MOLECULAR STUDIES OF GENE EXPRESSION IN DEVELOPMENT OF THE SEA STAR PISASTER OCHRACEUS(BRANDT)

by

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Molecular Analysis of Gene Expression in Development of the Sea Star Pisaster ochraceus (Brandt)

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ABSTRACT

The objectives of this study were to assess the informational content in the egg RNA from Pisaster ochraceus and to determine the gene type and abundance of actin transcripts during embryonic development.

The sequence complexity of egg RNA was measured by RNA excess hybridization to single-copy ^{125}I-DNA. 5.9% of the reactable single-copy DNA mass is present in DNA-RNA hybrids. Assuming asymmetric transcription, this represents 11.8% of the genomic single-copy complexity, or about 4.3×10^7 nucleotides of diverse sequence. The hybridization reaction is driven by 2-3% of the maternal RNA mass. Each single-copy transcript is represented approximately 1.7×10^4 times per egg.

Genomic and cDNA recombinant libraries were prepared and screened for actin specific sequences. Three major classes of genomic actin clones have been identified by restriction enzyme mapping and localization of actin coding regions. There are at most five non-allelic actin genes in the sea star genome. Southern blots of restriction enzyme digested genomic DNA from individual sea stars probed with actin sequences indicate extensive polymorphism in actin gene regions. Distinct actin clones were isolated from cDNA libraries. The genes from which actin transcripts originate have been identified by hybridization with 3' untranslated regions from each of the cDNA plasmids. 3' untranslated regions hybridized with genomic DNA indicate three putative alleles of each actin gene.

Coding and 3' non-coding sequences of actin subclones were used to measure actin transcript abundance in development using RNA transfer and dot-blot analyses. Hybridization of single-stranded probes with total and poly(A)$^+$ RNA determined the average number of actin transcripts per embryo as 3.1×10^5 per egg, 1.6×10^5 per 21 h blastula, 1.3×10^5 per 48 h gastrula and 1.8×10^5 per 72 h gastrula. These experiments show differential actin gene expression.

Purple, yellow, stars

Found you in the deep blue sea

Reveal your secrets

ACKNOWLEDGMENT

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A. GENERAL INTRODUCTION

The requirements for control of gene expression in early embryonic development are quite different from that of a terminally differentiated system. The development of a multicellular organism from an apparently simple looking egg involves the coordination of a large number of variables. To understand the developmental phenomena, it is necessary to know how the coordinate utilization of information occurs in a developmental system. In this study the mechanism of storage and stage specific utilization of informational macromolecules are investigated in sea star early development.

Investigations of echinoderm embryos established that there is a maternal program in the egg which determines the early pattern of development (reviewed by Davidson, 1976; Raff, 1980; Davidson et al., 1982a; Jeffery and Raff, 1983). Interspecies hybrid experiments conducted since the beginning of this century have shown that development follows the maternal pattern until the blastula stage. These observations were confirmed by analysing the patterns of embryonic protein synthesis (Tufaro and Brandhorst, 1982). The spermatozoon's contribution to the zygote is only the paternal haploid genome, therefore without the preformed maternal transcription and translation machinery the initiation of embryonic development would not be possible. Eggs are similar to storage houses with large quantities of

materials ready to begin development. Both protein and RNA are stored during oogenesis.

Initial evidence for the existence of maternal RNA came from experiments with chemically treated embryos. Inhibition of transcription with actinomyocin D established that there is a sufficient amount of RNA stored in the sea urchin egg to provide the templates required until the time of hatching (reviewed by Raff, 1980).

At fertilization the rate of protein synthesis increases dramatically which was shown to be due to utilization of the maternal RNA (Epel, 1967; Humphreys, 1969; 1971).

There are indications that a large fraction of the maternal RNA is mRNA, which is readily usable by the embryo. RNA extracted from sea urchin and Xenopus eggs were template active in translation systems (reviewed by Davidson, 1976). This RNA also shows typical messenger RNA size with a mean length of about 2000 nucleotides, primarily single-copy sequence and extensive poly(A) tails.

Alternatively, a considerable part of the maternal RNA has approximately 1000 nucleotides longer length, as well as being translated very poorly in a reticulocyte cell free system (reviewed by Thomas et al., 1981). The discovery of a large number of repetitive sequences on maternal RNA (Constantini et al., 1978; 1980) indicated that this RNA is still unprocessed, or has functions other than protein synthesis. It was also observed that maternal RNA contains unusual multiple

oligo(A) (Duncan and Humphreys, 1981) and oligo(U) sequences (Duncan and Humphreys, 1983). The role of these unique sequences are implied to have some regulatory or processing function (Thomas et al., 1981; Davidson, 1982b). Alternatively, Raff (1983) suggests, that these peculiar transcripts might represent incomplete processing and solely relics of oogenetic events.

Transcripts synthesized during oogenesis exist as masked RNP particles in eggs (reviewed by Raff, 1980; Jeffery and Raff, 1983). Fertilization in sea urchins begins a cascade of events (Epel, 1980) which includes a dramatic increase in protein synthesis (Humphreys, 1971) and polyadenylation of RNA (Wilt, 1973; 1977; Nemer, 1975; Dolecki et al., 1977). The controlled unmasking of the large amounts of maternal RNA provides the primary control over early development until the embryonic genome can supply sufficient quantities of transcripts (reviewed by Raff, 1980).

Transcription from the embryonic genome begins very early in development. Radioactive labeling of RNA proved that both new heterogeneous nuclear RNA and mRNA are synthesized in Xenopus as well as in sea urchin embryos (reviewed by Davidson, 1976). Kinetic determinations on newly synthesized RNA showed a very high rate of turnover. Paradoxically the embryonic transcripts appear to code for the same proteins as the maternal RNA and the embryonic transcription seems to make little qualitative change in the prevalent messenger RNA population (reviewed by Brandhorst et al., 1983).

Recent studies using specific cloned sequences confirmed that the dominant pattern in sea urchin embryogenesis is the replacement of maternal transcripts with embryonic transcripts around the time of gastrulation (Flytzanis et al., 1982). However, Cabrera et al. (1984) found that individual transcripts are replaced at different times and the parameters that determine the level of each transcript vary widely. The most important factors that control the transcript abundance are the amount and stability of the maternal RNA as well as the cytoplasmic entry rate and stability of the newly synthesized transcripts.

In the sea urchin embryo all the complex events associated with the reorganization of maternal components and new transcripts occur within a closed system. The turnover of proteins and specific RNA transcripts are high but the mass of the protein and RNA in the embryo remains essentially constant prior to feeding (reviewed by Davidson et al., 1982a).

Although the control of maternal RNA translation is not understood, it was demonstrated for specific sequences in mollusc (Rosenthal et al., 1980), sea star (Rosenthal et al., 1982) and in sea urchin (Cabrera et al., 1984). The role of maternal RNA in the determination of embryonic cell fates through its association with specific cytoskeletal domains was shown in ascidian development (Jeffery, 1983).

Maternal RNA plays a major part in early development, therefore its study is of fundamental interest. The complexity

of this stored RNA could indicate an initial transcript requirement at the beginning of development. The informational content was shown to be sufficient to encode approximately 20000 proteins (reviewed by Davidson, 1976). This complexity is surprisingly similar for a wide variety of species and bears no close correlation with genome size (Hough-Evans et al., 1980) or total egg RNA mass (Thomas et al., 1981). Conversely the concentration of transcripts per unit volume of egg cytoplasm is fairly constant (Kovesdi and Smith, 1982). There is clear evidence that a large fraction of maternal RNA is mature mRNA (Wilt, 1977; Brandhorst, 1976). The complexity of RNA decreases during development (Galau et al., 1976) but at the 16-cell cleavage stage approximately 73% of the maternal RNA complexity is still represented in polysomes (Hough-Evans et al., 1977). It is important to realize that about 90% of the polysomal RNA in Strongylocentrotus purpuratus gastrula is represented by prevalent messages that contain only 10% of the complexity (Galau et al., 1974; Lasky et al., 1980). The abundance of these transcipts suggests a central functional importance in early ontogeny. Therefore their study might lead to a greater understanding of the relationships between the expression of genes and developmental morphology.

The study of specific genes may ascertain the fate of maternal coding sequences and determine the onset of embryonic gene expression. Some of the most studied genes are present as multiple copies in the genome. These copies are related either

structurally or functionally to each other forming a gene family. The study of these families offer unique insights into gene structure, gene regulation and evolution.

The actin gene families have been very extensively examined in several organisms including echinoderms. To study gene expression during sea star development actin was chosen for several reasons: It is ubiquitous in eukaryotes and is highly conserved across phylogenetic lines. In addition to its role in muscle contraction, actin has been implicated in various cellular activities including cytokinesis, cytoskeletal structure and cell motility. The actin genes are members of a multigene family whose protein products and RNA intermediates are readily identifiable. It is therefore possible to determine the abundance and location of the gene products and to assess the possibility of differential gene expression during embryonic development.

A prerequisite for greater insight into the expression of specific sequences in development is a knowledge of their genomic organization. The structure of actin gene families has been reported from yeast (Ng and Abelson, 1980), Dictyostelium (Kindel and Firtel, 1978; McKeown et al., 1978; McKeown and Firtel, 1981), C. elegans (Files et al., 1983), Drosophila (Fyrberg et al., 1980; 1981; Tobin et al., 1980; Sanchez et al., 1983), S. purpuratus (Durica et al., 1980; Overbeek et al., 1981; Schuler and Keller, 1981; Scheller et al., 1981; Cooper and Crain, 1982; Schuler et al., 1983; Lee et al., 1984),

P. ochraceus (Kovesdi et al., 1984), chicken (Fornwald et al., 1982), rat (Nudel et al., 1982; 1983), human (Engel et al., 1981; Khalili et al., 1983), and soybean (Shah et al., 1982). The number of actin genes in these species vary between the two extremes of 1 for yeast and 17 for Dictyostelium. Most of these genes appear to be dispersed in the genomes, although there are a few linked genes which have been reported for C. elegans (Files et al., 1983) and S. purpuratus (Scheller et al., 1981; Schuler et al., 1983). Some actin genes have introns, but do not show the striking conservation observed in other gene families like the globins (Efstraditis et al., 1980).

Amino acid sequence studies have shown that actin is a highly conserved protein in evolution (Vanderkerchkov and Weber, 1978a; 1978b). Vertebrates exhibit characteristic amino acid sequence differences which allow for distinction between cytoplasmic and muscle actins (Vanderkerckhov and Weber, 1978b). All invertebrate actins analysed to date resemble vertebrate cytoplasmic actins (Vanderkerckhov and Weber, 1980; Fyrberg et al., 1981; Files et al., 1983), even though some of these are specifically expressed in muscle tissue (Fyrberg et al., 1983; Shott et al., 1984). Differential expression of identical actins in Dictyostelium (McKeown and Firtel, 1981) and the stage as well as tissue-specific expression of very similar actins in Drosophila (Zulauf et al., 1981; Sanchez et al., 1983; Fyrberg et al., 1983) incited speculations on actin gene evolution. Davidson et al. (1982a) hypothesized that in these animals

certain genes are probably producing specific actins as "they belong to muscle ontogenic regulatory module, rather than because the gene coding sequence specifies a muscle and not a cytoskeletal protein."

Actin gene expression has been extensively analysed in sea urchins (Crain et al., 1981; 1982; Merlino et al., 1981; Durica and Crain, 1982; Flytzanis et al., 1982; Crain and Bushman, 1983; Bushman and Crain, 1983; Garcia et al., 1984; Shott et al., 1984). The main conclusion of these studies is that most sea urchin eggs contain very little actin specific maternal RNA, and the transcript prevalence appears to increase approximately 50-fold from egg to gastrula. Therefore it is by no means insignificant that this study found the expression of actin genes to be very different in sea stars.

Echinoderm early development has been one of the most extensively studied systems at both the cellular and molecular levels (reviewed by Hinegardner, 1967; Davidson, 1976). For this study sea stars (Echinodermata: Asteroidea) were chosen as the experimental organisms. They present several distinct advantages for molecular investigations of early development. For example, they have small genome sizes (Hinegardner 1974; Smith and Boal, 1978), conservative early embryogeny, provide large quantities of eggs that develop synchronously and can be easily maintained (Fraser et al., 1981).

Oocyte maturation in sea stars can be readily studied, as oocytes are stored in the ovary arrested at the meiotic

prophase. Meiosis is reinitiated by a hormone produced in the follicle cells, which was identified as 1-methyladenine (1-MeAde) (Kanatani, 1969; 1975). Hormonal treatment causes the oocytes to mature and become fertilizable. The maturation process is correlated with extensive morphological changes involving actin polymerization (Schroeder and Stricker, 1983), derepression of protein synthesis (Houk and Epel, 1974), changes in RNA synthesis (Boylan et al., 1973) and in polyadenylation (Jeffery, 1977). Many of these changes appear to be similar to those observed with sea urchins at the time of fertilization (reviewed by Epel, 1980). In sea stars, fertilization does not cause further change in the pattern of protein synthesis (Rosenthal et al., 1982). The change in protein synthesis during oocyte maturation was shown to be the result of translational control over the cytoplasmic population of maternal RNA (Martindale and Brandhorst, 1984).

Molecular studies of development are enhanced by the availability of a very large number of sea star species. The range of phylogenetically related organisms with conservative macroscopic development allows us to look for correlations at the molecular level. It is assumed that if specific structures appear and behave similarly in closely related species, their control might also be conserved. By looking for the similarities, a pattern perhaps will be elucidated. Furthermore, comparisons of sea star and sea urchin systems could reveal evolutionarily important functions.

The overall objective of this thesis was to study the storage and developmental expression of RNA transcripts in sea stars.

The experimental approach followed was:

1. Assessment of the transcript informational content of P. ochraceus egg RNA and establishing basic data for this novel developmental system.

2. Application of a specific gene as a model in determining the fate of its transcripts in early sea star development.

The specific experimental goals were:

1. To ascertain the informational RNA content of P. ochraceus eggs using RNA driven hybridization kinetics.

2. To characterize the organization of actin genes in the P. ochraceus genome through the use of recombinant genomic and cDNA libraries.

3. To determine the number and type of actin genes through the use of gene specific probes in DNA transfer, dot-blot and hybridization kinetic experiments.

4. To evaluate the type and relative amounts of actin transcripts in various RNA populations through the use of gene specific cDNA probes.

5. To determine the actin transcript concentration in different RNA populations using both saturation and RNA driven hybridization, allowing for two independent estimates.

The format of this thesis follows the general outline of the experimental objectives described above. The first chapter

is concerned with the maternal RNA population of P. ochraceus in general. The remaining part of the thesis deals specifically with the actin multigene family. The second chapter focuses on the organization of actin genes, and the third chapter assesses the actin transcript concentration in early development and tube foot.

B. CHAPTER I

Sequence Complexity in the Maternal RNA of the Starfish *Pisaster ochraceus*(Brandt)

I. INTRODUCTION

The mature eggs of all eukaryotes contain a store of informational transcripts which are utilized in early development. The general significance of maternal RNA in a wide range of organisms has been reviewed (Davidson 1976). The complexity of the maternal RNA stored in eukaryote eggs is on the order of 10 million nucleotides and this value varies little among diverse eukaryotes (Hough-Evans et al., 1980). The most completely studied system in respect to the nature and fate of maternal transcripts is the sea urchin. For example, in Strongylocentrotus purpuratus, the mature egg contains 3.6×10^7 nucleotides of unique DNA transcript (Hough-Evans et al., 1977). The different RNA transcripts are found in a range of abundances (Lasky et al., 1980) and most of the sequence diversity is represented on RNA molecules which also contain a restricted range of repetitive sequences (Constantini et al., 1980). Sequences homologous to approximately 73% of maternal sequences are found in polysomal mRNA in early development, while as much as 56% of maternal sequence homologs are found in gastrula mRNA (Galau et al., 1976; Hough-Evans et al., 1977). Studies using cloned sea urchin maternal cDNA sequences indicate that the relative abundance of most maternal messages do not change radically during early development (Lasky et al., 1980). However there are notable exceptions which demonstrate a clear cut

transcriptional regulation, for example, actin gene transcripts (Crain et al., 1981; Scheller et al., 1981). It is apparent that the major event at fertilization is an increased utilization of stored maternal RNA rather than qualitative changes in RNA synthesis (Brandhorst, 1976; 1980).

There have been a number of molecular studies utilizing sea star eggs and embryos, but there has been no investigation to date of the sequence complexity and maternal RNA abundance in oocytes. The sequence organization in genomic DNA as well as the degree of sequence homology in the unique DNA of representative sea stars have been ascertained as a preliminary study to measures of transcriptional homology (Smith and Boal, 1978; Smith et al., 1980; Smith et al., 1982). The experiments in this section deal with the measurement of the amount and complexity of maternal RNA in the mature oocyte of Pisaster ochraceus (Brandt).

II. MATERIALS AND METHODS

Pisaster ochraceus (Brandt) were collected from the Indian Arm of Burrard Inlet, British Columbia, between February and June, 1980. This period encompasses the documented reproductive season of *P. ochraceus* (Fraser *et al.*, 1981).

RNA isolation

Mature oocytes were collected by injecting individual starfish with one to two mls per arm of 0.13 mM 1-methyladenine (1-MeAde). This compound induces both oocyte maturation and spawning in the starfish. The mature eggs were collected, passed through a triple layer of cheese cloth, and washed twice with millipore filtered seawater. The eggs were pelleted by centrifugation. Total egg RNA was isolated with guanidine-HCl according to Deeley *et al.*, (1977) and Chirgwin *et al.*, (1979). The size and integrity of extracted RNA was routinely analysed by isokinetic sucrose gradient sedimentation (Noll, 1967) or acid urea-agarose gel electrophoresis. RNA was sedimented in 15 to 30% sucrose gradients containing 10 mM Na-acetate, pH 5.0, 1 mM EDTA, and 10 μg ml^{-1} polyvinylsulfate for 20 h at 36,000 rpm in a Beckman SW40 rotor at 4°C. Parallel gradients containing *E. coli* ribosomal RNA were run as size standards. Alternatively, RNA samples were electrophoresed on 1.5% agarose slab gels

containing 6 M urea and 0.025 M citric acid, pH 3.5 (Woo et al., 1980). RNA samples were dissolved in urea-citrate loading buffer which also contained 20% sucrose and 0.005% Bromophenol Blue. The running buffer was 0.025 M citric acid, pH 3.5. Electrophoresis was for 4 hours at 100 V and 4°C with rapid recirculation of reservoir buffer. Gels were stained for 15 min in 1% Methylene Blue, 15% acetic acid solution, destained overnight in water and scanned at 600 nm using a Gilford 250 spectrophotometer.

Measurement of P. ochraceus egg RNA content

The RNA content of eggs was determined by three independent methods; by isotopic dilution, by direct spectrophotometric analysis of alkaline digests, and by orcinol colorimetric estimates of RNA content in egg homogenates and alkaline digests. In the isotope dilution method, a known amount of ^{3}H-uridine labeled E. coli RNA was mixed with the egg homogenate which was then extracted by the guanidine-HCl method. Based on recovered E. coli RNA cpms the extraction efficiency was 60%. Spectrophotometric estimates of egg RNA content were made on alkaline digestion extracts of measured numbers of eggs (Munro and Fleck, 1966). Between 97% and 100% of the RNA was extracted by 0.3 N KOH in 1 h at 37°C. This value was verified with E. coli ^{3}H-RNA extractions. Comparison of the UV absorption spectra of alkaline egg extracts with UV spectra of purified

Pisaster ochraceus RNA showed minimal contamination with proteins for which corrections were made by the method of Fleck and Begg (1965). Both homogenates and alkaline digests were assayed by the orcinol colorimetric test (Drury, 1948; Dische, 1955). Polysaccharides in the egg interfere with the orcinol RNA determination, therefore Drury's method of correction for glucose equivalent was used (Drury, 1948).

Preparation of polyadenylated RNA

Polyadenylated RNA was separated from rRNA by two cycles of binding to oligo(dT)-cellulose (Type T-2, Collaborative Research, Waltham, MA). The procedure of Aviv and Leder (1972) was followed as modified by Chirgwin et al., (1979). The binding buffer contained 0.5 M lithium chloride 0.2% SDS, and 10 mM triethanolamine hydrochloride, pH 7.4. Bound polyadenylated RNA was eluted with 10 mM triethanolamine hydrochloride, pH 7.4. RNA content of the eluate fractions was determined spectrophotometrically.

DNA isolation and preparation of single-copy ^{125}I-labeled DNA

DNA was isolated from ripe testes as described (Smith and Boal, 1978). The extracted DNA was sheared for 30 min in a Virtis 60K homogenizer (Britten et al., 1974). Single-copy DNA was isolated from total sheared DNA by initial denaturation and

reassociation to a Cot (M sec; moles of nucleotides per liter times seconds) greater than 10,000. The double-strand DNA (95%) was collected by hydroxyapatite chromatography, denatured, and reassociated a second time to Cot 1,000. The unreassociated single-copy DNA was collected by hydroxyapatite chromatography, dialyzed, precipitated with ethanol, and subsequently iodinated with ^{125}I (Amersham Corp., carrier-free, 557 mCi ml^{-1}) by a modified Commerford reaction (Commerford, 1971). Single-copy DNA was prepared from genomic DNA that had been randomly sheared to either 800 bp or 400 bp. The 400 bp single-copy DNA was iodinated on two separate occasions. The average fragment length of the resulting ^{125}I-DNA preparations were 130 bp and 150 bp with specific activities of 4×10^7 cpm μg^{-1} and 1.4×10^7 cpm μg^{-1} respectively. The single-copy DNA isolated from randomly sheared 800 bp genomic DNA fragments was iodinated to give a ^{125}I-DNA with a fragment length of 300 bp and a specific activity of 1.6×10^7 cpm μg^{-1}. DNA fragment length was determined by isokinetic alkaline sucrose sedimentation (Studier, 1965; Noll, 1967). These fragment length values are minimum estimates since alkaline treatment of iodinated DNAs can result in some strand scission (Chan et al., 1976). ^{125}I-DNA activity was determined in a Nuclear Chicago Gamma counter at 68% efficiency.

