Actin gene transcript concentrations were determined in the total RNA of these embryo populations. Titrations were carried out at a tracer excess of tenfold or greater, at the relatively high solution hybridization criterion of 50°C, 0·4 M-Na^+, 50% formamide, and the amount of RNA–RNA hybrid formed was assayed after digestion with RNase A and RNase T_1. Titration data for all five actin genes are collected in Figure 2. The top portion of each panel displays the least-squares solutions from the measurements of probe–mRNA hybrid content as embryo RNA content in the reactions is varied. The slope of each titration curve is converted into the mass of mRNA and number of mRNA molecules per embryo, as described in the legend to Table 2. The developmental changes in actin mRNA levels are illustrated in the bottom portion of each panel in Figure 2.

(i) *Actin maternal mRNA*

Though actin protein represents nearly 2% of the total protein in the unfertilized egg (Mabuchi & Spudich, 1980), actin mRNA is quite rare in the unfertilized egg. Table 2 shows that only three of the actin genes contribute any detectable maternal mRNA. Per egg, there are about $2{\cdot}1 \times 10^3$ transcripts of gene CyI; about $1{\cdot}2 \times 10^3$ transcripts of gene CyIIIa; and about 200 transcripts of gene CyIIb. As can be seen in the inset in Figure 2(c), this low concentration of transcript can still be detected clearly. Thus the absence of detectable transcripts of genes CyIIb and M means that, if present, these must exist at less than 50 copies per egg. The maternal transcripts of genes CyI and CyIIIa were detected earlier in gel blot hybridizations by Shott *et al.* (1984), whose experiments demonstrated also that the egg contains the same size mRNAs as found for each gene later in development (Table 1). In absolute terms, the prevalence of the maternal actin transcripts is similar to the average prevalence calculated for the high complexity, low abundance class of maternal transcripts, which is about $1{\cdot}5 \times 10^3$ copies of each sequence per egg (Hough-Evans *et al.*, 1977).

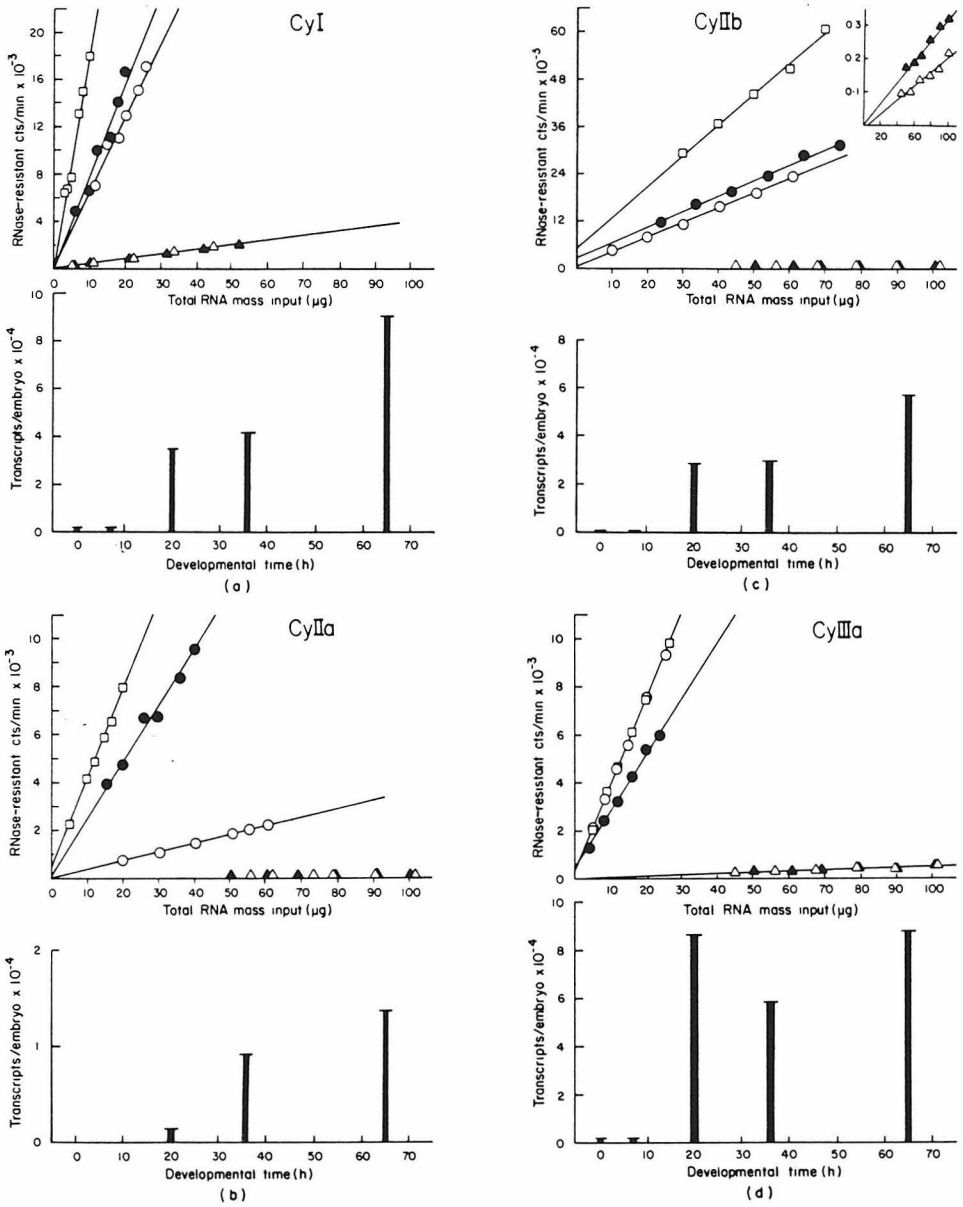

Figure 2. Measurement of actin transcripts with egg and embryo total RNA. RNA probes representing the actin genes (a) CyI; (b) CyIIa; (c) CyIIb; (d) CyIIIa; and (e) M were utilized in tracer excess titrations of the prevalence of the respective gene transcripts in unfertilized egg and embryo RNAs. The probes were generated from clones containing an Sp6 phage promoter and representing the anticoding stand of the 3′ trailer region of each gene. They were labeled internally with [α-^{32}P]UTP as described in Materials and Methods. Measurements were carried out on total RNAs prepared from (△) unfertilized eggs; and from embryos, (▲) 7 h, (○) 20 h, (●) 36 h and (□) 65 h post-fertilization. The upper portion of each section of the Figure represents probe hybridization (ordinate) as a function of increasing quantity of embryo or egg RNA (abscissa). Hybridizations were carried out at 50°C, 0·40 M-Na^+ in 50% formamide as described in Materials and Methods. The quantity of RNA–RNA hybrid was assayed in each sample by digestion with RNase A and RNase T_1 in 0·375 M-Na^+ followed by precipitation in 5% trichloroacetic acid. The data for each gene are presented as RNase-resistant cts/min *versus* total RNA input (μg). The slopes of the lines derived from these data were used to calculate the embryonic prevalence of the individual actin mRNAs (Table 2). The results of these calculations are displayed in the histograms shown in the lower portion of each section of the Figure.

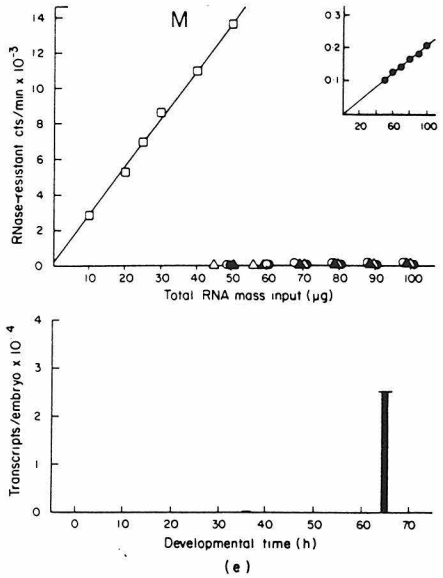

Fig. 2.

(ii) *Transcripts of genes CyI and CyIIb*

Figure 2 reveals four different patterns of transcript accumulation during embryogenesis. As shown in Figure 2(a) and (c), transcripts of genes CyI and CyIIb begin to accumulate before 20 hours post-fertilization, and continue to increase in prevalence throughout development. Examined qualitatively on a finer time-scale by RNA gel blot hybridization, an increase in the abundance of these mRNAs can be detected as early as about 10 to 12 hours (Crain *et al.*, 1981; Shott *et al.*, 1984; Garcia *et al.*, 1984). At this stage, the embryo is still engaged in rapid cell division (Cox *et al.* (1986) show in the accompanying paper that all the cells of the early blastula stage embryo express CyI (and probably CyIIb) actin genes. Though CyI is represented a little more extensively in the overall actin transcript pool than is CyIIb, both genes are major contributors in mid-stage and late-stage embryos. At gastrula stage, there are about 4×10^4 transcripts of the CyI gene, and about 3×10^4 of the CyIIb gene per embryo, and these values approximately double again by the late pluteus stage (Table 2). Expression of these genes in late embryos is restricted to cells other than the aboral ectoderm (Cox *et al.*, 1986). Since many of the cell types in which CyI and CyIIb transcripts are found continue to divide during gastrular and postgastrular development, it is likely that the postgastrular rise in CyI and CyIIb transcript level reflects, at least in part, the ongoing increase in the number of nuclear sites of CyI and CyIIb actin mRNA synthesis.

(iii) *Transcripts of the CyIIIa gene*

Accumulation of these transcripts also begins before 20 hours (Fig. 2(d)). The CyIIIa gene produces about 85,000 mRNA transcripts per embryo within the few hours intervening between the time of its activation and the 20 hour time-point. Furthermore, expression of this gene is confined strictly to the aboral ectoderm cell lineage, in both early and late embryos (Angerer & Davidson, 1984; Cox *et al.*, 1986). Shott *et al.* (1984) concluded from dot blot measurements that at the gastrula stage CyIIIa provides the largest component of actin message. This is substantiated by the data for 36-hour gastrulae shown in Table 2, where it can be seen that there are about 6×10^4 molecules of CyIIIa message per embryo at this stage. However, the net quantity of CyIIIa mRNA does not rise continuously during development, and indeed the final value has already been attained in the 20-hour blastula. This behavior probably reflects the population dynamics of the aboral ectoderm cells in which the CyIIIa gene is expressed. These cells largely withdraw from mitotic activity after about the tenth cleavage, i.e. the hatching blastula stage, and their number does not noticeably increase for the remainder of embryogenesis (Masuda, 1979; Masuda & Sato, 1984; Cox *et al.*, 1986; for a review, see Angerer & Davidson, 1984). Figure 2(d) displays a transient drop in CyIIIa transcripts at gastrula stage, suggesting degradation and subsequent resynthesis. This feature has been observed with three independent sets of RNA preparations, though it was not noticed for any other actin message. It is clear in any case that the level of CyIIIa transcripts found in late pluteus stage embryos is almost the same as that already present in early blastula-stage embryos.

(iv) *Transcripts of the CyIIa gene*

The major accumulation of CyIIa transcripts occurs later than 20 hours post-fertilization, though these mRNAs can first be detected at this time (Table 2; Shott *et al.*, 1984). These mRNAs are initially observed in primary mesenchyme cells within the blastocoel and in presumptive mesenchyme cells still embedded in the vegetal plate of the hatched blastula (Angerer & Davidson, 1984; Cox *et al.*, 1986). They are confined to secondary mesenchyme cells during gastrulation and primarily to certain regions of the gut of the differentiated pluteus (Cox *et al.*, 1986). CyIIa mRNAs never achieve the prevalence of CyI, CyIIb or CyIIIa transcripts, and in the late embryo this gene is represented by slightly more than 10^4 transcripts per embryo.

(v) *Transcripts of the M actin gene*

Figure 2(e) and Table 2 show that the muscle actin gene is the last to be expressed during development, at least as measured by accumulation of its transcripts. This was observed qualitatively by Shott *et al.* (1984). The explanation is clear from the accompanying study (Cox *et al.*, 1986). M gene transcripts initially occur only in the bilateral patches of presumptive muscle cells that coalesce on

Table 2
Actin transcript prevalence during sea urchin development

Actin gene	Time post-fertilization† (h)	Cts/min probe hybridized per μg of total RNA‡	mRNA per embryo§ (pg)	Number of transcripts per embryo‖
CyI	0	41·1	$2·6 \times 10^{-3}$	$2·1 \times 10^{3}$
	7	41·7	$2·6 \times 10^{-3}$	$2·1 \times 10^{3}$
	20	689	$4·3 \times 10^{-2}$	$3·5 \times 10^{4}$
	36	824	$5·1 \times 10^{-2}$	$4·1 \times 10^{4}$
	65	1805	$1·1 \times 10^{-1}$	$9·0 \times 10^{4}$
CyIIa	0	—	—	—
	7	—	—	—
	20	38·1	$1·8 \times 10^{-3}$	$1·4 \times 10^{3}$
	36	231	$1·1 \times 10^{-2}$	$9·4 \times 10^{3}$
	65	367	$1·7 \times 10^{-2}$	$1·4 \times 10^{4}$
CyIIb	0	2·09	$1·9 \times 10^{-4}$	$1·6 \times 10^{2}$
	7	3·14	$2·8 \times 10^{-4}$	$2·4 \times 10^{2}$
	20	369	$3·3 \times 10^{-2}$	$2·8 \times 10^{4}$
	36	395	$3·5 \times 10^{-2}$	$3·0 \times 10^{4}$
	65	765	$6·9 \times 10^{-2}$	$5·8 \times 10^{4}$
CyIIIa	0	4·75	$1·2 \times 10^{-3}$	$1·2 \times 10^{3}$
	7	4·81	$1·2 \times 10^{-3}$	$1·2 \times 10^{3}$
	20	354	$8·8 \times 10^{-2}$	$8·6 \times 10^{4}$
	36	239	$5·9 \times 10^{-2}$	$5·8 \times 10^{4}$
	65	360	$9·0 \times 10^{-2}$	$8·8 \times 10^{4}$
M	0	—	—	—
	7	—	—	—
	20	—	—	—
	36	2·06	$2·1 \times 10^{-4}$	$1·7 \times 10^{2}$
	65	300	$3·1 \times 10^{-2}$	$2·5 \times 10^{4}$

† Embryonic stages corresponding to these culture periods are given in Materials and Methods, section (a).

‡ Slopes of the titration curves presented in the upper sections of Fig. 2. The slopes were determined by a linear least-squares analysis of the data points.

§ Mass of mRNA (pg)/embryo for a given actin gene is calculated as follows. Mass of mRNA (pg)/embryo = $xy/\alpha\beta\delta$: where x is cts/min of probe/μg of total RNA (slope of titration curves present in column 3 of this Table); y is mass of total RNA/embryo, here taken as 2·8 ng (Goustin & Wilt, 1981); α is ^{32}P scintillation counting efficiency (97·3%); β is specific activity of the RNA probe (disint/min per pg); δ is fraction of mature mRNA represented on the probe (the quotient of columns 2 and 3 of Table 1).

‖ The number of transcripts/embryo is derived by dividing pg of mRNA/embryo (column 4) by the molecular weight of the actin mRNA in question (pg/transcript). The molecular weights used in this study are: 2200 nt mRNA = $1·24 \times 10^{-6}$ pg/transcript; 2100 nt mRNA = $1·19 \times 10^{-6}$ pg/transcript; 1800 nt mRNA = $1·02 \times 10^{-6}$ pg/transcript.

either side of the esophagus following gastrulation (Ishimoda-Takagi *et al.*, 1984). This gene is very active in the late embryo and, as shown in the following section, when the relatively small number of cells in which it is expressed is taken into account, M actin transcripts are seen to be highly prevalent mRNA species.

(c) *Actin mRNA levels per cell*

We can now combine knowledge of the diverse cell types in which the various actin genes are expressed (Cox *et al.*, 1986) with the measurements just presented, and thus calculate the average prevalence of each transcript species in the cells in which they are present. This analysis is shown in Table 3 for the 65-hour pluteus stage embryo. To determine the approximate number of cells of each type, the appropriate nuclei were counted in whole mounts and sections of embryos stained with hematoxylin. The cell number of each region was derived from measurements of the number of nuclei per unit area or volume, and estimates of the total area or volume of each region.

Table 3 shows that, on a per cell basis, the actin mRNAs all fall within the moderate or high prevalence class of embryo transcripts (Lasky *et al.*, 1980; Flytzanis *et al.*, 1982). CyI and CyIIb mRNAs are found in about 60% of the embryonic cells at the late pluteus stage. These include a variety of cell types, primarily oral ectoderm and gut, where the transcripts are present at approximately uniform concentrations of 90 and 60 molecules/cell, respectively. Messages encoded by the CyIIa gene are expressed at similar concentration in about 10% of the embryonic cells comprising portions of the gut. All three of these mRNAs are detectable also in a small number of other cells, e.g.

Table 3
Approximate quantities of actin gene transcripts per cell in late pluteus

Actin gene	Predominant cell types in which gene is expressed at late pluteus†	Approximate number of cells transcribing actin mRNA per embryo‡	Average number of actin transcripts per embryo§	Average number of actin gene transcripts per cell
CyI	Oral ectoderm, stomach and intestine	$1{\cdot}0 \times 10^3$	$9{\cdot}0 \times 10^4$	90
CyIIa	Stomach and intestine	$1{\cdot}6 \times 10^2$	$1{\cdot}4 \times 10^4$	90
CyIIb	Oral ectoderm, stomach and intestine	$1{\cdot}0 \times 10^3$	$5{\cdot}8 \times 10^4$	60
CyIIIa	Aboral ectoderm	470	$8{\cdot}8 \times 10^4$	190
M	Muscle cells derived from 2° mesenchyme cells and the bilateral coelomic pouches	20–40	$2{\cdot}5 \times 10^4$	650–1300

† Cox *et al.* (1986).
‡ There are about 1800 cells in the late pluteus stage embryo.
§ Column 5 of Table 2.

mesenchyme cells, at significantly lower levels (Cox *et al.*, 1986). The actin messages whose expression is restricted to single cell types of the embryo are found at higher concentrations. Thus, CyIIIa transcripts are present at almost 200 molecules per cell in aboral ectoderm, and the closely related CyIIIb gene may contribute almost as many transcripts (Shott *et al.*, 1984). M actin gene transcripts are present at about 650 to 1300 copies/cell in about 20 to 40 cells that form muscle filaments around the esophagus. For comparison, at their peak levels in late-cleavage-stage embryos consisting of about 100 cells, each species of early histone mRNAs is present at about 10^7 molecules per embryo, or 10^5 per cell (Mauron *et al.*, 1982). There are about 900 histone genes per diploid genome, and thus each gene is represented by approximately 100 histone mRNA molecules, compared to 300 to 650 messages per cell for each M actin gene. It is thus evident that the M actin gene is a very intensely expressed sequence for the sea urchin embryo.

4. Discussion

For measurement of specific transcript concentrations in a developing system such as the sea urchin embryo, single-stranded probe excess titration is clearly the method of choice (Scheller *et al.*, 1978; Wallace *et al.*, 1977; Lev *et al.*, 1980; Lynn *et al.*, 1983). The accuracy of the measurement is limited only by the recovery of the probe–mRNA hybrids, the data scatter, and knowledge of the probe specific activity. Use of RNA probes synthesized *in vitro* from precursors of known specific activity assists in all three respects (Melton *et al.*, 1984). The high stability of RNA–RNA duplexes, and their near absolute resistance to ribonuclease at adequate concentrations of monovalent cation, renders this procedure less sensitive to particular assay conditions compared, for example, to measuring S_1 nuclease resistance of DNA–RNA hybrids. In addition, in our experience the background level of acid-precipitable, RNase-resistant radioactive RNA is reproducibly less than 0·5% of the input tracer, which, combined with the high specific activity commercially available for ribonucleotide precursors, means that extremely low transcript levels can be measured. Thus the measurement of CyIIb maternal transcripts (see inset, Fig. 2(c)) is equivalent to the detection of less than 0·2 transcript per average cell, since the mass of cytoplasm in the egg is divided into more than 10^3 cells as development proceeds. The principles of the method aside, it is reassuring that the agreement with less accurate earlier estimates, where they exist, implies the absence of any significant systematic errors. For example, Shott *et al.* (1984) estimated from RNA gel blot hybridizations that there are a few thousand maternal mRNA molecules deriving from gene CyIIIa, and about 150,000 transcripts of this gene in the advanced embryo. Both estimates fall within a factor of two of the values measured in this study, about 2100 maternal mRNA molecules and 85,000 mRNA molecules in the pluteus stage embryo. Similarly, the overall amount of actin mRNA of all species, about 4×10^5 molecules in the late embryo (Table 2; this estimate includes the likely contribution of CyIIIb in late embryos (Shott *et al.*, 1984)), is about 0·8% of the total mRNA of the embryo (i.e. about 5×10^7 molecules; Lasky *et al.*, 1980), which again more or less conforms to earlier estimates (Merlino *et al.*, 1980; Infante & Heilmann, 1981; Garcia *et al.*, 1984; Shott *et al.*, 1984). The absolute error of the titration measurements is probably less than a factor of 2, and the internal comparisons are considerably more accurate than this.

As more is learned of the diverse and often lineage-specific pattern of expression in the embryo, the developmental regulation of individual members of the sea urchin actin gene family becomes an increasingly interesting problem. In the accompanying paper, Cox *et al.* (1986) show that each of these genes functions in a specific set of

embryonic cells. Their measurements, and those described here, as well as several previous studies (e.g. Crain *et al.*, 1981; Scheller *et al.*, 1981; Angerer & Davidson, 1984; Shott *et al.*, 1984) demonstrate that each actin gene operates independently, under the control of its own regulatory apparatus. The issues thus raised are both evolutionary and developmental, as discussed elsewhere (e.g. see Davidson *et al.*, 1982*b*; Lee *et al.*, 1984; Angerer & Davidson, 1984; Cox *et al.*, 1986). In the following, we focus on specific developmental aspects of actin gene utilization that are indicated by the measurements shown in Table 2.

Different strategies are employed to provide the necessary proteins in the early development of the sea urchin embryo. Some proteins, such as the early histones, are largely synthesized from maternal messages stored in the unfertilized egg (e.g. Maxson & Wilt, 1982; Mauron *et al.*, 1982). Others, such as the tubulins, are largely stored in the egg as proteins and, though there is maternal message, it is present in relatively low quantities and its translation accounts for only a small fraction of the required protein molecules. The cytoskeletal actins clearly belong to the latter class. Until the first cytoskeletal actin genes are activated late in cleavage, the quantity of maternal message could add only a minute fraction of the massive store of preformed actin in the egg. Furthermore, mobilization of the maternal actin message is delayed at the translational level, relative to the bulk of the maternal message (Infante & Heilmann, 1981). Functions carried out by cleavage-stage cells that might require cytoskeletal actins, such as cleavage, cytoplasmic movement and maintenance of the characteristic intercellular geometry of the embryo, evidently must be performed with structural assemblages that are already in place or that readily self-assemble from the preformed components.