Hybridization of single-copy ^{125}I-DNA with egg RNA

^{125}I-DNA was reacted with a mass excess (>2x10^4:1) of unlabeled oocyte RNA in 0.41 M PB (equimolor mono- and disodium phosphate buffer), 0.16% SDS, at 67°C, after denaturation for 1 to 2 min at 105°C. All RNA Cot (Rot) values were corrected for acceleration in reaction rate relative to the rate in 0.12 M PB at 60°C due to higher Na^+ concentration (Britten et al., 1974). A comparison of 0.12 M PB reaction rates with the 0.41 M PB rates (data not shown) justifies a rate correction factor of 5 for 0.41 M PB instead of the recently published 2.5 determined by Van Ness et al. (1979). A rate correction value 2.5 would double the observed hybridization rate constant and the amount of RNA driving the reaction.

The above contradicts results found in Chapter III, where a rate correction factor of 2.5 was applied. Similarily to Van Ness et al. (1979), Chapter III deals with homogeneous cloned DNA fragment probes. This chapter used a heterogeneous population of randomly sheared DNA as probes. Theoretically it is not clear as to how this difference could account for the discrepancy. Only further experimentation may resolve the exact nature of the problem.

Hybridization reactions were analysed by procedures described earlier (Galau et al., 1974). In short, the reaction mixture was divided, and half was used to measure the fraction of ^{125}I-DNA bound to hydroxyapatite as DNA-DNA duplex subsequent

to degradation of RNA-DNA hybrids by RNase treatment under low salt conditions. The second half was used to measure the total ^{125}I-DNA bound in DNA-DNA plus RNA-DNA duplex. The RNA-DNA hybrid content was calculated by subtracting the DNA-DNA duplex content from the total. Data were analysed by a least-squares fitting procedure (Pearson, et al., 1977).

Recovery of hybridized ^{125}I-DNA

^{125}I-DNA was recovered from egg RNA-DNA hybrids as described previously (Galau et al., 1974; Hough-Evans et al., 1980). Single-copy ^{125}I-DNA was incubated with excess egg RNA to Rot 11,000. The hybridization mixture was treated with 10 μg ml^{-1} Ribonuclease A in 0.24 M PB for 1 h at 4°C to hydrolyze unhybridized RNA, and extracted with chloroform:isoamyl alcohol (24:1), before chromatography on hydroxyapatite. The hybridized tracer was eluted with 0.5 M PB, 0.16% SDS, dialyzed into 0.3 M Na acetate, and co-precipitated with a mass excess of randomly sheared genomic DNA with which it was then reassociated.

III. RESULTS

RNA content of sea star eggs

The mature P. ochraceus egg contains 15 ng of RNA. Spectrophotometric and orcinol colorometric assays gave values of 15.1 ng (S.D. = 0.5; n = 5) and 14.9 ng (S.D. = 0.9; n = 7) respectively. These values were verified by an isotope dilution experiment which gave a value of 15 ng. The greatest proportion of the egg RNA is ribosomal.

Characteristics of single-copy ^{125}I-DNA

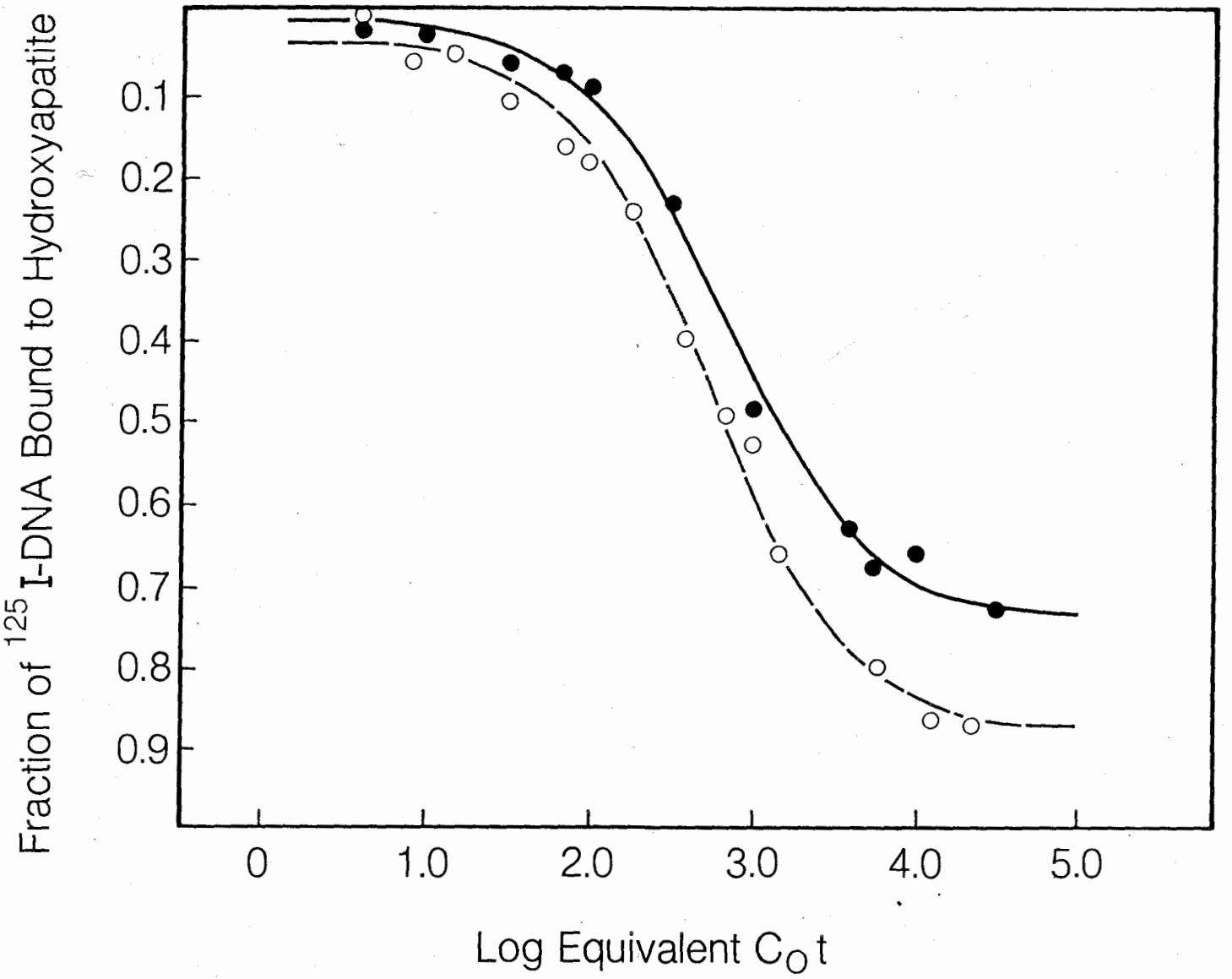
The haploid genome size of Pisaster ochraceus is 0.65 pg or about 6.0×10^8 bp (Smith and Boal, 1978). P. ochraceus DNA is organized in a short period or 'Xenopus' interspersion pattern (Smith and Boal, 1978). Reassociation kinetic analyses reveal that about 50% of the 300 nucleotide DNA fragments contain only single-copy sequences. S1 nuclease estimates indicate that at most one-third of the nucleotides are found in repetitive sequences. Kinetic estimates of genomic, unique DNA content, based on hydroxyapatite chromatography of randomly sheared DNA fragments, can result in an underestimate of the true single-copy sequence content. In this study 60% was used as a

conservative estimate for the single-copy sequence content of P. ochraceus genomic DNA. This is equivalent to about 3.6×10^8 bp.

To assay the kinetic homogeneity of the single-copy ^{125}I-DNA preparations, each was reassociated with an excess of total Pisaster DNA (Figure 1.1). The least squares solution for the reassociation of the single-copy DNA with total DNA give rate constants of 1.54×10^{-3} M^{-1} s^{-1}, 1.59×10^{-3} M^{-1} s^{-1} and 1.76×10^{-3} M^{-1} s^{-1}, with 74%, 61%, and 65% of the ^{125}I-DNA reacting respectively. In Figure 1.1 the reassociation of 130 bp ^{125}I-DNA with excess sheared genomic DNA is shown. There is no obvious repetitive DNA component in this reaction, nor that of the other two tracer preparations. The observed tracer reassociation rates are in good agreement with that expected for a 0.65 pg genome size (Smith and Boal, 1978). Although the two shorter tracer preparations react somewhat slower than the third preparation, the difference is not significant. This result indicates that in this case a length correction which is linearly proportional to the tracer length (Chamberlin et al., 1978) is not necessary. The reactibility of each tracer was determined at the beginning and the end of the hybridization experiments. There was no detectable loss of tracer reactivity over that period.

Figure 1.1 Reaction of single-copy ^{125}I-DNA with excess genomic DNA.

All incubations were in 0.41 M PB at 67°C. Reassociation was monitored by binding to hydroxyapatite. Closed circles show data for the reassociation of isolated 130 nucleotide long single-copy ^{125}I-DNA with large excess of total 310 nucleotide long genomic DNA. The least square solution (solid curve) gives a second order reaction rate constant of 1.54×10^{-3} M^{-1} s^{-1}. Open circles show the reassociation of egg DNA (DNA isolated from DNA-RNA hybrids) with large excess of genomic DNA. The dashed curve is a least-squares fit to the data with a rate constant of 1.84×10^{-3} M^{-1} s^{-1}.


Fraction of ^{125}I-DNA Bound to Hydroxyapatite
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
1.0
2.0
3.0
4.0
5.0
Log Equivalent $C_O t$

Single-copy sequence content of Pisaster egg RNA

Hybridization of the single-copy DNA with excess Pisaster egg RNA is shown in Figure 1.2. Experiments were carried out with at least 2 x 10^4 RNA mass excess as described in Materials and Methods. The hybridization data have been normalized at each point to the reactivity of the tracer. Data were fit with a single pseudo-first order hybridization function by a least squares fitting program (Pearson et al., 1977). The results are listed in Table 1.1. The pooled hybridization data were best fit with a rate constant of 3.64 x 10^{-4} M^{-1} s^{-1}. At kinetic termination, 5.9% of the Pisaster single-copy tracer is hybridized by Pisaster egg RNA.

In these types of experiments there is always the possibility that a small amount of repetitive sequence might be present in the single-copy DNA tracer. A small amount of repetitive DNA transcript in the RNA population could introduce large errors in estimation of the hybridization saturation value. To determine whether the sequences reacting with the egg RNA are actually single-copy, oocyte RNA was incubated with single-copy ^{125}I-DNA to an RNA Cot of 11,000 and the DNA which was in an RNA-DNA hybrid was isolated. This tracer was reassociated with an excess of randomly sheared 310 bp genomic DNA fragments and the second-order reassociation rate constant for the tracer DNA was determined. This reaction is shown for

Figure 1.2 Hybridization of oocyte RNA to single-copy ^{125}I-DNA.

Hybrids were analysed by hydroxyapatite chromatography (see Materials and Methods). Two methods for presenting the data are shown: in Panel A, the data are plotted on a linear scale which best demonstrates hybridization termination values; in Panel B, the data are presented on a log scale which best demonstrates the reaction kinetics. The RNA used in this experiment was extracted from the eggs of nine animals. Three preparations of single-copy tracer were used as detailed above. These preparations are indicated by solid circles for the 130 nucleotide long, open circles, for the 150 nucleotide long and triangles for the 300 nucleotide long iodinated tracer. The solid line represents the best least square solution for the data according to the pseudo-first order equation D/Do = exp(-kRot), where D/Do is the fraction of ^{125}I-DNA remaining single-stranded at time t, Ro is the RNA concentration, and k is the pseudo-first order rate constant. The rate constant obtained for the combined data fit is 3.64×10^{-4} M^{-1} s^{-1}, and the terminal value is 5.9% of the ^{125}I-DNA in hybrid (corrected for tracer reactibility).

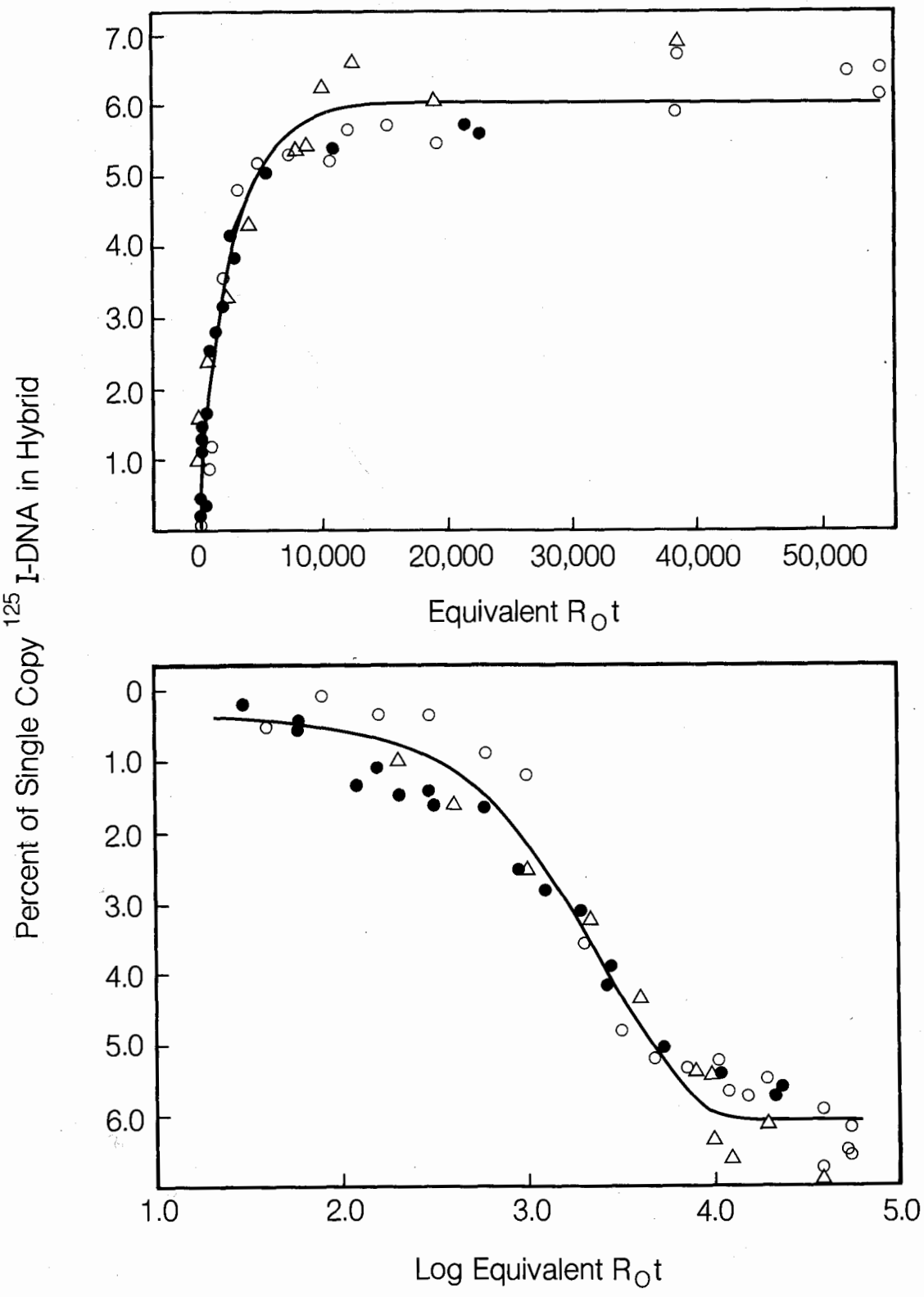

Percent of Single Copy ^{125}I-DNA in Hybrid
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
10,000
20,000
30,000
40,000
50,000
Equivalent $R_O t$
0
1.0
2.0
3.0
4.0
5.0
6.0
1.0
2.0
3.0
4.0
5.0
Log Equivalent $R_O t$

Table 1.1 Sequence complexity of Pisaster ochraceus mature oocyte RNA.

Single-copy ^{125}I-DNA fragment length (nucleotids)	Percent of ^{125}I-DNA hybridized ± SD[a]	Hybridization rate K obs (M^{-1} s^{-1})	RMS (%)	RNA complexity[b] (nucleotides)
130	5.5 ± 0.2	0.000464	0.3	4.0×10^7
150	6.0 ± 0.2	0.000379	0.4	4.3×10^7
300	6.2 ± 0.2	0.000248	0.3	4.5×10^7
Mean	5.9 ± 0.1	0.000364	0.4	4.3×10^7

a) The extent of hybridization was corrected for ^{125}I-DNA reactivity. SD signifies the standard deviation from the least square solution and not the errors in the experimental data.

b) Complexity (C RNA) = (Fraction of single-copy sequence hybridized x 2) x (3.6×10^8), where 3.6×10^8 bp is the complexity of single-copy DNA from Pisaster ochraceus (Smith and Boal, 1978).

the 130 bp ^{125}I-DNA in Figure 1.1 (open circles). Least squares analyses give a second-order rate constant of 1.84×10^{-3} M^{-1} s^{-1} for this reaction, a value which is close to the expected rate constant for the single-copy sequence in the Pisaster ochraceus genome. Figure 1.1 shows that 88% of the hybrid recovered ^{125}I-DNA tracer reassociated with total genomic DNA.

These data were submitted to a second least squares analysis to determine if the ^{125}I-DNA could have also contained a small fraction of slow repetitive sequences. The maximum fraction of the ^{125}I-DNA which could be assigned to repetitive frequencies is 10%. The data indicate therefore that a minimum of 90% of the hybridized DNA is single-copy sequence.

Sequence complexity and transcript prevalence in P. ochraceus egg RNA

At hybridization saturation (Rot>10,000), 5.9% of the single-copy ^{125}I-DNA hybridized to the oocyte RNA (Figure 1.2). Assuming asymmetric transcription this means that 11.8% of the single-copy DNA in P. ochraceus is represented in the egg RNA which corresponds to 4.3×10^7 nucleotides of RNA coding sequences or 2.2×10^4 diverse RNA molecules of average length of 2,000 nucleotides (Table 1.1). The minimum estimate of complexity is calculated at 3.8×10^7 nucleotides if 10% of the hybridized tracer is repetitive sequence.

As noted, the greatest proportion of the egg RNA mass is ribosomal RNA. The contribution of ribosomal RNA to the total complexity of egg RNA is very low. The fraction of the RNA mass driving the RNA excess hybridization can be calculated from the measured RNA complexity and the rate constant of hybridization (reviewed in Davidson, 1976).[1] In our calculations no tracer length correction was used for the two short preparations as discussed above. The driver RNA length was estimated by assuming that the average length of polyadenylated RNA is a representative of the entire hybridizing RNA population. Polyadenlyated RNA was isolated from an aliquot of egg RNA by passage over oligo(dT)-cellulose. The average fragment length of poly(A)$^+$ RNA was determined by denaturing 1.5% urea-agarose gel electrophoresis to be 1250 nucleotides. The pseudo-first-order hybridization constant expected at a final complexity of 4.3 x 10^7 nucleotides with a driver RNA length of 1250 nt is 1.36 x 10^{-2} M^{-1} s^{-1}. The ratio of the observed rate (K obs) to the expected hybridization rate (K exp) demonstrates that the

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[1] A pseudo-first order rate constant can be predicted for a RNA population of a known complexity by the relationship (Galau et al., 1977): K exp = (5374 x 200)/C RNA x (LT/300) x $(360/LD)^{0.5}$ where 5374 is the complexity of ΦX174 RNA (Sanger et al., 1977a), 200 M^{-1} s^{-1} is the pseudo-first order rate constant for an RNA excess hybridization between 360 nucleotide (+) strand ΦX174 RNA and 300 nucleotide tracer RF DNA (Galau et al., 1977). The correction for the tracer length difference is (LT/300), and the correction for the driver length difference is $(360/LD)^{0.5}$ (Chamberlin et al., 1978). LD is the average length of the P. ochraceus driver RNA (1250 nt) and LT is the tracer DNA length used in the calculation (300 nt). The fraction of the RNA that is driving the reaction (the most complex RNA in the population) is calculated from the ratio of K obs to K exp.

fraction of the egg RNA mass driving the reaction is 2.7%. The above calculation, using E. coli rather than ΦX174 as a rate and complexity standard (Galau et al., 1974; Hough et al., 1975) indicates that a lower fraction, 2%, of the egg RNA mass is driving the hybridization. In conclusion, the most complex RNA in the population comprises approximately 2-3% of the oocyte RNA or about 14,000-20,000 copies (average = 17,000) of each unique sequence per egg (Table 1.2).

It can be observed from the data presented in Figure 1.2 that the reaction proceeds somewhat faster at low Rot values than would be expected for this type of hybridization. This effect could be due to a minor slow repetitive contamination of the tracer which was discussed above. It should be pointed out that the driving RNA is neither homogeneous in its length nor in individual sequence abundance. In the sea urchin the prevalent RNA class contains approximately 10% of the total complexity of the maternal RNA (Lasky et al., 1980), but comprises probably 50-60% of the mass of RNA which is capable of reacting with single-copy DNA sequences. The effect of this prevalent RNA class on the overall hybridization reaction would be two-fold. The reaction will begin faster and later proceed somewhat slower than would be expected for complex RNA sequences which are homogeneous in abundance. A two component least-square fit analysis to the data shows that 0.6% of the 5.9% saturation value could be assigned to a prevalent class of sequences (data not shown). This means that this class would have approximately

Table 1.2 Complexity Characteristics of Mature Oocyte RNA

Species	Egg diameter[a] (μm)	Egg RNA (ng)	RNA complexity ($\times 10^7$ nucleotides)	Unique transcript as percentage of egg RNA mass[b]	Average transcript frequency per egg[c]	Transcript concentration (pM)
Pisaster ochraceus	163	15	4.3	2–3	1.7×10^4	12.4
Strongylocentrotus purpuratus[d]	80	3.3	3.7	0.5–1.5	1.6×10^3	9.9
Arbacia punctulata[d]	79	2.3	3.0	1–2	1.4×10^3	9.0
Lytechinus pictus[f]	111	1.8	3.1	2	2.0×10^3	4.6
Tripneustes gratilla[g]	90	1.8	3.4	2	1.9×10^3	8.1
Urechis caupo[d]	130	14	4.7	0.2	1.6×10^3	2.3
Xenopus laevis[d]	1350	4000	4.0	1	1.8×10^6	2.3
Drosophila melanogaster[h]	500 × 200	190	1.2	0.2	4.5×10^4	4.8
Musca domestica[h]	1000 × 400	1200	2.5	1	7.6×10^5	10.0

Footnotes Table 1.2

Table reproduced from Kovesdi and Smith (1982).

a) Data from Czihak (1975) and Wilt and Wessells (1967). For the purpose of volume calculations D. melanogaster and M. domestica eggs were considered to be simple cylinders of the lengths and diameters given.

b) The fraction of the RNA that is driving the hybridization reaction is discussed in the text.

c) The average number of copies of each single-copy sequence in the egg is calculated from the measured mass of unique transcript and the complexity of that RNA.

d) Data from Davidson (1976).

e) Calculated from Whitely (1949).

f) Data are derived from the poly(A)$^+$ RNA sequence analysis of Wilt (1977). 2% of egg RNA mass was assumed to be unique transcript. If the amount of RNA per egg is 3.9 ng (Brandhorst, 1980) the average transcript frequency per egg would be 4.3×10^3 and the transcript concentration would be 10.0 pM.

g) Cited in Hough-Evans et al. (1980).

h) Data from Hough-Evans et al. (1980).

4.3×10^6 nucleotide complexity compared with the 3.82×10^7 nucleotide complexity of the complex class. The other effect of the abundant RNA on the complex class is a reduction of the observed rate constant to 3.24×10^{-4} M^{-1} s^{-1} indicating that 2.1% of the complex class drives the reaction. As this reduced number is still within the precision of the one component analysis, the presence of a prevalent class of RNA will not change the basic conclusions.

IV. DISCUSSION

The oocytes of sea stars undergo meiotic maturation preceding fertilization in response to 1-MeAde stimulation (Kanatani, 1969). Houk and Epel (1974) showed that the hormonal stimulation results in a partial synthetic derepression of the egg. The resulting marked increase in protein synthesis is not affected by subsequent fertilization. At the same time, the number of poly(A)$^+$ RNA present in the egg increase threefold (Jeffery, 1977). Jeffery (1977) argues that during 1-MeAde induced meiosis polyadenylation of newly-synthesized mRNA, rather than maternal mRNA, is observed. If this is the case, our complexity measures would include this newly synthesized RNA as well as maternal RNA transcripts since the spawning induction method used here was 1-MeAde stimulation.

Kiper (1979) suggested that single-copy DNA prepared from randomly sheared fragments by hydroxyapatite chromatography could be preferentially enriched for coding sequences and therefore produce excessive saturation values in RNA-DNA hybridization experiments. He proposed that calculations of single-copy sequence content should be based on the fraction of unique DNA resolvable in reassociation kinetics rather than the amount which is estimated in interspersion analysis. Accordingly, our 300 bp unique ^{125}I-DNA which was prepared from a 800 bp single-copy fraction, should contain a significantly

greater proportion of expressed single-copy complexity than our shorter tracer DNA preparations. However, the termination of the RNA excess hybridization reactions for each of the tracer preparations was not significantly different (Figure 1.2;Table 1.1) indicating that the single-copy tracers were neither enriched for coding sequences nor had lost complexity.

The complexity of the P. ochraceus oocyte RNA is approximately 4.3×10^7 nucleotides, which is very similar to values found in a large variety of species (Table 1.2). This complexity represents a vast amount of information (about 10 times the genome size of E. coli) and if it functions as stored maternal message, it could code for 22,000 diverse proteins of average size. At gastrula stage in sea urchin embryos about half of the complexity of maternal sequences is represented as polysomal mRNA and at the 16-cell cleavage stage about 73% of the maternal RNA complexity in sea urchin egg RNA can be recovered from polysomes (Hough-Evans et al., 1977). It is possible that not all the single-copy sequence complexity stored in eggs serves as future mRNAs, but in seas urchins, at least 70-80% represent sequences which are in active translation during early development.

The number of genes in Drosophila are estimated as approximately 5000 (reviewed by Watson, 1977) from a variety of cytological and biochemical data. The maternal RNA complexity could account for 6000 diverse proteins (Hough-Evans et al., 1980), indicating that most of the transcripts which the genome

could code for are probably represented in the egg. If this is a general phenomenon, then it would indicate that the number of protein coding genes in metazoans are between 5000 to 20000 genes regardless of genome size. Furthermore, all or most of the transcripts from these genes are present in the egg.

Listed in Table 1.2 are the maternal RNA egg complexities which have been determined for a number of species. These animals have egg RNA mass and egg volumes which differ more than 1,000 fold, but the amount of complexity stored in the maternal RNAs is essentially on the same order of magnitude. The major uncertainty in these values is caused by the determination of the amount of single-copy DNA in the genomes. Hough-Evans et al.(1980) pointed out that the egg RNA complexity is essentially independent of genome size. The amount of single-copy egg RNA transcript, that is, the fraction of the RNA that drives the reaction appears to range from 1 to 3%. Exceptions are Urechis caupo and Drosophila melanogaster which have significantly lower values. Besides the inherent uncertainty in the observed hybridization rate constants, other factors which make the comparison more difficult are the different experimental conditions and kinetic rate corrections used by various researchers. Despite these limitations it is still clear that the average number of copies of each sequence per egg varies greatly between species although the number of copies per microgram of total RNA fluctuates less. What is striking is that the concentration of transcripts per unit

volume of egg cytoplasm is fairly constant; the range between organisms for sequence concentration is only about five-fold (Table 1.2). Within this context it should be remembered that for example the S. purpuratus gastrula at 36 hours has about 600 cells, whereas P. ochraceus at 36 hours has about 2,000 cells per gastrula (Fraser et al., 1981). The number of transcripts of each sequence per cell, assuming that 50% of maternal RNA is functional at gastrula and that each cell has equal transcript representation, would be 1-2 for sea urchin and about 4 for sea star. It is more likely that, as in sea urchin micromeres (Rodgers and Gross, 1978; Ernst et al., 1980), the distribution of maternal transcripts among embryonic cells will not be uniform. In that case, the concentration of particular sequences in a given cell could be considerably higher or lower than the average values. These facts argue strongly for a functional significance for the rare complex class of maternal RNAs. The requirement for the vast diversity of relatively rare transcripts in early embryos is still unexplained.

C. CHAPTER II

Number of Actin Genes and Actin Gene Expression in Sea Stars

I. INTRODUCTION

The actin multigene family offers a unique opportunity for the study of gene regulation and evolution. The presence of multiple genes which are differentially expressed facilitates the intraspecific study of gene regulation at the DNA level. Multigene families also lend themselves to investigations of the mechanisms that control the duplication and persistence of multiple gene copies. The actin genes represent a highly conserved family of functional DNA sequences. The genomic organization, genomic frequency, intron number and position, and the 3' sequences adjacent to and transcribed with coding regions are certainly less well conserved between eukaryotes of remote phylogenetic relationship (Firtel, 1981;Davidson et al., 1982b)

The study of the origin and evolution of multiple genes can be enhanced by using animal systems which display a diversity of species of increasing phyletic divergence. The sea stars (Phylum, Echinodermata; Class, Asteroidea) are widely distributed and are represented by a large number of species. The total single-copy DNA homology between a number of Pacific sea star species was described and relative divergence times were proposed (Smith et al., 1982). The sequence complexity and maternal RNA abundance in oocytes of P. ochraceus was also determined (Kovesdi and Smith, 1982).

A study involving the structure and organization of actin genes has been undertaken in order to achieve a clearer understanding of the mechanisms which control multigene family evolution and transcriptional regulation. The experiments in this section deal with the number of actin genes in sea stars and the type of actin genes in Pisaster ochraceus. With this information on hand a detailed study of actin gene expression in development is possible.

II. MATERIALS AND METHODS

Construction and screening of P. ochraceus genomic library

Pisaster ochraceus genomic DNA was prepared from sperm as described by Blin and Stafford (1976). Total genomic DNA from several individual sea stars was partially digested with EcoRI restriction endonuclease, size selected from linear sucrose gradients, and cloned in the λ vector Charon 4 (Blattner et al., 1977) essentially as described by Maniatis et al. (1978). The Charon 4 library was initially screened (Benton and Davis, 1977) with a ^{32}P-labeled nick translated HindII excised fragment of the plasmid pSpG2. This plasmid contains 188 bp of 3' terminal coding region of the actin gene expressed in sea urchin gastrulae (gift of Dr. E.H. Davidson) (Scheller et al., 1981) Positive plaques were selected and screened a second and third time with a ^{32}P-labeled 1.8 kb HindIII fragment of plasmid pDMA2, a Drosophila genomic subclone (gift of Dr. N. Davidson) (Fyrberg et al., 1980) which contains all but 70 bp of the entire Drosophila actin coding region. Ten genomic equivalents of P. ochraceus DNA, calculated as described by Clark and Carbon (1976), were screened and gave 164 positive plaques. Actin positive phage clones were grown in the host E. coli K802. After lysis phage were isolated by polyethylene glycol precipitation

followed by isopycnic CsCl sedimentation in vertical gradients. DNA was isolated from phage by sodium dodecylsulfate phenol extraction and ethanol precipitation, or directly from CsCl using the formamide method of Davis et al. (1980).

Construction and screening of tube foot cDNA plasmid library

Tube feet were removed from adult P. ochraceus and total RNA was extracted by the guanidine-HCl method (Deeley et al., 1977). The ethanol precipitated RNA was twice passed over oligo(dT)-cellulose columns and bound poly(A)$^+$ RNA was collected (Aviv and Leder, 1972). Complementary DNA, cDNA, was synthesized with AMV reverse transcriptase (gift of J. Beard, Life Sciences Inc.) using oligo(dT) as primer. The resultant cDNA was treated with alkali to destroy the RNA, neutralized, and the second DNA strand was synthesized with E. coli polymerase I (Higuchi et al., 1976). The double-strand cDNA, dscDNA, was treated with S1 nuclease to clip hair-pin loops and passed over Biogel A-150m. The fractions containing dscDNA longer than 500 bp were collected. The dscDNA was tailed with dCTP and inserted into the PstI site of pBR322 (Bolivar et al., 1977) which had been extended with dGTP (C. Flytzanis, personal communication; Roychoudhury and Wu, 1980). E. coli MC1061 (Casadaban and Cohen, 1980) transformants were selected for tetracycline resistance and ampicillin sensitivity. The 627 transformants were screened with the actin coding sequence from the plasmid pDMA2. Five

recombinants containing actin coding sequence were obtained; i.e. slightly less than 1% of the transformants.

Construction and screening of λgt10 cDNA libraries

P. ochraceus embryos were grown as described by Fraser et al. (1980). Total RNA was extracted from eggs and gastrula(48 hr) by the CsCl centrifugation method (Glisin et al., 1974). RNA from tube foot, egg and gastrula was passed over oligo(dT)-cellulose columns and bound poly(A)$^+$ RNA was collected (Aviv and Leder, 1972). Complimentary DNA, cDNA, was synthesized with AMV reverse transcriptase (gift of J. Beard, Life Sciences Inc.) using oligo(dT) as primer. The resultant cDNA was heated to 95°C to separate it from the RNA, and the second DNA strand was synthesized with E. coli polymerase I (Higuchi et al., 1976). The double-strand cDNA, dscDNA, was treated wtih S1 nuclease to clip hair-pin loops. After methylation with EcoRI methylase the fragment ends were filled in with Klenow fragment and dNTPs. The dscDNA was ligated with kinased EcoRI linkers and extensively digested with EcoRI endonuclease. Short linker fragments were eliminated by repeated isopropanol precipitation at room temperature and the dscDNA was ligated with EcoRI cut λgt10 DNA (B. Wold personal communication). The packaged phage was plated on E. coli C600ΔHfl. 2000 phage from the tube foot,3000 from the egg and 6000 from the gastrula libraries were screened with the actin coding sequence from pPoTA9/650 (see

Subcloning below), yielding 5, 9 and 16 positive recombinants respectively; i.e. about 0.3% of the recombinant phage. Selected λgt10 clones, three from tube foot, three from egg, five from gastrula, were subcloned into the EcoRI site of pUC9 (Messing and Vieira, 1982).

Restriction endonuclease mapping

Genomic and plasmid DNA samples were restriction endonuclease mapped by a series of total and partial digestions using single or multiple combinations of enzymes. The restriction enzyme digestion conditions were those recommended by the suppliers. Restriction endonuclease digested clone DNAs were electrophoresed on agarose gels (McDonell et al., 1977) and transferred to nitrocellulose membranes by the method of Southern (1975) or by the method of Smith and Summers (1980). Filters were hybridized at 68°C in 4X SET (1X SET is 0.15 M NaCl, 30 mM Tris, pH 8.0, 2 mM EDTA), 0.1% sodium pyrophosphate, 0.1% sodium dodecylsulfate (SDS), 25 mM Na phosphate, pH 6.8, 5X Denhardt's solution (1X Denhardt's solution is 0.02% BSA, 0.02% Ficoll, 0.02% Polyvinylpyrrolidone) (Denhardt, 1966) and 50 μg ml^{-1} sheared denatured calf thymus DNA or in 5X SSPE (1X SSPE is 0.18 M NaCl, 10 mM Na phosphate, 1 mM EDTA, pH 7.4), 0.3% SDS, 5X Denhardt's solution. After hybridization the filters were washed, dried, and exposed to preflashed Kodak XAR-5 or BB1 film with intensifying screens at -70°C. Essentially the same method

was used for genome blots. Washing of the filters was done at moderate stringency (down to 1X SET 0.2% SDS at 68°C) or at high stringency (0.2X SET 0.2% SDS at 68°C). Restriction mapping of pPoTA5 and pPoTA9 clones with four nucleotide recognizing enzymes were done by the method of Smith and Birnstiel (1976).

DNA dot-blot hybridization

The number of actin genes in genomic DNA was assayed by dot-blot hybridization (Kafatos et al., 1979). DNA was serially diluted and denatured for 10 min at room temperature by adding 1 M NaOH to 0.33 M. The samples were neutralized by adding 2 M ammonium acetate to 1 M before application to nitrocellulose membranes. Nitrocellulose membranes were presoaked in 1 M ammonium acetate, and samples were applied using a BRL dot-blot manifold. Hybridization was done, as described for Southern transfers.

Subcloning

DNA fragments were subcloned into the plasmid vector pUC9 and grown in E. coli JM83 as described by Crouse et al. (1983). Eleven λgt10 actin specific clones were subcloned into the EcoRI site of pUC9 and restriction mapped (see Figure 2.3 below). Tube foot, egg and gastrula clones were named as pPoTA, pPoEA and pPoGA respectively.

Construction of actin coding region subclone pPoTA9/650: The tube foot cDNA clone pPoTA9 (Figure 2.2) was cut with HinfI and the 650 bp coding region fragment isolated. After blunt-ending with Klenow fragment and dNTPs it was ligated to EcoRI linkers and digested with EcoRI. DNA was precipitated with spermine (Hoopes and McClure, 1981) and ligated to EcoRI digested pUC9.

Construction of the 3' non-coding region subclone pPoTA5/460: F. Preugschat constructed this subclone by ligating the 460 bp HincII/PstI fragment of the pPoTA5 clone (Figure 2.2) with HincII/PstI cut pUC9.

Construction of the 3' non-coding region subclone pPoTA13/210: Digestion of pPoTA13 (Figure 2.3) with KpnI and BamHI left the 3' end of this clone attached to the pUC9 vector. Using Klenow fragment and dNTPs the fragments were blunt-ended, and religated recreating a BamHI site. This clone was called pPoTA13/440. The 440 bp insert was excised by EcoRI/BamHI digestion and partially digested with HinfI. The 260 bp EcoRI/HinfI fragment was isolated and the EcoRI ends were ligated to an EcoRI/HincII digested pUC9. The HinfI end was blunt-ended with Klenow fragment and this linear construct was circularized by blunt-end ligation. This clone (pPoTA13/260) was initially used as a gene specific probe. However it was suspected to contain about 40 bp coding sequence which caused some cross reaction at moderate stringency washing conditions. To eliminate this problem a sequencing search was done for a

suitable restriction site outside the coding region where an MspI site was found. The 260 bp fragment was isolated from pPoTA13/260 by EcoRI/HindIII digestion and redigested with MspI. The 210 bp EcoRI/MspI fragment was ligated to EcoRI/AccI digested pUC9 producing the pPoTA13/210 clone.

Construction of the 3' gene specific region subclone pPoA137/630: The 3.9 Kb SalI/HindIII actin specific region of the λPoA137 genomic clone (Appendix A) was subcloned into pUC9 (by B. Ydenberg) and named pPoA137. The coding and 3' non-coding junction was further localized to the 1100 bp SstI/SalI fragment. The region was subcloned by cutting pPoA137 with HindIII/SstI and religating after blunt-ending with Klenow fragment and dNTPs. The 630 bp 3' SalI/PstI fragment of this clone (pPoA137/1100)(Figure 2.5) did not show any reaction with actin coding probes and was cloned into the SalI and PstI sites of pUC9, producing the subclone pPoA137/630 (Figure 2.5).

Subcloning into M13 single-stranded DNA phage

Cloning with the M13 phages M13mp9 and M13mp8 was essentially as described by Messing (1983) using E. coli JM103 as the host. The pPoTA9/650 actin coding and pPoTA13/210 3' non-coding fragments were cloned into M13mp9 and the pPoTA5/460 3' non-coding fragment was cloned into M13mp8. Fragments were simply excised from the pUC9 vector and recloned in the same restriction sites into M13. The orientation of the inserts was

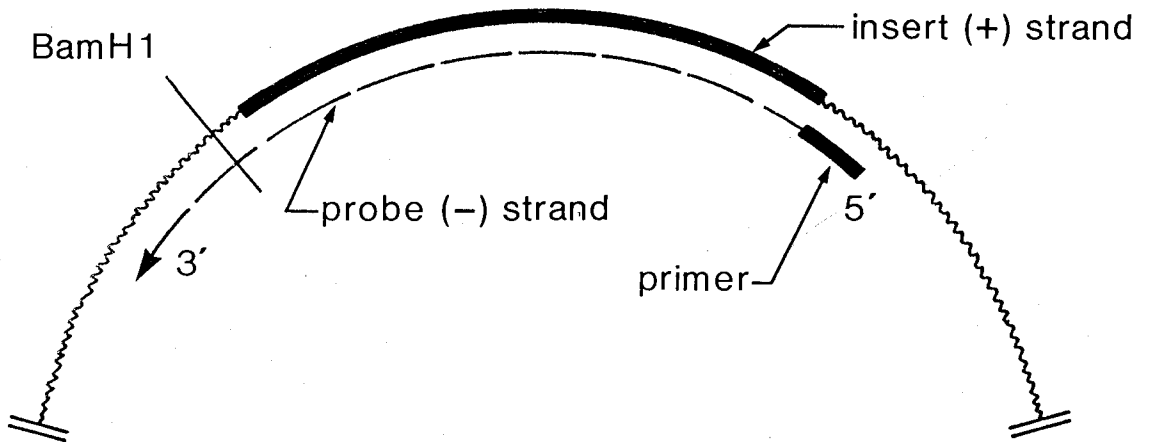
chosen so that the recombinant phages produced the template DNA strand that would serve as a probe complementary to RNA transcripts.