The cytoskeletal actin mRNAs fall into an important minority class of embryonic transcripts. A large amount of evidence, including hybridization analyses of the mRNA populations (reviewed by Davidson, 1976), comparison of the spectrum of proteins coded by maternal mRNAs with those synthesized in the early embryo (Brandhorst, 1976; Bedard & Brandhorst, 1986), and measurements of transcript prevalence based on cDNA clone populations have shown that most embryo transcript species are represented also in the maternal RNA at very similar abundances on a per egg or embryo basis (Flytzanis *et al.*, 1982; reviewed by Davidson *et al.*, 1982*a*). Thus about 90% of the mRNA species found in mid-stage embryos, at all levels of prevalence, are represented also by equally prevalent maternal mRNAs. The remaining 10% or so of embryonic messages appear to attain their embryonic levels of expression as the result of a sharp accumulation of zygotic gene products that are initially absent, or nearly so, from the maternal RNA. All of the actin gene transcripts included in this study belong to this 10% class of zygotically activated genes. It is noteworthy that this is true also of the other genes known to be expressed only in certain given embryonic cell lineages. These include: the Spec1 and Spec2 genes, which produce aboral ectoderm Ca^{2+} binding proteins (Bruskin *et al.*, 1981, 1982); the spicule matrix protein gene, which is expressed in primary mesenchyme cells (Sucov, Benson, Wilt & Davidson, unpublished results); the hyalin gene, expressed in ectoderm cells (McClay & Fink, 1982; Robinson *et al.*, unpublished results); and the muscle actin and the myosin heavy chain gene (Garcia *et al.*, 1984; Shott *et al.*, 1984; this paper; Rose & Davidson, unpublished results).

The patterns of transcript accumulation described by the measurements shown in Figure 2 almost certainly result from transcriptional activation of the respective genes. This is a particularly compelling implication for examples such as the CyIIa and M actin genes, for which no transcripts can be detected before the specific stages late in development, when the accumulation of these transcripts is initiated in particular cells. In the absence of any other evidence, it could perhaps be maintained that the accumulation pattern results from transcript stabilization rather than increase in the rate of transcriptional initiation. However, unpublished transcriptional run-off experiments (carried out in our laboratory by S. Johnson) demonstrate the activation of CyIIIa and CyI actin genes by the blastula stage, and the absence of transcriptional activity in these genes earlier. Experiments are now in progress to measure throughout development the transcription rates, and cytoplasmic entry and turnover rates, for the five actin genes included in the present analysis. With the measurements reported here, these studies should provide a quantitative description of the transcriptional activation of this specifically expressed set of zygotic genes, and show exactly how this accounts for the levels of expression that we have measured.

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CHAPTER 5

Activation of Sea Urchin Actin Genes During Embryogenesis: Nuclear Synthesis and Decay Rate Measurements of Transcripts from Five Different Genes

Activation of Sea Urchin Actin Genes during Embryogenesis: Nuclear Synthesis and Decay Rate Measurements of Transcripts from Five Different Genes

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1. Introduction

Previous studies have provided a wealth of information about the actin gene family of the sea urchin *Strongylocentrotus purpuratus*. The haploid genome of this species contains eight different actin genes of which six are known to be functional (Lee *et al.*, 1984; Shott *et al.*, 1984). These genes display distinct patterns of expression in adult sea urchin tissues, and while they are all transcribed to some extent during embryogenesis (Shott *et al.*, 1984; Garcia *et al.*, 1984), they function in different cell lineages or sets of cell lineages at diverse stages of development (Cox *et al.*, 1986). Moreover, molecular titration assays of embryonic total RNA have established the absolute steady state prevalence of transcripts from each of five different actin genes (Lee *et al.*, 1986). In this report we provide evidence of the transcriptional activation of these actin genes as well as quantitative measurements of the rate of nuclear synthesis and decay during embryogenesis.

The details of transcript accumulation and cell lineage expression can be summarized as follows. As in previous papers, we utilize the nomenclature of Lee *et al.* (1984), in which the actin genes are classified according to homologies in the 3' nontranslated trailer sequences of their messages. Actin gene transcripts make up a small fraction of total egg RNA. The genes CyI (2100 transcripts), CyIIb (160 transcripts), and CyIIIa (1200 transcripts) are the only actin genes that contribute transcripts to the maternal pool. Transcripts from the cytoskeletal actin genes (CyI, CyIIa, CyIIb, CyIIIa) begin to accumulate during cleavage stage, 10-12 h postfertilization. The genes CyI and CyIIb are initially transcribed in all blastomeres of the early embryo. It is not until the mesenchyme blastula stage (22 h postfertilization) that the transcription of these genes becomes restricted to cell lineages which give rise to the oral ectoderm, the embryonic stomach and intestine. The prevalence level of CyI and CyIIb transcripts increases throughout

development, reaching maxima of 9.0×10^4 transcripts/embryo and 5.8×10^4 transcripts/embryo, respectively.

The CyIIa gene contributes no transcripts to egg RNA. Transcripts from this gene begin to accumulate specifically in the vegetal plate cells of the late blastula. This accumulation continues in a cell lineage specific manner throughout development and, in contrast to the other members of its linkage group (CyI and CyIIb, Scheller *et al.*, 1981; Schuler and Keller, 1981), CyIIa transcripts never accumulate to very high per embryo levels.

The pattern of accumulation of CyIIIa transcripts is unique among the *S. purpuratus* cytoskeletal actin genes. Transcripts from this gene accumulate specifically in the cells of the aboral ectoderm. The accumulation of CyIIIa transcripts occurs very rapidly, reaching its highest embryonic prevalence level (8.5×10^4 transcripts) within 10 h. This high level of prevalence remains throughout the rest of development.

Transcripts from the M actin gene do not begin to accumulate until after the gastrula stage (36 h postfertilization). The number of cells in which this gene accumulates is small (restricted exclusively in a bilateral set of muscle anlage cells (Cox *et al.*, 1986)), yet M gene transcripts accumulate to a high level (2.5×10^4 transcripts per embryo) by 65 h postfertilization. The measurements described in this paper show that the accumulation of actin gene transcripts is a direct consequence of gene activation and continued transcription during embryogenesis.

2. Materials and Methods

(a) *Sea urchin embryos and labeling conditions*

Gametes of *Strongylocentrotus purpuratus* were collected by intracoelomic injection of 0.5 M KCl. Eggs were washed by repeated settling in Millipore-

filtered sea water. Embryos were fertilized and cultured at 15°C with constant stirring and aeration as described previously (Smith *et al.*, 1974; Hough-Evans *et al.*, 1977). The embryos were grown at a concentration of 1×10^4/ml in Millipore-filtered sea water containing 40 μg gentamicin sulfate/ml (Schering Corporation).

The developmental stages relevant to the experiments in this paper are described in detail elsewhere (Davidson, 1986; Davidson *et al.*, 1982; Cox *et al.*, 1986). Briefly, at 7 h postfertilization (pf) the embryos contain 32-64 cells and the number of cells per embryo is growing exponentially. Hatching of early blastulae occurs at 18.5 h (pf), and ingression of primary mesenchyme cells into the blastocoel cavity occurs about 24 h (pf). By 36 h the embryos have initiated archenteron invagination (gastrulation) and skeletal formation has begun. The embryos reach the final morphological stage of early development (pluteus) by 65 h (pf). The number of cells per embryo at stages of development relevant to this work were determined from the data of Hinegardner (1967). These stages include: 8 h (pf) - 64 cells, 10 h (pf) - 120 cells, 21 h (pf) - 450 cells, 36 h (pf) - 600 cells, 65 h (pf) - 1200 cells.

Embryos were labeled during three distinct periods spread throughout early development. In each case the labeling period was 2 h, 21-23 h (pf), 36-38 h (pf), and 65-67 h (pf). The labelings were initiated with the addition of [8-^{3}H] guanosine (14.4 Ci/mMole; Dupont-NEN Products) to the cultures to a final concentration of 1.0 μM (7.1×10^7 dpm/ml). No unlabeled guanosine was introduced. The development of embryos in this amount of [^{3}H] guanosine was normal at the level of phase microscope observation (see also Galau *et al.*, 1977; Cabrera *et al.*, 1984). In addition to these labelings a fourth experiment was done at an earlier 2 h interval, 7-9 h (pf). Unlike later stages of development these early embryos are surrounded by a proteinaceous sheath known as the fertilization

envelope (Czihak, 1975). This barrier was removed before the labeling period began. Eggs from *S. purpuratus* were washed, resuspended, and fertilized in Millipore-filtered sea water containing 1 mM 3-amino-1,2,4-triazole (Sigma) (Showman and Foerder, 1979). This reagent is a peroxidase inhibitor that prevents the cross-linking of fertilization envelope proteins, which "hardens" this structure. After 30 min the embryos were concentrated five-fold by settling and the fertilization envelopes were removed by filtration through 54 μM nitex mesh (Tetko). The envelope-free embryos were washed three times by settling through Millipore-filtered sea water and then returned to a concentration of 10^4 per ml. Embryos treated in this fashion continued to cleave normally and were labeled 7-9 h (pf) under the same conditions used for later stages.

(b) *Specific activity of the internal GTP pools*

Portions of the labeled cultures (5 ml) were withdrawn (in duplicate) at the times indicated and the embryos were pelleted in a tabletop centrifuge for 30 sec. The embryo pellets were immediately frozen in liquid N_2 and stored at -80°C for determination of the GTP pool specific activity. No variations were observed as a result of such storage over 12 months. Purification of intracellular GTP and determination of the GTP pool specific activity were performed using an RNA polymerase assay, according to the procedure of Sasvári-Székely *et al.* (1975) (see Maxson & Wu, 1976). Small intracellular molecules such as nucleotide triphosphates were extracted from the 5 ml aliquots of frozen embryos via homogenization (off and on vigorous vortexing for 5 min) in 400 μl of ice cold 0.5 M perchloric acid. The acid extracted embryos were pelleted and the supernatants from this centrifugation were neutralized on ice (check using pH paper) with the addition of 1 M NH_4OAc and 2 M KOH. The neutralized extracts were kept at -20°C for 1-2 h to allow complete precipitation of potassium

perchlorate crystals. The perchlorate-free extracts were used directly in the polymerase assay system or could be stored for long periods of time at -20°C with no change in activity. The basis of the polymerase assay system is the equimolar incorporation of [^{3}H] GTP (from the embryonic extract) and exogenously added [^{14}C] CTP in the presence of a poly(dG-dC):poly(dC-dG) alternating co-polymer. Assays were carried out in 50 µl reaction volumes which included 40 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5 µg of poly(dG-dC):poly(dC-dG), 30 µl neutralized embryonic extract, and 7 U of *E. coli* RNA polymerase (holoenzyme) (New England Biolabs). The reactions were incubated at 37°C for 1 h and incorporation was determined by TCA precipitation at 0°C. The specific activity of the [^{3}H] GTP is the product of the specific activity of the exogenously added [^{14}C] CTP and the ratio of incorporated [^{3}H] and [^{14}C] dpm's.

(c) *Preparation and characterization of total nuclear RNA*

(i) ***Isolation of nuclei and preparation of total nuclear RNA.*** Samples of 2.5×10^6 embryos were harvested 30 sec before the indicated times by low speed centrifugation in conical bottles. The embryo pellets were resuspended in 1x homogenization buffer [homogenization buffer is 0.3 M glycine, 120 mM potassium gluconate, 100 mM Pipes (titrated to pH 6.8 with potassium hydroxide), 20 mM NaCl, 10 mM ethylene-glycol-bis(β-aminoethyl ether) (titrated to pH 6.8 with Na-hydroxide), 1 mM $MgCl_2$, 10 mM glycerophosphate, 100 mM betaine], at -2°C (sea water/ice water bath), and pelleted immediately. Washing the embryos in this manner took no more than 40 sec. The washed embryo pellet was resuspended in 20 ml of 1x homogenization buffer, flash-frozen in liquid N_2, and stored at -80°C. Embryos were successfully stored in this fashion for at least six months. It is important to note that it took no more than 75 sec to take the embryos from

culture to the point of flash-freezing in liquid N_2. This is of special interest because of the small time intervals (15 min) necessary to study nuclear RNA synthesis. The frozen embryos were thawed slowly on ice with occasional vigorous vortexing. This freeze/thaw results in greater than 98% cell lysis and at the level of phase microscope observation (staining with methyl green), the nuclei retained their spherical shape and did not swell. These nuclei were pelleted by low speed centrifugation (4000 revs /min) for 15 min. The nuclei were then washed free of cytoplasm and cellular debris by resuspending and pelleting them in 25 ml of 1x homogenization buffer. This was followed by two additional washes with homogenization buffer containing 1% Triton X-100 and 0.5% Na-deoxycholate. The latter washes removed most residual yolk platelets and any remaining unlysed cells. RNA was isolated from these nuclei essentially as described by Lee *et al.* (1986). The pellets were suspended in a buffer containing 7 M urea, 50 mM sodium acetate (pH 5.5), 10 mM EDTA, 15 mM ethylene-glycol-bis(β-aminoethyl ether), 1% sarcosine and 10 µg polyvinyl sulfate/ml. Proteinase K (Merck) was added to a final concentration of 300 µg/ml of lysate and the homogenate was incubated at 65°C for 30 min. RNA was purified from this homogenate by the CsCl pelleting method of Glisin *et al.* (1974). The homogenate was diluted 1:1 with diethylpyrocarbonate-treated distilled water, and solid CsCl (1 gm/ml) was added. The homogenate was transferred to Beckman Quick-Seal tubes and underlayered with a cushion of 5.7 M CsCl in 10 mM sodium acetate (pH 5.5). These CsCl step gradients were centrifuged for 24 h at 39,000 revs/min at 26°C in a 60 Ti rotor. The RNA pellets were dissolved in diethylpyrocarbonate-treated distilled water at 4°C and precipitated from 0.2 M sodium acetate (pH 5.5) with the addition of 2 vol ethanol. The RNA was again dissolved in distilled water and precipitated overnight at -20°C with 2 vol 4.5 M sodium acetate, pH 5.5 (Childs

et al., 1979). These RNA pellets were redissolved in distilled water, precipitated a final time with 2 vol ethanol at -20°C, and the resulting pellets were dissolved in distilled water and stored at -70°C.

(ii) *Determination of nuclear RNA specific activity.* Aliquots of the urea/sarcosine nuclear lysates were taken at the time of RNA isolation and stored frozen at -20°C. The number of nuclei (i.e., the amount DNA) represented by the measured quantities of nuclear RNA were determined by DAPI fluorescence measurements (Brunk *et al.*, 1979) of the nuclear lysates taking into account the isolation efficiency of nuclear RNA. The nuclear RNA isolation efficiency is calculated as the ratio of recovered RNA dpm vs. total RNA dpm put onto the CsCl step gradients. The number of nuclei contributing to a preparation of nuclear RNA is the product of the nuclear RNA isolation efficiency and the DAPI fluorescence measurement of the total number of nuclei lysed. This calculation assumes a DNA content per nucleus of 1.60 pg (Graham *et al.*, 1974).

(d) *Preparation of cytoplasmic RNA and total RNA*

Total and cytoplasmic RNAs were prepared from labeled embryos by a procedure similar to that used to prepare nuclear RNA. Thirty seconds before the indicated times, samples of 5×10^6 embryos were harvested in conical bottles. The embryo pellets were washed once by resuspending and pelleting in 1x homogenization buffer. The washed embryos were divided in half and each processed separately. To prepare cytoplasmic RNA, embryos were resuspended in homogenization buffer and flash frozen in liquid N_2. The embryos were slowly thawed with vigorous vortexing to lyse greater than 98% of the embryonic cells. After low speed centrifugation (4000 revs/min), to remove nuclei, the lysate was cleared of mitochondria and most cellular debris by high speed centrifugation (10,000 revs/min) for 20 min. Ultrapure urea (BRL) and Na-sarcosine (Sigma) were

added to 7 M and 1% respectively. Proteinase K (Merck) was added to a final concentration of 300 µg/ml of lysate and the homogenate was incubated at 25°C for 30 min. Cytoplasmic RNA was purified from this lysate as described for nuclear RNA (see Materials and Methods, section (c)). Preparation of total RNA from the washed embryo pellets was carried out as described by Lee *et al.* (1984).

(e) *In vitro transcription using isolated nuclei in a run-off system*

(i) *Isolation of transcriptionally competent nuclei.* Nuclei were prepared by a modified version of a previously published protocol (Marzloff, 1983). Early blastulae (21 h (pf)) or 8 h embryos, whose fertilization envelopes were removed (see Materials and Methods, section (a)), were harvested and then repeatedly washed with Ca-Mg free sea water (Ca-Mg free sea water is 0.44 M sodium chloride, 9.0 mM potassium chloride, 16 mM sodium sulfate, 1 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 8.0). The embryos were then resuspended, at 0°C, in dextrose washing buffer (400 mM dextrose, 40% Ca-Mg free sea water) and disassociated into single cells. The disassociated cells were pelleted at 3000 revs/min for 5 min. The loosened cell pellet is homogenized (vigorous shaking) with 10 pellet volumes of cell lysis buffer (cell lysis buffer is 0.32 M sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM DTT, 1 mM spermidine, 0.1% NP-40). The nuclei were collected by low speed centrifugation (4000 revs/min) for 10 min. These nuclei were extensively washed by resuspending and pelleting them repeatedly with cell lysis buffer (at least five times). The washed nuclei were then resuspended in nuclei storage buffer (nuclei storage buffer is 25% glycerol, 50 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM spermidine, 1 mM DTT, 5 mM $MgCl_2$), counted, and flash frozen with liquid nitrogen in 250 µl aliquots (~10^9 nuclei/ml). These nuclei were stored at -80°C until use.

(ii) *In vitro transcription and purification of run-off transcripts.* Run-off transcripts were synthesized from 1-3×10^8 nuclei. In each experiment, 4 mCi of ^{32}P-α-GTP (410 Ci/mMole, Amersham) were dried down and resuspended in 200 µl of the following mix: 180 mM-potassium chloride, 20 mM magnesium chloride, 1 mM ATP, 1 mM CTP, 1 mM UTP. The synthesis reaction is initiated with the addition of 200 µl of slowly thawed (on ice) nuclei. The nuclei are incubated at 18°C for 20 min and the reaction is stopped by the addition of 10 ml of urea/sarcosine lysis buffer containing 300 µg/ml proteinase K. Labeled RNA transcripts were isolated from these nuclei as described in Materials and Methods, section (c). Note that for these experiments 300 µg of total *E. coli* RNA was added to the lysates as carrier.

(f) *DNA excess filter hybridizations*

Plasmid supercoils were either linearized by digestion with HindIII or insert DNA was isolated from preparative agarose gels. The DNA was then proteinase K treated, extracted with phenol, precipitated with ethanol, and dissolved in water. Filters were prepared as described by Kafatos *et al.* (1979). An aliquot (6 µg) of DNA was loaded per 0.75x 8 mm slot of the Manifold II™ apparatus (Schleicher and Schuell). The nitrocellulose filter-slots were then cut into three equal sized pieces which were further trimmed down to 1.0×2.5 mm chips (~2 µg of cloned DNA/filter). The filters were prehybridized for 2 h at 68°C in 4x SET (SET is 0.5 M-NaCl, 30 mM-Tris-HCl (pH 8.0), 1 mM EDTA), 5x Denhardt's solution (Denhardt, 1966), 0.2% (w/v) sodium dodecyl sulfate and 5 mg/ml yeast RNA. Hybridizations were performed at 68°C for 62 h; typical reaction mixtures contained six to eight filters, containing the different actin gene specific DNAs (Lee *et al.*, 1986). The reactions were done under mineral oil in 100 µl drops of 4x SET, 5x Denhardt's, 0.2% (w/v) sodium dodecyl sulfate and [^{3}H] RNA. Yeast RNA

was added to maintain a 5 mg/ml concentration of RNA in the hybridization reaction. This hybridization mix was boiled for 2 min and then added to the prehybridized filters. Amounts of RNA were used such that the filter-bound DNA was always in effective excess over the homologous transcripts in the [^{3}H] RNA preparation, as determined in separate control experiments (see Cabrera *et al.*, 1984). Following hybridization, the reaction mixes were removed and the filters were washed at 68°C with strong shaking. The filters were washed twice in 4x SET/0.2% SDS followed by two washes each in 2X SET/0.2% SDS and 1x SET/0.2% SDS. Finally, the filters were treated with 5.6 µg RNase A/ml and 12 U RNase T_1/ml in 2.5x SET at 37°C for 1 hr. After a brief wash in 4x SET/0.2% SDS (at 37°C) they were air dried and placed individually in glass scintillation vials. The filters were solubilized (37°C, overnight) with 1 ml of NCS™ tissue solubilizer (Amersham) and then counted with the addition of 10 ml of spectrafluor/toluene (Amersham). Under these conditions the [^{3}H] counting efficiency is 28.1%.

To measure the fraction of labeled RNA that could be bound in filter-driven reactions carried out as described here, SP6-RNA polymerase generated [^{32}P]-labeled RNA probes (Lee *et al.*, 1984) were hybridized back to filter bound DNA. The reactions were set up to mimic the sequence concentrations of the RNAs labeled *in vivo* included in the following studies. Hybridization of the [^{32}P] RNA proceeded to 77% completion in 60 h of reaction, as determined by comparison of the amount of input [^{32}P] with that bound to the filter at each time point. Similar experiments done with [^{125}I]-labeled rRNA proceeded to 85% completion (Cabrera *et al.*, 1984).

(g) *Mathematical approach and data reduction procedures*

(i) Synthesis rate and turnover equations. Our objective is to obtain molecular rate constants for the nuclear synthesis and decay of transcripts from

the different actin genes of *S. purpuratus*. Units of the nuclear synthesis rate constant, K_s, are molecules min^{-1} $cell^{-1}$, and for the decay rate constant, k_d, the units are min^{-1}. In some cases, the synthesis rate constant is expressed in mass rather than molecular units, i.e., as pg min^{-1} $embryo^{-1}$. Expressed in these terms, the synthesis rate constant is called k'_s in this paper. The number of newly synthesized (i.e., labeled) RNA molecules accumulated in the nucleus at any given time after the experiment begins, C(t) (assuming zero labeled molecules of this species are present initially), is:

$$C(t) = \frac{k_s}{k_d}(1-e^{-k_d t}), \qquad (1)$$

in mass terms (pg/embryo):

$$C(t) = \frac{k'_s}{k_d}(1-e^{-k_d t}). \qquad (1')$$

The specific activity of the GTP pool in sea urchin embryos labeled with [^{3}H] guanosine changes with time as previously discussed (Galau *et al.*, 1977; and Cabrera *et al.*, 1984). This specific activity time function, here called S(t), was measured experimentally as described above, and total nuclear RNA was extracted from samples of the same embryos as were used for the pool determinations. The amount of [^{3}H] RNA hybridizing to filters containing each actin gene-specific clone can be described as follows:

$$\frac{dR(t)}{dt} = k'_s\, S(t) - k_d\, R(t),$$

(2)

the solution to which, for our purposes, is:

$$R(t) = k'_s \int_L^t S(t')e^{-k_d(t-t')}dt'. \qquad (3)$$

Here, L is the time lag preceeding the appearance of labeled transcript in the nucleus and t' is a variable of integration. L was determined graphically by extrapolation from the early measured values of R(t), and was 0 to 7 min for all the experiments described in this paper. Equations (2) and (3) were presented initially by Galau *et al.* (1977), and their application has been discussed in detail by Cabrera *et al.* (1984) and Davidson (1986).

(ii) Data reduction. The computer program, MESSAGE (available on request), and the manipulation of the derived kinetic parameters are described in detail by Cabrera *et al.* (1984) (see also Pearson *et al.*, 1977).

3. Results

(a) Labeling kinetics of the GTP pools

The observation that the intracellular GTP pool specific activity quickly rises when blastula or gastrula stage embryos are exposed to exogenous [^{3}H]-guanosine (Galau *et al.*, 1977; Cabrera *et al.*, 1984) has been confirmed and expanded to other stages of development in the present study. The GTP pool specific activity time-courses are shown in the first panels of each section (a-d) of

Figure 1. The initial increase in specific activity occurs as the embryos rapidly remove all the available [^{3}H] -guanosine from the medium and convert a portion of it to [^{3}H] GTP. Uptake of the precursor is essentially complete within 20 minutes and labeled [^{3}H] GTP continues to flow into the pool during the remainder of the labeling period.