Preparation of single-stranded DNA Template

Preparation of the single-stranded probes were as described by Murre et al. (1984) and H. Jacobs (personal communication)(Figure 2.1). A universal 17 base long primer was annealed with 0.5 μg of the single-stranded M13 recombinant. The primer was extended by Klenow fragment using [α-^{32}P]dATP (800 Ci mmol^{-1}, Amersham) and unlabeled dTTP, dCTP and dGTP. Synthesis of the second strand completed a unique BamHI site downstream from the insert. After synthesis of the second strand, digestion with BamHI (PstI for pPoTA13/210; Chapter III) released a ^{32}P-labeled single-stranded copy of the insert. This labeled fragment was very different in size from the linear unlabeled template. The single-stranded labeled probe was purified on 3.5% denaturing acrylamide gel and recovered by the diffusion method of Maxam and Gilbert (1980). It was further purified from low molecular weight contaminants on a G-100 Sephadex column. Specific activity of the probes ranged from 1.2 x 10^8 to 2.5 x 10^9 cpm μg^{-1}.

Figure 2.1 Preparation of single-stranded probes using M13 phage.

Single-stranded probes were prepared as described in the Materials and Methods. The dashed line indicates the strand filled in with Klenow and ^{32}P-labeled dATP and dNTPs. The specific activity ranged from 1.2 x 10^8 cpm μg^{-1} to 2.5 x 10^9 cpm μg^{-1} depending on the DNA sequence and the amount of ^{32}P-dNTP used.



M13 Single-Strand Probe Preparation

DNA sequence analysis

The coding strand of the M13mp9 clone of pPoTA9/650 was sequenced from the conserved HinfI site (middle HinfI site in pPoTA9, Figure 2.2) by the dideoxy nucleotide sequencing method of Sanger et al. (1977b). The single-stranded template was annealed to a 17 nt universal primer and extended with Klenow fragment in the presence of [α-^{32}P]dATP and dideoxy nucleotide mixtures from P-L biochemicals. The reactions were analysed on a 8% denaturing urea gel.

The subclone pPoTA13/440 (described above) was sequenced by the method of Guo and Wu (1983). The plasmid first was cut with BamHI and then digested with exonuclease III (25 u, 1 h). The reaction was stopped by phenol and Sevag extraction. The DNA was filled back with Klenow fragment in the presence of [α-^{32}P]dATP and dideoxy mixtures from P-L biochemicals. The second restriction enzyme cut was done with HincII (5 u, 10 min. at 37°C), and the products analysed on a 8% denaturing urea gel. Therefore the coding strand, starting from the HincII site (Figure 2.3) was sequenced towards the 5' end of the clone.

Labeling of DNA probes

Nick translation: Large DNA probes were nick-translated (Maniatis et al., 1975) using [α-^{32}P]dNTPs to a specific activity of 5 x 10^7 to 2 x 10^8 cpm μg^{-1}. The reaction was stopped as described for exonuclease III labeling below.

End labeling: Small probes with 5' protruding ends were end-labeled by filling in with [α-^{32}P]dNTPs and Klenow fragment (Wartell and Resnikoff, 1980) to a specific activity of 5 x 10^7 to 1 x 10^8 cpm μg^{-1}. The reaction was stopped as described for exonuclease III labeling below.

Exonuclease III labeling: Most of the probes were labeled by this method (Guo and Wu, 1983). Approximately 0.1 μg insert DNA with 5' protruding ends were incubated at room temperature for 10 minutes with 5-10 u of exonuclease III. The reaction was stopped by heating the sample at 80°C for 10 minutes. Nucleotides were filled back with Klenow fragment, 30 μCi [α-^{32}P]dATP (800 Ci mmole^{-1}, Amersham) and each of the three cold dNTPs at room temperature for 20 minutes followed by a cold dATP chase for 5 minutes. The reaction was stopped by the addition of 5 μl 0.5 M EDTA, 5 μl 1 M Tris pH 8.0, 50 μl H_2O and 85 μl of phenol : Sevag (1:1) (saturated with 1 M Tris pH 8.0). Unincorporated nucleotides were removed by passage of the aqueous fraction through a 1.5 ml Sephadex G-25 column. The specific activity was 1 x 10^8 to 3 x 10^8 cpm μg^{-1}.

DNA reassociation kinetics

DNA was isolated from ripe testes as described by Smith and Boal (1978). The extracted DNA was sheared for 30 min in a Virtis 60K homogenizer (Britten et al., 1974). The average fragment length was determined on denaturing alkaline agarose gels (McDonell et al., 1977). ^{32}P-labeled single-stranded actin coding probe (pPoTA9/650) was reacted with a mass excess (>4 x 10^6 : 1; this is a greater than 20 fold actin sequence excess) of unlabeled genomic DNA in 0.41 M PB (equimolor mono- and disodium phosphate buffer) at 67°C. All Cot values were corrected for acceleration in reaction rate relative to the rate in 0.12 M PB at 60°C due to higher Na^+ concentration (Britten et al., 1974).

The fraction of the DNA in duplex bearing fragments was determined by hydroxyapatite chromatography at each Cot value as described in Chapter I. Reassociation kinetic data were analysed by a least-squares fitting procedure (Pearson, et al., 1977).

III. RESULTS

Genomic clones containing actin sequence

The primary screening of 10 genome equivalents of the *P. ochraceus* lambda library with ^{32}P-labeled pSpG2 insert gave 164 positive recombinants. Of these 34 gave particularly strong autoradiographic signals and were subjected to secondary and tertiary screening with ^{32}P-labeled pDMA2 insert. Eighteen clones were amplified finally and subjected to either EcoRI or EcoRI plus BamHI digestion. Five distinctive restriction patterns were identified and representative clones were restriction enzyme mapped by M. Stuerzl and F. Preugschat (Appendix A). Actin coding regions were demonstrated by blotting and hybridizing with ^{32}P-labeled actin coding sequence from pDMA2. It was further verified that these five lambda clones represented distinct genomic regions by Southern blotting EcoRI digests of the clones and hybridizing with ^{32}P-labeled *P. ochraceus* actin coding region from the tube foot cDNA clone pPoTA9. After restriction enzyme cleavage within the actin coding regions of the five genomic clones, the transcriptional directionality in the DNA segment was determined by Southern blot analysis using the 3' coding fragment from the sea urchin gastrula cDNA clone pSpG2 (Scheller *et al.*, 1981). The following

restriction enzymes were used in these analyses: EcoRI for λPoA145; EcoRI and SstI for λPoA128, λPoA68, and λPoA137; KpnI for λPoA160. The five genomic actin clones from P. ochraceus fall into three classes or categories. The first of these is exemplified by the clones λPoA145 and λPoA128. These two clones have a striking homology of EcoRI, KpnI, BamHI, and SstI sites (Appendix A). λPoA145 has an additional EcoRI site within the actin coding fragment bounded by KpnI and SstI sites. A second category of actin genomic clones is represented by λPoA68 and λPoA137. Here again there is a conservation of EcoRI and SstI sites. In addition there is a conserved HindIII site at the 5' limit of the actin positive region. Both of the above categories of actin gene regions contain a SstI site which appears to lie within the actin coding region. A third category or class of genomic clones is typified by λPoA160. This class of actin bearing recombinant is distinctive from both of the above classes in its distribution of restriction enzyme sites. It does not have internal EcoRI, BamHI, nor SstI sites (Appendix A).

Each of the five clones represents a distinct DNA region. Within the λPoA145,128 category, the restriction map 3' to the 3' SstI is unique in the individual clones. λPoA128 has a pair of KpnI sites and a HindIII site whereas λPoA145 is terminated by an EcoRI site. Also the λPoA145 recombinant has an EcoRI site which lies within the actin-containing fragment bounded by SstI and KpnI. λPoA68 and λPoA137, although closely related, also represent distinctive DNA regions. λPoA68 has a BamHI site 3' to

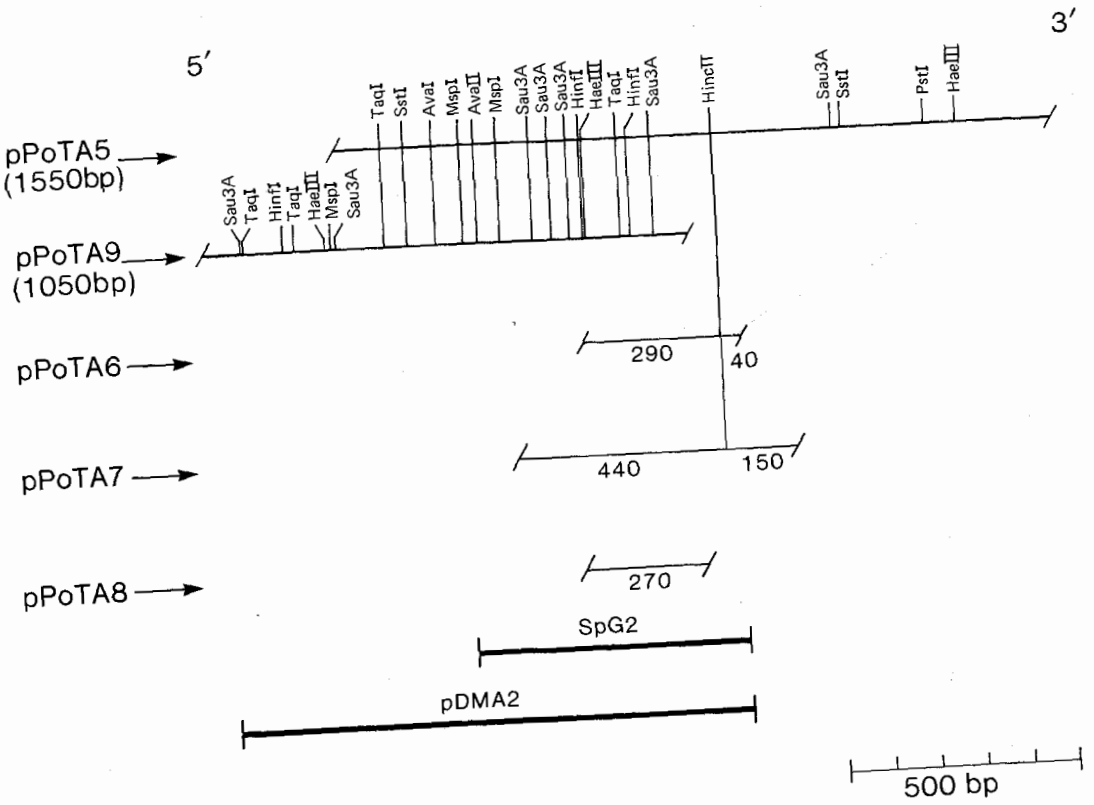
its coding region as shown in Appendix A. λPoA137 lacks this site but has a SalI site. As stated, 18 genomic clones were selected for examination. Of these, six, including λPoA128, are related as judged by restriction digestion with EcoRI, BamHI, or a combination of the two enzymes with subsequent Southern blot hybridization to actin coding sequence probes. All of these clones contain an 8.8 kb BamHI/EcoRI fragment which hybridizes with ^{32}P-labeled actin probes. Each of these genomic clones is 12.7 kb long except λPoA36 which is homologous to λPoA128 but has an additional 4.9 kb EcoRI fragment (not shown in Appendix A). In this category, λPoA145 is unique with its internal EcoRI site. There are 4 recombinants in the λPoA137 group based upon EcoRI/BamHI digestion and Southern blot hybridization. The related genomic clone λPoA68 is distinct and has no homologous members among the 18 isolates. The λPoA160 group has the largest representation among the screened recombinants. The seven members of this group are characterized by a lack of internal BamHI sites and are limited at 17.1 kb by EcoRI sites.

Tube foot cDNA Library

Tube foot actin cDNA clones were amplified and restriction endonuclease mapped with several enzymes (Figure 2.2). The 5' to 3' orientation of the inserted cDNA was determined by blotting restriction digested recombinants and hybridizing the filters with the 3' coding segment of the sea urchin actin cDNA clone

Figure 2.2 Restriction enzyme maps of sea star tube foot actin cDNA clones.

The five cDNA clone inserts are aligned where possible on common restriction enzyme sites. The solid bars at the bottom of the Figure indicate fragments resulting from AvaI plus HincII digestion which hybridize with the 3' actin sea urchin probe pSpG2 and the Drosophila total actin coding sequence probe pDMA2 respectively. Restriction fragments 3' to the HincII site of pPoTA5 do not contain actin coding sequence. The distance between the SstI sites of pPoTA5, 930 bp, is the same as that found in the genomic clones λPoA145 and λPoA128. The 460 bp 3' non-coding HincII/PstI fragment of pPoTA5 extends 3' from the HincII site to the internal PstI site. pPoTA5 and pPoTA9 are more extensively mapped to demonstrate their homology. Note that the SstI hexamer recognition site contains the AluI tetramer site. The following enzymes do not cut the inserts from pPoTA5 and pPoTA9: BamHI, BglI, BglII, EcoRI, HindIII, KpnI, PvuI, and SalI. Preliminary DNA sequence analysis of the 5' PstI/HinfI fragment of pPoTA9 indicates, in addition to 80 dC linker residues, amino acid codon homology with sea urchin actin from amino acid 114 (Cooper and Crain, 1982). The second HinfI site cuts the sequence at amino acid 363 which places the presumptive stop codon in the 3' direction from this site by 38 bp:


5′
3′
TaqI
SstI
AvaI
MspI
AvaII
MspI
Sau3A
Sau3A
Sau3A
HinfI
HaeIII
TaqI
HinfI
Sau3A
HincII
Sau3A
SstI
PstI
HaeIII
pPoTA5
(1550bp)
Sau3A
TaqI
HinfI
TaqI
HaeIII
MspI
Sau3A
pPoTA9
(1050bp)
pPoTA6
290
40
pPoTA7
440
150
pPoTA8
270
SpG2
pDMA2
500 bp

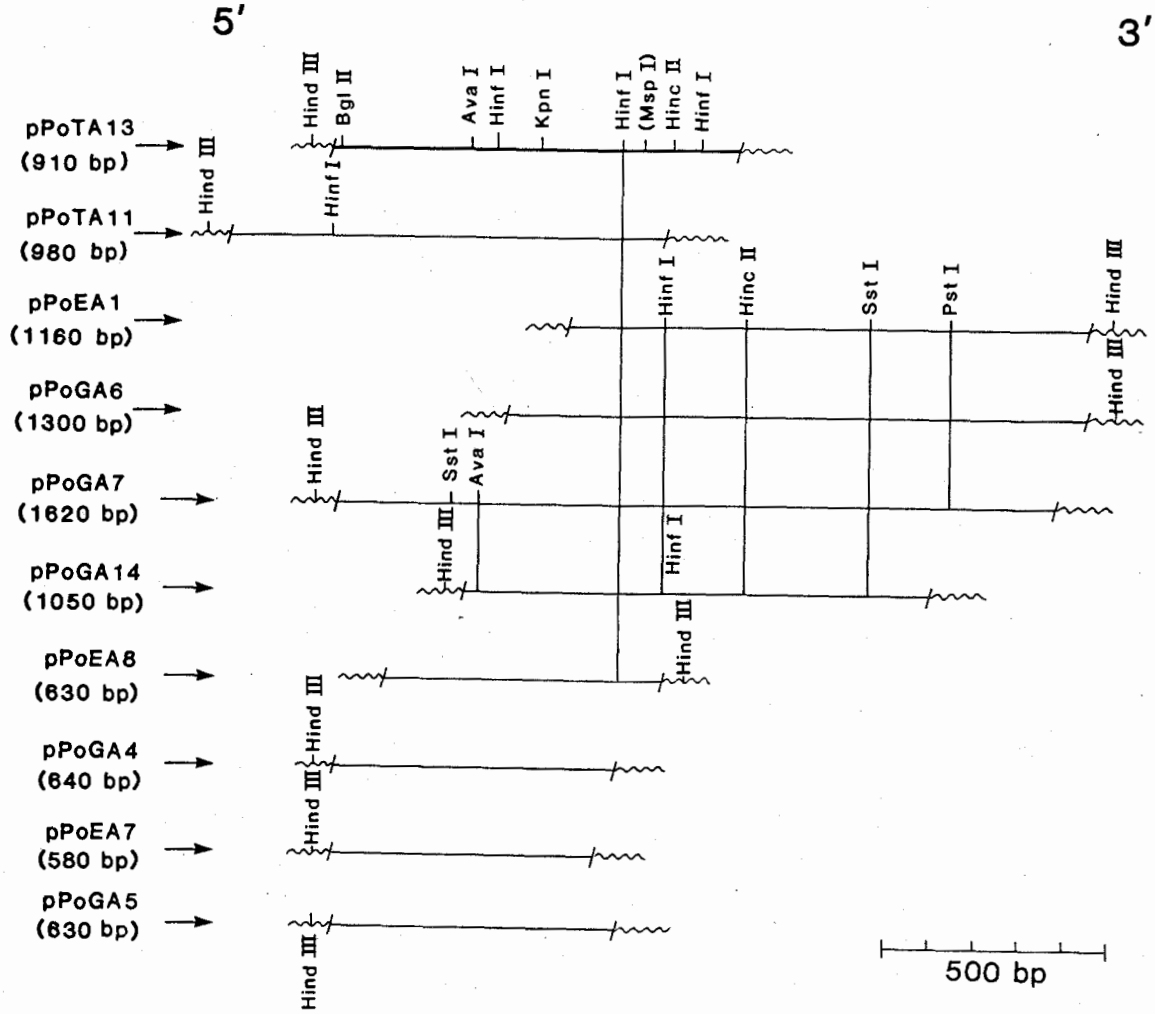
pSpG2. All five of the sea star actin cDNA clones hybridized with the sea urchin 3' actin clone. Three of the cDNA clones, pPoTA5, 6, and 7, contain 3' sequences that do not react with the sea urchin 3' pSpG2 probe. Two of the actin cDNA clones, pPoTA5 and pPoTA9, contain 5' regions that react with the actin coding sequence of pDMA2 but not with the 3' coding segment of the sea urchin pSpG2 clone. In addition both pPoTA5 and pPoTA9 contain a SstI site 50-60 bp 5' to an AvaI site. The plasmid pPoTA5 has a second SstI site in the region 3' to actin coding sequence. The distance between the SstI sites in this cDNA clone is 930 bp which corresponds to the length of the fragment limited by SstI sites in the genomic clones λPoA145 and λPoA128. To verify that the cDNA plasmids pPoTA5 and pPoTA9 were derived from transcripts of the same gene region, each was mapped with a large number of restriction enzymes. All of the enzyme sites in the overlapping regions of these plasmids are coincident (Figure 2.2). There are no sites unique to either plasmid from the common 3' Sau3A site to the 5' TaqI site of pPoTA5.

λgt10 cDNA libraries

The eleven subclones derived from the three λgt10 libraries were amplified and restriction endonuclease mapped with several enzymes (Figure 2.3). The tube foot clone pPoTA12 is not shown in this figure, as it is identical to pPoTA13, except that it is in the opposite orientation with regard to the pUC9 vector.

Figure 2.3 Restriction enzyme maps of sea star actin cDNA clones from tube foot, egg and gastrula.

Tube foot clones(pPoTA),egg clones(pPoEA) and gastrula(48 hr) clones(pPoGA) were aligned, where possible on the conserved HinfI site 38 bp 5' from the stop codon. All clones with the exception of pPoTA13 are similar to pPoTA5 and pPoTA9. The pPoTA13 is the only clone which contains a KpnI site in the coding region. The 210 bp 3' non-coding fragment of pPoTA13 extends from the MspI site to the 3' end of the clone. The MspI site was deduced from sequencing data. The 3' actin sea urchin probe pSpG2, the Drosophila total actin coding sequence probe pDMA2 and the 650 bp HinfI/HinfI coding region sequence from pPoTA9 (pPoTA9/650) were used to determine the 5'-3' orientation. The wavy lines denote pUC9 sequences with the HindIII site indicating the orientation of the vector. The length of inserts in bp is given in parentheses.


5′
3′
Hind III
Bgl II
Ava I
Hinf I
Kpn I
Hinf I
(Msp I)
Hinc II
Hinf I
pPoTA13
(910 bp)
Hind III
Hinf I
pPoTA11
(980 bp)
Hinf I
Hinc II
Sst I
Pst I
Hind III
pPoEA1
(1160 bp)
Hind III
pPoGA6
(1300 bp)
Hind III
Sst I
Ava I
pPoGA7
(1620 bp)
Hind III
Hinf I
pPoGA14
(1050 bp)
Hind III
pPoEA8
(630 bp)
Hind III
pPoGA4
(640 bp)
Hind III
pPoEA7
(580 bp)
pPoGA5
(630 bp)
Hind III
500 bp

The 5' to 3' orientation of the inserted cDNA was determined by blotting restriction digested recombinants and hybridizing the filters with the 3' coding segment of the sea urchin actin cDNA clone pSpG2. All eleven of the sea star actin cDNA clones hybridized with the sea urchin 3' actin clone. Three of the cDNA clones, pPoGA4, 5 and pPoEA7 appeared to contain only coding sequences and their 5' to 3' direction was not determined. Clones pPoTA11, pPoEA7, 8, pPoGA4, 5, were not mapped with AvaI and SstI. All clones with the exception of pPoTA12 and 13 are similar to pPoTA5 and pPoTA9. The only two restriction sites which seem to be conserved between the two types of actin cDNA clones are one of the HinfI sites and possibly the AvaI site (Figure 2.3). The partial nucleotide sequence of pPoTA13 and pPoTA9 (Figure 2.4) demonstrates that these HinfI sites are indeed the same. Clones pPoTA12 and 13 also contain a BglII and a KpnI site. The only genomic clone which has a KpnI site in its coding region is λPoA160 (Appendix A).

DNA sequence analysis

The two tube foot cDNA clones pPoTA9 and pPoTA13 were partially sequenced (Figure 2.4), and compared with pSpG17 a sea urchin genomic clone (Cooper and Crain, 1982). The 40 nucleotides sequenced from the coding region of pPoTA9 show only 1 nucleotide change compared to pSpG17 and 2 compared to pPoTA13.

Figure 2.4 Nucleotide sequence comparision of sea urchin and sea star actin clones.

Partial sequence of the tube foot cDNA clones pPoTA13 and pPoTA9 is compared to the sea urchin genomic clone pSpG17 (Cooper and Crain, 1982). The 650 bp HinfI/HinfI fragment from pPoTA9 was cloned into M13mp9 and analysed by the dideoxy sequencing method of Sanger et al.(1977b). The dideoxy sequencing method of Guo and Wu (1983) was used on pPoTA13 as described in the Materials and Methods. Homogenity between the sequences is indicated by stars(*). The restriction enzyme sites refer to the pPoTA13 sequence.

```
a.a.#        335                 340                 345                 350
a.a.         lys tyr ser val trp ile gly gly ser ile leu ala ser leu ser thr phe gln gln met
pSpG17       *** *** *** *** *** *** *** *** *** *** *** *** *** **C *** *** *** *** *** ***
pPoTA13      AAA TAC TCC GTA TGG ATC GGA GGC TCC ATC CTT GCC TCT CTG TCC ACC TTC CAA CAG ATG
pPoTA9                                                                   *** *** *** ***
R.enzyme                      Sau3A
```

```
a.a.#        355                 360                 365                 370             374
a.a.         trp ile ser lys gln glu try asp glu ser gly pro ser ile val his arg lys cyc phe
pSpG17       *** *** *** *** *** *** *** **T *** **C *** *** **C *** *** *** A*G **G *** ***
pPoTA13      TGG ATC AGC AAG CAG GAA TAC GAC GAG TCT GGC CCA TCG ATC GTC CAC CGC AAA TGC TTC
pPoTA9       *** *** *** *** *** **G *** **T *** *
R.enzyme       Sau3A                          HinfI  HaeIII ClaI,TaqI,Sau3A,PvuI
```

```
             stop
pSpG17       *** CAACTC*C*T TT*TTCTACA **T*TTGAGC ACAA*AACGA *C*C**G*G* TGGG*A*A** G*TCG
pPoTA13      TAA ACGTGTGTTG CCGGAAATAT TCAGACTGTA TTTTTTTTTC AATTATTGTG CATTTTATTG TGCAT
R.enzyme        SacIII      MspI                              EcoRI*
```

The 123 nucleotides sequenced from the coding region of pPoTA13 show 7 nucleotide changes compared to pSpG17. None of these changes caused any amino acid replacements. The 65 nucleotide 3' non-coding sequence of pPoTA13 is 48% thymine and indicates no homology to the pSpG17 sequence in the region assessed.

Dot-blot hybridization

The number of genomic sequences homologous to the pPoTA9 actin coding sequence and to the non-coding 3' fragments was determined by dot-blot hybridization. Denatured genomic DNA was serially diluted and applied to a nitrocellulose filter. Denatured unlabeled pPoTA9 insert or 3' fragments from pPoTA5/460 (Figure 2.2), pPoTA13/210 (Figure 2.3) and pPoA137/630 (Figure 2.5) were serially diluted and each applied to the nitrocellulose filter in separate lanes. The filters were hybridized with ^{32}P-labeled inserts from the appropriate clones and exposed to preflashed X-ray film. The results of the experiment are shown in Figure 2.6. Visual inspection and densitometric scanning of the autoradiographs demonstrates that the actin sequences homologous to pPoTA9 insert are present approximately 5 times per haploid genome. Autoradiographs of filters probed with the 460 bp and 210 bp fragments indicate that these sequences are present approximately once per haploid genome. Filters probed with the 630 bp fragment indicate approximately two copies for this sequence.

Figure 2.5 Restriction enzyme map of sea star genomic actin subclone pPoA137/1100.

The 3' end of the coding region and sequences adjacent to it were subcloned from the genomic clone λPoA137. Sequences 3' from the PstI site did not react with the coding region probe pPoTA9/650. The 630 bp PstI/SalI fragment was subcloned and used as a gene specific probe.

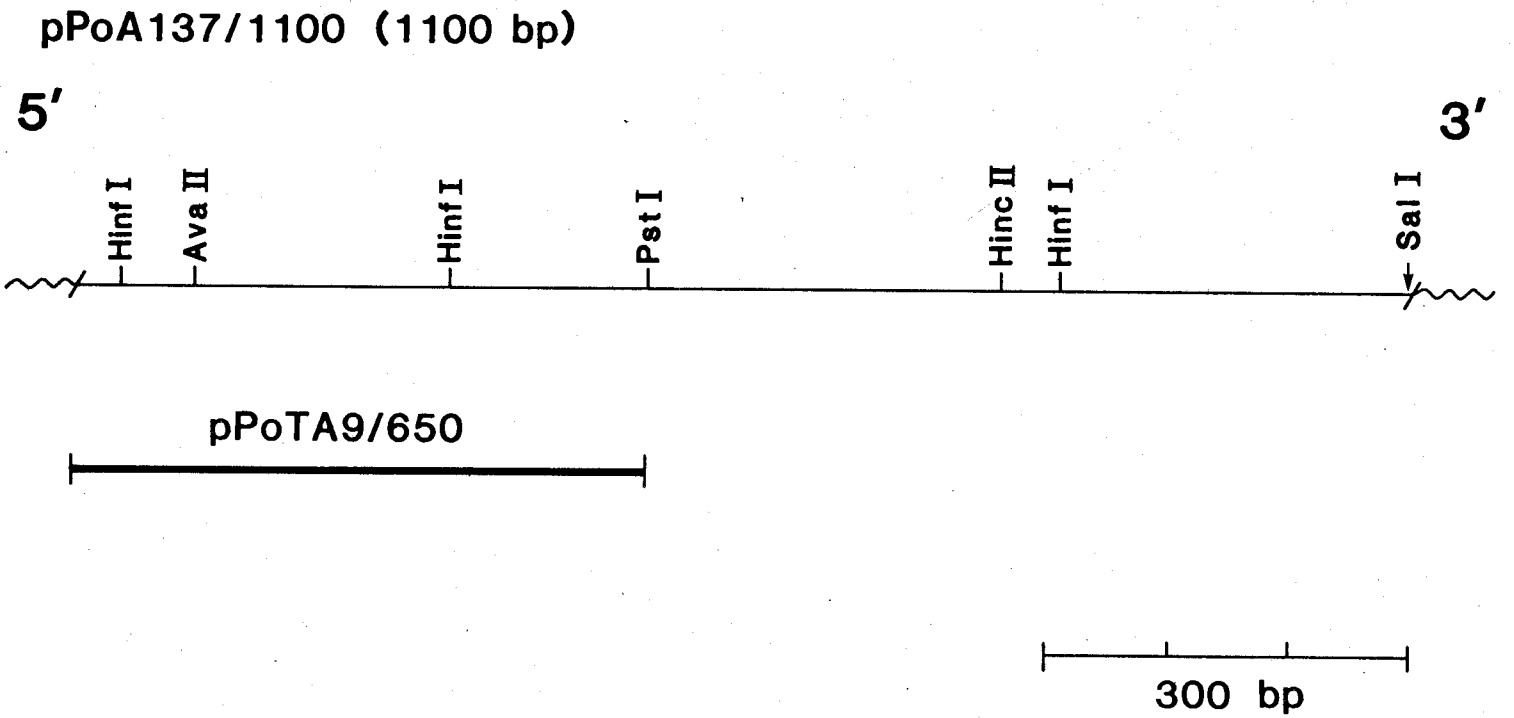
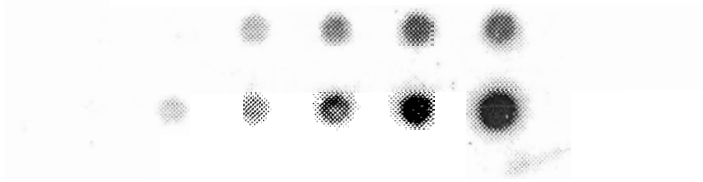

pPoA137/1100 (1100 bp)
5′
3′
Hinf I
Ava II
Hinf I
Pst I
Hinc II
Hinf I
Sal I
pPoTA9/650
300 bp

Figure 2.6 Dot-blot hybridization of actin coding and gene specific probes with genomic DNA.

Actin coding sequence probe was prepared from pPoTA9 insert which had been excised from the vector with PstI and restricted with AvaII. The AvaII fragments were end-filled with ^{32}P-dNTPs using Klenow fragment. The preparation of the gene specific clones are described in Materials and Methods. The fragments were isolated and end-filled with ^{32}P-dNTPs and Klenow fragment. The probe specific activities were approximately 1 x 10^8 cpm μg^{-1}. DNA extracted from the sperm of a single individual was serially diluted to mass amounts equivalent to approximately 5 x 10^5 to 16 x 10^6 haploid genomes, upper row of panels. The 1050 bp actin coding insert from pPoTA9 was excised by PstI digestion. Mass amounts of the insert equivalent to 2.7 x 10^6 to 87 x 10^6 copies of the actin sequence were dot-blotted, lower row of first panel. The 460 bp non-coding region of the tube foot cDNA pPoTA5 was prepared by digestion with HincII and PstI. Mass amounts of the 460 bp insert equivalent to 3.1 x 10^6 to 100 x 10^6 were dot-blotted on nitrocellulose filters, lower row of second panel. The 210 bp non-coding region of the tube foot cDNA pPoTA13 was prepared by digestion with EcoRI and HindIII. The 630 bp non-coding region of the genomic clone λPoA137 was prepared by digestion with BamHI and HindIII. Both of these fragments were isolated from agarose gels and mass amounts of insert equivalent to 5 x 10^5-16 x 10^6 were dot-blotted on

nitrocellulose filters, lower row of third and fourth panels respectively. The filters were hybridized with the appropriate ^{32}P-labeled probe, dried, and exposed to preflashed Kodak XAR-5 or BB1 film. The intensity of the autoradiographic signal indicates that there are approximately five actin genes, one 460 bp, one 210 bp and two 630 bp homologous sequences per haploid genome.

0.5 1 2 4 8 16 NO. GENOMES x10^6



2.7 5.5 11 22 44 87 NO. ACTIN SEQUENCES x10^6

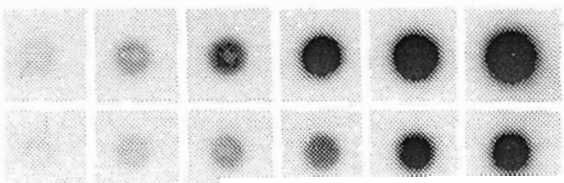
0.5 1 2 4 8 16 NO. GENOMES x10^6

3.1 6.3 12.5 25 50 100 NO. 460 bp FRAGMENTS x10^6

0.5 1 2 4 8 16 NO. GENOMES x10^6

0.5 1 2 4 8 16 NO. 210 bp FRAGMENTS x10^6

0.5 1 2 4 8 16 NO. GENOMES x10^6



0.5 1 2 4 8 16 NO. 630 bp FRAGMENTS x10^6

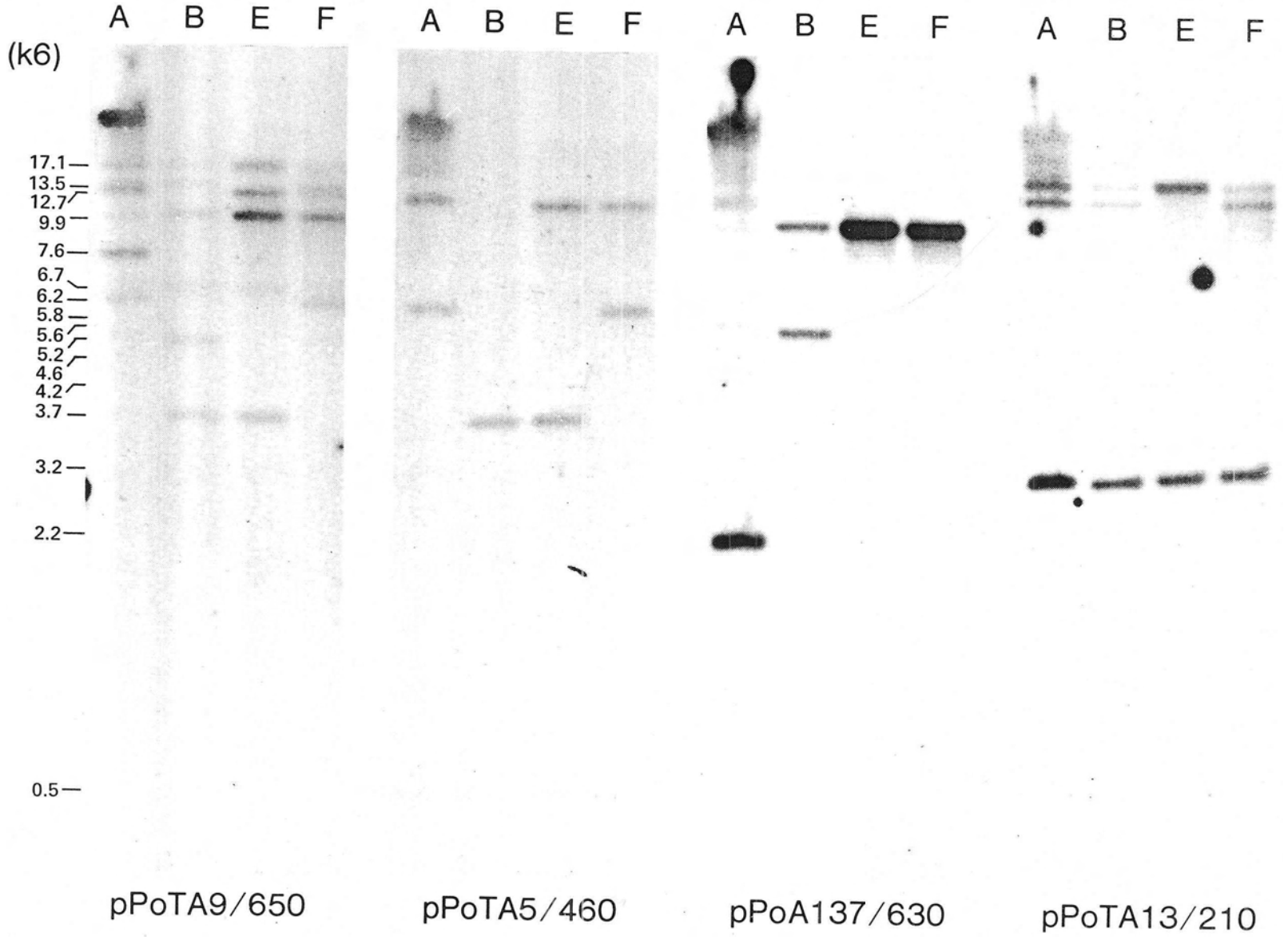
Polymorphism and gene number in sea stars

In order to assess actin gene number and polymorphism in actin gene regions, genomic DNA was isolated from individual sea stars. The DNA from each of these individuals was digested with EcoRI and electrophoresed in separate lanes of a 0.8% agarose gel in duplicate. Bidirectional blots of the gel were hybridized with ^{32}P-labeled actin coding segment from the tube foot cDNA clone pPoTA9/650 and with the three 3' probes pPoTA5/460, pPoTA13/210 and pPoA137/630. The results of this experiment are shown in Figure 2.7.

Between the four individuals there are 9 actin-containing EcoRI bands. Genomic DNA from an individual sea star has five or six actin sequence-containing EcoRI segments reacting with the pPoTA9/650 probe. Similar experiments done previously with complete insert of pPoTA9 (Kovesdi *et al.*, 1984) showed 11 actin sequence-containing EcoRI segments (Appendix B) at high stringency washing conditions. Moreover, when EcoRI digested DNA pooled from six individuals was probed with the actin coding region of pPoTA9 or pDMA2 at moderate stringency washing conditions, an additional strong band at 2.2 kb was observed. The total of these genomic digests demonstrate that there are at least 12 actin-containing EcoRI fragments in *P. ochraceus* genomes with fragment length of 17.1, 13.5, 12.7, 9.9, 7.6, 6.7, 6.2, 5.2, 4.6, 4.2, 3.7 and 2.2 kb.

Figure 2.7 Southern blot hybridization of EcoRI-digested genomic DNA from individual sea stars.

Genomic DNA from four individual sea stars was digested with EcoRI. Each DNA sample was incubated with a four-fold unit excess of EcoRI per μg^{-1} of DNA for four hours. The completeness of reaction was judged by the appearance of characteristic EcoRI bands visible in ethidium bromide stained agarose gels.(Although all samples appeared to be totally digested judging by this criteria, individual A did produce some smear on the gel.) The digested DNA (10 μg/lane) was electrophoresed in 0.8% agarose gel in duplicate and transferred bidirectionally to two pieces of nitrocellulose paper (Smith and Summers, 1980). The duplicate filters were cut and the four filters were hybridized with the indicated probes at 68°C in 5X SSPE for 24 hours. The probes were isolated fragments of pPoTA9/650, pPoTA5/460, pPoTA13/210 and pPoA137/630 prepared as described in Materials and Methods, and in Figure 2.6. The isolated fragments were labeled with ^{32}P-dNTPs using Exonuclease III and Klenow fragment to specific activities: 9×10^7 cpm μg^{-1}, 1.7×10^8 cpm μg^{-1}, 3.2×10^8 cpm μg^{-1} and 1.8×10^8 cpm μg^{-1} respectively. After hybridization, the filters were washed sequentially in 4X SET 0.2% SDS, 2X SET 0.2% SDS, 1X SET 0.2% SDS at 68°C with two 30 minute changes per salt condition. The filters were washed finally for 20 minutes with 0.2X SET 0.2% SDS at 68°C.


A B E F
A B E F
A B E F
A B E F
(k6)
17.1
13.5
12.7
9.9
7.6
6.7
6.2
5.8
5.6
5.2
4.6
4.2
3.7
3.2
2.2
0.5
pPoTA9/650
pPoTA5/460
pPoA137/630
pPoTA13/210

Hybridization of the genomic blot with the 3' cDNA probe pPoTA5/460 shows that there are at least three EcoRI fragments (12.7, 6.2 and 3.7 kb) containing regions homologous to the 460 bp fragment. An individual sea star however displays at most two such fragments. There are faint bands in all individuals at 0.5 kb, which were not noticed previously (Kovesdi et al., 1984) and are not represented in the coding sequence bands. These might be indicating another actin gene, but most probably represent unspecific hybridization or cross reaction to a different sequence.

Hybridization of the genomic blot with the 3' cDNA probe pPoTA13/210 shows that there are at least three EcoRI fragments containing regions homologous to the 210 bp fragment. Three out of the four individual sea stars display all three of the 17.1, 13.5 and 3.2 kb fragments, however, the 3.2 kb fragment is not represented in the coding sequence bands. This can be explained by cross reaction to a different gene or by an intron in the non-coding 3' region, which contains an EcoRI site.

Hybridization of the genomic blot with the 3' genomic probe pPoA137/630 shows that there are at least three EcoRI fragments (9.9, 5.6 and 2.2 kb) containing regions homologous to the 630 bp fragment. An individual sea star however displays only two such bands. The 5.6 and 2.2 kb bands are not represented by coding sequence bands displayed with pPoTA9/650 and their presence can be explained by cross reaction or introns as above for the 3.2 kb 210 bp specific band. It is worth noting, that

the 2.2 kb band appears with the pPoTA9 probe when moderate stringency is used.

Identification of the genomic origins of the 3' non-coding gene specific sequences

The five actin genomic clones were digested with EcoRI, blotted, and hybridized with the actin coding segment from the tube foot cDNA clone pPoTA9 and with the three 3'probes pPoTA5/460, pPoTA13/210 and pPoA137/630 as described in Figure 2.8.

In Figure 2.8A the results of the coding probe experiment are shown. Five out of the 12 actin-containing EcoRI genomic fragments can be assigned, on the basis of their length, to the actin-bearing genomic clones: the 17.1 kb band to λPoA160; the 12.7 kb band to λPoA128; the 9.9 kb band to λPoA68 and λPoA137; and both the 3.7 kb and 6.7 kb bands to λPoA145. However, the rest of the genomic bands are not represented in the selected recombinants.

To ascertain which of the genomic lambda clones represented the gene region from which the pPoTA5 tube foot actin transcript originated, the pPoTA5/460 probe was used. This region of the cDNA clone is 3' to the coding region and 5' to regions which would contain poly(dA/dT) and GC linker residues (Figure 2.2). The results of this experiment are shown in Figure 2.8B. It is clear that this 3' non-coding segment is derived from λPoA145 or

Figure 2.8 Blot hybridization of genomic clones.

A/ Blot hybridization of genomic clones with actin coding sequence from pPoTA9.