The GTP pool-specific activity determinations for the earlier labeling experiments (7-9 hour (pf), 21-23 hour (pf), 36-38 hour (pf)) were made directly by an RNA polymerase assay as described in Materials and Methods. The GTP pool specific activity for 65-67 hour embryos, however, could not be assayed directly. Attempts to purify and recover GTP from perchloric acid-extracted plutei were unsuccessful. The nucleotide triphosphate pools of these late embryos degrade sometime after the time points are taken (data not shown). We suspect that this degradation is the result of phosphatases and perhaps other digestive enzymes which are made in the newly developed larval gut. The GTP pool specific activity time-course for 65-67 hour embryos was therefore estimated by normalizing the GTP pool specific activity time-courses of the earlier labelings (21-23 hour (pf) and 36-38 hour (pf)). The normalization is based on the relative difference between the specific activity of the nuclear RNAs involved. Since we were studying primary transcripts (i.e., total nuclear RNA) any immediate changes in the specific activity of this RNA should reflect changes in the specific activity of the GTP from which the RNA was made. The 65-67 hour (pf) GTP pool specific activity time-course derived via this analysis is shown in the first panel of section (d) in Figure 1.

The GTP specific activity time-courses shown in Figure 1 (a-d) are the function S(t) in equation (3). As described in detail in Materials and Methods, the

rate constants for the synthesis (k_s) and decay (k_d) of a nuclear RNA species can derived from measurements of S(t) and the amount of [^{3}H] GMP incorporated in that RNA over the same period in the same embryos. There are two assumptions implied in the treatment used here. These are, first, that if the RNA decays it does so with first-order kinetics, as indicated by several previous measurements for most sea urchin embryo RNA species (reviewed by Davidson, 1986), and, second, that the GTP pool in sea urchin embryos is not compartmentalized. The latter assumption is supported by several lines of evidence (including additional kinetic labelings) which are discussed in detail by Cabrera *et al.* (1984).

(b) Kinetic parameters for newly synthesized nuclear RNA

The kinetics with which [^{3}H] GMP appears in the nuclear RNA of early embryos is illustrated in the center panel of each section (a-d) of Figure 1. The interpolated lines indicate the least-squares solution for the function R(t) in equation (3), calculated by varying the values of the synthesis and decay rate constants k'_s and k_d, as described in Materials and Methods. The rate constants extracted from these experiments are displayed in Table 1. Table 1 also lists values for the same kinetic parameters, independently derived, using different isotopes and experimental protocols. In agreement with these earlier measurements, the per-nucleus rate of RNA synthesis drops approximately three-fold during the course of embryonic development. The nuclear decay rates of these same transcripts, however, remain relatively constant.

The last panel of each section (a-d) of Figure 1 describes the mass accumulation of newly synthesized nuclear RNA. This function is generated from the values of k'_s and k_d listed in Table 1 according to equation (1'). It can be seen

that the steady state per-nucleus content of RNA drops slightly as development proceeds; however, because of the rapid increase in the number of cells per embryo, the per embryo content of nuclear RNA increases. In the last stage of development examined (65-67 hour (pf)), each early pluteus larva contains 75.3 pg of rapidly turning over nuclear RNA (Table 1), or approximately 3% of total embryo RNA (Davidson, 1986). Previous measurements (Washburn, 1971) have shown that total nuclear RNA (i.e., including stable nuclear transcripts; for example, nucleolar rRNA) represents 10% of total embryo RNA. Our results therefore demonstrate that approximately one-third of total nuclear RNA belongs to that class of transcripts which rapidly turn over.

Previous studies (Devlin, 1976; Ruderman and Schmidt, 1981; Cabrera *et al.*, 1983) have shown that mitochondria are transcriptionally active during early development. This was a potential source of error. Therefore we determined whether the isolated nuclear RNAs in the present study were contaminated by newly synthesized transcripts of non-nuclear origin (i.e., mitochondria). Pilot labeling experiments (data not shown) demonstrated that intact cellular components could be successfully fractionated using the procedure detailed in Materials and Methods, section (c). The use of a low salt homogenization buffer lacking detergent prevented membrane bound organelles from lysing, and allowed the isolation of nuclear RNA free of any contaminating mitochondrial RNA.

(c) Nuclear synthesis and turnover kinetics for actin gene transcripts

DNA of the actin gene-specific clones (Lee *et al.*, 1984, 1986) was bound to nitrocellulose filters and hybridized with RNA preparations that had been labeled

metabolically. The specific activity of the [^{3}H] GTP pool was measured on samples of the same labeled embryos. The quantities of filter-bound DNA and of [^{3}H] RNA used in each hybridization were demonstrated to provide a significant DNA excess, as described in Materials and Methods. Model hybridization tests showed that the reactions proceeded to about 80% of completion. The amounts of [^{3}H] RNA hybridized to filters of each cloned DNA sequence as a function of labeling time, and the corresponding pool specific activity data, were used to calculate the rate at which that species of transcript is synthesized (k'_s), and the rate at which it then decays (k_d), according to equation (3). Kinetic data for each of five actin gene-specific clones are shown in Figure 2. This figure is divided into three main sections (a-c) representing 2 hour labeling periods (21-23 hour (pf), 36-38 hour (pf), 65-67 hour (pf)). [^{3}H] nuclear incorporation data are shown for each actin gene in a given labeling period (left-hand panels). In addition the accumulation of newly synthesized nuclear actin transcripts are also presented (right-hand panels).

(i) Actin gene transcription during early cleavage. RNA gel blots (Shott *et al.*, 1984) and molecular titration assays (Lee *et al.*, 1986) have shown that during the early stages of embryonic cleavage there is no net increase in the number of actin transcripts in the embryo. In fact, transcripts from only three actin genes (CyI, CyIIb, and CyIIIa) are present at all in the maternal population of sequences found in the egg. As indicated in Table 2, gene-specific nuclear transcription was not detected from any actin gene during embryonic cleavage (7-9 hour (pf)). The *in vivo* labeling experiment during this time period does, however, have some constraints. In particular, the number of cells per embryo is sufficiently low (~64 cells) as to seriously decrease the sensitivity of this labeling

assay. Despite using the maximum amount of embryos (as well as isotope) that could be handled at each time point, the low number of cells per embryos at 7-9 hour (pf) allows for the isolation of nuclear RNA from only 5×10^7—1×10^8 nuclei. This is nearly 50-fold less than the number of nuclei recoverable at later stages. Each actin gene might therefore be transcriptionally active at a level too low to be detected. To address this issue, *in vitro* nuclear run-off experiments were performed.

For early cleavage stage embryos *in vitro* nuclear run-off experiments afford higher sensitivity as compared to the *in vivo* labeling system. This increase in sensitivity results from three independent factors. First, the practical upper limit for the number of nuclei which can be used in the *in vitro* assay is $2\text{-}4\times10^8$. This is nearly fivefold the input number of nuclei that can be achieved in the 7-9 hour (pf) *in vivo* labeling. In addition, the [^{3}H]GTP precursor pool of *in vivo* labeled embryos (Figure 1) is approximately 1.5 Ci/mMole. The use of high specific activity (3000 Ci/mMole) [^{32}P]-α-GTP as the label in the *in vitro* experiments therefore dramatically increases the sensitivity of this assay. Finally the use of [^{32}P] vs. [^{32}H] also increases the sensitivity of the *in vitro* system (i.e., a threefold increase in counting efficiency, 100% vs. 28%). Together with the fact that nuclei *in vitro* transcribe at about 1% efficiency (Stallcup *et al.*, 1978; Morris and Marzluff, 1983; data not shown), suggests that during early cleavage the *in vitro* assay provides a 300-fold increase in sensitivity over the *in vivo* measurement. Note, however, that this marked increase in sensitivity is limited to cleavage stage. At later stages the increase in the number of cells per embryo is such that nuclear RNA could be recovered from 5×10^9 nuclei. This represents an increase of 25-fold over the number of nuclei used in the *in vitro* assay. As a

result, the sensitivity of the *in vitro* assay, using nuclei from later staged embryos, is only twofold greater than corresponding *in vivo* experiments (i.e., the sensitivity of both assays is essentially the same). In agreement with this argument is the observation that transcription from the CyIIa actin gene could be detected at 21 hour (pf) with the *in vivo* system (Table 2) but was not detected by the *in vitro* assay (Table 3).

Even with the enhanced level of sensitivity afforded by the *in vitro* assay, actin gene transcription was not detected from cleavage-stage embryos (8 hour (pf)). As shown in Table 2, label was not incorporated into transcripts from any of the actin genes in this study. Since the actin gene-specific probes used in this study represent the 3' trailer sequence of the RNA (Lee *et al.*, 1984), these experiments do not rule out the possibility that transcription ititiation still occurs with the subsequent degradation of the nascent RNAs (e.g., a modified version of transcript attenuation, Bertrand *et al.*, 1975). However, in the absence of any other evidence it is assumed here that the labeling systems (both *in vivo* and *in vitro*) assay for the initiation of transcription.

An additional filter, containing cloned DNA of an early histone cluster (pCO_2, see Overton and Weinberg, 1978), was also hybridized to the labeled transcripts made from isolated 8 hour (pf) nuclei. This experiment detected 4.5×10^6 cpm (Table 2). The amount of histone RNA sequence on the pCO_2 probe is 2600 nt and the total number of early histone genes of all five species per embryonic cell is 4500 (Kedes, 1979). If an actin gene were transcribed at the rate of a histone gene (~1 transcript per gene•min, reviewed by Davidson, 1986), then its specific probe would have hybridized ~1900 cpm in the run-off experiment. Since the limit of detection in the *in vitro* system is ~30 cpm above

background, this implies that the *S. purpuratus* actin genes are not active or at a maximum they are being transcribed at a rate ≤0.016 transcripts per gene•min, i.e., less than one transcript per gene•hour.

(ii) Transcription from the CyI actin gene. Figure 2(a-c) reveals the kinetic behavior of CyI nuclear transcripts at different times during development. CyI is among four actin genes transcriptionally active at the blastula stage (21 hour (pf)). Table 3 shows that from 21 hour (pf) through the rest of pre-feeding development, the per embryo transcription rate of CyI is the highest of the actin genes in this study. At 21-23 hour (pf) the CyI actin gene is transcribed at a rate of 23.3 molecules/embryo•minute. This rate remains approximately the same during the blastula-gastrula transition, increasing slightly to 34.1 molecules/embryo•minute. By 65-67 hour (pf) CyI is being transcribed at a rate nearly nine times higher, 298 molecules per embryo•min.

The nuclear decay rate of CyI transcripts changes only by a factor of 2-3 during the same periods of development. These changes cause a 20-25 minute change in the nuclear half life of these transcripts (Table 3). The synthesis and decay kinetic parameters provide an estimate of the steady state level of nuclear CyI transcripts (k_s/k_d, column 7, Table 3). The prevalence level of these nuclear transcripts, on a per-embryo basis, increases during embryogenesis (Table 3). This level is maximum at the latest time period studied (65-67 hour (pf)), accounting for 5.09×10^3 nuclear transcripts per embryo or about 10^{-4} of total nuclear RNA at this stage.

(iii) Transcription from the CyIIa actin gene. The major accumulation of CyIIa transcripts occurs later than 20 hour postfertilization though these mRNAs

can first be detected at this time (Shott *et al.*, 1984; Lee *et al.*, 1986). These mRNAs are initially observed in primary mesenchyme cells within the blastocoel and in presumptive mesenchyme cells still embedded in the vegetal plate of the hatched blastula (Angerer and Davidson, 1984; Cox *et al.*, 1986). At this time newly synthesized CyIIa transcripts are being made at a rate of only 3.15 molecules per embryo•minute (Table 3). This nuclear synthesis rate increases threefold at 36-38 hour (pf) and finally, by 65-67 hour (pf), the rate of CyIIa transcription has increased more than 15-fold over the 21-23 hour (pf) level (Table 3). Like CyI, however, CyIIa nuclear transcripts decay at a rate similar to that of total nuclear RNA, i.e., these transcripts turnover with a half-life of about 52 minutes throughout embryogenesis.

(iv) Transcription from the CyIIb actin gene. The pattern of accumulation of CyIIb transcripts (both in time and space) was previously shown to be similar to that of the actin gene CyI (Shott *et al.*, 1984; Cox *et al.*, 1986; Lee *et al.*, 1986). The presence of sequence homology in the 5'-flanking regions of these two genes (Durica *et al.*, unpublished observations) suggests that this similarity in transcript accumulation may reflect homologies in upstream regulatory sequences. This is in part substantiated by the nuclear synthesis data presented in Table 3. Transcripts from CyIIb begin to accumulate as the result of the activation of transcription sometime after the 7-9 hour (pf) labeling period. Nuclear transcription rate measurements at 21-23 hour (pf) show that at this time CyIIb transcripts are made at a rate of 11.3 molecules per embryo•minute. This level of transcription remains constant through the blastula-gastrula transition, but increases more than sixfold by 65-67 hour (pf). Although the absolute magnitude of CyIIb nuclear synthesis is different from CyI, the relative changes in transcription from one

stage to another during development are essentially the same. In addition, the nuclear decay rate of CyIIb transcripts is roughly the same as the decay rate of CyI (or CyIIa) transcripts. The resulting half-life of CyIIb nuclear transcripts is about 48 minutes (Table 3).

(v) Transcription from the CyIIIa actin gene. CyIIIa per embryo transcription is unique among actin genes in that the rate of transcription increases less than two fold during the entire post-hatching period of early development (Table 3). The rate of transcription of the CyIIIa gene was found to be 14.7 molecules per embryo•minute at 21-23 hour (pf). This nuclear synthesis rate increases slightly at the gastrula, but by 65-67 hour (pf) has increased only 50% over the 21-23 hour (pf) rate, 22.7 molecules per embryo•minute (Table 3). This is in marked contrast to the other cytoskeletal genes in this study, and reflects the fact that unlike the cells of other embryonic lineages, the cells of the aboral ectoderm do not increase significantly in number after the blastula stage.

The nuclear synthesis and decay rate parameters of CyIIIa transcription were used to determine the nuclear steady state quantity of CyIIIa transcripts (Equation (1'), Table 3). The solution of equation (1'), using the kinetic parameters measured at 36-38 hour (pf), yields a nuclear steady state prevalence level of 827 transcripts per embryo. This result agrees well with the number of CyIIIa nuclear transcripts measured with the molecular titration assay described in Lee *et al.*, 1986 (1080 transcripts per embryo, data not shown). Unlabeled nuclear RNA was isolated by the procedure described in section (c) of Materials and Methods, and used in the titration assay. The close agreement of these two independent methods lends support to the validity of the kinetic measurements in the present study.

(vi) Transcription from the M actin gene. Table 3 shows that the muscle actin gene is the last to be expressed during development. Nuclear transcription from this gene is first detected after 36-38 hour (pf) (Shott *et al.*, 1984; Lee *et al.*, 1986; Table 2) and *in situ* hybridization data shows that this gene is expressed in presumptive muscle cells that coalesce on either side of the esophagus following gastrulation (Ishimoda-Takagi *et al.*, 1984; Cox *et al.*, 1986). At 65-67 hour (pf) the M actin gene is transcribed at a rate of 11.6 molecules per embryo•minute. This rate of transcription is comparable to the rate of synthesis from the cytoskeletal actins in this study (Table 3). However, as described later (section (e) results), if the relatively small number of cells in which it is expressed is taken into account, the transcription rate from the M actin gene at 65-67 hour (pf) is higher than that of any other actin gene in this study.

(d) Comparison of transcription rate and cytoplasmic entry rate of actin protein coding sequence

Figure 3 shows the total RNA transcription rate (K'_T) and cytoplasmic entry rate (K'_C) measurements for p9-17E1, an actin protein coding sequence probe, at 36-38 hour (pf). It was previously demonstrated that the nuclear synthesis rates of a particular sequence could be obtained by examining the incorporation of [^{3}H] GMP into total RNA at points early in the labeling period (Cabrera *et al.*, 1984). Rates obtained for given sequences could then be compared to the rates of entry of these transcripts into the cytoplasm, measured in samples of the same cultures of labeled embryos. Equation (3) shows that the initial rate of incorporation of [^{3}H] RNA is the synthesis (or entry) rate which, in all the kinetic experiments reported in this paper, is determined early in the

measurement. The rise of the specific activity of the [^{3}H] GTP pool shortly after label addition is illustrated in Figure 3(a), and the time-course of accumulation of newly synthesized RNA that hybridizes to p9-17E1 is shown in Figure 3(b). Under the hybridization conditions used, this clone is sufficiently homologous to cross-react with transcripts from all members of the actin gene family. Since for this sequence there is little contribution from labeled cytoplasmic molecules early in the labeling period, the data obtained in the total RNA samples before 30-45 minutes suffice to determine the primary transcription rate. The ratio K'_T/K'_C, extracted from the data presented in Figure 3, is 0.95. Consequently, there is no evidence that the rate at which actin protein coding sequences are transcribed greatly exceeds the rate at which these transcripts enter the cytoplasm. It follows that all, or almost all, of the nuclear precursor molecules transcribed from actin genes ultimately give rise to cytoplasmic transcripts.

(e) Transcription rates per nucleus and accumulation of actin transcripts among cells actively synthesizing actin mRNA

The actin nuclear transcription data, expressed on a per-cell basis, is presented in Table 4. These data are the result of dividing the per-embryo nuclear transcription rates (columns 3 and 4, Table 3) by the number of cells actively transcribing each actin gene. These cell numbers were determined from the *in situ* hybridization data of Cox *et al.* (1986).

(i) Synthesis and accumulation of CyI actin gene transcripts. Table 4 shows that there is little change in the per-cell transcription rate of CyI transcripts during development. The average per cell rate of transcription of this gene is 0.22 molecules per cell•minute. This transcription rate was used to calculate the

amount of newly synthesized actin transcripts made during the time intervals discussed in this study. Table 5 shows that for CyI the amount of new synthesis (in the basence of turnover) is sufficient to account for the increase in the prevalence of these transcripts (Lee *et al.*, 1986).

Since the cytoplasmic entry rate of actin transcripts equals their per-cell nuclear synthesis rate (Figure 3; Results, section (d)), the CyI transcription rate measurement can be used to calculate the cytoplasmic decay rate of CyI transcripts by applying equation (1) in the limit of $t = \infty$ (i.e., steady state). Evaluating equation (1) late in development (our closest approximation to steady state), where the prevalence of CyI transcripts is 9×10^4 molecules per embryo (Lee *et al.*, 1986) and 780 cells expressing this gene (column 3, Table 4), results in a calculated decay rate of CyI transcripts of 0.12 $hour^{-1}$. This rate of decay gives CyI transcripts a cytoplasmic half life of ~5.8 hours.

(ii) Synthesis and accumulation of CyIIa actin gene transcripts. The rate of transcription from the CyIIa gene varies less than two-fold during all of prefeeding development (Table 4). On average, CyIIa is transcribed at a rate of 0.21 molecules per cell•minute and the projected accumulation of newly synthesized transcripts (Table 5) is again sufficient to account for the observed increase in CyIIa transcript prevalence. Cytoplasmic decay calculations for CyIIa mRNA shows that these transcripts turnover with a half-life of 4.8 hours.

(iii) Synthesis and accumulation of CyIIb actin gene transcripts. Table 4 shows that, as with the other members of the CyI-CyIIa-CyIIb linkage group (Scheller *et al.*, 1981; Schuler and Keller, 1981), the per-cell transcription rate of CyIIb does not noticeably change during development. This gene is transcribed in embryonic blastomeres at an average rate of 0.064 molecules per cell•minute and

this rate of synthesis is the lowest of these three linked cytoskeletal actin genes. CyIIb is, however, transcribed in a sufficient number of cells to account for the per-embryo increase in transcript prevalence as development proceeds (Table 5). In addition, the per-cell data presented in Table 4 allows us to estimate a steady state cytoplasmic decay rate for CyIIb transcripts. The calculated decay rate gives CyIIb transcripts a cytoplasmic half life of 13 hours. This is 2-3 times longer than the half-lives measured for transcripts from CyI or CyIIa.

(iv) Synthesis and accumulation of CyIIIa actin gene transcripts. The CyIIIa gene is expressed only in the aboral ectoderm cells of the embryo (Cox *et al.*, 1986). As shown in Table 4, the resulting per-cell transcription rate of CyIIIa is on average 0.076 molecules per cell•minute. This is the lowest of all the actin transcription rates measured. The striking consequence of this low rate of transcription is that the embryo could not possibly accumulate enough transcripts to account for the rapid accumulation in CyIIIa mRNA prevalence in early blastula stage embryos. Table 5 shows that in the time interval in which the embryo accumulates 8.5×10^4 CyIIIa transcripts, it could have only made 5300 transcripts at the measured rate of synthesis. This result suggests either that the number of cells actually transcribing the CyIIIa gene at 21-23 h (pf) is much lower than the *in situ* estimate of 200 cells (i.e., before the kinetic measurement many cells of this lineage have turned off their CyIIIa actin gene) or that the per-cell rate of CyIIIa gene transcription has decreased significantly. To account for the accumulation of transcripts, the CyIIIa gene would have to be transcribed at a rate of at least 1.2 molecules per cell•minute or 16 times higher than the rate measured at 21-23 hour (pf).

(v) Synthesis and accumulation of M actin gene transcripts. The *in situ* hybridization data of Cox *et al.* (1986) provides evidence that as many as 30 cells are expressing the M actin gene. This observation allows us to make the minimum per-cell transcription rate estimate appearing in Table 4. Though a minimum estimate, this level of transcription (0.39 molecules per cell•minute) is the highest measured per-cell rate of transcription from any actin gene.

4. Discussion

(a) Kinetic behavior of total nuclear RNA

The methodology described in this study allows us to make quantitative measurements of the kinetic behavior of total nuclear RNA. In addition, the measurements made in this paper provide values of the absolute synthesis and decay rates of gene specific nuclear transcripts. The accuracy of the procedure rests mainly with our ability to isolate nuclear RNA free of contaminating RNA from other cell fractions. Kinetic labeling data described here, for example, show that labeled mitochondrial sequences were excluded from the isolated nuclear RNA. These observations, however, could not rule out the possibility that the labeled nuclear RNA is contaminated with sequences from the large pool of embryo cytoplasmic RNA (Goustin and Wilt, 1981). While our experiments do not to specifically address this possibility, results of the CyIIIa molecular titration studies bear on this point. Using the same nuclear RNA isolation procedure as described in Materials and Methods section (c), it was found that the quantity of nuclear CyIIIa transcripts was less than 2% of the total number of CyIIIa transcripts. This result is similar to an observation made with the ovalbumin gene in the tubular gland cells of the chick oviduct (Tsai *et al.*, 1979). These authors estimated the prevalence of ovalbumin mRNA and primary nuclear transcripts

corresponding to intervening sequences by hybridization to cloned DNA probes. This analysis showed that ovalbumin nuclear transcripts represented about 4% (2500/58000) of the total number of ovalbumin transcripts. In light of this agreement the assumption is made that our nuclear isolation/labeling procedure assays only ***bona fide*** nuclear transcripts.