Five genomic lambda clones were digested with EcoRI, electrophoresed in a 0.8% agarose gel, and transferred to a nitrocellulose filter. The filter was hybridized with ^{32}P-labeled insert from the actin cDNA pPoTA9. The genomic clones λPoA128, λPoA145, and λPoA160 have distinctive blot patterns. λPoA68 and λPoA137 each contain a 9.9 kb actin positive fragment but differ in their restriction maps (Appendix A) The left panel is a photograph of the ethidium bromide stained gel of the EcoRI digests prior to Southern transfer. The right panel is the autoradiograph of the hybridized nitrocellulose filter.

B/ Southern blot hybridization of sea star genomic clones with pPoTA5/460 3' non-coding region.

Intense hybridization occurs with the 3.7 kb 3' terminal fragment from λPoA145 and with the 12.7 kb EcoRI fragment from λPoA128. When blots are given a final wash in 0.2X SET 0.2%SDS at 68°C the faint hybridization with the 9.9 kb fragment of λPoA68 and λPoA137 and with the 17.1 kb λPoA160 insert is no longer evident.

C/ Southern blot hybridization of sea star genomic clones with pPoA137/630 3' non-coding region.

Intense hybridization occurs only with the 9.9 kb fragment from

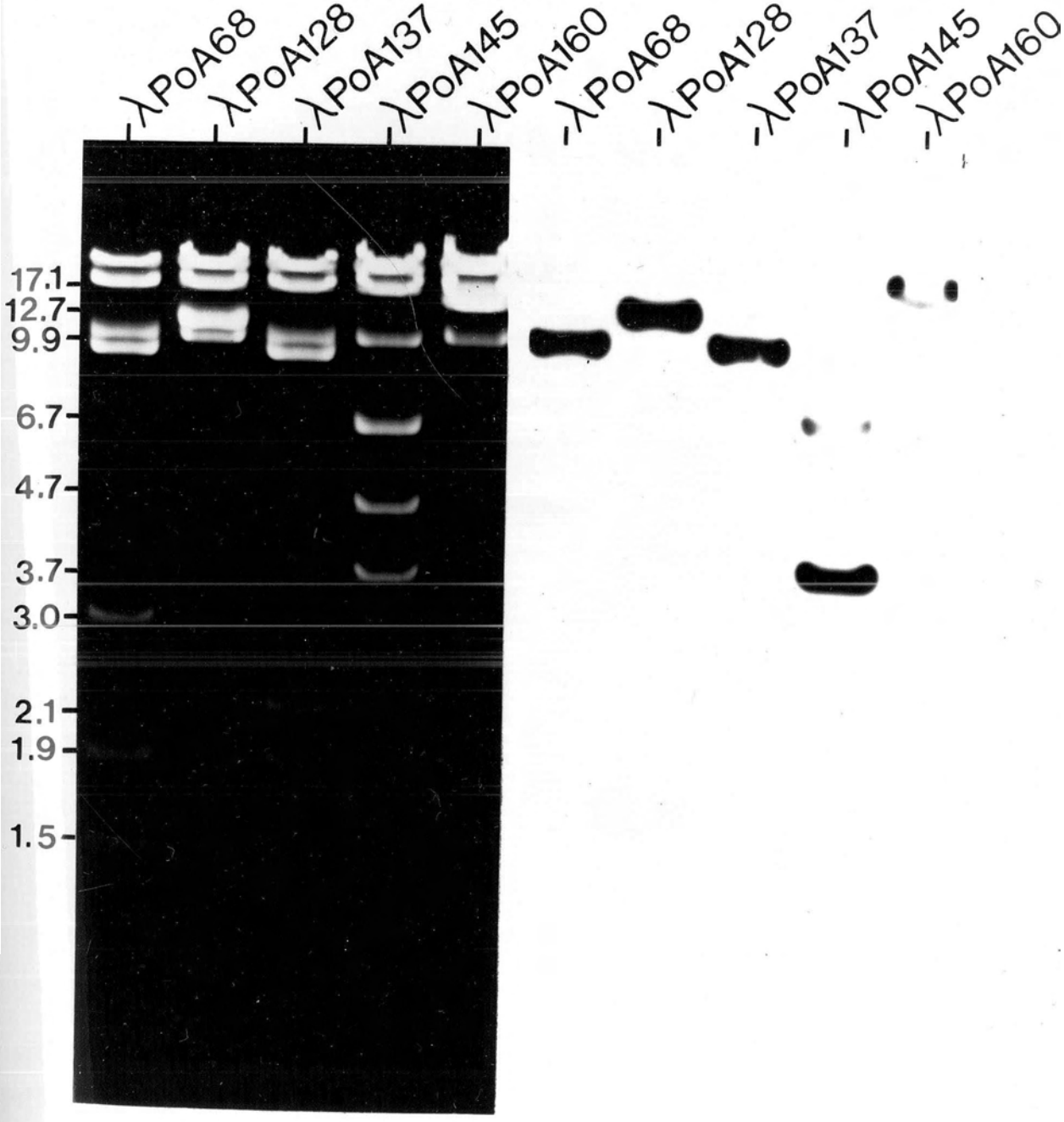
λPoA137 and λPoA68.

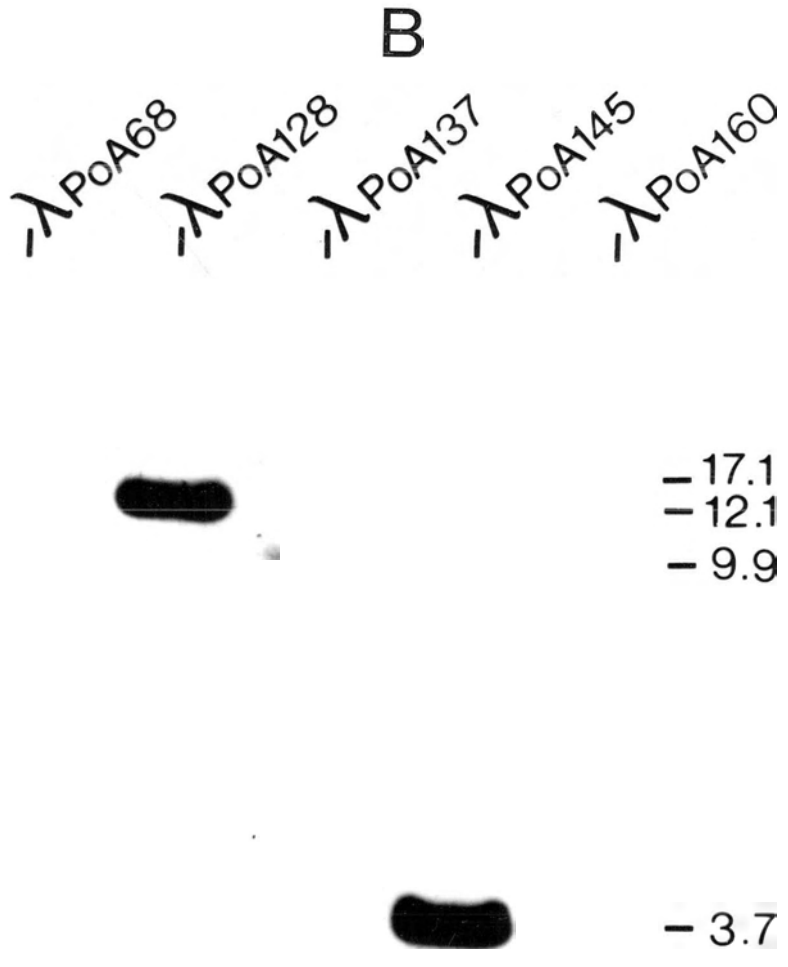
D/ Southern blot hybridization of sea star genomic clones with pPoA13/210 3' non-coding region.

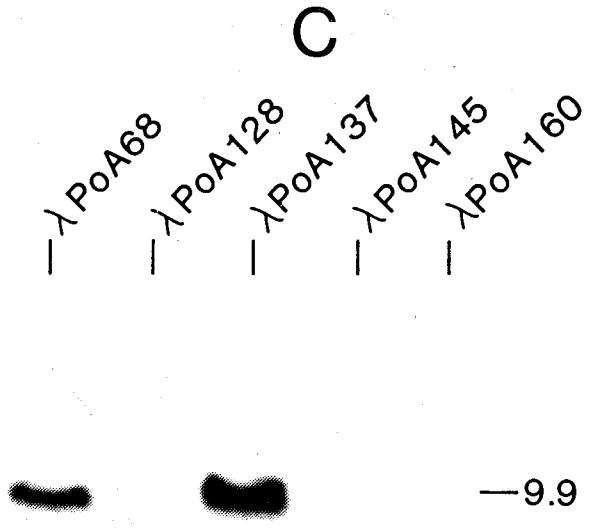
Intense hybridization occurs only with the 17.1 kb fragment from λPoA160.

All the blots were hybridized at 68°C in 5X SSPE and washed sequentially with decreasing salt concentration to 1X SET 0.2% SDS at 68°C.

A

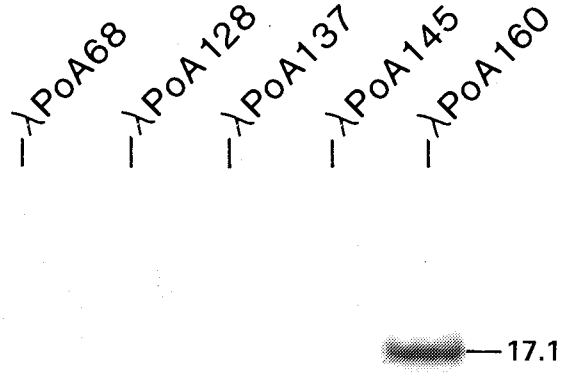

λPoA68
λPoA128
λPoA137
λPoA145
λPoA160
λPoA68
λPoA128
λPoA137
λPoA145
λPoA160
17.1
12.7
9.9
6.7
4.7
3.7
3.0
2.1
1.9
1.5


B
λPoA68
λPoA128
λPoA137
λPoA145
λPoA160
17.1
12.1
9.9
3.7



pPoA137/630

D



pPoTA13/210

λPoA128 gene regions. The intensity of the autoradiographic signal is highest in the 3.7 kb EcoRI fragment derived from λPoA145. The reaction with the 12.7 kb EcoRI fragment from λPoA128 is only slightly less intense. The 460 bp 3' fragment of pPoTA5 retains some homology to the clones λPoA68 and λPoA137 as well as just detectable hybridization with the 17.1 kb λPoA160 recombinant. The blots of the λ clones with the 460 bp 3' fragment were washed with 1X SET 0.2% SDS at 68°C for two hours. When blots are washed with 0.2X SET 0.2% SDS at 68°C for two hours, the homology of the 460 bp fragment with λPoA68, 137, and 160 is not apparent. The above analyses indicated that at least two gene regions represented in the λ clones could be the origins of this expressed tube foot actin.

To verify the specificity of the pPoA137/630 probe, the 630 bp fragment originating from λPoA137 was hybridized to the five λ genomic clones (Figure 2.8C). Intense hybridization occurred only with the 9.9 kb fragment from λPoA137 and λPoA68, although washing of the filters was done at moderate stringency.

To verify the genomic origin of the tube foot expressed actin transcript from which the pPoTA13 cDNA clone was derived, the five λ genomic clones were hybridized with the pPoTA13/210 probe (Figure 2.8D) It is clear that this 3' non-coding segment is derived from λPoA160 gene region as intense hybridization occurred only with the 17.1 kb fragment. There is no cross reaction between the other clones even at moderate stringency wash.

Kinetic determination of the number of actin genes in sea stars

The reassociation kinetics for sheared genomic DNA of *P. ochraceus*, *P. brevispinus* and *D. imbricata* with ^{32}P-labeled single-stranded actin coding probe are shown in Figure 2.9. The probe used is the pPoTA9/650 actin coding sequence prepared from an M13mp9 clone, as described in Materials and Methods. The single-copy DNA reassociation rates for *P. ochraceus*, *P. brevispinus* and *D. imbricata* have been determined (Smith and Boal, 1978; Smith *et al.*, 1980; Smith *et al.*, 1982) and listed with the genomic and tracer DNA fragment lengths in Table 2.1. Least-squares analysis of the reassociation kinetics demonstrate a single component second order reaction. The ratio of the observed rates to the expected single-copy rates (corrected for tracer and driver length according to Chamberlin *et al.*, 1978) provides the number of tracer homologous sequences per haploid genome. The insets of Figure 2.9 illustrate the change in the root mean square error for the least-square fit, when the rate constant is fixed for a specific number of actin sequences per haploid genome. The best fit curves indicate approximately five actin genes for *P. ochraceus* and *P. brevispinus*, and about eight genes for *D. imbricata*.

Figure 2.9 Hybridization of actin coding sequence probe with excess genomic DNA from three sea stars.

Large excess of sheared genomic DNA was used from three different species of sea stars to drive the ^{32}P-labeled single-stranded actin coding sequence probe in hybridization kinetic experiments. The probe was the pPoTA9/650 actin coding sequence prepared from an M13mp9 clone, as described in Materials and Methods. The specific activity of the probe was approximately 1×10^9 cpm μg^{-1}. (See Table 2.1 for the second order rate values and the fragment length of the tracer and driver DNAs.) The solid lines are computer least-square best fits to the data. The insets illustrate the change in the root mean square error for the least-square fit, when the rate constant is fixed for various numbers of actin sequences per haploid genome.

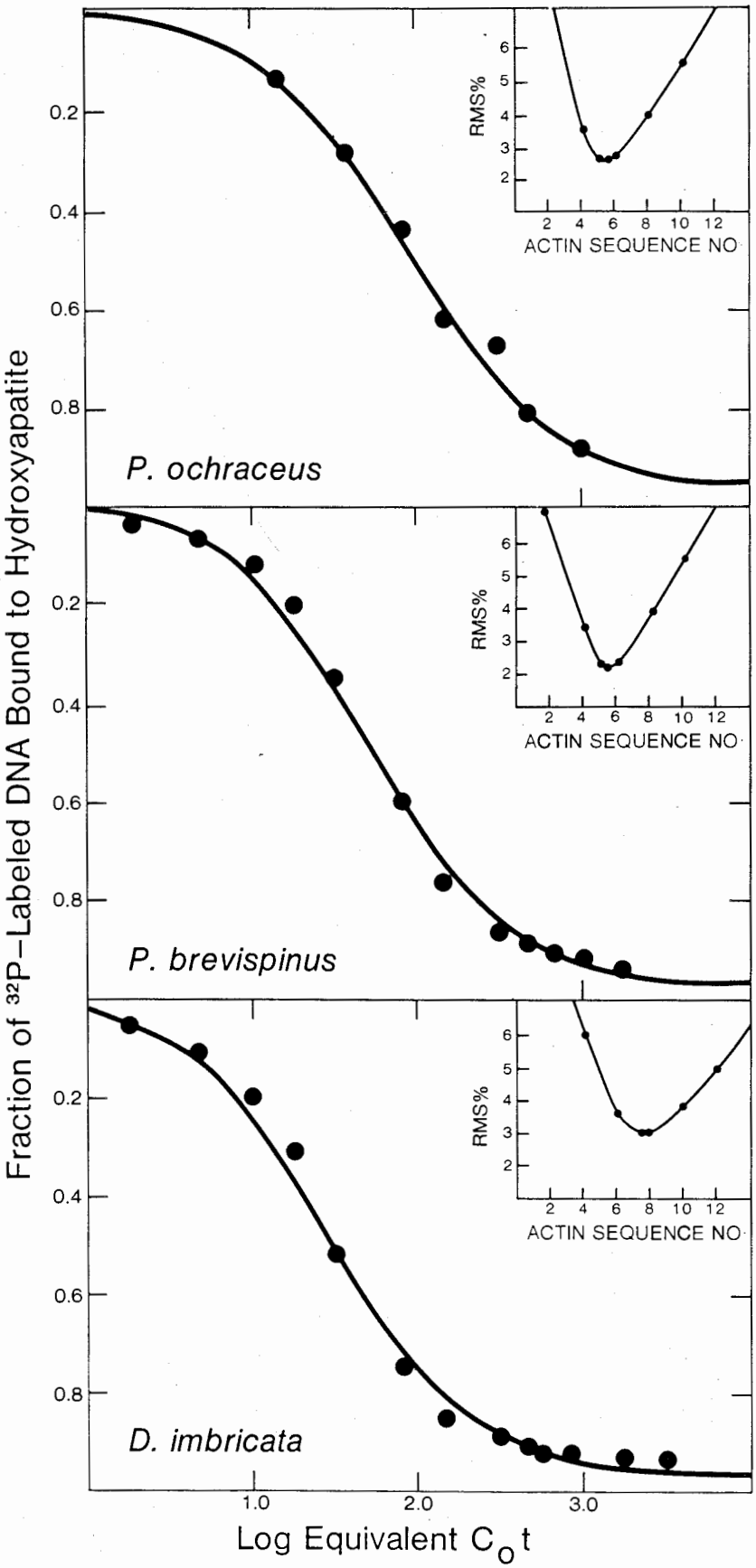

Fraction of ^{32}P–Labeled DNA Bound to Hydroxyapatite
0.2
0.4
0.6
0.8
P. ochraceus
RMS%
2 3 4 5 6
2 4 6 8 10 12
ACTIN SEQUENCE NO
P. brevispinus
D. imbricata
1.0
2.0
3.0
Log Equivalent $C_o t$

Table 2.1 Kinetic determination of the number of actin genes in sea stars.

Species	DNA fragment length(nt)		Hybridization rate ($M^{-1}s^{-1}$)		RMS[b]	No. of actin genes/ haploid genome[c]
	^{32}P-tracer	genomic driver	K exp[a]	K obs	(%)	
P. ochraceus	400	250	0.0021	0.0105	2.5	5.1
P. brevispinus	600	300	0.0035	0.0180	2.2	5.2
D. imbricata	600	250	0.0042	0.0345	3.0	8.2

a) The expected single-copy rate of hybridization (K exp) was calculated from the single-copy DNA reassociation constant (K sc) corrected for length (Chamberlin et al., 1978) as described in Chapter I. Data are derived from:

(Smith and Boal, 1978) for P. ochraceus with K sc = 0.0014 and tracer and driver length of 300 nt;

(Smith et al., 1982) for P. brevispinus with K sc = 0.0020 and tracer and driver length of 400 nt;

(Smith et al., 1980) for D. imbricata with K sc = 0.0022 and tracer and driver length of 400 nt.

b) RMS is the root mean square error for the least-square fit.

c) Calculated as K obs/K exp.

IV. DISCUSSION

Analyses of the genomic DNA blots (Figure 2.7) indicate that there are at most five distinct non-allelic actin genes in P. ochraceus. For an individual sea star the greatest number of actin positive bands in EcoRI digested genomic DNA is six. If two of these are due to the actin alleles represented by the λ145,128 clones, and all other positive genomic bands represent distinct genes, the total is five genes. For example, individual B in Figure 2.7 displays six EcoRI bands two of which can be assigned to specific actin coding fragments (3.7 and 6.7 kb bands) in the genomic clone λPoA145. If the other 4 bands represent 3 genes there are only 4 genes in sea stars. This argues for four or a maximum of five actin genes per haploid genome.

Based on genomic and dot-blot analyses with complete insert of the pPoTA9 probe, the maximum number of non-allelic actin genes in P. ochraceus was estimated to be six (Kovesdi et al., 1984). Hybridization with the pPoTA9 probe showed a maximum of eight actin positive bands in EcoRI digested genomic DNA from an individual sea star (Appendix B).

The different results with the two probes indicate that the 4.6 and 4.2 kb bands which are not seen in genomic blots hybridized with the pPoTA9/650 probe are probably specific for sequences 5' to the 650 bp fragment. The pPoTA9 insert does not

contain the nucleotides coding for the 113 5' amino acids of actin. Intervening sequences occur in most echinoid actin genes at codon locations 121 and 203 and in some cases at codon 41. (Scheller et al., 1981; Cooper and Crain, 1982; Schuler and Keller, 1981). If there has been a conservation of the intron 121 location in sea stars, the pPoTA9 probe terminates about 20 nucleotides 5' of the intron junction. Therefore, polymorphic EcoRI sites present within introns could account for the extra two bands observed when this probe was used.

The disparity in signal strength between autoradiographic bands within individual genomic blots may indicate multiple copies of actin genes. However, a comparison of Figure 2.7 and Appendix B shows that the pPoTA9/650 probe produces much less signal strength difference than the complete pPoTA9 insert, indicating that most of the disparity is probably due to an artifact resulting from the nature of the probe. Disparity in blot signal strength is certainly not evident for a given band between individuals when the DNA is digested with PstI and other restriction enzymes recognizing hexamers, e.g. BamHI and HindIII (Kovesdi et al., 1984).

In Figure 2.7 every band hybridizing to the pPoTA9/650 coding probe can be assigned as well to a 3' specific band on the basis of their length with the exception of the 6.7 kb and 5.2 kb bands. The 6.7 kb band is probably the actin coding fragment from λPoA145 (see above). This genomic clone also contains the 3.7 kb 3' fragment reacting with the pPoTA5/460

probe. The 5.2 kb band is present in individual B when probed with the coding region probe pPoTA9/650. When probed with the 3' non-coding region of pPoA137/630 a band appears at 5.6 kb. It can be assumed then that the 5.2 kb fragment represents a coding region which has an EcoRI site near or adjacent to its 3' end. This fragment could originate from the λPoA137,68 family as the coding portion of the 5.6 kb 3' specific fragment. The 5.2 kb band is not represented in the selected recombinant λ clones.