The measured kinetic parameters for total nuclear RNA agree with previous determinations of these values (Table 1), implying the absence of any significant systematic errors. The measured nuclear kinetic values confirm that the nuclear RNA of sea urchin embryos turns over rapidly, with an average half-life of 30 min. Very similar rates have been measured for the heterogenous nuclear RNA of other animal cells, e.g., mouse L cells (Brandhorst and McConkey, 1974). In addition, the nuclear kinetic values also confirm the unexpected conclusion that as development proceeds the rate of transcription per nucleus steadily declines. The cause of this decline remains largely unknown. However, influence on the embryonic transcriptional machinery by factors (e.g., proteins) of zygotic origin is a likely explanation. These factors could act very early in embryogenesis as it was previously shown that transcription occurs as early as fertilization (Wilt, 1963, 1964; Poccia *et al.*, 1985).

(b) Kinetic characterization of newly synthesized actin transcripts

Molecular titration assays and RNA gel blots of embryo total RNA have shown that actin transcripts do not begin to accumulate until mid-cleavage stage (10-12 hr pf). In fact only three actin genes were shown to contribute any transcripts at all to the maternal pool (Shott *et al.*, 1984; Lee *et al.*, 1986). Genes whose transcripts display this accumulation pattern are described by Davidson (1986) as "late genes". Operationally, this class of mRNA is identified as transcripts not detectable in unfertilized egg RNA, or present at extremely low

levels compared to their ultimate prevalence in the embryo. The assumption is that mRNAs from late genes begin to accumulate with the transcriptional activation of these genes during embryogenesis. The kinetic experiments reported here support this assumption for the actin gene family of *S. purpuratus*. *In vivo* kinetic labelings and *in vitro* nuclear run-off data establish that actin transcripts begin to accumulate during embryogenesis as a direct consequence of activation and continued transcription of each actin gene. The data of Table 4 shows that actin gene transcripts are synthesized at rates of 0.1-0.4 molecules/cell•min or per gene 0.05-0.2 molecules/min. By means of comparison, an examination of a random set of sequences from which moderately prevalent mRNAs arise shows that the actin genes are "typical" with respect to their rate of transcription (Cabrera *et al.*, 1984). Specifically, sequences which give rise to transcripts whose prevalence is comparable to actin mRNA, were found to be transcribed at rates of 0.05-0.15 molecules per gene•min. Members of the early histone gene family are transcribed at a somewhat rate, 0.5-1 molecules per gene•min (Maxson and Wilt, 1981; Weinberg *et al.*, 1983). This difference in rate reflects the large difference in per cell transcript prevalence (Mauron *et al.*, 1982) and confirms earlier inferences that the rate of transcriptional initiation is the major variable in determining the prevalence of a cytoplasmic mRNA sequence.

Measurements presented in Tables 2 and 3 show that newly synthesized actin nuclear transcripts turnover with kinetics similar to the decay kinetics of total nuclear RNA. In both cases the respective RNAs turnover with half-lives of approximately 30-40 min. This result is especially interesting because 90% of total nuclear RNA rapidly turns over in the nucleus and is not exported to the cytoplasm (reviewed by Davidson, 1986), i.e., the vast majority of nuclear transcripts do not go on to form mature (processed) cytoplasmic RNAs. Since actin nuclear transcripts are processed with an efficiency of one (Figure 3), an

extension of the observed decay kinetics of actin nuclear RNAs to most polymerase II-transcribed RNAs would imply that these transcription units code for transcripts which belong exclusively to the minority fraction of total nuclear RNA. Therefore, the nucleus contains at least two distinct populations of sequences (those that simply turnover vs. transcripts which are exported to the cytoplasm) yet both populations exhibit similar decay kinetics.

(c) Interrelation of the kinetic parameters controlling the accumulation of actin gene transcripts

Actin gene expression is at best a complicated process, the rate of which depends on a number of diverse kinetic parameters. The embryo apparently utilizes all of these parameters in solving the logistical problem presented by its requirement for each actin transcript species, and a variety of different solutions evidently coexist. The strategies thus far known can be summarized as follows.

(i) The amount of maternal actin RNA. Molecular titration assays provide decisive evidence that actin gene transcripts are not a major component of egg RNA and as a result do not play a significant role in actin metabolism of the very early embryo (Lee *et al.*, 1986). In that study as well as the present work only five of the six transcriptionally active actin genes were studied. The behavior of the sixth gene, CyIIIb, has not been examined in detail for lack of a gene specific probe (Akhurst *et al.*, manuscript in preparation). Using probes which react to the the CyII subtype, however, has shown that like the other actin genes, CyIIIb transcripts are rare if present at all in egg RNA (Shott *et al.*, 1984).

(ii) The initiation of actin transcript accumulation. The *in vitro* nuclear run-off data presented in Table 2 and the *in vivo* kinetic labeling experiments of Figure 2 show that early cleavage stage embryos do not synthesize full length actin transcripts from any of its genes. Transcripts appear to be synthesized as a

result of ontogenically programmed signals, i.e., each actin gene is transcribed in a particular set of cells at a specific moment in time. Transcripts from the cytoskeletal actin genes, for example, are not synthesized until midcleavage (10-12 hour (pf)), while M gene transcripts do not appear until late gastrulation (~36 hour (pf)). In every case examined, transcripts from an actin gene do not accumulate before the observed point of transcriptional activation of that gene. Thus, our previous assumption (Shott *et al.*, 1984; Lee *et al.*, 1986) appears to be true in that the initiation of transcription is a prominent kinetic parameter governing the accumulation of actin transcripts during embryogenesis.

(iii) The rate at which actin nuclear transcripts enter the cytoplasm. The kinetic labeling experiment presented in Figure 3 shows that an actin protein coding sequence probe is exported to the cytoplasm at a rate equal to its rate of transcription, i.e., each initiation event leads to the production of mature mRNA. The implication is that post-transcriptional events are not rate limiting. Therefore, the regulation of the cytoplasmic level of actin transcripts is not a consequence of selective mechanisms imposed on actin nuclear RNA.

(iv) The rate of transcription of actin genes. Our kinetic analysis of actin gene transcription shows that this gene family uses at least two major mechanisms to regulate their level of expression. The results presented in Tables 4 and 5 show that the mechanism used by four of the five genes studied (CyIIIa is the exception) is one in which the per cell transcription rate is constant. Once these actin genes are transcriptionally activated they are transcribed at a constant rate for as long as transcription occurs. Of major interest, however, is the number of cells which express these genes and how it changes as development proceeds. Genes of the group CyI, CyIIa, CyIIb, and M depend on the cell division rate to increase the number of sites (i.e., nuclei) which transcribe these genes. The observed per embryo increase in transcription and ultimately the level at which these actin

gene transcripts accumulate is a result of the interplay between the rate of transcription and the number of cells transcribing that gene. The remaining gene, CyIIIa, displays a transcription pattern distinctly different than the other *S. purpuratus* actin genes. Table 5 shows that the accumulation of CyIIIa transcripts cannot be accounted for with the measured rates of transcription. Cells expressing this gene either regulate the rate of initiation (e.g., the rate of transcription of the CyIIIa gene would have to change by 15-20 fold in order to account for the accumulation of transcripts) or a subset of the cells of the lineage transcribe the genes for a transient period of time. The latter case implies that all the cells of a given lineage are not necessarily identical. This difference in the transcriptional behavior of the cells of the aboral ectoderm may simply reflect cells in a particular point of the cell cycle. Consequently if the cells of this lineage are at different points in the cell cycle and transcription of CyIIIa occurs only in a transient period of time after cell division (allowing the cells to attain their steady-state level of approximately 200 CyIIIa transcripts/cell), the measured rate of CyIIIa transcription at any moment in time is probably an underestimate of the true per gene level of CyIIIa transcription.

(v) The cytoplasmic decay rate of actin mRNA. Though limited data exist for this parameter it is known that all actin mRNAs do not turnover at the same rate. The kinetic data presented here allowed us to estimate the decay rate of the cytoplasmic mRNAs of CyI, CyIIa, and CyIIb. The observation made was that the half-life of CyIIb mRNA was nearly twice that of either CyI or CyIIa. The consequence of this difference is that to achieve the CyIIb steady state mRNA level (Lee *et al.*, 1986), CyI and CyIIa are transcribed at a rate twice that of CyIIb. This implies that actin transcripts, as with other embryonic sequences (Cabrera *et al.*, 1984), also depend on cytoplasmic decay to regulate their final prevalence level.

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TABLE 1

Synthesis and turnover rates for total nuclear RNA of sea urchin embryos

	Nuclear Transcription Rate[+]		Nuclear Decay Rate		Nuclear Kinetic[§] Steady-State		Rates of Transcription of Total Nuclear RNA from Previous Studies	
Labeling[*] Period Hrs pf	K'_s pg emb•min	K_s pg cell•min	K_d min^{-1}	$t_{\frac{1}{2}}$ min	pg emb	pg nuc	pg min•nuc	pg min•emb
7-9	7.7×10^{-1}	1.2×10^{-2}	0.042	24	18	2.9×10^{-1}	1.0×10^{-2}[b]	0.48[b]
21-23	8.8×10^{-1}	2.0×10^{-3}	0.029	34	30	6.8×10^{-2}	6.7×10^{-3}[b]	2.3[b]
36-38	1.0	1.7×10^{-3}	0.026	39	40	6.7×10^{-2}	3.5×10^{-3}[b]	2.3[b]
65-67	3.4	2.3×10^{-3}	0.045	22	75	5.0×10^{-2}	3.1×10^{-3}[e]	4.1[e]

[*]The indicated labeling times refer to hours postfertilization at a ***carefully*** maintained 15°C.

[+]Assumes that the per embryo cell number follows the data of Hinegardner ***et al.*** (1967), i.e., for ***S. purpuratus*** at 15°C the number of cells per embryo at each labeling time is: 7-9 h (pf), 64 cells; 21-23 h (pf), 450 cells; 36-38 h (pf), 600 cells; 65-67 h (pf), 1200 cells.

[§]The kinetic steady-state content is given by K_s/K_d or K'_s/K_d (see equations (1) and (1')).

[b]Roeder and Rutter (1970); Davidson (1986) where more than one observation for a given stage is reported, the data are averages.

[e]Data from Brandhorst and Humphreys (1971, 1972).

TABLE 2

Kinetic parameters for individual actin nuclear transcripts during embryogenesis

Gene	Labeling Period (Hrs pf)	Nuclear Synthesis Rate‡ K'_s pg of Trans Emb•Min	Nuclear Synthesis Rate‡ K_s Molecules Emb•Min	Nuclear Decay Rate‡ K_d (min^{-1})	Nuclear Decay Rate‡ $t_{\frac{1}{2}}$ (min)	Steady-State Nuclear RNA Prevalence Transcripts/Embryo
CyI	7-9	N.D.	N.D.	N.D.	N.D.	N.D.
	21-23	2.9×10^{-5}	23	0.018	39	1.3×10^{3}
	36-38	4.3×10^{-5}	34	0.030	23	1.1×10^{3}
	65-67	3.8×10^{-4}	298	0.059	12	5.1×10^{3}
CyIIa	7-9	N.D.	N.D.	N.D.	N.D.	N.D.
	21-23	4.0×10^{-6}	3.2	0.014	51	2.3×10^{2}
	36-38	1.3×10^{-5}	9.9	0.013	53	7.6×10^{2}
	65-67	6.1×10^{-5}	49	*	*	*
CyIIb	7-9	N.D.	N.D.	N.D.	N.D.	N.D.
	21-23	1.4×10^{-5}	11	0.019	38	6.1×10^{2}
	36-38	1.2×10^{-5}	10	0.013	55	7.9×10^{2}
	65-67	8.0×10^{-5}	66	*	*	*
CyIIIa	7-9	N.D.	N.D.	N.D.	N.D.	N.D.
	21-23	1.5×10^{-5}	15	0.018	38	8.1×10^{2}
	36-38	1.9×10^{-5}	19	0.023	31	8.3×10^{2}
	65-67	2.3×10^{-5}	23	0.015	48	1.6×10^{3}
M	7-9	N.D.	N.D.	N.D.	N.D.	N.D.
	21-23	N.D.	N.D.	N.D.	N.D.	N.D.
	36-38	N.D.	N.D.	N.D.	N.D.	N.D.
	65-67	1.5×10^{-5}	12	*	*	*

Kinetic parameters are calculated as described in Materials and Methods, section (g). N.D., not detected.

‡The experimental data for these values are shown in Figure 2.

*The amount of scatter in the data presented in Figure 2 prevented an accurate determination of the decay rate of these nuclear transcripts.

TABLE 3

Transcription derived from isolated nuclei of sea urchin embryos

	Isolated Nuclei from 7 hour Embryos Input: 1.5×10^8 cpm RNA		Isolated Nuclei from 21 hour Embryos Input: 1.3×10^8 cpm RNA		
Gene* Specific Probe	cpm+ Hybridized	cpm+ Hybridized	Hybridization Data Normalized for the Amount of Probe on Transcript (cpm/bp)	*In Vitro*§ Synthesis Relative to CyI	*In Vivo*‡ Synthesis Relative to CyI
pCyI	N.D.	276	0.53	1.0	1.0
pCyIIa	N.D.	N.D.	-	-	0.1
pCyIIb	N.D.	20	0.049	0.1	0.5
pCyIIIa	N.D.	29	0.24	0.5	0.6
pM	N.D.	N.D.	-	-	-
pCO_2 Early Histone Cluster	4.5×10^6				

*The actin gene specific clones are each described in detail elsewhere (see Materials and Methods, Lee *et al.*, 1984). For the purpose of this table the relevant information about each probe is the amount of sequence actually present on mRNA: pCyI, 517 bp; pCyIIa, 720 bp; pCyIIb, 400 bp; pCyIIIa, 131 bp; pM, 300 bp. Early histone clone, pCO_2 (Overton and Weinberg, 1978) contains about 2600 bp of mRNA sequences.

+Raw data after background subtraction. The backgrounds for these experiments, filters containing either linearized pUC8 or pBR322, were generally about 4×10^{-5}% of total input cpm, i.e., 50-60 cpm; N.D., not detected.

§The amount of transcription derived from each actin gene relative to CyI *in vitro* is the ratio of the raw cpm hybridized after adjusting each for the number of bases actually present on mRNA.

‡The amount of transcription derived from each actin gene relative to CyI *in vivo* is the ratio of K_s values appearing in the fourth column of Table 2.

TABLE 4

In vivo actin gene transcription rates

Actin Gene	Labeling Period Hours (pf)	Approximate No. of Cells* Actively Transcribing Actin mRNA per Embryo	Actin Gene Transcription Rates+ in Cells Synthesizing Actin mRNA	
			k'_s pg/cell•min	k_s Molecules/cell•min
CyI	7-9	N.D.	N.D.	N.D.
	21-23	200	1.5×10^{-7}	1.2×10^{-1}
	36-38	200	2.2×10^{-7}	1.7×10^{-1}
	65-67	780	4.8×10^{-7}	3.8×10^{-1}
CyIIa	7-9	N.D.	N.D.	N.D.
	21-23	18	2.2×10^{-7}	1.8×10^{-1}
	36-38	60	2.1×10^{-7}	1.7×10^{-1}
	65-67	160	3.8×10^{-7}	3.0×10^{-1}
CyIIb	7-9	N.D.	N.D.	N.D.
	21-23	200	6.9×10^{-8}	5.7×10^{-2}
	36-38	200	4.7×10^{-8}	5.0×10^{-2}
	65-67	780	1.0×10^{-7}	8.5×10^{-2}
CyIIIa	7-9	N.D.	N.D.	N.D.
	21-23	200	7.6×10^{-8}	7.4×10^{-2}
	36-38	200	9.6×10^{-8}	9.4×10^{-2}
	65-67	380	6.2×10^{-8}	6.0×10^{-2}
M	7-9	N.D.	N.D.	N.D.
	21-23	N.D.	N.D.	N.D.
	36-38	N.D.	N.D.	N.D.
	65-67	30	4.9×10^{-7}	3.9×10^{-1}

*The number of cells actively transcribing mRNA was determined from the *in situ* hybridization data of Cox *et al.* (1986).

+Actin gene transcription rates were determined utilizing the embryo synthesis rate data (column 4, Table 2) together with the number of cells per embryo expressing the different actin mRNAs (column 3 of this table).

Table 5

Accumulation of actin transcripts during early development

Actin Gene	Developmental Time Interval (Hours Post-Fertilization)	Projected Amount of Actin* RNA Synthesized during the Time Interval (No. of Transcripts per Embryo)	Accumulation of Actin// Transcripts during the Time Interval as Measured by Molecular Titration (No. of Trans/Embryo)
CyI	0-8	0	0
	8-20	9.8×10^4	3.3×10^4
	20-36	4.2×10^4	6.0×10^3
	36-65	1.5×10^5	4.9×10^4
CyIIa	0-8	0	0
	8-20	1.9×10^3	1.4×10^3
	20-36	8.2×10^3	8.0×10^3
	36-65	4.1×10^4	4.6×10^3
CyIIb	0-8	0	0
	8-20	2.9×10^4	2.8×10^4
	20-36	1.2×10^4	2.0×10^3
	36-65	4.4×10^4	2.8×10^4
CyIIIa	0-8	0	0
	8-20	5.3×10^3	8.5×10^4
	20-36	1.5×10^4	$(-2.8\times10^4)^+$
	36-65	3.8×10^4	3.0×10^4
M	0-8	0	0
	8-20	0	0
	20-36	0	1.7×10^2
	36-65	1.3×10^4	2.5×10^4

*The amount of actin RNA synthesized assumes that these transcripts do not turnover (i.e., $K_d = 0$). The number of newly synthesized actin transcripts was determined for each gene (in each time interval) by multiplying the actin gene transcription rates (column 5, Table 4) with the number of cells synthesizing a given actin gene as a function of the time interval. The *in situ* hybridization data of Cox *et al.*, 1986 showed that once activated, the genes CyI and CyIIb are transcribed in all of the blastomeres of the pre-twenty hour embryo. The accumulation of transcripts in these exponentially dividing cells is calculated as in Cabrera *et al.* (1984):

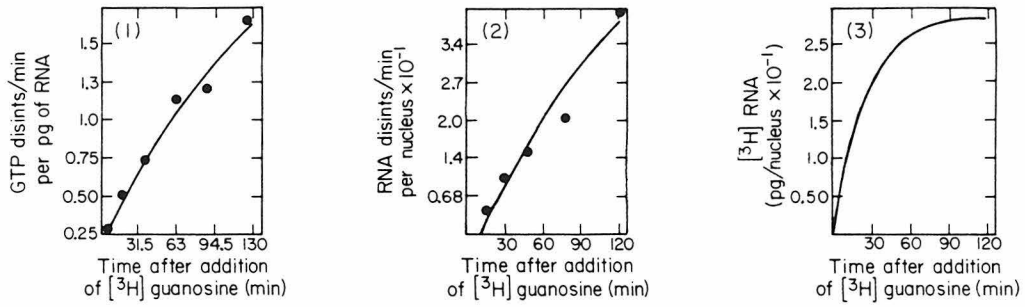
$$C(t) = \frac{K_s N_o}{K_g} [e^{K_g t} - 1]$$

where K_g is the cell multiplication rate constant, N_o is the number of cells at the beginning of the phase, and K_s is the molecular transcription rate constant (Materials and Methods, section (g)). The cell number data indicate two phases of cell growth, the first lasting from 5 h to about 15 h (pf), during which the least-squares solution to the data of Hinegardner for K_g is 0.38 $hour^{-1}$; and the second from 15 hour, when there are about 325 cells. For the second phase, the least-squares value of K_g used in the calculation was 0.25 $hour^{-1}$. The remaining genes in this study were shown to be activated and transcribed only in the cells of particular cell lineages as listed in column 3 of Table 4.

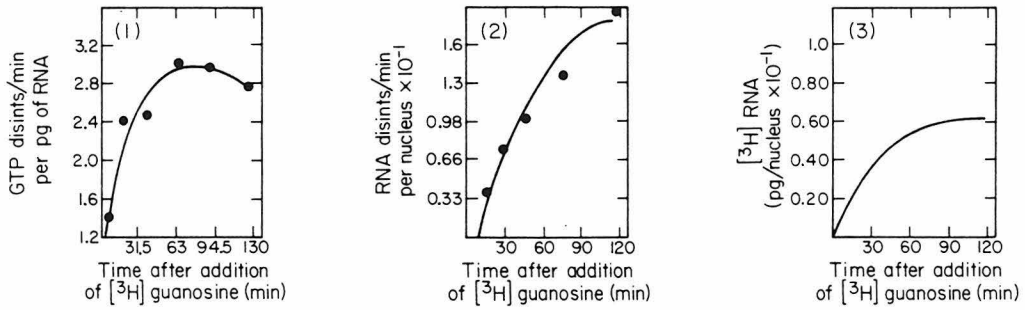
//Lee *et al.* (1986)

+The minus sign indicates that during this time interval the steady-state prevalence level of CyIIIa transcripts has decreased.

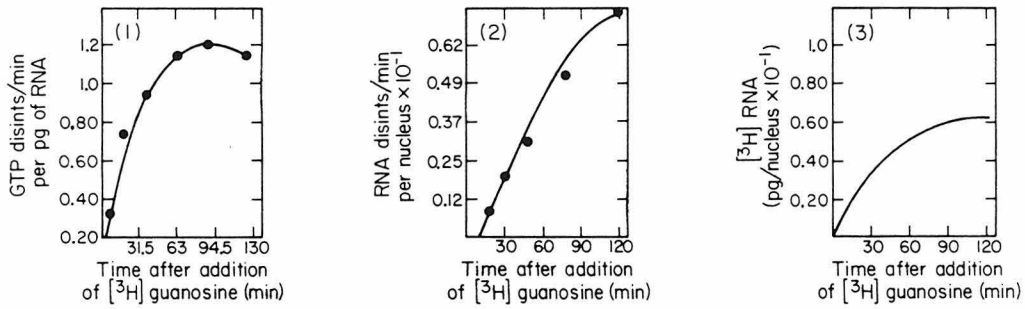
(a) Labeling period: 7-9 hours postfertilization



(b) Labeling period: 21-23 hours postfertilization



(c) Labeling period: 36-38 hours postfertilization



(d) Labeling Period: 65-67 hours postfertilization

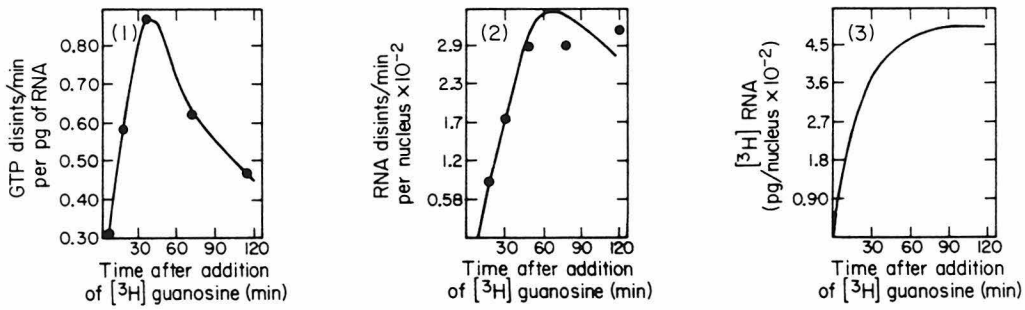
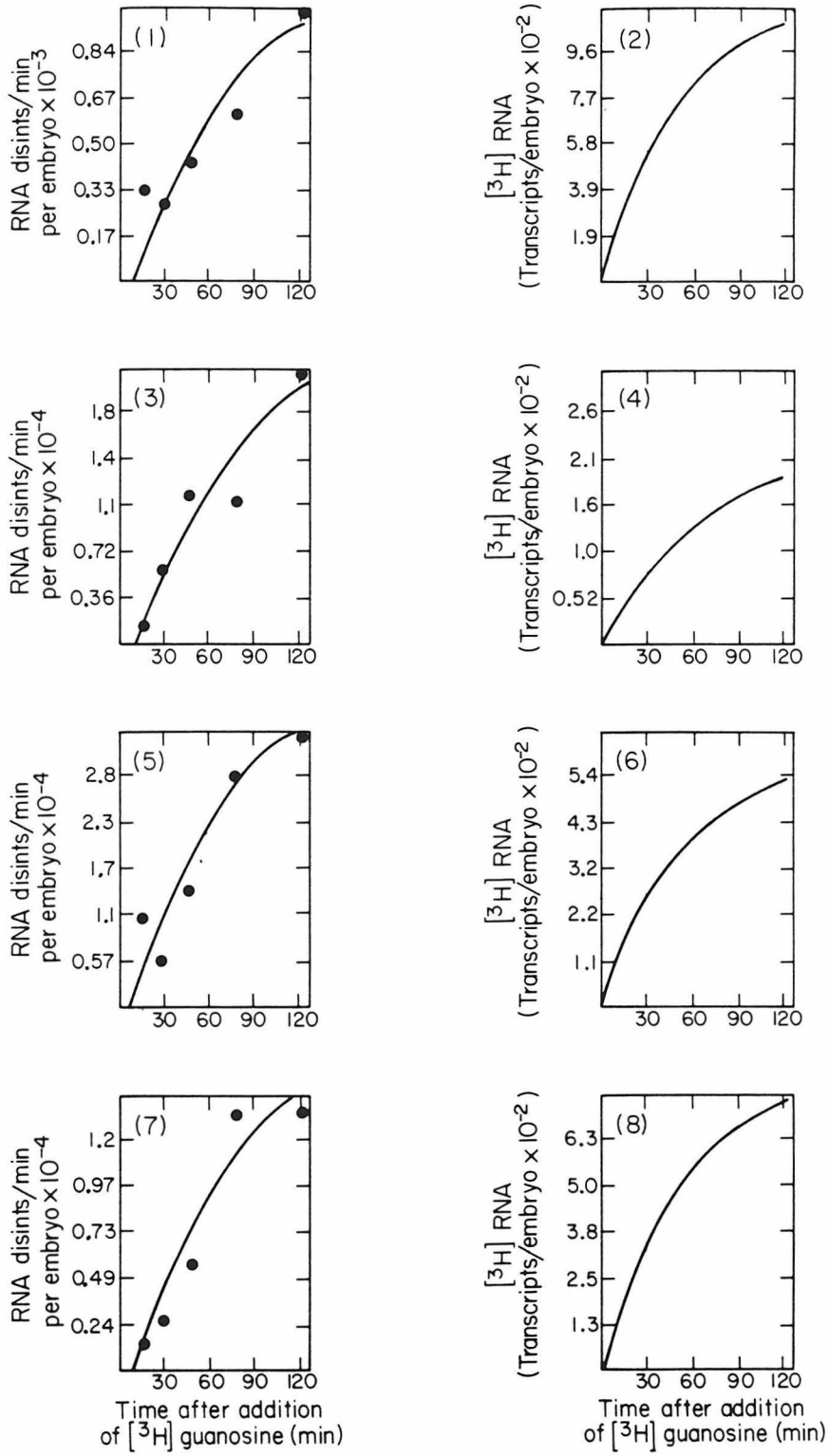
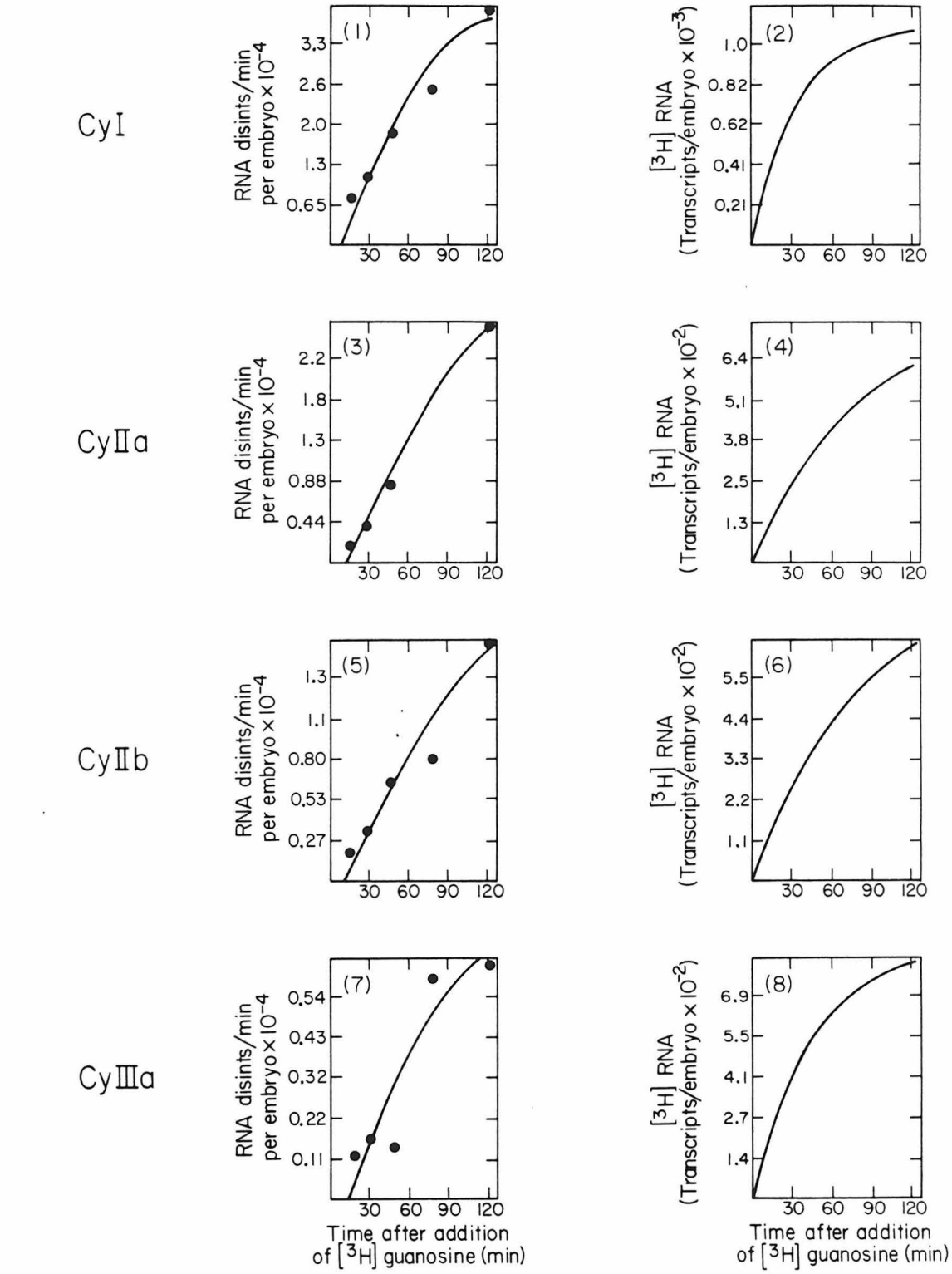


FIG. 1. The kinetics of *in vivo* labeled total nuclear RNA. The four sections (a-d) of the figure each contain three plots which are from left to right the precursor pool specific activity during the labeling, the kinetics of incorporation of [^{3}H]GMP into nuclear RNA, and the mass accumulation kinetics of newly synthesized nuclear RNA. Labeling periods were begun at the times indicated on top of each section and the time-scales shown start at these points. In the first panel of each section the filled circles indicate the pool specific activities which determine the interpolated form of the time function S(t). These were used for the synthesis rate calculations of equation (3). Nuclear RNA was extracted from portions of the same labeled embryos as used for the GTP pool-specific activity measurements, and the radioactivity per nucleus was measured. These data, the measured RNA specific activities (as RNA disints/min per nucleus), are plotted in the center panel of each section. The fitted curves represent the least-squares solutions that describe the form of the functions R(t) (Equation (3)). The mass accumulation kinetics for newly synthesized nuclear RNA during each labeling period were derived from the least-squares values of K_s and K_d obtained from the solution shown in the center panels. This function is given by equation (1'). K'_s is the initial slope of this curve, and the steady-state value for the newly synthesized RNA is the ratio K'_s/K_d. The least-squares solution for K'_s, K_d, and related parameters are listed in Table 1.

(a) Labeling period: 21-23 hours postfertilization



(b) Labeling period: 36-38 hours postfertilization



(c) Labeling period: 65-67 hours postfertilization

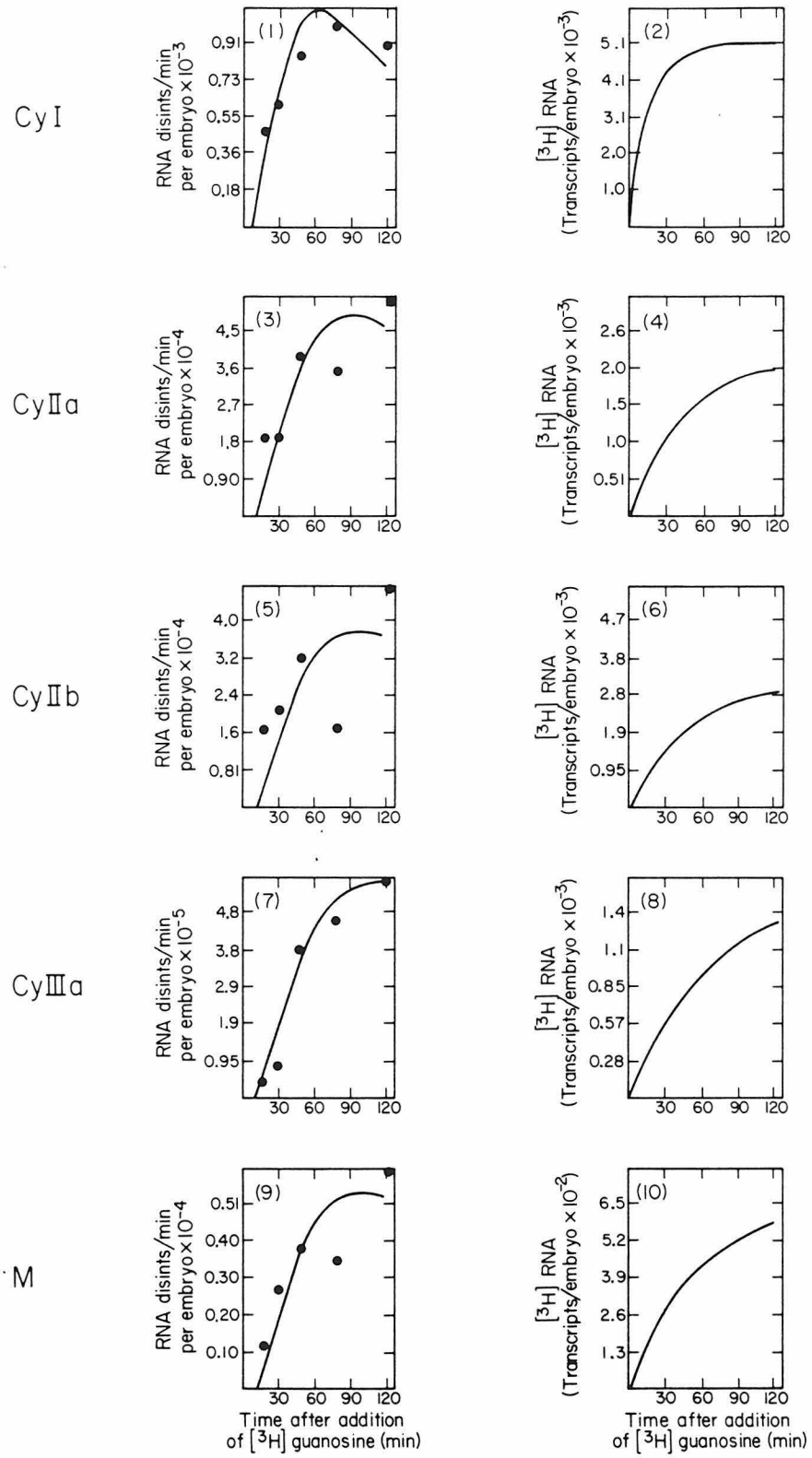


FIG. 2. Kinetics of accumulation of newly synthesized actin transcripts. Data are shown for the five actin genes included in Table 2. The figure is divided into three sections representing the three labeling periods in which newly synthesized actin transcripts could be detected. The nuclear RNAs used to generate Figure 1 were used for these experiments. As in Figure 1 the labeling periods were begun at the times indicated on the top of each section and the time scales shown start at these points. The left-hand columns show incorporation data for transcripts derived from each actin gene. The continuous lines represent the least-squares solutions for the function R(t) of Equation (3). Nuclear RNAs from the same batch of embryos were used for all determinations within a given labeling period. These RNAs were reacted with filters containing the various actin gene specific clones, and the amount of radioactivity hybridized, after appropriate washes and treatment with RNase is shown, normalized on a per embryo basis (see Materials and Methods, sections (f,g), for details and data reduction procedures). In the right-hand column, the mass accumulation of newly synthesized nuclear [^{3}H] RNAs complementary to the cloned sequences are shown as a function of time after label addition. These functions were generated by application of equation (1), using the values for K_s and K_d obtained in the least-squares solutions.

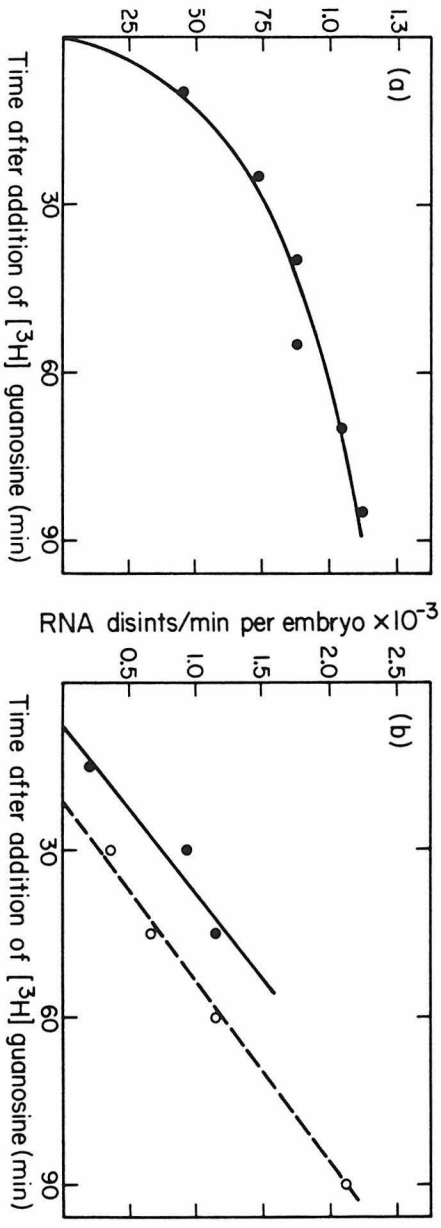

(a)
1.3
1.0
75
50
25
30
60
90
Time after addition of [3H] guanosine (min)
RNA disints/min per embryo ×10-3
(b)
2.5
2.0
1.5
1.0
0.5
30
60
90
Time after addition of [3H] guanosine (min)

FIG. 3. Initial kinetics of synthesis and cytoplasmic entry of an actin protein coding sequence probe. Kinetic data were obtained for total embryo RNA, and total cytoplasmic RNA at 36-38 hour (pf). The RNA preparations were extracted from the same embryos, and the [^{3}H]GTP pool-specific activity was measured on these embryos as well. (a) Time-course of [^{3}H]GTP pool-specific activity, as in Figure 1 (panel 1). (b) Initial radioactivity accumulation functions (R(t)) in Equation (3)) for p9-17E1, a 700 base pair clone containing sequences corresponding to the first 121 amino acids of the actin gene Cyl. The filled circles and solid line represent incorporation of [^{3}H]GTP into actin protein-coding sequence of total RNA. The open circles and dashed line represent incorporation of radioactivity into the same sequence of cytoplasmic RNA.

APPENDIX TO CHAPTER 5

Regulation of Cytoplasmic mRNA Prevalence in Sea Urchin Embryos:

Rates of Appearance and Turnover for Specific Sequences

Reprinted from *J. Mol. Biol.* (1984) **174**, 85–111

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Regulation of Cytoplasmic mRNA Prevalence in Sea Urchin Embryos

Rates of Appearance and Turnover for Specific Sequences

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Complementary DNA clones representing cytoplasmic poly(A) RNAs of sea urchin embryos were hybridized with metabolically labeled cytoplasmic RNA preparations and the rates of appearance and of decay for each transcript species were determined at the blastula-gastrula stage of development. The prevalence of the transcripts chosen for this study ranged, on average, from about one molecule per cell to a few hundred molecules per cell. The embryos were labeled continuously for 18 hours with [^{3}H]guanosine, beginning at 24 hours post-fertilization. The amount of cytoplasmic [^{3}H]poly(A) RNA that hybridized to each cloned sequence was determined and the specific activity of the [^{3}H]GTP pool was measured in the same embryos. Rate constants for the entry of each transcript species into the cytoplasm, and for its decay were extracted from these data. The embryo transcript species identified by the cloned probes displayed a range of stabilities. Half-lives of only a few hours were measured both for a very rare sequence and for a moderately prevalent sequence. Other newly synthesized transcripts, including sequences that first appear during embryonic development, as well as sequences also represented in maternal RNA, are far more stable. We conclude that cytoplasmic RNA turnover rate is a major variable in the determination of the cytoplasmic level of expression of embryo genes. The entry rates of the transcripts into the cytoplasm also varied, from a few molecules per embryo per minute to several hundred, depending on the sequence. By comparing the mass of transcripts of a given sequence in the embryo to the mass of transcripts of that sequence accumulating as a result of new synthesis, the point at which embryo transcription accounts for the major fraction of the cytoplasmic molecules could be estimated. This calculation showed that for some sequences maternal transcripts persist well beyond gastrulation, while other embryo poly(A) RNA species are largely the product of transcription in the embryo nuclei from the blastula stage onwards. There is no single stage at which all maternal transcripts are suddenly replaced by newly synthesized embryo transcripts. Primary transcription rates were measured for two sequences by determining accumulation of label in these RNA species soon after addition of [^{3}H]guanosine to the cultures. Comparing these rates to the cytoplasmic entry rates, we did not detect a significantly greater nuclear transcription of the sequence homologous to the cloned probe.

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1. Introduction

Transcripts of a wide range of abundance occur in the sea urchin embryo. Galau *et al.* (1974,1976) showed that at the gastrula stage these embryos contain a highly diverse class of relatively rare polysomal RNA species, in which there are only a few copies on average of each sequence per cell. From complementary DNA (cDNA) clone colony hybridization measurements, we estimated that rare species (≤ 10 molecules per cell) constitute about 40% of the mass of the cytoplasmic poly(A) RNA (Lasky *et al.*, 1980). The same data indicate that the remaining 60% of the poly(A) RNA mass is distributed among sequences whose prevalence ranges, on average, from about 10 to over 10^3 molecules per embryo cell. Similar conclusions are drawn from measurements of cDNA hybridization kinetics with embryo poly(A) RNAs (Wilt, 1977; McColl & Aronson, 1978; and unpublished data from this laboratory). In general, the rate of synthesis of each protein species is expected to be proportional to the number of available mRNA molecules coding for that species. Thus, to a first approximation, the abundance of the cytoplasmic embryo transcripts determines the level of expression of the various genes whose products are being translated in the embryo. As would be expected from the broad distribution of poly(A) RNA prevalence, the rates at which different newly synthesized proteins appear in the sea urchin embryo are found to vary enormously. Bedard & Brandhorst (1983) found that of the approximately 10^3 protein species whose synthesis is detectable by two-dimensional electrophoretic procedures, some are synthesized at as much as 200 times the rate of others.

In this study, we have sought to define the specific kinetic parameters that determine the prevalence of cytoplasmic poly(A) RNA transcripts in sea urchin embryos. We chose a series of nine cDNA clones, representing embryo transcripts that differ markedly in their cytoplasmic abundance, and measured for these sequences both the rate of entry of newly synthesized transcripts into the cytoplasm, and their rate of turnover. Perhaps not surprisingly, the results show that the cytoplasmic abundance of each sequence is modulated differently. Maternal transcripts of some sequences persist well into development, while for others new transcripts become dominant at an early stage. Different cytoplasmic entry and decay rates are also observed for each individual sequence. These molecular rate constants, together with the number of surviving maternal transcripts, define the cytoplasmic levels of expression for genes active in the embryo.

2. Materials and Methods

(a) *Sea urchin embryos and labeling conditions*

Strongylocentrotus purpuratus eggs were collected and fertilized, and embryos were grown in sea-water containing antibiotics at 15°C, at a concentration of 10^4/ml, as described (Smith *et al.*, 1974). The eggs used for these experiments exhibited $\geq 98\%$ fertilization, as determined by the appearance of fertilization envelopes within 10 min of the addition of sperm. The embryos were labeled at the mesenchyme blastula stage (26 h) or the early gastrula stage (36 h). [8-^{3}H]guanosine (16 Ci mmol^{-1}; ICN) was added to the cultures to a final concentration of 0·6 to 0·9 μM ($2{\cdot}1 \times 10^7$ to $3{\cdot}2 \times 10^7$ disints/min per ml). No unlabeled

guanosine was introduced. The development of embryos exposed to this amount of [^{3}H]guanosine was normal at the level of phase microscope observation (see also Galau *et al.*, 1977). Uptake of the label was followed by thin-layer chromatography on PEI plates (Bakerflex, J. T. Baker Chem. Co.). As described earlier (Galau *et al.*, 1977), the embryos absorb essentially all of the exogenous label within minutes, but over the next hour the majority of the ^{3}H is excreted back to the medium in a different chemical form, which migrates more slowly on PEI plates than does guanosine.

(b) *Specific activity of the internal GTP pool*

Portions of the labeled culture (5 ml) were withdrawn at the times indicated and the embryos were immediately pelleted by centrifugation at 1200 *g* for 5 min, and frozen in an ethanol/solid CO_2 bath. They were stored at −70°C for determination of the GTP pool specific activity. No variations were observed as a result of such storage over 6 months. Purification of intracellular GTP and determination of the GTP pool specific activity by the luciferase assay were performed as described (Dolecki *et al.*, 1976; Galau *et al.*, 1977). In some experiments, an alternative procedure for measuring the GTP pool specific activity was utilized, according to the procedure of Sasvári-Székely *et al.* (1975). (See Maxson & Wu (1976).) This procedure gave results that were closely comparable to those obtained by the luciferase method. The GTP pool size measured by the polymerase method was 0·035 pmol in the blastula stage embryo, compared to 0·025 pmol/embryo estimated earlier (Galau *et al.*, 1977).