The 3.2 kb pPoTA13/210 and the 2.2 kb pPoA137/630 specific bands are missing in the blots hybridized with the pPoTA9/650 coding probe. Therefore these fragments probably contain the 3' non-coding region of an actin gene. The coding portions of these sequences may be represented by the 9.9 kb and 6.7 kb bands respectively. Therefore, it can be concluded that the three gene families (Appendix A) could account for all the genes in P. ochraceus.

Dot-blot and kinetic hybridization of genomic DNA with actin coding sequence probe demonstrate that the number of actin genes is on the order of five. Also, dot-blot hybridization with the gene specific probes indicates (Figure 2.6), that there are one pPoTA5/460, one pPoTA13/210 and two pPoA137/630 homologous sequences per haploid genome. These results agree with the genomic blot data (Figure 2.7) regarding the pPoTA5/460 and pPoA137/630 specific probes (minimum of one homologous gene each), but contrary to the minimum of two genes expected for the pPoTA13/210 probe.

P. ochraceus shows an overall polymorphism of single-copy DNA between individuals on the order of 5% (Smith et al., 1982). As a result we would expect that approximately one in four hexamer recognition sites could contain a single base change. There would not be any phenotypic selection for such changes if they occurred in non-functional regions between and within genes. The genomic blot data were used to estimate polymorphism in actin gene regions by comparing the restriction fragment distribution between pairs of individuals by the method of Engels (1981). All unique pair combinations were estimated, e.g. estimates were made for A vs. B through F individuals, as well as estimates of B vs E, F etc., resulting in a range of estimates from 1.3% to 3.8%. Previous estimates by Kovesdi et al. (1984) were 1.0% to 3.6% for EcoRI digestions and 1.6% to 2.8% for PstI digestions. All of the estimated values were lower than estimates of total single-copy DNA polymorphism observed for P. ochraceus (Smith et al., 1982).

The five actin clones characterized (Appendix A) represent three classes or categories of genes. There are 13 other recombinants whose preliminary restriction maps and Southern blot hybridization patterns relate them to these five clones. The three classes of genes are distinguished by homology in restriction enzyme sites particularly in the regions 5' to the coding fragments. This homology of restriction site location is most striking in the gene class represented by λPoA145,128 where there is only one site difference in the 8 kb fragment bound on

the 3' end by an SstI site and at the 5' end by an EcoRI site (Appendix A). In the gene class represented by clones λPoA68 and λPoA137, there is homogeneity in the location of EcoRI, SstI, and some KpnI, HindIII, and SalI sites. However there is considerably more divergence than that found in the λPoA145,128 category. Kovesdi et al. (1984) estimated the sequence divergence between the different gene families, assuming that there has been no major insertion or deletion of sequences within the fragments under consideration. The distribution of restriction sites between fragments was used to estimate genetic divergence by the method of Engels (1981) and Jefferys (1981). It was estimated that there is on the order of 1% divergence between the 8 kb regions of clones λPoA145 and λPoA128 indicated by the heavy arrows in Appendix A. In these segments there are eight sites common to both clones and one divergent. The divergence between λPoA137 and λPoA68 was calculated for the 13 kb region indicated by the heavy arrows in Appendix A. The sequence divergence in that region is on the order of 10%. This analysis suggests that λPoA145,128 are alleles and λPoA137,68 are related and probably represent a fairly recent duplication event.

The conclusion of the above analysis is that in P. ochraceus there are probably four or five genes per haploid genome, one from the λPoA145,128 two from the λPoA137,68 and one or two from the λPoA160 families (Appendix A).

The classes of genes found in the sea star genome are reminiscent of the four categories of actin genes found in sea urchin (Scheller et al., 1981; Lee et al., 1984). The sea star clones described here do not display linked genes reported for Dictyostelium (McKeown et al., 1978), S. purpuratus (Scheller et al., 1981; Schuler and Keller, 1981) and C. elegans (Files et al., 1983). The data do not, however, exclude the possibility of actin gene linkage in P. ochraceus.

Lee et al. (1984) defined an actin gene subtype in the sea urchin system as those actin genes which react at a 55°C, 0.75 M Na^+ (5X SET), 1X SET wash at the same temperature condition with a specific 3' terminal probe. In P. ochraceus the pPoTA5/460 probe cross reacts with the λPoA137,68 and λPoA160 gene families at a 13°C higher temperature (Figure 2.8B). It is best to be cautious when inferring nucleotide homologies from hybridization results (discussed by Patient, 1984). However, due to lack of sequencing data, it could be assumed that there are 3' non-coding regions which are conserved through large evolutionary distances. The same 460 nt sequence also appears to be highly conserved between P. ochraceus and P. brevispinus (C. Cuddeford and M.J. Smith unpublished observations). This is a situation very similar to that described for the CyI cytoplasmic S. purpuratus actin gene (Lee et al., 1984). The untranslated 3' region of this gene has been specifically conserved during a minimum of 150 million years of sea urchin evolution. Ordahl and Cooper (1983) showed that rat and chicken skeletal muscle

a-actin genes has conserved blocks of homologous sequences in the 3' terminal regions. They concluded similarly to Lee *et al.* (1984), that the localized conservation is probably functional and maintained by selection pressure.

DNA hybridization kinetic experiments determined the number of actin genes per haploid genome as five for *P. ochraceus* and *P. brevispinus*. The sequence homology of single-copy DNA was used to establish phylogenetic relatedness between different sea stars (Smith *et al.*, 1982). The divergence time was estimated for these two closely related species to be between 4 and 11 Myr, therefore the similarity in the number of actin genes is not suprising. The fossil record as well as sequence divergence of 5S RNA indicate a divergence time for sea urchins from sea stars of at least 500 Myr (Paul, 1977; Ohama *et al.*, 1983). The divergence times between *P. ochraceus* and *D. imbricata* are also in this time range (Spencer and Wright, 1966; Smith *et al.*, 1982). Although *D. imbricata* has a smaller genome size, the number of actin genes appear to be about eight. This is the same number that was determined for *Strongylocentrotus purpuratus* (Lee *et al.*, 1984).

It has been reported (Kabat-Zinn and Singer, 1981) based upon in vitro translation and RNA-driven hybridization of cDNA, that as much as 60% of the sea urchin tube foot mRNA is actin transcript. Tube foot is a contractile tissue and as such should be enriched in actin mRNA, however these results indicate that the tube foot plasmid cDNA library described here contains about

0.8% and the λgt10 library about 0.3% actin sequence clones. These values are higher than the more accurate estimates presented in Chapter III, and may reflect an efficiency of cloning or reverse transcription of actin mRNA. The λgt10 egg and gastrula cDNA libraries contain approximately 0.3% actin sequence clones. This value agrees with the proportion of actin transcripts found in sea urchin gastrula or pluteus RNA populations. In these embryonic systems the fraction of transcripts or cDNA clones which contain actin sequence is in the range 0.1 to 0.3% (Lasky et al., 1980; Crain et al., 1981; Flytzanis et al., 1982; Shott et al., 1984).

The amino acid sequence of actin from evolutionarily diverged organisms is highly conserved (Vandekerckhove and Weber, 1978a; 1978b) and therefore most of the base substitutions are expected in the third position of a codon. The partial comparision of the two expressed actin sequences from tube foot, pPoTA9 and pPoTA13 with the sequence of pSpG17, a genomic sea urchin actin clone (Cooper and Crain, 1982) shows no amino acid replacements (Figure 2.4). Coding region differences between the three sequences are: 7 out of 123 between pSpG17 and pPoTA13, 2 out of 40 between pPoTA9 and pPoTA13 and 1 out of 40 between pPoTA9 and pSpG17. Earlier reports have documented the uniqueness of 3' non-coding regions of actin genes in several organisms (reviewed in Davidson et al., 1982b). As expected,the 3' region of pPoTA13 is highly diverged from the pSpG17 sequence. There are 47 nucleotides which differ out of 65,

indicating complete randomness in the region assessed.

Three different 3' fragments were used in this study to identify the specific genes in a Charon 4 library from which these sequences originated. Hybridization of these 3' non-coding region probes with genomic DNA from several individuals under stringent criteria has demonstrated that there are at least three restriction fragment polymorphs for each of the three actin gene families in the sea star population. The band intensity of positive EcoRI fragments in genomic blots as well as the dot-blots probed with these sequences show that the pPoTA5/460 specific gene is single-copy in the genome, and the other two gene families are represented probably by two copies each. This study also shows that in P. ochraceus there are approximately five non-allelic actin genes. This finding is in agreement with reports for other eukaryotes (reviewed in Firtel, 1981). At least two of the actin genes have been shown here to be present in tube foot transcript populations and one of them also prevalent in egg and gastrula. The restriction fragment length polymorphism in the vicinity of actin genes reflects the total polymorphism observed for single-copy DNA found in earlier studies (Smith et al., 1982).

D. CHAPTER III

Actin Transcript Concentration in *Pisaster ochraceus* Early Development and Tube Foot

I. INTRODUCTION

Early embryonic development is dependent on the large store of maternal RNA accumulated in the egg through oogenesis (reviewed by Davidson, 1976; Brandhorst et al., 1983). The complexity of this RNA could be sufficient to code for approximately 20000 diverse proteins (Kovesdi and Smith, 1982). However, early cleavage stage polysomal RNA in sea urchins can account for only approximately 70% of the sequence set found in eggs (Hough-Evans et al., 1977). Also, many of the stored molecules contain multiple tracts of oligo(A) (Duncan and Humphreys, 1981) and at least 65% of the mass of egg poly(A)$^+$ RNA consists of covalently linked single-copy and repeat sequence transcripts in both sea urchin and Xenopus eggs (Constantini et al., 1978; 1980). These transcripts might be processed during embryogenesis and in this way may provide the necessary mRNA concentration until the embryo's own transcriptional system takes over.

Studies on prevalent cytoplasmic poly(A)$^+$ RNAs from sea urchins show that the abundance of most of these transcripts are similar in eggs and plutei but have an intervening stage when they decline (Flytzanis et al., 1982; Lasky et al., 1980) indicating probably a switch from the maternal supply to new transcripts.

Cabrera et al. (1984) showed recently that the parameters which 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. It appears that these parameters vary for each transcript. In sea urchins the maternal store of histone transcripts comprise 5 to 10 percent of the total quantity of mRNA (reviewed in Davidson, 1976; Raff, 1983). On the other end of the scale is actin, which is not represented significantly in the maternal RNA of sea urchins (Crain et al., 1981; Merlino et al., 1981; Shott et al., 1984). The patterns of expression of these highly conserved structural genes are maintained through large evolutionary distances (Bushman and Crain, 1983) and a change in their regulation might indicate major modification in a developmental pathway (Raff et al., 1984).

In order to investigate the storage and stage-specific utilization of RNA transcripts, the expression of actin genes was studied. The experiments in this chapter deal with the measurement of relative abundance and absolute number of actin transcripts in P. ochraceus early development and in the adult tube foot tissue.

II. MATERIALS AND METHODS

RNA dot-blots

RNA from tube foot, egg, 21 h post-fertilization blastula, 48 h early gastrula and 72 h late gastrula was denatured with glyoxal (Thomas, 1983), serially diluted and blotted onto a 20X SSPE soaked nitrocellulose paper with a BRL dot-blot manifold. The filters were hybridized with the appropriate ^{32}P-labeled probe at 42°C in 5X SSPE, 0.3% SDS, 5X Denhardt's solution, 50% formamide and washed sequentially with decreasing salt concentration to 0.2X SSPE 0.2% SDS at 68°C and exposed to preflashed Kodak XAR-5 or BB1 film.

RNA transfers

RNA from tube foot, egg, 21 h blastula, 48 h early gastrula and 72 h late gastrula was electrophoresed in 1.0 or 1.5% formaldehyde agarose gel (Maniatis et al., 1982) or RNA was glyoxalated and electrophoresed according to the method of Thomas (1980). The RNA was transferred to nitrocellulose paper or to Pall Biodyne A nylon membrane. The filters were hybridized with the appropriate ^{32}P-labeled probe at 42°C in 5X SSPE, 0.3% SDS, 5X Denhardt's solution, 50% formamide, washed sequentially

with decreasing salt concentration to 0.2X SSPE 0.2% SDS at 68°C and exposed to Kodak XAR-5 or BB1 film.

When rehybridization was planned, the filters were exposed damp in saran wrap. After visualization of the autoradiograph bands the filters were washed in 99% formamide 10mM PB at 70°C for one hour or heated to 100°C in H_2O, rinsed in 1X SSPE at room temperature and rehybridized with the next probe. P. ochraceus and E. coli ribosomal RNA was used for size markers.

Densitometry

Autoradiograms of RNA dot-blots were scanned by an ACD-18 Automatic computing densitometer, Gelman Scientific Inc.; autoradiograms of RNA transfers were scanned by a Jarrell-Ash Recording Microphotometer.

M13 single-stranded probes

The construction and preparation of the single-stranded probes were described in Chapter II.

RNA driven hybridization kinetics

RNA driven hybridization with single-stranded DNA was performed as described in Chapter I. The only deviation from the original protocol was the omission of the RNase treatment, as parallel reactions without RNA indicated negligible amount of contaminating RF DNA from the M13 clones.

^{32}P-labeled DNA was reacted with a mass excess (>1 x 10^6 : 1) of total or (>3 x 10^4 : 1) poly(A)$^+$ RNA.

Reactions were performed in 0.12 M PB (equimolar mono- and disodium phosphate buffer) at 60°C and in 0.41 M PB or 0.38 M PB at 67°C. All Rot values were corrected for acceleration in reaction rate relative to the rate in 0.12 M PB at 60°C due to higher Na^+ concentration (Van Ness et al., 1979). A comparison of 0.12 M PB reaction rates with the 0.41 M PB rates for the actin coding sequence indicated that the rate correction factor of 2.5 (Van Ness et al., 1979) should be used for 0.41 M PB instead of the previously published 5 determined by Britten et al. (1974) for randomly sheared DNA.

The above contradicts results found in Chapter I, where a rate correction factor of 5 was applied. Experiments in Chapter I used a heterogeneous population of randomly sheared DNA as probes. Similarily to Van Ness et al. (1979), this chapter deals with homogeneous DNA fragment probes. Theoretically it is not clear as to how this difference could account for the discrepancy. Only further experimentation may resolve the exact

nature of the problem.

Hybridization reactions were analysed by procedures described earlier in Chapter I. Data were analysed by a least-squares fitting procedure (Pearson, et al., 1977).

Saturation hybridization

Hybridization, hydroxyapatite chromatography and the quantitative treatment of data were done essentially as described by Scheller et al. (1978).

The specific activities of the 3' specific probes were calculated by standard curves obtained by titrating the probe against increasing quantities of unlabeled cold DNA inserts. This procedure is explained in detail by Mauron et al. (1981). The reactable sequence in the mixture is the strand of the insert which is of messenger polarity and its mass fraction is therefore $1/a = 0.5$. The proportion of the probe in hybrid form, T/To, was assayed by hydroxyapatite chromatography. The titration is expresed as T/To vs R', where R' is the ratio of unlabeled DNA mass to labeled probe cpm. The titration data were fitted by a least-squares program (provided by D. Wilson) to the equation $T/To = (1 + (a'/R'))^{-1}$ (Scheller et al., 1978; Mauron et al., 1982) which has a linear initial slope, 1/a', for low R' values. Therefore, the specific activity of the probe can be calculated as $S = (1/a')/(1/a)$

Using RNA, the initial titration curve can be described by the equation $T/To = R/a = (R' \times S)/a = R'/a'$. Knowing the specific activity (S), the fraction of total RNA mass present as probe specific RNA can be calculated as $1/a = (1/a')/S$ and the transcript as fraction of RNA mass becomes $((1/a')/S \times (LR/LP)$, where LR is the RNA transcript length and LP is the length of the probe.

All RNA samples used in the experiments were tested for contaminating DNA by subjecting an aliquot of the hybridization reaction corresponding to the highest RNA/DNA ratio to RNase A digestion in 0.02 M PB (50 $\mu g\ ml^{-1}$) at 37°C for 1 h. The samples were adjusted to 0.12 M PB before chromatography. RNase treatment reduced hybridization values to background levels, demonstrating that there was no detectable DNA contamination.

The specific activity of the actin coding probe was determined as follows: The maximum possible specific activity was calculated to be 1.27×10^9 cpm μg^{-1} by using the sea urchin pSpG17 actin sequence. Probes were sized on denaturing agarose gels just after extension with the Klenow fragment (see Materials and Methods, Chapter II). The change in length of the probe was evaluated periodically on denaturing agarose gels. This length was compared with the expected theoretical decay of a probe with different calculated specific activities. The theoretical and experimental values agreed with an initial specific activity of 1×10^9 cpm μg^{-1} ($\pm$ 0.2×10^9 cpm μg^{-1}).

III. RESULTS

Actin gene specific probes

The expression of actin genes during early devlopment and in tube foot were analysed by the gene specific probes described in Chapter II. The coding region probe pPoTA9/650 was used to assess the total actin sequence concentrations. The presence of gene products originating from the λPoA145,128 family, the λPoA137,68 family and the λPoA160 family was investigated by probes pPoTA5/460, pPoA137/630 and pPoTA13/210 respectively (see Appendix A for description of gene families).

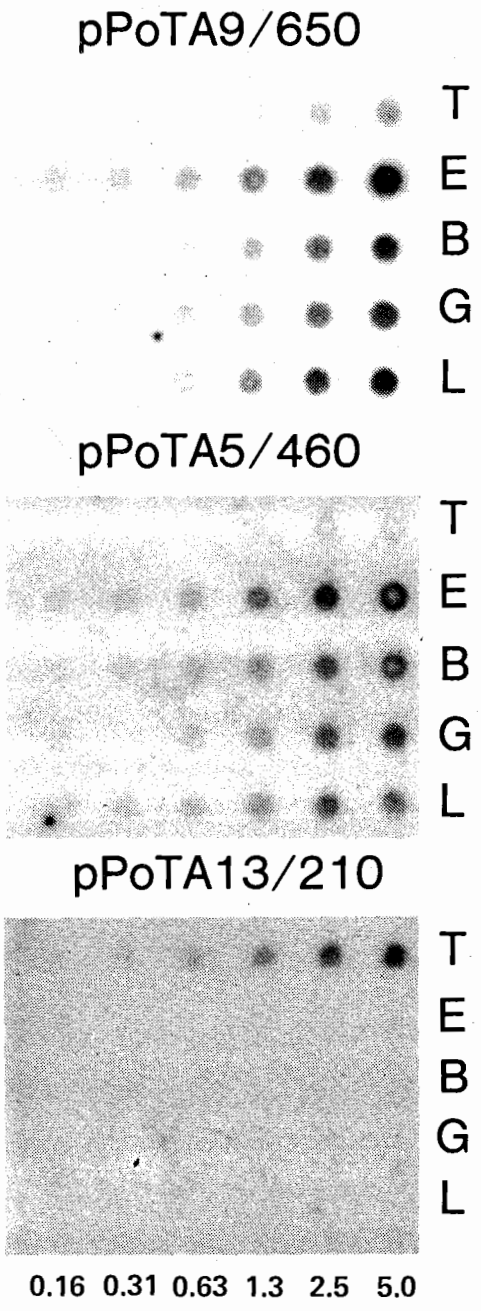
Expression of actin genes in tube foot tissue

To investigate the expression of the different actin genes in tube foot, glyoxalated RNA was serially diluted, dotted onto nitrocellulose and reacted with ^{32}P-labeled gene specific probes (Figure 3.1).

Both λPoA160 and the λPoA145 gene families are expressed in tube foot, as was expected. Unexpectedly the relative amount of reaction with the pPoTA5/460 probe was much lower than with the pPoTA13/210 fragment, indicating that most of the transcripts originate from λPoA160. The tube foot cDNA libraries from which

Figure 3.1 Dot-blot hybridization of actin coding and gene specific probes with total RNA.

The probes pPoTA9/650, pPoTA5/460, pPoTA13/210 and pPoA137/630 were described in Chapter II and in Figure 2.7. Total RNA from tube foot(T), egg(E), 21 h blastula(B), 48 h early gastrula(G) and 72 h late gastrula(L) was denatured with glyoxal (Thomas, 1983), serially diluted and blotted onto a 20X SSPE soaked nitrocellulose paper with a BRL dot-blot manifold. The filters were hybridized with the appropriate ^{32}P-labeled probe at 42°C in 5X SSPE, 0.3% SDS, 5X Denhardt's solution, 50% formamide and washed sequentially with decreasing salt concentration to 0.2X SSPE 0.2% SDS at 68°C and exposed to preflashed Kodak XAR-5 or BB1 film. Results of the pPoA137/630 hybridization are not shown, because this probe did not react with any of the RNA samples.


pPoTA9/650
T
E
B
G
L
pPoTA5/460
T
E
B
G
L
pPoTA13/210
T
E
B
G
L
0.16 0.31 0.63 1.3 2.5 5.0
RNA / spot (µg)

these sequences originate from showed a large excess of λPoA145 class over λPoA160 class of specific clones. These results indicate a preferential selectivity on oligo(dT) columns or in cloning for the λPoA145 product. The pPoA137/630 probe did not show any reaction to total or poly(A)$^+$ tube foot RNA even at two weeks of exposure. This indicates that if λPoA137 transcripts are present, they must be less than 0.3% of the actin transcript population. It also must be kept in mind, that this is a genomic DNA probe, and there is always the possibility that this gene has a very short 3' end or has a large 3' intron not transcribed into RNA.