(c) *Preparation of cytoplasmic poly(A) RNA and total RNA*

Samples of 4×10^6 embryos were harvested at the indicated times, and the embryos collected and lysed by Dounce homogenization (Hough-Evans *et al.*, 1977). The nuclei were pelleted at 7500 revs/min, the supernatant treated with sodium deoxycholate and Triton-X, and again centrifuged at 10,000 revs/min, all as described (Hough-Evans *et al.*, 1977). Solid urea (Ultrapure; Schwarz-Mann) was added to 7 M, together with an equal volume of 2× urea extraction buffer (Hough-Evans *et al.*, 1977). Samples were extracted twice with phenol-cresol-8-hydroxyquinoline/Sevag (1:1, v/v), once with Sevag (Mallinckrodt) solution, and the cytoplasmic RNA was precipitated with 2·5 vol. ethanol following addition of NaCl to 0·3 M. The precipitated RNA was resuspended in diethylpyrocarbonate-treated water and reprecipitated at −20°C overnight by the addition of 2·0 vol. 4·5 M-sodium acetate, pH 5·5 (Childs *et al.*, 1979). The RNA was again dissolved in diethylpyrocarbonate-treated water and precipitated a final time with 2·5 vol. ethanol, after addition of NaCl to 0·3 M. Poly(A) RNA was obtained by 2 passages of the cytoplasmic RNA through an oligo(dT)-cellulose column under the conditions described by the manufacturer (Collaborative Research, type 3). The final yield was 0·6 to 0·9% of the total mass of cytoplasmic RNA. Preparation of total cytoplasmic RNA was carried out by centrifugation through a CsCl cushion, as described by Posakony *et al.* (1983).

To determine the fraction of RNA radioactivity in [^{3}H]GMP, a portion was hydrolyzed in 0·3 M-KOH at 37°C for 16 h, and then neutralized with perchloric acid. After the $KClO_4$ precipitate was removed by centrifugation, the hydrolyzate was loaded on a Dowex column and nucleosides eluted with 0·5 M-HCl. This eluate was lyophilized to dryness, spotted on a PEI plate and developed as described above. Radioactivity was found exclusively in the GMP spot of the chromatographs. Similar results have been reported by Kijima & Wilt (1969) and Galau *et al.* (1977).

(d) *DNA excess filter hybridizations*

Plasmid supercoils were linearized by digestion with *Pst*I, and the DNA was extracted with phenol, precipitated with ethanol and dissolved in water. Filters were prepared as described by Kafatos *et al.* (1979). A portion (1 μg) of plasmid DNA was loaded per 4 mm^2

of the filter (Schleicher & Schuell). The filters were prehybridized for 4 h at 68 to 70°C in 4 × SET (SET is 0·15 M-NaCl, 30 mM-Tris (pH 8·0), 1 mM-EDTA), 5 × Denhardt's solution (Denhardt, 1966), 0·1% (w/v) sodium dodecyl sulfate and 5 mg poly(rA)/ml. Hybridizations were performed at 68°C for 40 h; typical reaction mixtures contained six 4 mm^2 filters, usually containing different cloned DNAs, per 50 μl of a mixture of 4 × SET, Denhardt's solution, and the [^{3}H]poly(A) RNA (previously boiled for 30 s). Amounts of RNA were used such that the filter-bound DNA was always in effective excess over the homologous transcripts in the [^{3}H]poly(A) preparation, as determined in separate control experiments (see below). Following hybridization, the reaction mix was removed and the filters were washed in 50 ml of 4 × SET, 0·1% SDS† at 68 to 70°C with strong shaking. The buffer was changed every hour until no counts could be detected in 1 ml. The filters were then washed with SET, 0·1% SDS following the same procedure. Finally, they were treated with 50 μg RNase A/ml (preboiled for 5 min in 2 × SSC: SSC is 0·15 M-NaCl, 0·015 M-sodium citrate) at 37°C for 30 min, briefly washed with 4 × SET, 0·1% SDS, air-dried, and counted in a toluene-based liquid scintillation fluid (Liquifluor; New England Nuclear). Background levels of radioactivity were assayed on filters counting pBR322 DNA. Backgrounds were about 5 to 10 cts/min above machine background or 10^{-5} to 10^{-6} of input cts/min.

(e) *Technical controls on the filter hybridization procedure*

(i) *Effective DNA excess*

The following experiments were carried out to determine whether a sufficient fraction of the filter-bound DNA was able to react with the labeled RNA to provide an effective DNA excess. Filters containing 1 μg of bound DNA were reacted with increasing amounts of RNA presented in the same standard volumes of solution. This measurement was performed with a clone, SpG30, that contains a fragment of the sea urchin mitochondrial cytochrome oxidase I gene (Jacobs *et al.*, 1983). The absolute concentration of transcripts from this gene were determined previously by titration (Lasky *et al.*, 1980). Transcripts represented by this clone are in the moderately high prevalence class of sea urchin embryo poly(A) RNAs. The same fractions of [^{3}H]poly(A) RNA were observed to bind to the filters over a 15-fold range of input RNA concentrations, thus demonstrating effective DNA excess (data not shown). The absolute quantities of poly(A) RNA used in this experiment were 0·1 to 1·5 μg of poly(A) RNA per 50-μl reaction mixture. The reactions with prevalent sequences shown in the text all included less than or 1 μg of poly(A) RNA, and slightly larger amounts were used for rare sequence determinations. The ratio of the mass of SpG30 transcripts to the mass of filter bound DNA was several-fold higher in this control experiment than for any of the other hybridization reactions included in this paper.

(ii) *Extent of reaction*

To measure the fraction of a labeled RNA that could be bound in filter-driven reactions carried out as described here, 18 S ribosomal RNA was isolated from velocity sedimentation gradients, and labeled *in vitro* with ^{125}I. This RNA was reacted with a filter-bound cloned DNA carrying the gene for this rRNA species (data not shown). The clone utilized was pLv1334, isolated from a genomic library of the sea urchin *Lytechinus variegatus* by Blin *et al.* (1979) and kindly provided for our use by Dr Darrel Stafford. The reactions were set up to mimic the sequence concentrations of the RNAs labeled *in vivo* included in the following studies. Hybridization of the [^{125}I]rRNA proceeded to 85% completion in 40 h of reaction, as determined by comparison of the amount of input [^{125}I]RNA with that bound to the filter at each time point.

(iii) *Fidelity of filter-bound hybrids*

The hybrids formed between cytoplasmic [^{3}H]RNA and filter-bound cloned DNA were verified by means of a stepwise thermal elution procedure. The filters were washed free of

† Abbreviation used: SDS, sodium dodecyl sulfate.

scintillation fluid with chloroform, and melted incrementally in SSC. Two 0·7 ml washes were carried out at each temperature step. The temperature was increased in 2·5 deg.C intervals, and at each step the buffer was counted in Ready-Solv (Beckman). The t_m values for these thermal elution profiles were all about 83°C (data not shown). No counts were irreversibly bound to the filters after the 100°C wash. The hybrids were also treated with RNase A under low salt conditions (50 μg/ml at 37°C for 30 min, in 0·1 × SSC) and in every case complete digestion was obtained, as expected for *bona fide* RNA–DNA hybrids.

(f) *Mathematical approach and data reduction procedures*

(i) *Entry rate and turnover equations*

Our object is to obtain molecular rate constants for the entry of RNA transcripts into the cytoplasm, and for the decay of these transcripts. Units of the cytoplasmic entry rate constant, k_s, are molecules min^{-1} $cell^{-1}$, and for the decay rate constant, k_d, the units are min^{-1}. In some cases, the entry rate constant is expressed in mass rather than molecular units, i.e. as pg min^{-1} $embryo^{-1}$. Expressed in these terms, the entry rate constant is called k'_s in this paper. The number of newly synthesized (i.e. labeled) RNA molecules accumulated in the cytoplasm at any given time after the experiment begins, $C(t)$ (assuming zero labeled molecules of this species are present initially), is:

$$C(t) = \frac{k_s}{k_d}(1 - e^{-k_d t}). \tag{1}$$

Here, $C(t)$ is in molecular terms, i.e. molecules/cell; in mass terms (pg/embryo):

$$C(t) = \frac{k'_s}{k_d}(1 - e^{-k_d t}). \tag{1'}$$

The specific activity of the GTP pool in sea urchin embryos labeled with [^{3}H]guanosine changes sharply with time, as discussed by Galau *et al.* (1977). This specific activity time function, here called $S(t)$, was measured experimentally as described above, and cytoplasmic poly(A) RNA was extracted from samples of the same embryos as used for the pool determinations. The amount of [^{3}H]RNA hybridizing to filters containing each cloned DNA ($R(t)$) was then determined as a function of time of labeling. The rate of change $R(t)$ can be described as follows:

$$\frac{dR(t)}{dt} = k'_s S(t) - k_d R(t), \tag{2}$$

the solution to which, for our purposes, is:

$$R(t) = k'_s \int_L^t S(t') e^{-k_d(t-t')}\, dt'. \tag{3}$$

Here, L is the time lag preceding the appearance of labeled transcript in the the cytoplasm, and t' is a variable of integration. L was determined graphically by extrapolation from the early measured values of $R(t)$, and was 30 to 40 min for all the experiments described in this paper. Equations (2) and (3) were presented initially by Galau *et al.* (1977), and their application has been discussed in detail by Davidson (1976).

(ii) *Data reduction*

A computer program, MESSAGE (available on request), was constructed for the purpose of extracting the best estimate of k'_s and k_d from the values of S and R measured in each experiment, assuming the relation shown in eqn (3). A non-linear least-squares algorithm was used in MESSAGE to obtain k'_s and k_d (Pearson *et al.*, 1977). In MESSAGE, these rate constants are treated as parameters and are varied continuously from initial guesses made by the operator. The parameters are changed until the root-mean-square difference

between the measurements of R and the calculated values of R using eqn (3) is minimized. This calculation is carried out by means of a numerical integration procedure based on Simpson's rule, in which the time between L and t in eqn (3) is divided into subintervals until no significant change in result is obtained by further subdivisions, and the integrand is evaluated by Simpson's rule in each subinterval. The values of S required for this process are obtained by linear interpolation between the measured points. When the best values of k'_s and k_d have been calculated, the program generates the time function for the accumulation in the cytoplasm of the newly synthesized RNA molecules, i.e. $C(t)$, using eqn (1'). The steady state content of the newly synthesized RNA (i.e. k'_s/k_d), is then computed. At the end of the calculation, the best fit $R(t)$ curve is evaluated by numerical integration at selected time points, and then interpolated for plotting a "spline" method. The linear interpolation of the precursor specific activity curve, $S(t)$, and the calculated newly synthesized RNA molar accumulation curve, $C(t)$, are also plotted. Examples of these plots are shown in the Figures presented below.

(iii) *Unit conversions*

Data are fed to the MESSAGE program as files that must be presented in compatible mass units. For most purposes, the desirable terms are the number of molecules or mass of RNA synthesized, rather than of GMP incorporated. Thus the values of $S(t)$ actually fed to the MESSAGE program are reduced by a factor of 4 to allow for this conversion.

(iv) *Accumulation of newly synthesized transcripts*

Data for cell number (N) as a function of developmental time published by Hinegardner (1967) were utilized for this calculation. Estimates of transcript accumulation were made for the period 5 to 54 h. We assumed that values of the cellular entry and decay rate constants are the same between 5 h (when a logarithmic rate of division is established) and 24 h as measured thereafter, though no direct measurements were actually carried out before 24 h. For stable sequences not displaying visible turnover, the rate of accumulation of new transcripts during a phase of logarithmic growth would be:

$$\frac{dC(t)}{dt} = k_s N_0 e^{k_g t}, \tag{4}$$

where k_g is the cell multiplication rate constant, N_0 is the number of cells at the beginning of the phase, and k_s is the molecular entry rate constant as above. The cell number data indicate two such phases, the first lasting from 5 h to about 15 h, during which the least-squares solution to the data of Hinegardner for k_g (k_{g_1}) is 0·38 h^{-1}; and the second from 15 h, when there are about 400 cells, to 54 h (or later). For the second phase, the least-squares value of k_g (k_{g_2}) used in the calculation was 0·25 h^{-1}. If the newly synthesized transcript is not stable, the steady state value is used in the calculation, assuming each cell of the embryo to contain the same steady state content of transcript; i.e. the number of transcripts per embryo is calculated at each time period as the product of the cell number (computed according to the values of k_{g_1} and k_{g_2}) and the steady state number of transcripts per cell.

3. Results

(a) *Labeling kinetics of the GTP pool*

Galau *et al.* (1977) found that when *S. purpuratus* blastula or gastrula stage embryos are exposed to exogenous [^{3}H]guanosine, the intracellular GTP pool specific activity quickly rises to a peak value, and then decays until a steady state is attained many hours later. These observations have been reproduced quantitatively in the present study, although different methods were used to assay the pool specific activity. A typical GTP pool specific activity time-course is

shown in Figure 1(a). Three processes combine to produce specific activity curves of this form. The initial increase in specific activity occurs as the embryos rapidly remove all the available [^{3}H]guanosine from the medium and convert a portion of it to [^{3}H]GTP. Uptake of the precursor is essentially complete within 20 minutes and labeled [^{3}H]GTP continues to flow into the pool for up to an hour. In the meantime, however, the nuclear RNA (nRNA) synthesized earlier from unlabeled precursors is undergoing rapid turnover. The unlabeled GMP thus released presently enters the GTP pool, and results in the relatively steep decline in the GTP pool specific activity observed in Figure 1(a) after about 60 minutes of labeling. The kinetics of this decline at first directly reflect the decay rate of the embryo nRNA. This phase is followed by a period of more gradual specific activity decline, dominated quantitatively by the turnover of more stable RNA species, mainly cytoplasmic mRNAs. Ultimately, as shown below, the GTP of the triphosphate pool and the GMP of the newly synthesized cytoplasmic poly(A) RNA approach a common steady state specific activity value.

The GTP specific activity time-course shown in Figure 1(a) is the function $S(t)$ in equation (3). As described in detail in Materials and Methods, the rate constants for the entry (k_s) and decay (k_d) of a cytoplasmic RNA species can be derived from measurements of $S(t)$ and the amount of [^{3}H]GMP incorporated in that RNA over the same period in the same embryos. There are two assumptions implied in the treatment used here. These are, first, that if the RNA decays it does so with first order kinetics, as indicated by several previous measurements for most sea urchin embryo mRNA species (reviewed by Davidson, 1976); and, second, that the GTP pool in sea urchin embryos is not compartmentalized. The following observations are almost certainly inconsistent with any significant nucleotide triphosphate pool compartmentalization in sea urchin embryos. (1) Total RNA synthesis rates in sea urchin embryos have been derived from incorporation data obtained with three different nucleoside precursors, and the results are found to agree closely irrespective of the particular nucleoside employed. Therefore, either none of the pools is compartmentalized or all are to the same extent. The sizes and labeling kinetics of these triphosphate pools are so diverse, however, that this restriction essentially excludes compartmentalization (Davidson, 1976). (2) The same synthesis rates were obtained by measuring directly the mass of newly synthesized embryo RNA, separated from pre-existent RNA by heavy nucleoside labeling (Grainger & Wilt, 1976), as are calculated from the precursor pool specific activity. (3) Galau *et al.* (1977) showed that the absolute specific activity of the [^{3}H]GTP in *S. purpuratus* embryos is almost the same as that of newly synthesized polysomal mRNA after long periods of labeling. The evidence thus demonstrates that the measured intracellular GTP pool specific activity is an acceptable index of the specific activity of the GTP actually being utilized for RNA synthesis in these embryos.

(b) *Kinetic parameters for newly synthesized cytoplasmic poly(A) RNA*

Figure 1(b) illustrates the kinetics with which [^{3}H]GMP appears in the cytoplasmic poly(A) RNA. The interpolated points indicate the least-squares

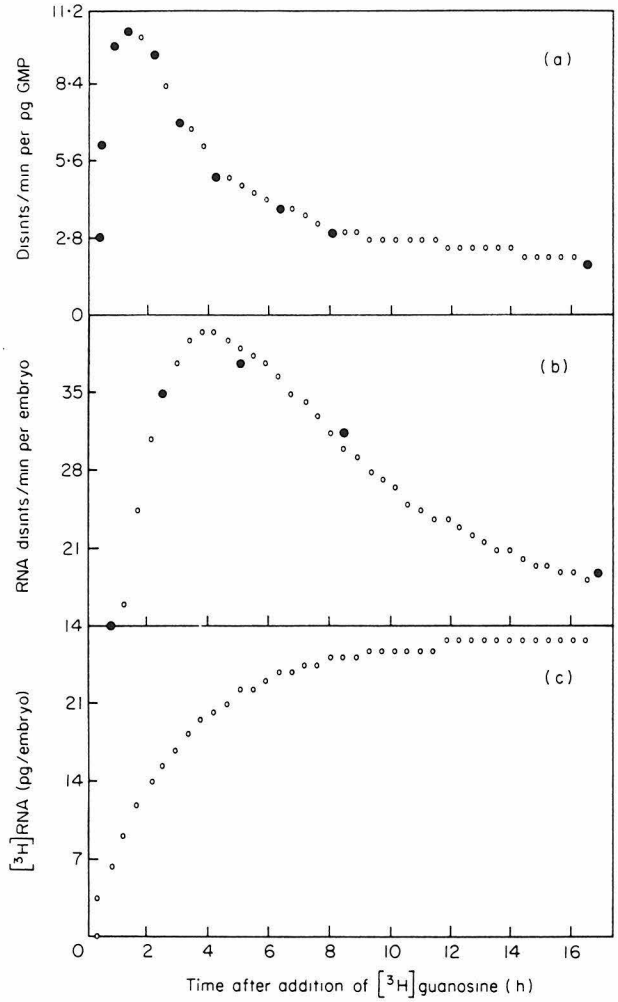

FIG. 1. Pool specific activity, [^{3}H]GMP incorporation, and entry kinetics for cytoplasmic poly(A) RNA. (a) Precursor pool specific activity during the labeling period. Embryos were labeled for 18 h with 0·9 μM of [^{3}H]guanosine (16 Ci/mmol) as described in Materials and Methods, and samples were removed for GTP pool specific activity determination at the times indicated. Labeling was begun at 26 h post-fertilization and the time-scale shown starts at this point. The peak specific activity value in this experiment was about 1·6 Ci/mmol, or 10% of the exogenous tracer specific activity. Specific activity values measured originally as cts/min per pmol of GTP were converted to disints/min per pg of GMP taking into account the counting efficiency of the liquid scintillation system used (0·29), since it is GMP that is incorporated into RNA. For calculation of RNA synthesis rates (i.e. rather than GMP incorporation rates), as in the remaining portions of the Figure, the specific activity values shown were divided by 4 and were supplied to the MESSAGE program described in Materials and Methods. Filled circles indicate measured pool specific activities and open circles indicate the interpolated form of the time function $S(t)$ used for the synthesis rate calculation of eqn (3). (b) Kinetics of incorporation of [^{3}H]GMP into cytoplasmic poly(A) RNA. Cytoplasmic poly(A) RNA was extracted from portions of the same labeled embryos as used for (a) at the indicated times, and the radioactivity per unit mass of RNA was measured. The number of embryos represented by the measured quantities of RNA is known from the content of poly(A) RNA per embryo, about 40 pg (see the text). Filled points indicate the measured RNA specific activities, as RNA disints/min per embryo. The data were supplied directly to the MESSAGE program as the values of R to be used for the least-squares fit. The least-squares

solution for the function $R(t)$ in equation (3), calculated by varying the values of the entry and decay rate constants k'_s and k_d, as described in Materials and Methods. For this particular calculation, the $S(t)$ data shown in Figure 1(a) were utilized. Table 1 displays the rate constants extracted from this and other experiments carried out on the total embryo cytoplasmic poly(A) RNA.

Two features of the curve illustrated in Figure 1(b) deserve note. First, it will be observed that radioactive poly(A) RNA does not appear in the cytoplasm until after 30 to 40 minutes of labeling. Of this, approximately 15 minutes are required for sufficient [^{3}H]GMP to enter the GTP pool and first appear in newly synthesized *nuclear* RNA, and the remainder is evidently the minimum nuclear transcription, processing, and transport time necessary for the production of cytoplasmic poly(A) RNAs. Secondly, it is apparent from the downward curvature of the function $R(t)$ that most of the newly synthesized cytoplasmic poly(A) half-life measured in these experiments is 2·0 to 2·2 hours. Table 1 also shows that at 18 hours the specific activity of the GTP pool and that of the poly(A) RNA [^{3}H]GMP are about the same, verifying the use of the GTP pool to determine the specific activity of the labeled RNA precursor for these particular experiments.

Figure 1(c) describes the accumulation of newly synthesized poly(A) RNA flowing into the cytoplasm. This function is generated from the values of k'_s and k_d listed in Table 1 according to equation (1′). It can be seen that a steady state is achieved after about 12 hours of labeling. The steady state content of newly synthesized cytoplasmic poly(A) RNA is about 30 pg per embryo, or approximately 5×10^4 molecules of average length per cell (Table 1). This is just about half the steady state content of newly synthesized polysomal mRNA according to Galau *et al.* (1977). The entry rate of the cytoplasmic poly(A) RNA is the same or slightly higher than that of total polysomal mRNA (Table 1), but the steady state quantity is half as great because the turnover rate of the cytoplasmic poly(A) RNA is apparently twice as high. One possible interpretation is that essentially all the newly synthesized cytoplasmic poly(A) RNA becomes polysomal but that, as Nemer (1975) reported, only half of the total newly synthesized polysomal mRNA of the gastrula is polyadenylated. However, for this explanation to be fully consistent, the entry rate for total polysomal RNA should be twice that for total poly(A) RNA, whereas the measured entry rates are almost identical. Thus, both the calculated entry rate of the cytoplasmic poly(A) RNA and its turnover rate would have to be about twofold too high as a result of experimental error (or the kinetic parameters for total polysomal RNA reported by Galau *et al.* (1977) would have to be about twofold too low). While systematic experimental errors of this magnitude cannot be excluded out of hand, an

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solution describes the form of the function $R(t)$ (eqn (3)), as indicated by the open circles. (c) Entry kinetics for newly synthesized cytoplasmic poly(A) RNA. The least-squares values of k_s and k_d obtained from the solution shown in (b) are used to generate a mass accumulation curve for the newly synthesized cytoplasmic poly(A) RNA. This function is given by eqn (1′). k'_s is the initial slope of this curve, and the steady state value for the newly synthesized RNA is the ratio k'_s/k_d. The least-squares solutions for k'_s, k_d and the related parameters obtained in this particular experiment are listed in the first row of Table 1.

TABLE 1

Entry and turnover rates for cytoplasmic poly(A) RNA of sea urchin embryos

Labeling period† post-fertilization (h)	Entry rate k'_s (pg embryo^{-1} min^{-1})	Entry rate k_s‡ (mol cell^{-1} min^{-1})	Decay rate k_d (min^{-1})	Decay rate $t_{\frac{1}{2}}$ (h)	Kinetic steady state§ (pg/embryo)	Kinetic steady state§ (mol./embryo)	Kinetic steady state§ (mol./cell)	Specific activity at 18 h [^{3}H]GMP of poly(A) RNA (disints min^{-1} pg^{-1})	Specific activity at 18 h Precursor pool (disints min^{-1} pg^{-1})
26–43	0·16	245	$5{\cdot}8\times10^{-3}$	2·0	28	$2{\cdot}5\times10^{7}$	$4{\cdot}2\times10^{4}$	2·0	2·3
26–44	0·11	170	$5{\cdot}8\times10^{-3}$	2·0	19	$1{\cdot}7\times10^{7}$	$2{\cdot}9\times10^{4}$	1·7	2·4
36–54	0·17	260	$5{\cdot}2\times10^{-3}$	2·2	33	$3{\cdot}0\times10^{7}$	$5{\cdot}0\times10^{4}$	1·2	1·0
Polysomal mRNA‖ 24–42 (average value)	0·13	200	$2{\cdot}0\times10^{-3}$	5·7	65	$5{\cdot}9\times10^{7}$	$9{\cdot}9\times10^{4}$		

† The 26 to 44 h labeling period begins at the mesenchyme blastula stage and the 36 to 54 h period begins at early gastrula stage.

‡ Assuming a mean poly(A) RNA (or mRNA) length of 2000 nucleotides, and 600 cells per embryo. The actual cell number varied from 450 cells/embryo at the beginning of the earliest labeling experiment to about 800 to 900 cells/embryo. However, the final third of each experiment has little effect on the measured parameters; hence the choice of 600 cells as a reasonable approximation of cell number at the critical period of the experiments for use in these conversions.

§ The kinetic steady state content is given by k_s/k_d or k'_s/k_d (see eqns (1) and (1′)).

‖ All data in this row are from Galau *et al.* (1977). The steady state mass is the kinetic estimate from the measurements listed. Independent estimates of the mass of polysomal mRNA in gastrula stage embryos are in the range 60 to 85 pg (Davidson, 1976). According to Nemer (1975), about half of the newly synthesized polysomal mRNA is polyadenylated.

alternative model may be considered. Possibly there is a more rapidly turning over cytoplasmic component that is never loaded on polysomes, including either the same or a different set of sequences than those found on the polysomes (e.g. see Salditt-Georgieff *et al.*, 1981). In this case, the fraction of the newly synthesized cytoplasmic poly(A) RNA that is loaded on polysomes would have a longer half-life than the average value listed in Table 1. Since there is a 30 to 40-minute lag in the appearance of any radioactive poly(A) RNA, such a rapidly decaying component would have to be an authentic cytoplasmic transcript class rather than a nuclear RNA contaminant. It is not possible to determine whether the overall decay rate measured for the cytoplasmic poly(A) RNA is in fact an average of two (or more) not very different kinetic components, as in this example. Neither of these interpretations can be discarded without further experimentation.

Irrespective of the intracellular disposition of the newly synthesized poly(A) RNA, its steady state quantity (about 30 pg per embryo) is an appreciable fraction of the total amount of poly(A) RNA in the cytoplasm. According to independent measurements, the quantity of cytoplasmic poly(A) RNA in *S. purpuratus* gastrulae is about 40 pg per embryo (calculated from data of Wilt (1977), and data reviewed by Davidson (1976), and data of Lasky *et al.* (1980)). Thus, much of the embryo poly(A) RNA, though probably not all, is in kinetic steady state at the stage when the measurements reported in Table 1 were carried out. This result shows that the majority of the poly(A) RNA molecules in blastula-gastrula stage embryos has been synthesized in the embryo genomes, and is not maternal in origin.

(c) *cDNA clones utilized for kinetic studies*

In the following, we describe measurements of flow rate into the cytoplasm, turnover rate, and kinetic steady state for a set of six cloned sequences. Characteristics of the six cDNA clones, and estimates of the prevalence of the transcripts they represent, are given in Table 2. These clones all represent genomic rather than mitochondrial sequences. Their DNAs display no homology with purified mitochondrial DNA in blot hybridizations, while (except for one that was not tested) they react clearly with genome blots prepared from the sperm DNA of a single individual sea urchin. The Spec1 clone contains a 3′ repetitive sequence element (see note (3) of Table 2) but, as Table 3 indicates, the other clones all display a small number of reactive restriction fragments in genome blots. In the highly polymorphic genome of *S. purpuratus*, it is usual for single copy probes to react with two different restriction fragment alleles in any given DNA preparation (Britten *et al.*, 1978; Thomas *et al.*, 1982; Posakony *et al.*, 1983; Lee *et al.*, 1984), as do SpG6 and SpG61. SpG6 is known to be single copy (unpublished observations), and this could be true also of SpG61. There may be a small number of genomic sequences homologous to SpG20 and SpG16, respectively. We made no further effort to determine the exact number of copies of these sequences per haploid genome, as this parameter is not directly relevant to the main concerns of the present study. As detailed in the notes to Table 2, two

of the clones react with a single poly(A) RNA size class, and a fifth, Spec1, with a small family of related transcripts (Bruskin *et al.*, 1981). Data are not available either for the very rare transcripts represented by clone SpG20 or for clone SpG25.

Table 2 shows that the set of six clones represent transcripts of a very wide range of abundance in gastrula stage cytoplasmic poly(A) RNA. Titration measurements indicate that there are only about 300 SpG20 transcripts per embryo, or about 0·5 per cell, while Spec1 transcripts are over 600 times more prevalent per embryo (Lynn *et al.*, 1983). Spec1 mRNAs are confined to ectoderm cells, where their prevalence is estimated at 500 molecules per cell (Lynn *et al.*, 1983). The object of the following experiments was to determine how the different

TABLE 2

cDNA clones utilized for kinetic determinations

cDNA clone†	Insert length (nt)‡	Genome blots§ (no. bands)	Approximate prevalence‖, ¶ molec/emb	molec/cell
SpG20	910	6	$3 \times 10^{2(1)}$	0·5
SpG25	450	N.A.	N.A.	N.A.
SpG6	765	2	$1{\cdot}1 \times 10^{4(1)}$	18
SpG16	1200	6	$3{\cdot}8 \times 10^{4(2)}$	60
Spec1	670	Rep.	$1{\cdot}9 \times 10^{5(1)}$	500/ectoderm cell
SpG61	720	2	$9 \times 10^{4(3)}$	150

N.A., data not available.

† Clones were derived from the *Strongylocentrotus purpuratus* gastrula library described by Lasky *et al.* (1980). This library was constructed by direct cloning of poly(A) RNA–cDNA hybrids (Zain *et al.*, 1979). The population of embryonic sequences represented in this library is described by Lasky *et al.* (1980) and was further studied by Flytzanis *et al.* (1982) and Xin *et al.* (1982). The cDNA clones listed represent distinct sequences, according to restriction map and gel blot hybridization data.

‡ Lengths are derived from restriction map data (not shown) and electrophoretic gel measurements.

§ Genome blots were carried out with sperm DNA isolated from a single individual sea urchin.

The Spec1 clone carries a repetitive sequence that is located in the 3′ non-translated region of the message from which the clone was transcribed (Carpenter *et al.*, 1982). Bruskin *et al.* (1981,1982) showed that the Spec1 gene is a member of a small gene family specifying a set of acidic embryonic ectoderm proteins.

‖ Prevalence estimates were derived as indicated below. The molecules/cell calculation assumes 600 cells in the gastrula stage embryo, and also that the transcripts are randomly distributed amongst these cells. This is of course known not to be true for Spec1 transcripts, which are confined to ectoderm cells (about 1/3 of the total cells in the embryo).

(1) Molecular titration carried out by solution hybridization of RNA with excess anticoding strand probe. Titration data for SpG20 and SpG6 are from Lasky *et al.* (1980). Data for Spec1 are from Lynn *et al.* (1983).

(2) Estimated by densitometry of the RNA gel blot obtained with this probe by Flytzanis *et al.* (1982). The clone is designated Sp64-B9 by Flytzanis *et al.* The same blot contained a Spec1 reaction. Since the number of Spec1 transcripts is known from molecular titration, it could be used as a standard for the quantitation of the SpG16 transcripts.

(3) Based on reactions of excess filter-bound DNA with labeled cDNA.

¶ RNA gel blots (not shown) indicate that single embryo cytoplasmic transcripts 1800 and 1200 bases in length are complementary to the SpG6 and SpG16 sequences, respectively. The Spec1 sequence reacts predominantly with transcripts of 1500 and 2200 bases though hybrid selection experiments demonstrate that it is homologous with at least 7 different ectoderm mRNAs (Bruskin *et al.*, 1982). Gel blot data were not obtained for the very rare sequence coded by SpG20 or for SpG25.

TABLE 3

Kinetic parameters for individual transcripts

Clone	Labeling period (h)	Cytoplasmic RNA	Entry rate k'_s (pg embryo^{-1} min^{-1})	Entry rate k_s (mol./embryo^{-1} min^{-1})	k_s (mol./cell^{-1} min^{-1})	Decay rate k_d (min^{-1})	Decay rate $t_{\frac{1}{2}}$ (h)	Kinetic steady state (pg/embryo)	Kinetic steady state (mol./embryo)	Kinetic steady state (mol./cell)	Independent prevalence estimates (mol./embryo)
SpG20	36–54 post fertilization	Poly(A) RNA	$3{\cdot}9\times10^{-6}$†	7·8	0·013	$5{\cdot}2\times10^{-3}$	2·2	$7{\cdot}5\times10^{-4}$	$1{\cdot}5\times10^{3}$	2·5	$0{\cdot}3\times10^{3}$
SpG25	24–40	Total	$3{\cdot}1\times10^{-5}$†	260	0·25	$2{\cdot}4\times10^{-3}$	4·8	$1{\cdot}3\times10^{-2}$	$6{\cdot}2\times10^{4}$	104	N.A.
			$1{\cdot}8\times10^{-5}$	73	0·12	$1{\cdot}2\times10^{-3}$	9·6	$1{\cdot}5\times10^{-2}$	$6{\cdot}1\times10^{4}$	101	N.A.
SpG6	24–40	Total	$1{\cdot}1\times10^{-4}$†	260	0·44	$<10^{-3}$	Stable	—	—	—	$1{\cdot}1\times10^{4}$
	26–43		$4{\cdot}3\times10^{-5}$	102	0·17	$<10^{-3}$					
SpG16	24–40	Total	$2{\cdot}5\times10^{-5}$	38	0·06	$1{\cdot}3\times10^{-3}$	8·8	$1{\cdot}9\times10^{-2}$	$2{\cdot}9\times10^{4}$	50	$3{\cdot}8\times10^{4}$
			$6{\cdot}6\times10^{-5}$†	100	0·17	$1{\cdot}1\times10^{-3}$	10·4	6×10^{-2}	9×10^{4}	150	
			$4{\cdot}5\times10^{-5}$	68	0·11	$1{\cdot}4\times10^{-3}$	8·2	3×10^{-2}	$4{\cdot}8\times10^{4}$	81	
Spec1	24–40	Total	$1{\cdot}3\times10^{-4}$†	350	1·8†	$<10^{-3}$	Stable	—	—	—	$1{\cdot}9\times10^{5}$
SpG61	26–44	Poly(A) RNA	6×10^{-7}	1·5	2×10^{-3}	$<10^{-3}$	Stable	—	—	—	9×10^{4}
			2×10^{-7}†	0·5	$0{\cdot}8\times10^{-3}$	$<10^{-3}$	Stable	—	—	—	

Kinetic parameters are calculated as described in Materials and Methods, section (f). Since the labeling period was only 18 h in most cases, where the turnover rate constant was less than 10^{-3} min^{-1} it could not be estimated accurately (i.e. $t_{\frac{1}{2}} > 11{\cdot}5$ h). In these cases, the word Stable is inserted under $t_{\frac{1}{2}}$. N.A., data not available. It is not ruled out that these messages in fact turn over with a half-life greater than could be measured here. Transcript prevalence estimates in the last column are from Table 2.

† The experimental data for these values are shown in Fig. 2.

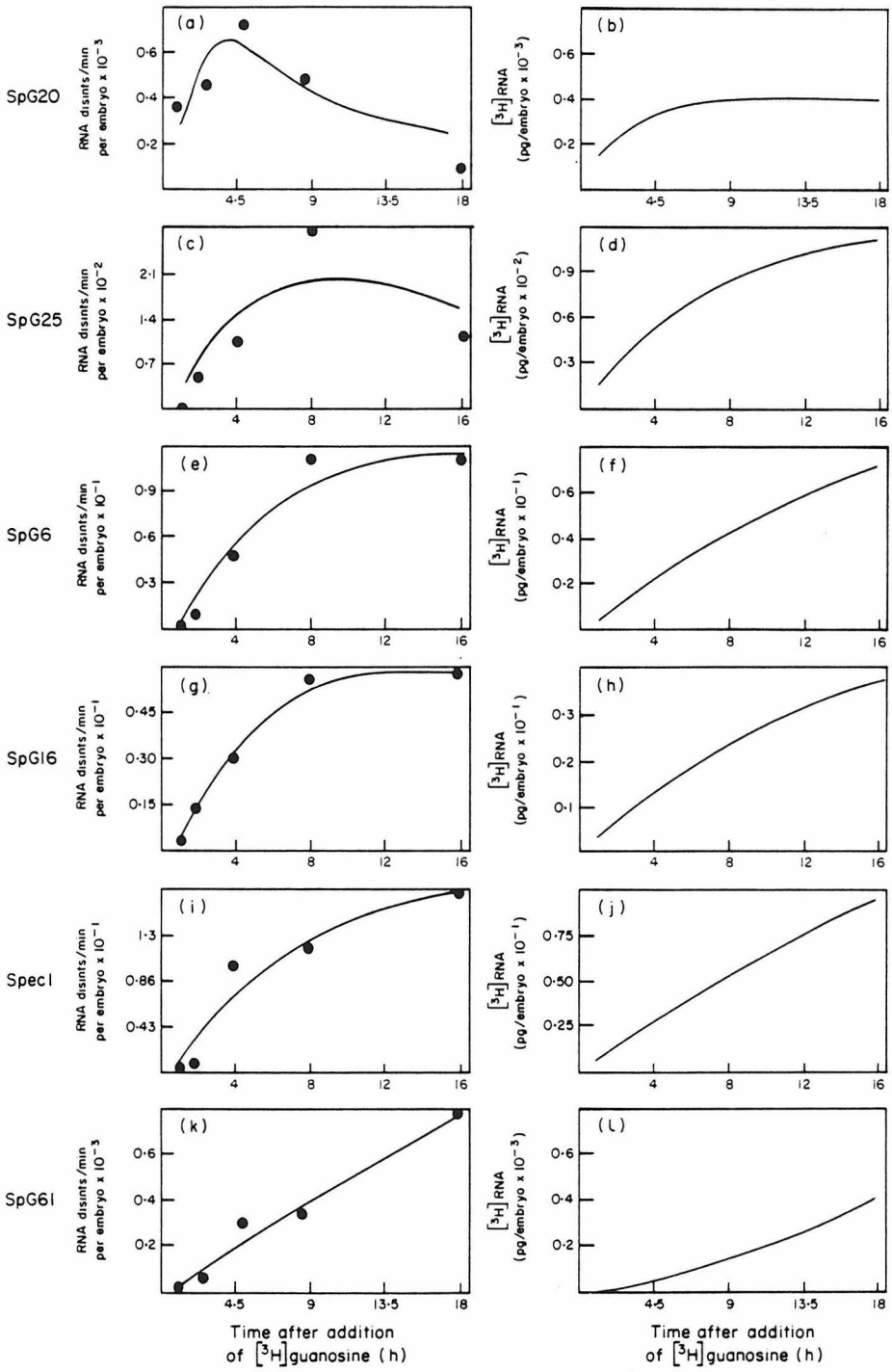
SpG20
SpG25
SpG6
SpG16
Spec1
SpG61
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
RNA disints/min per embryo x 10⁻³
[³H]RNA (pg/embryo x 10⁻³)
RNA disints/min per embryo x 10⁻²
[³H]RNA (pg/embryo x 10⁻²)
RNA disints/min per embryo x 10⁻¹
[³H]RNA (pg/embryo x 10⁻¹)
Time after addition of [³H]guanosine (h)

Fig. 2.

prevalence levels of these cloned transcripts are established and maintained in the embryo.

(d) *Synthesis and turnover kinetics for six specific sequences*

DNA of the six clones was bound to filters and hybridized with RNA preparations that had been labeled metabolically and, as before, the specific activity of the [^{3}H]GTP pool was measured on samples of the same labeled embryos. The quantities of filter-bound DNA and of [^{3}H]RNA used in each hybridization was demonstrated in previous experiments to provide a significant DNA excess, as described in Materials and Methods. Model hybridization tests showed that the reactions proceeded to about 80% of completion. The amounts of [^{3}H]RNA hybridized to filters of each cloned DNA sequence as a function of labeling time, and the corresponding pool specific activity data, were used to calculate the rate at which that species of transcript enters the embryo cytoplasm (k'_s), and the rate at which it then decays (k_d), according to equation (3). Kinetic data for each of the six clones are shown in Figure 2, and the parameters extracted from these analyses are listed in Table 3. It can be seen here that in replicate determinations on the same sequence, the entry rate constants may vary as much as threefold from experiment to experiment. This error includes biological variability, since replicate measurements were made on [^{3}H]RNA preparations extracted from different batches of embryos, and also the combined experimental errors incurred in the pool measurements, the hybridization procedures, and the data reduction. Where it is determined, the values of k_d tend to vary somewhat less in replicate measurements. The magnitude of the error on individual k_s and k_d measurements is to be considered in comparison to the range of values shown in Table 3 for these parameters. The entry rate constants differ amongst the sequences by a factor of greater than 100, as do the estimated prevalences of the respective transcripts.

The sequences included in Table 3 fall into three kinetic classes. The SpG20 sequence is a low abundance poly(A) RNA that displays a relatively high turnover rate. The SpG25 and SpG16 sequences are moderately prevalent in the embryo cytoplasm, but also decay measurably, while in the same embryos the prevalent cytoplasmic RNAs hybridizing with clones SpG6, Spec1 and SpG61 are

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FIG. 2. Kinetics of accumulation of specific newly synthesized transcripts. Data are shown for the 6 clones included in Table 3. The left-hand column shows incorporation data for these clones. The continuous lines represent the least-squares solutions for the function $R(t)$ of eqn (3). Cytoplasmic [^{3}H]poly(A) RNAs or total cytoplasmic RNAs from the same batch of embryos (see Table 3) were used for all determinations within a given labeling period. These RNAs were reacted with filters containing the various cloned DNAs, and the amount of radioactivity hybridized after appropriate washes and treatment with RNase is shown, normalized on a per embryo basis (see Materials and Methods, section (f), for details and data reduction procedures). In the right-hand column, the mass accumulations of newly synthesized [^{3}H]RNAs complementary to the cloned sequences are shown as a function of time after label addition. These functions were generated by application of eqn (1'), using the values for k'_s and k_d obtained in the least-squares solutions. The initial slopes of the curves in (c) and (d) are equal to k'_s and, where attained, the terminal plateau value is the steady state content (i.e. k'_s/k_d).

stable. Each of these examples evidently represents a different strategy for the control of transcript prevalence.

Of the six sequences, only the SpG20 sequence decays at a rate similar to that measured for the total newly synthesized poly(A) RNA (Fig. 1). The low abundance of SpG20 poly(A) RNA increases the difficulty of measurement, and thus we regard the kinetic steady state value observed, about 2·5 molecules of SpG20 transcript per cell, to be in fair agreement with the titration value, 0·5 molecules per cell. Table 3 shows that, despite its relatively rapid turnover, only a low cytoplasmic entry rate is needed to maintain the steady state concentration of this sequence. Were every cell of the gastrula to function equally in synthesizing SpG20 RNAs, the minimum rate of transcription required per cell would be only one productive initiation each 75 minutes. The minimum per gene transcription rates would be half of these values, since the cells are diploid. Such a low entry rate is consistent with other available information. Thus, Lasky *et al.* (1980) concluded that there are about $4{\cdot}8 \times 10^3$ species of poly(A) RNA in the three to ten copies per cell prevalence class, and there are probably an approximately equal number of species in the less than three copy per cell class, since there are at least 10^4 species of low abundance message in the embryo (Galau *et al.*, 1974,1976; Davidson & Britten, 1979). If $4{\cdot}8 \times 10^3$ species of poly(A) RNA entered the cytoplasm at the rate we report for SpG20 transcripts, they would account for about 60 molecules per cell per minute. This value is not unreasonable for very rare sequences, given the *total* cytoplasmic poly(A) RNA entry rates measured in this study, i.e. <260 molecules per cell per minute (Table 1).

Not all sequences that turn over detectably in the embryo are of low abundance. This is shown by the example of the SpG25 sequence, the half-life of which in one measurement is as low as 4·8 h. Yet the kinetic steady state estimate for this sequence indicates that it is at least 40 times more prevalent than is the SpG20 transcript, as a consequence of its high entry rate. There is a steady state level of about 100 molecules of this transcript per cell. The minimum transcription rate per nucleus needed to maintain the cytoplasmic population of SpG25 RNAs is one initiation every four to eight minutes, or per gene, half this rate. The parameters for the SpG16 transcript are similar, except that it has a two to four times greater half-life. Table 3 shows that there is reasonable agreement between the kinetic steady state of the SpG16 sequence and its independently estimated prevalence. Moderately prevalent sequences such as the transcripts hybridizing with clones SpG25 and SpG16 could account for a large fraction of the mass of the unstable cytoplasmic poly(A) RNA detected in the experiments of Figure 1.

The Spec1 and SpG6 RNAs enter the cytoplasm at the highest rates measured in our sample. Taking into account the known restriction of Spec1 transcription to ectoderm cells, we estimate that the minimum per cell initiation rate required is 1·8 per minute. However, several related genes are probably included in this estimate (see the notes to Table 2). For SpG6, which is known to be single copy, the minimum rate is about one initiation per two to five minutes. Though these are both stable transcripts at the blastula-gastrula stage, when our measurements were carried out, neither accumulates in later development. A subsequent increase in turnover rate is almost certainly implied for the Spec1 transcripts, since

Bruskin *et al.* (1982) and Lynn *et al.* (1983) noticed about a twofold *decrease* in the total number of Spec1 transcripts per embryo between gastrula and pluteus stages. This may occur also with the SpG6 transcript since, on the assumption of unchanged entry and turnover rates, the projected amount of newly synthesized SpG6 RNA would eventually exceed the measured amount. Thus, decrease in the synthesis rates for these transcripts might well take place also in the post-gastrula embryo. It may be relevant that in *S. purpuratus* the overall nuclear transcription rate per cell is known to decrease several-fold in the course of embryonic development (reviewed by Davidson, 1976).

(e) *Comparison of transcription rates and cytoplasmic entry rates for two specific sequences*

Figures 1 and 2 demonstrate that labeled RNA does not enter the cytoplasm until 40 to 60 minutes have elapsed, though the embryos contain radioactive [^{3}H]GTP less than five minutes after addition of label to the culture. Incorporation of [^{3}H]GMP into total embryo RNAs during this lag period could thus be used to monitor nuclear rates of transcription, without actually extracting the nuclei. This approach would avoid any perturbation of nuclear metabolism that might result from a nuclear isolation procedure, and should provide a measurement of transcription under completely normal physiological conditions. Rates obtained for given sequences in this way could then be compared to the rates of entry of these transcripts into the cytoplasm, measured as above in samples of the same cultures of labeled embryos. These experiments required an increased number of RNA samples taken within the first 90 minutes after label addition, as well as a more detailed measurement of the [^{3}H]GTP pool specific activity during this period.

As a preliminary control (data not shown), we used the ribosomal DNA (rDNA) clone pLv1334 (Blin *et al.*, 1979) to measure the rate of rRNA transcription, which has been determined in two previous kinetic studies carried out on *S. purpuratus* embryos. Galau *et al.* (1977) utilized a procedure similar to that reported here, also based on measurements of the [^{3}H]GTP pool specific activity time-course, and concluded that in blastula stage embryos the rate of ribosomal RNA synthesis is 12 to 18 molecules per minute per cell. In their experiments, the ribosomal RNA was separated from other cellular RNAs in velocity sedimentation gradients. Surrey *et al.* (1979) measured the [*methyl*-^{3}H]*S*-adenosylmethionine pool specific activity, and from the time-course of incorporation of tritium into rRNA methyl groups, calculated a rRNA synthesis rate of 40 molecules per minute per cell. The estimates we obtained from measurements on the total RNA of blastula stage embryos with the pLv1334 probe were about 15 molecules of rRNA synthesized per minute per cell.

In Figure 3 are shown total transcription rate (k'_T) and cytoplasmic entry rate (k'_s) measurements for two of the cloned sequences considered above, SpG16 and SpG25. Data are presented only for the initial portion of the labeling period. Equation (3) shows that the initial rate of incorporation of [^{3}H]RNA is the synthesis (or entry) rate which, in all the kinetic experiments reported in this

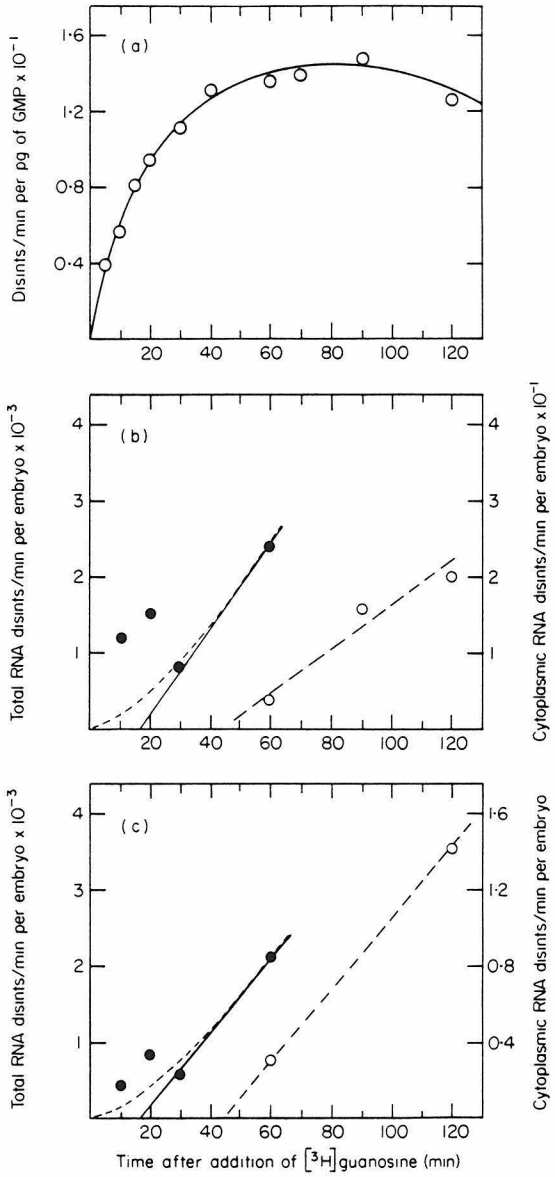

FIG. 3. Initial kinetics of synthesis and cytoplasmic entry for 2 specific sequences. Kinetic data were obtained for the total embryo RNA, and for the total cytoplasmic RNA. Both RNA preparations were extracted from the same embryos, and the [^{3}H]GTP pool specific activity was measured on these embryos as well. These experiments differed from those in Fig. 2 in that a greater number of data points were obtained at very early times in the pool specific activity and the total RNA measurements. (a) Time-course of [^{3}H]GTP pool specific activity, as in Fig. 1(a). (b) and (c) Initial portions of radioactivity accumulation functions ($R(t)$ in eqn (3)) for Spec1 and SpG16, respectively. The dotted lines show the rate of accumulation of radioactivity in the specific transcript at early times after addition of label. The concave upward curvature is due to the rising specific activity of the [^{3}H]GTP pool. The extrapolation (continuous line) indicates the linear portion of the curve, which is appropriate for comparison to the message radioactivity accumulation function shown to the right of each panel. The slope of this line is $k_T \times S(t)$, for t around 60 min (see (a)).

paper, is determined early in the measurement. The rise and fall of the specific activity of the [^{3}H]GTP pool shortly after label addition is illustrated in Figure 3(a), and the time-course of accumulation of newly synthesized RNA that hybridizes to SpG25 and SpG16 DNAs is shown in Figure 3(b) and (c). Since for these sequences there is virtually no early contribution from labeled cytoplasmic molecules, the data obtained in the total RNA samples before 60 minutes suffice to determine the primary transcription rate. Cytoplasmic entry rates in the experiments shown were measured as in the earlier experiments, though only the initial points are reproduced in Figure 3. The values obtained are given in the legend to Figure 3. In two sets of measurements carried out on SpG16, the ratio k'_T/k'_s was measured as 1·6 and 0·9, and in two sets of measurements on SpG25, this ratio was 0·52 and 1·3. That is, for both clones the values of k'_s fall within a factor of two of the values of k'_T, and the average of all the k'_s/k'_T ratios measured is about one. Thus, there is no evidence that the rate at which SpG25 and SpG16 sequences are transcribed greatly exceeds the rate at which these transcripts enter the cytoplasm. It follows that all, or almost all, of the nuclear precursor molecules transcribed from these two regions of the genome ultimately give rise to cytoplasmic transcripts.

4. Discussion

(a) *Kinetic characteristic of the newly synthesized embryo cytoplasmic poly(A) RNA*

We first consider the overall synthesis and turnover kinetics of the embryo poly(A) RNAs, and of other comparable RNA fractions studied earlier. Table 1 provides kinetic data for total newly synthesized cytoplasmic poly(A) RNA obtained on the same labeled RNA preparations as used for hybridization with the cloned sequences. Overall half-life of the cytoplasmic poly(A) RNA was measured in several experiments as about two hours. The decay rate of the total cytoplasmic poly(A) RNA thus appears to be about 2·5 times higher than was reported for total polysomal mRNA by Galau *et al.* (1977), using a similar method, or for either poly(A)$^+$ or poly(A)$^-$ polysomal RNA by Nemer *et al.* (1975), using a "chase" procedure. We do not know if this kinetic distinction between cytoplasmic poly(A) RNA and polysomal mRNA is real, though the ranges of the $t_{\frac{1}{2}}$ values obtained in different experiments by Galau *et al.* (1977) (5·3 to 5·8 h) and those listed in Table 1 (2·0 to 2·2 h) do not overlap. As pointed out in Results, section (b), this difference could be the result of experimental error, or it could indicate the presence in the embryo cytoplasm of relatively unstable newly synthesized poly(A) RNA molecules other than polysomal mRNAs.

Table 3 shows that both moderately prevalent and rare sequences are included in the class of short-lived transcripts. This class clearly dominates the overall kinetics for the total newly synthesized cytoplasmic poly(A) RNA illustrated in Figure 1, though examples such as the Spec1 and SpG6 transcripts show that the embryo contains stable, newly synthesized transcripts of significant prevalence as well. On the other hand, the case of the SpG61 transcript (see below) indicates

that there exist in the cytoplasm of the embryo some very stable high prevalence maternal poly(A) RNAs, that would not be labeled at all in experiments such as those of Figure 1. It follows that the prevalence distribution of newly synthesized transcripts may not be the same as that of the total transcript population, in that the latter may include a lower proportion of unstable RNAs. The rare polysomal mRNAs of the late blastula are essentially all unstable (Galau *et al.*, 1977), as is the specific low abundance SpG20 poly(A) RNA studied here. Thus, the turnover kinetics of the newly synthesized poly(A) RNA shown in Figure 1 could reflect to a major extent the behavior of low abundance species. It should be recalled here that there are ten times the number of genes producing rare mRNAs than produce prevalent mRNAs (Davidson & Britten, 1979), and that more than 40% of the mass even of the total steady state cytoplasmic poly(A) RNA consists of low abundance species (Lasky *et al.*, 1980; Xin *et al.*, 1982). This discussion affects the interpretation of many previous measurements carried out on the labeled poly(A) RNA or polysomal mRNA of sea urchin embryos (e.g. see Brandhorst & Humphreys, 1971; Nemer *et al.*, 1974,1975; Nemer, 1979; Galau *et al.*, 1977; see review in Davidson, 1976). Such measurements may overemphasize the characteristics of rare and moderately low prevalence RNAs relative to their quantitative contribution to the overall transcript population.

(b) *Cytoplasmic entry rates for the specific clones sequences*

The entry rates listed in Table 3 are essentially determined within a few hours of the beginning of each experiment, since these are essentially the initial rates of transcript accumulation, and the majority of the data are obtained then. The turnover kinetics depend on the ultimate curvature of the labeled RNA accumulation fractions. Our experiments were carried out after the termination of the exponential phase of rapid cell division that occurs in late cleavage. Thus there is only a negligible error in the calculated values of the entry rate constants due to increase in cell number during the initial labeling periods when the entry rate is being measured. A related argument applies if the rate of cytoplasmic entry increases due to more rapid transcription (or processing) in the course of the experiment. A sufficiently large increase would produce an illusion of greater transcript stability. If the transcript is relatively stable, and there is an increase in entry rate during the experiment, the result will be a mass accumulation curve with an upward changing slope as can be seen, for example, for the SpG61 transcript (Fig. 2). The uncertainty in the degree of stability of the transcript in this case is unimportant, since were it less stable than assumed, the disparity between the calculated amount of newly synthesized transcript that would be accumulated and the total quantity of the transcript (Fig. 2) would only be more severe. The mass accumulation curves for the SpG6 and Spec1 sequences are nearly linear. Were the apparent stability of these sequences to be due to a combination of increasing entry rates and rapid transcript decay, it would be necessary for there to be a very steep upward change in k_s during the labeling period, and of exactly the right magnitude to compensate for the turnover. Measurements of the entry rates for SpG6 transcripts carried out over two

different 18-hour intervals, one beginning two hours after the other, in fact reveal no such increase in entry rate (Table 3). The overall result of the metabolism of each transcript is in any case approximated by the mass accumulation curves of Figure 2, whether these represent a composite effect of compensating changes of k_s and k_d during the experiment, or are the simple result of nearly constant k_s and k_d values, as assumed here.

Table 3 demonstrates that some newly synthesized sequences enter the cytoplasm at intervals of only a few minutes in each cell, while others appear only occasionally. The case of the SpG20 transcript verifies the conclusion reached by Galau *et al.* (1977), that genes producing rare cytoplasmic poly(A) RNA sequences need function only intermittently to account for the flow of such sequences into the cytoplasm. We calculated that a new initiation event must occur on the individual genes coding for this rare sequence no more often than once every 2·5 hours. Since in sea urchin embryo nuclei the chain elongation rate is six to ten nucleotides per second (Aronson & Chen, 1977), genes initiated at this rate, or even ten times more frequently, would lack any nascent transcripts much of the time, and would never or only rarely contain more than one transcript. Since by far the major fraction of genes that produce any cytoplasmic poly(A) RNAs or polysomal mRNAs produce rare ones (Davidson & Britten, 1979), this result is consistent with the electron microscope observations made by Busby & Bakken (1979), who found that about 82% of the transcription units they observed bore single nascent transcripts. They also concluded that a majority of transcription units represented in embryo RNA display no nascent transcripts in any given nucleus at any one time.

Other sequences enter the cytoplasm much more rapidly, as do the Spec1 and SpG6 transcripts. The minimum nuclear initiation frequency required by the cytoplasmic entry rates for the single copy SpG6 sequence, about 0·5 molecules per minute per cell, would produce a transcription complex bearing a nascent RNA molecule about every 2000 nucleotides. Spacings of this order were observed among the minor fraction of sea urchin embryo transcription complexes observed by Busby & Bakken (1979) that displayed multiple transcripts.

(c) *The cytoplasmic stability of embryo poly(A) RNAs is a major determinant of their prevalence*

These measurements show that the turnover rates of the newly synthesized transcripts are significant in determining their cytoplasmic prevalence, just as are their diverse entry rates. Transcript-specific intracellular mechanisms apparently set the rates of decay differently for some transcripts than for others. Several (non-exclusive) classes of such mechanisms can be envisioned. The primary sequence of each RNA could determine its decay rate, e.g. according to its preferred secondary structure, the proteins it can bind, or its terminal sequence elements. Alternatively, specific transcripts could be localized in different subcellular compartments or embryonic structures, among which the activity of degradation enzymes varies, or in which there is some structural protection against nucleases (as perhaps exists in polysomes; see above). It is possible that

there is a "default" cytoplasmic turnover rate that results generally in a half-life of several hours, and that the relatively small number of diverse transcripts included in the high prevalence classes are the beneficiaries of special protective mechanisms. Coexistence within the same cells of specific mRNAs displaying many diverse half-lives has been observed in other systems as well, e.g. in yeast (Chia & McLaughlin, 1979; Koch & Friesen, 1979) and in animal cells (Wilson & Darnell, 1981). Stabilization of mRNAs of extremely high prevalence during terminal differentiation was discussed by Kafatos (1972), and several examples have been described more recently (see for instance, Guyette *et al.*, 1979). The present results suggest that the transcript prevalence depends in part on cytoplasmic RNA stability across the whole range of cytoplasmic poly(A) RNA abundances observed in sea urchin embryos.

(d) *Genomic control of the cytoplasmic poly(A) RNA population in the sea urchin embryo*

During embryonic development, the maternal transcripts are gradually replaced by newly synthesized RNAs transcribed in the embryo genomes. This process results in the transfer of control over biosynthetic activity to the embryonic nuclei and the embryonic transcriptional apparatus. The measurements reported here provide insight into some of the mechanisms underlying this fundamental developmental change.

In Figure 4 are presented calculations of the projected accumulations of three of the transcript species we studied, assuming the kinetic parameters listed in Table 3, and the known rate of cell division during cleavage, which increases exponentially the number of potential synthesis sites per embryo. The filled points indicate the actual prevalence of each transcript, as estimated from direct measurements independent of kinetic factors (see Table 2). A sharp developmental increase in the total amount of the transcript is observed only in the cases of the SpG16 and Spec1 sequences. RNAs complementary to these sequences have been shown to be absent or present only at very low levels in the maternal RNA (for SpG16, Flytzanis *et al.*, 1982; for Spec1, Bruskin *et al.*, 1981,1982). In contrast, the SpG61 sequence is represented in maternal RNA at about the same levels as in gastrula and pluteus RNAs, as are the SpG6 and SpG20 transcripts (Lasky *et al.*, 1980). The sharp rise in the calculated quantities of newly synthesized transcripts in Figure 4 is solely due to the increase in numbers of cells, since the cytoplasmic entry rates per cell are here assumed to be constant. The general implication of the calculations is therefore that a significant increase in transcript concentration during early embryogenesis does not necessarily require an increase in transcription rate per nucleus, as would be the case for a system with a fixed nucleus to cytoplasm ratio. Thus from Figure 4(a) it is evident that the large early accumulation of SpG16 transcripts could be accounted for solely as the consequence of developmental increase in the number of cells synthesizing these molecules at an unchanging rate. On the other hand, Spec1 transcripts do not accumulate until after the 400-cell stage (Bruskin *et al.*, 1981), and several additional examples are known of sequences that become

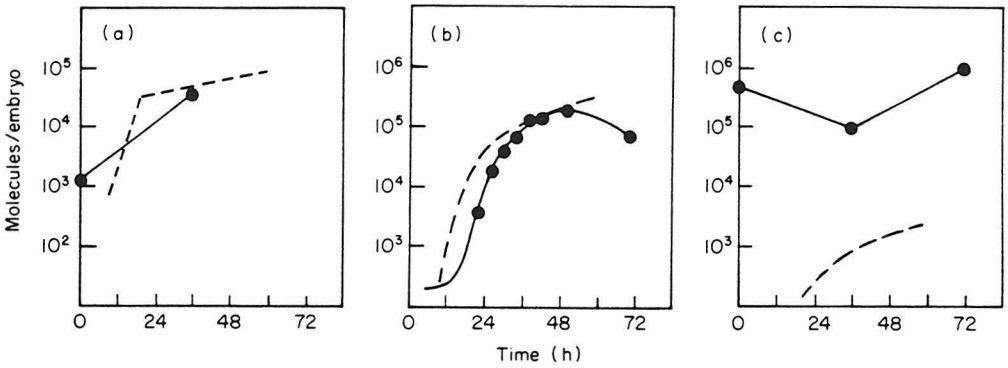

FIG. 4. Estimated accumulation of newly synthesized transcripts compared to measured total transcript quantities. Accumulation of the newly synthesized specific transcripts is calculated for the whole period of embryonic development, assuming that the synthesis and decay rates measured for the 24 to 42 h period remain constant. These calculated functions are shown as broken lines. The total amounts of each transcript measured in the cytoplasmic RNAs are shown as continuous lines and filled points. (a) SpG16, prevalence data calculated from Flytzanis *et al.* (1982). The SpG16 sequence is difficult to detect in maternal RNA, and the net quantity of this sequence rises sharply during the blastula stage. (b) Spec1. The prevalence values plotted for Spec1 are from Bruskin *et al.* (1982). (c) SpG61. This sequence is present throughout early development, according to colony hybridization measurements (Flytzanis *et al.*, 1982; Lasky *et al.*, 1980). The accumulation of new transcripts was calculated according to eqn (4), using the values of k_s or k_s/k_d given in Table 3 (see Materials and Methods). To carry out this calculation, several assumptions were necessary, as detailed in Materials and Methods. Briefly, the calculation represents the accumulation of new cytoplasmic RNAs, assuming the same per cell entry rates obtained before 24 h as thereafter, even though these rates were not actually determined in the period before 24 h. However, there are so few cells early in development that the cleavage stage estimates are relatively insensitive to the value used for the entry rate constant (i.e. k_s). Essentially the same final values are obtained if accumulation is calculated from 15 h post-fertilization. To affect the conclusions, synthesis rates for the cleavage period would have had to be at least 10 times the blastula-gastrula rates, which is extremely unlikely.

prevalent only at later times, following the period of rapid cell division. Among these are certain of the sea urchin actin genes (Shott *et al.*, 1983). In such cases, transcriptional activation and/or a sharp increase in transcript stability must occur.

Calculations for the SpG61 sequence shown in Figure 4(c) demonstrate that maternal RNAs may persist far longer during development than has been recognized. It is clear that the rate of entry of newly synthesized molecules of this transcript is insufficient to account for its high concentration in the embryo. As late as the pluteus stage (72 h post-fertilization), over 90% of the cytoplasmic molecules of SpG61 poly(A) RNA are probably still maternal in origin. We may conclude that in the sea urchin embryo there is no one particular stage at which new transcripts suddenly replace all maternal transcripts. Diverse evidence, ranging from molecular observations to species hybrid and enucleation experiments, shows that for many gross characters, the embryo remains essentially a creature of the maternal constitution well into blastulation (reviewed by Davidson, 1976). On the other hand, in this organism transcription is fully active even in cleavage, and several specific examples demonstrate productive mRNA synthesis very early in development. Thus transcription of embryo histone

genes accounts for most histone mRNA soon after the 16-cell stage (Goustin & Wilt, 1981), and new embryo messages from at least two different actin genes appear during cleavage (Shott *et al.*, 1983). The examples considered in this paper range from replacement of maternal transcripts by early blastula, as for the SpG20 and SpG6 sequences, to almost the complete retention of maternal transcripts throughout embryogenesis.

The parameters that determine the level of expression of any given gene in the embryo are the amount and stability of the maternal RNA, and the cytoplasmic entry rate and stability of the newly synthesized embryo transcripts. The embryo apparently utilizes all of these parameters in solving the logistical problem presented by its requirement for each transcript species, and a variety of different solutions evidently coexist. The strategies thus far known can be summarized as follows.

(1) Appearance of a new transcript species not represented significantly in the maternal RNA. Several of the embryo actin mRNAs (Crain *et al.*, 1981; Merlino *et al.*, 1980; Scheller *et al.*, 1981; Shott *et al.*, 1983), the ectoderm-specific messages produced by the Spec genes (Bruskin *et al.*, 1981,1982), and the late histone genes (reviewed by Childs *et al.*, 1979) provide examples. However, only about 10% of embryo poly(A) RNA species appear for the first time after fertilization, as do these sequences. The vast majority of sea urchin embryo cytoplasmic poly(A) RNAs, or polysomal messages, are already represented in the RNA stored in the egg at fertilization (reviewed by Davidson *et al.*, 1982).

(2) Replacement of low abundance maternal sequences by similarly rare, unstable embryo transcripts of the same species. As shown here and by Galau *et al.* (1977), newly synthesized rare transcripts are in steady state by the blastula stage, if not earlier, and the steady state accounts for most if not all of the molecules of these transcripts present in the embryo. Within a few hours after the embryo has been divided into several hundred cells, new synthesis could easily provide all of the low abundance transcripts present. It follows that low abundance transcripts could be regulated by the embryo, either up or down, from early on in development, as indeed has been observed (e.g. Lev *et al.*, 1980; see Galau *et al.*, 1976).

(3) Replacement of prevalent maternal transcripts by embryo transcripts of the same species. The rate at which new transcripts enter the cytoplasm, and their survival, determine for each sequence when in development this replacement occurs. The concentration of newly synthesized transcripts that turn over in the cytoplasm may be controlled by variations in the entry and decay rates, in either direction, but the embryo can only regulate the number of newly synthesized stable transcripts upward. In a study of several hundred cloned, moderately prevalent embryo poly(A) RNA sequences, Flytzanis *et al.* (1982) showed that in fact the dominant pattern is increased in prevalence after gastrulation, and decreased earlier, presumably due to maternal RNA degradation.

(4) Persistence of abundant maternal transcripts well beyond gastrulation. The example of SpG61 illustrates this case. In this example, embryo dominance of the cytoplasmic RNA is not achieved at all before larval stages, even though measurable transcription indeed occurs during development. Sequences of this

nature might be found only among the high abundance maternal poly(A) RNAs. The embryo apparently has no control over the aggregate number of such transcripts until later in development, though of course the situation in any given cell type could be quite different.

(e) *Conservation of newly transcribed sequences*

The rate of heterogeneous nuclear RNA synthesis is 10 to 20 times the rate of flow of newly synthesized transcripts into the cytoplasm of sea urchin embryos (reviewed by Davidson, 1976). One interpretation proposed earlier (Davidson & Britten, 1979), is that there is a "kinetic gate" in the processing of nuclear RNAs beyond which a certain regulated fraction of molecules of each species never pass, depending on the cell type and the sequence. The remainder would simply be degraded in the nucleus. Our comparisons of transcription rate to cytoplasmic entry rate are inconsistent with this idea, though of course they concern only two sequences. Within the limited accuracy of the measurements, i.e. about a factor of two, we found that all the newly synthesized molecules of each of these species are transferred to the cytoplasm. That is, those regions of the precursors represented in the cDNA probe are conserved, at least for a large fraction of the molecules. On the other hand, the nuclear RNA of adult sea urchin cells contains sequences that are absent from the cytoplasm of those cells, but present in the polysomes of embryo cells (Wold *et al.*, 1978; Lev *et al.*, 1980). Similarly, the nuclear RNA of mammalian cells has been observed to contain nuclear transcripts that belong to a different set of sequences than any of those contributing to the cytoplasmic message populations of those cells (Salditt-Georgieff *et al.*, 1981; Saldatt-Georgieff & Darnell, 1982; Schibler *et al.*, 1983). Transcription of such nucleus-confined sequences could add significantly to the high ratio of nuclear RNA synthesis rates to cytoplasmic RNA entry rates, the remainder being attributable to the intervening sequences and other elements of true message precursors that are removed during processing. The implication of the results in Table 4, and a similar observation made on a series of cloned hamster cell sequences by Harpold *et al.* (1981), is that if a sequence is going to be processed, this step is not a rate limiting one in gene expression. Expression of some sequences could be regulated by a mechanism that controls whether processing occurs at all in a given cell type. No evidence has been obtained that either supports or refutes this possibility.

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