The size of the transcripts were assayed by RNA transfers (Figure 3.2) of 1.5% denaturing agarose gels. E. coli (Brosius et al., 1978; 1980) and P. ochraceus ribosomal RNA was used as size markers. Glyoxalated P. ochraceus RNA was previously measured against λ HindIII and EcoRI digests (McMaster and Carmichael, 1977). The ribosomal peaks were determined to be approximately 3850 nt and 1800 nt respectively.

Two bands, a 2300 nt and a 2200 nt were homologous to the actin coding probe. The 2300 nt band, which is specific to the λPoA145 gene family, is observable only on long autoradiographic exposures. The 2200 nt band is specific to tube foot, and probably accounts for most of the actin transcripts present in this tissue. Comparison of the relative abundance of the coding and λPoA145 specific transcripts (Figure 3.3) would indicate that this gene could be responsible for as much as 30% of the

Figure 3.2 Expression of actin genes in sea star development and tube foot.

The probes pPoTA9/650, pPoTA5/460, pPoTA13/210 and pPoA137/630 were as described in Chapter II and in the legend to Figure 2.7. Total RNA from tube foot(T), egg(E), 21 h blastula(B), 48 h early gastrula(G) and 72 h late gastrula(L) was electrophoresed in 1.5% formaldehyde agarose gel for seven hours at 90V, transferred to Pall Biodyne A nylon membrane. The filter was hybridized with the first ^{32}P-labeled probe at 42°C in 5X SSPE, 0.3% SDS, 5X Denhardt's solution, 50% formamide, washed sequentially with decreasing salt concentration to 0.2X SSPE 0.2% SDS at 68°C and exposed to Kodak BB1 film. After visualization of the bands the filter was washed in 99% formamide 10mM PB at 70°C for one hour, rinsed in 1X SSPE at room temperature and rehybridized with the next probe. Results of the pPoA137/630 hybridization are not shown, because this probe did not react with any of the RNA samples. *P. ochraceus* and *E. coli* ribosomal RNA was used for size markers.

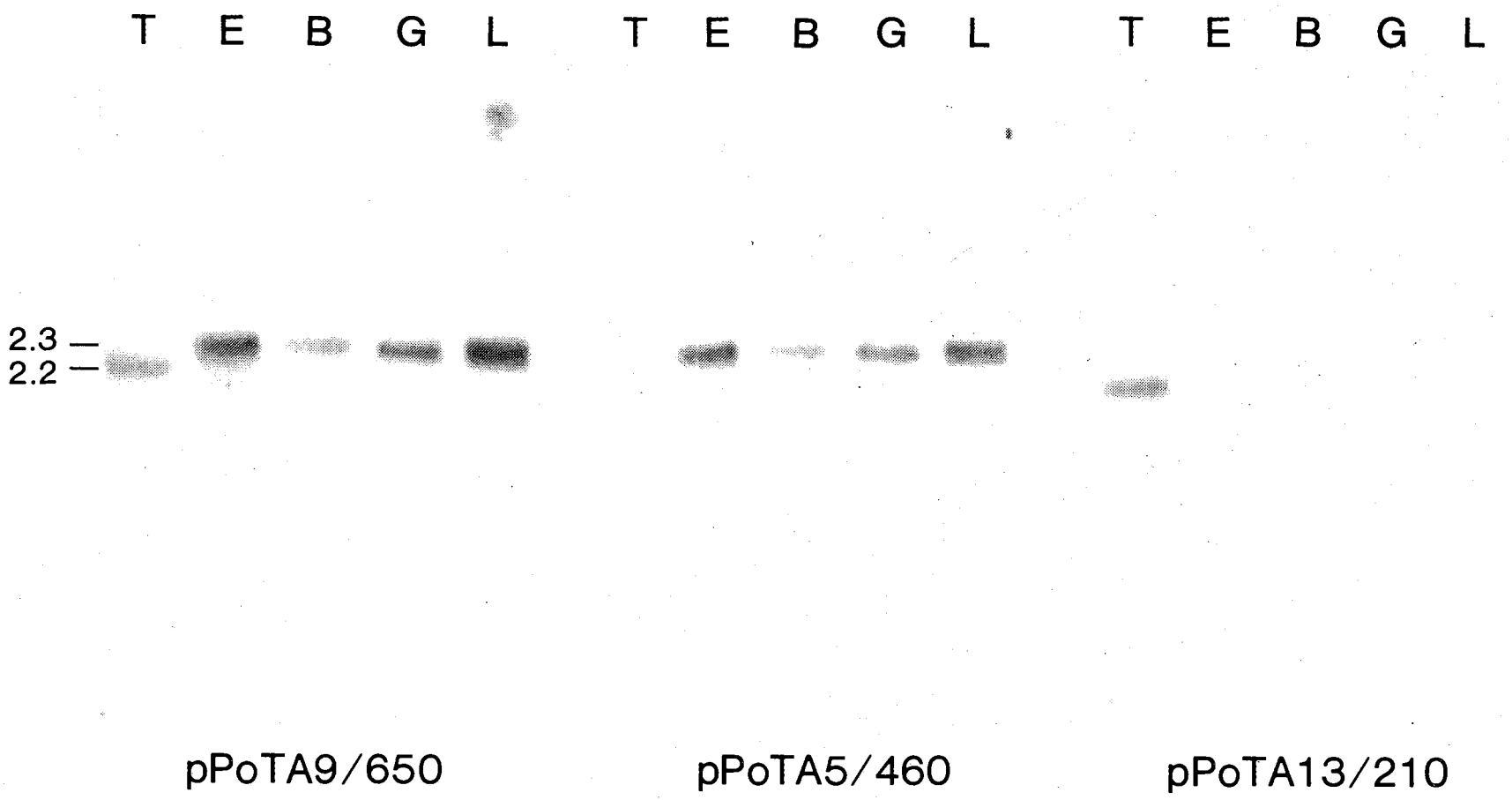

T E B G L
T E B G L
T E B G L
2.3
2.2
pPoTA9/650
pPoTA5/460
pPoTA13/210

Figure 3.3 Relative abundance of actin transcripts in sea star development and tube foot.

Autoradiograph bands in RNA gels and dot-blots were analysed by densitometry to obtain estimates of relative transcript abundance. Data are presented as the average percentage of the transcript prevalence observed for each probe relative to egg. Error bars denote the range of the values measured from five independent experiments for the coding region probe (pPoTA9/650) and from four independent experiments for the 3' probe pPoTA5/460. A comparision could not be made for the probes pPoTA13/210 and pPoA137/630 as the first reacted only with RNA from tube foot and the second did not react with any of the RNAs.

RELATIVE ABUNDANCE OF ACTIN TRANSCRIPTS AS PERCENT OF EGG VALUE

transcripts in tube foot. The data, however, were derived from densitometric scans of a large number of dot-blots and RNA transfers, some of which were done at moderate stringencies.

It was previously indicated that the pPoTA5/460 probe shows some cross reaction with the λPoA160 gene at these conditions (Figure 2.8B). Using only high stringency data, the λPoA145 gene family could account for a maximum of 10% of the transcripts.

Embryo specific expression of an actin gene

In order to study the expression of the actin gene family during early development, RNA was prepared from eggs, blastula (21 h), early gastrula (48 h) and late gastrula (72 h). RNA samples were serially diluted and dotted onto nitrocellulose (Figure 3.1) or electrophoresed in denaturing gels (Figure 3.2) as previously described. Hybridization with ^{32}P-labeled probes shows that P. ochraceus eggs contain large amounts of actin transcripts. The intensity of reaction indicates that the level of actin specific RNA decreases in blastula but increases again in early and late gastrula. The only transcript detectable in any of the embryonic stages tested was originating from the λPoA145 gene family with a transcript length of 2300 nt. Very long autoradiographic exposures with total and poly(A)$^+$ RNA failed to detect the λPoA137 or λPoA160 specific transcripts.

The relative abundance of the total and λPoA145 specific actin transcripts were assessed by densitometry (Figure 3.3).

Data from five independent experiments were pooled for the total amount of actin transcripts and from four experiments for the λPoA145 specific probe. The change in the relative transcript prevalance for the two different probes are indistinguishable from each other indicating that all actin transcripts in the embryo might originate from this gene. The transcript prevalence drops to approximately 50% of the egg value at blastula stage and rises back to nearly egg level in the late gastula.

To ensure equal loading of RNA and transfer, the filters were subsequently hybridized with a nick translated ribosomal λ clone (λPoR1) or with P. ochraceus mitochondrial DNA (gift of A. Beckenbach). λPoR1 was derived from a P. ochraceus λ EMBL3 genomic library which was screened with a ribosomal clone of Heterorhabitis heliothidis (gift of J. Curren). Mitochondrial transcripts are represented as highly prevalent transcripts in egg and embryo poly(A)$^+$ RNA of sea urchin (Cabrera et al., 1983). Densitometric scans of the autoradiographs showed that the amount of RNA available for hybridization varied less than 10% between applications.

To confirm the accuracy of the densitometric scans, dot-blots were hybridized with ribosomal DNA. After autoradiography the dots were cut out from the nitrocellulose filters and counted in scintillation fluid. The relative densitometric measurements were found to be within 10% of those obtained by scintillation counting.

Expression of actin genes in the eggs of different species of sea stars

The relative amount and size of maternal actin specific transcripts were evaluated in four widely diverged species of sea stars: Pisaster ochraceus, Evasterias troschelii, Pycnopodia helianthoides, Dermasterias imbricata.

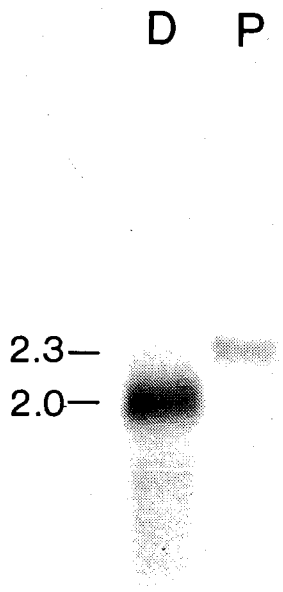
Total RNA was extracted from eggs and 20 μg of each was electrophoresed on 1.5% denaturing agarose gels (Maniatis et al., 1982). After transferring the RNA to solid support, it was hybridized with ^{32}P-labeled actin coding probe. The result of P. ochraceus and D. imbricata experiment is shown on Figure 3.4. Densitometric scans of the actin specific bands indicate that D. imbricata egg RNA contains approximately six times more of these sequences by mass than P. ochraceus. D. imbricata also shows only a single positive band, but the transcript length is significantly shorter (2000 nt) than that of the P. ochraceus. E. troschelii and P. helianthoides have the same size transcripts as P. ochraceus (data not shown).

RNA driven hybridization and saturation hybridization to determine the number of actin homologous sequences in different RNA populations

Two methods were used to determine the average number of actin and 3' non-coding homologous sequences in different

Figure 3.4 Expression of actin genes in the eggs of two different sea star species.

Total egg RNA (20μg/lane) from *P. ochraceus*(P) and from *D. imbricata*(D) was electrophoresed in 1.5% formaldehyde agarose gel and transferred to Pall Biodyne A nylon membrane. The filter was hybridized with the pPoTA9/650 ^{32}P-labeled probe at 42°C in 5X SSPE, 0.3% SDS, 5X Denhardt's solution, 50% formamide, washed sequentially with decreasing salt concentration to 0.2X SSPE 0.2% SDS at 68°C and exposed to Kodak BB1 film. Densitometric scanning of the bands indicates that *D. imbricata* egg RNA contains six times more actin sequence by mass than *P. ochraceus*.


D
P
2.3—
2.0—

embryonic cells and in tube foot.

In RNA driven hybridization (described in Chapter I; Materials and Methods), the expected rate of hybridization for the specific probe length and complexity is compared to the observed rate. Therefore, this method is sensitive to parameters which influence the rate of reassociation; that is length of probe and driver, salt concentration, RNA concentration and some other variables which are discussed in more detail by Wetmur (1976). However, it is not important to know the exact specific activity of the tracer as long as the mass of the driver is in large excess.

Saturation hybridization is based on titration of excess single-stranded ^{32}P-labeled DNA probe with increasing amounts of RNA (Scheller et al., 1978; Mauron et al., 1981). As the amount of RNA increases a greater amount of the ^{32}P-DNA is hybridized and can be bound to hydroxyapatite as described in Materials and Methods. Calculations of transcript prevalence carried out by this method are not subject to the particular uncertainties which affect the kinetic estimates, as long as the reactions are carried out to completion, that is $\geq$10 x Cot(1/2). The length of the reacting strands has no effect on the final results, if S1-nuclease digestion is used. However evaluation by hydroxyapatite could cause significant overestimates if the RNA transcripts are broken within sequences tested by the ^{32}P-DNA probe. The accuracy of titration measurements are most dependent on the specific activity determination of the radioactive probe.

Number of actin sequences in different developmental stages and in tube foot

Single-stranded M13 coding region probe was driven by a large excess of total or poly(A)$^+$ RNA and the amount of hybrids were analysed by hydroxyapatite chromatography (Figure 3.5). Data were fit with a single pseudo-first-order hybridization function by a least-squares fitting program (Pearson et al., 1977). Data from repeat experiments were combined by correcting for probe reactivities and rate of reassociation in different salt conditions. Probe reactivities ranged between 70 and 90%. The rate constants extracted from these data, and the calculated values of the number of transcripts per embryo or cell are listed in Table 3.1. The specific parameters and the calculation methods used are described in the footnotes.

There are no accurate estimates of the amount of total RNA content of a tube foot cell, therefore the number of transcripts were calculated in 15 ng RNA, in order to allow some comparison with the embryonic stages.

For example, the observed rate of hybridization (K obs) for total egg RNA is 0.00668 $M^{-1}s^{-1}$ (Figure 3.5). The expected rate (K exp) for the pPoTA9/650 probe corrected for tracer and driver length is 937 $M^{-1}s^{-1}$ (See Chapter I for details of this calculation). The ratio (K obs/K exp) = 7.1×10^{-6} and provides the estimate of the fraction of RNA mass which is homologous

Figure 3.5 RNA driven hybridization to single-stranded actin coding probe.

Total and poly(A)$^+$ RNA from different developmental stages and from tube foot was used to drive a ^{32}P-labeled single stranded probe (M13mp9/pPoTA9/650) in reassociation kinetics. Hybrids were analysed by hydroxyapatite chromatography (see Materials and Methods, Chapter I). The solid line represents the best least-square solution for the data assuming pseudo-first-order reaction (see figure legend, Figure 1.2). Different symbols indicate independent experiments. The rate constants obtained from these data fits are listed in Table 3.1.

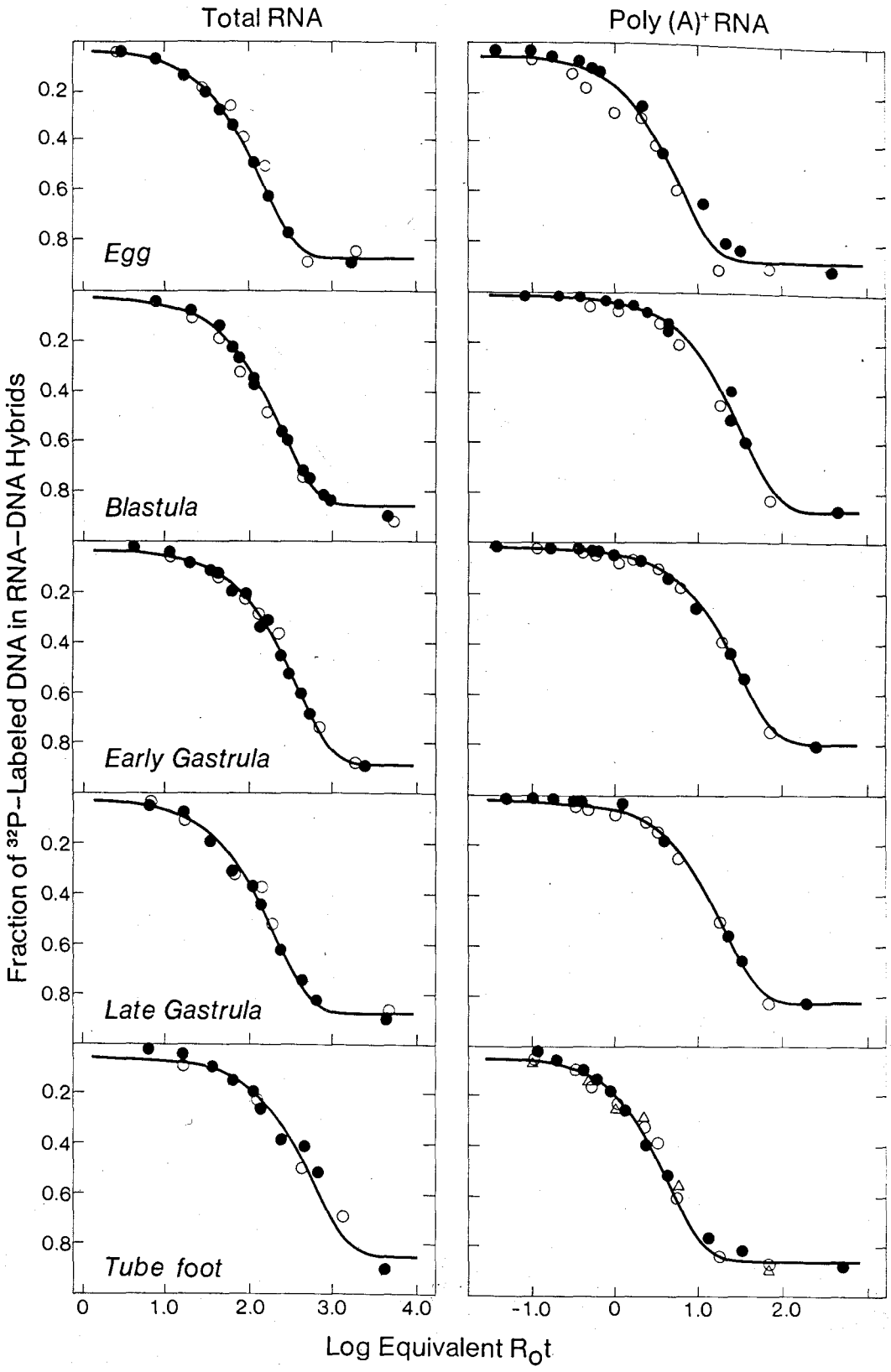

Total RNA
Poly (A)+ RNA
Egg
Blastula
Early Gastrula
Late Gastrula
Tube foot
0.2
0.4
0.6
0.8
0
1.0
2.0
3.0
4.0
-1.0
Fraction of 32P-Labeled DNA in RNA-DNA Hybrids
Log Equivalent R0t

Table 3.1 Determination of the number of actin coding sequences in Pisaster ochraceus RNA.

Stage/or Tissue	Hybridization rate K obs(RMS) ($M^{-1}s^{-1}$)(%)	Slope(1/a') (cpm ug^{-1})	Transcripts as[a] fraction of RNA	No.transcripts[b] per embryo	No.transcripts[c] per cell
RNA driven hybridization					
Total RNA					
Egg	0.00668(3.7)		$2.5x10^{-5}$	$2.9x10^{5}$	$2.9x10^{5}$
Blastula	0.00446(3.4)		$1.6x10^{-5}$	$1.9x10^{5}$	317
E.Gastrula	0.00272(3.0)		$1.0x10^{-5}$	$1.2x10^{5}$	30
L.Gastrula	0.00492(3.1)		$1.8x10^{-5}$	$2.2x10^{5}$	14
Tube foot	0.00180(4.6)		$6.6x10^{-6}$	$7.7x10^{4}$	
Poly(A+) RNA					
Egg	0.164 (5.0)		$6.0x10^{-4}$	$2.1x10^{5}$	$2.1x10^{5}$
Blastula	0.0358 (2.7)		$1.3x10^{-4}$	$7.5x10^{4}$	125
E.Gastrula	0.0365 (1.0)		$1.3x10^{-4}$	$8.0x10^{4}$	20
L.Gastrula	0.0530 (1.5)		$2.0x10^{-4}$	$1.2x10^{5}$	8
Tube foot	0.211 (3.3)		$7.8x10^{-4}$	$7.3x10^{4}$	
Saturation hybridization					
Total RNA					
Egg		$8.3x10^{3}$	$3.7x10^{-5}$	$4.3x10^{5}$	$4.3x10^{5}$
Blastula		$4.0x10^{3}$	$1.9x10^{-5}$	$2.3x10^{5}$	383
E.Gastrula		$3.5x10^{3}$	$1.7x10^{-5}$	$2.0x10^{5}$	50
L.Gastrula		$3.3x10^{3}$	$1.6x10^{-5}$	$1.9x10^{5}$	12
Tube foot		$2.8x10^{3}$	$1.4x10^{-5}$	$1.6x10^{5}$	

Footnotes Table 3.1

a) RNA driven hybridization: calculated as (K obs/K exp) x (LR/CP), where K exp is the expected first order rate of hybridization for the probe length and complexity, LR is the RNA transcript length and CP is the probe complexity. The details of K exp calculations are described in Chapter I.

Saturation hybridization: Calculated as ((1/a')/S) x (LR/LP), where 1/a' is the initial slope, S is the specific activity of the probe at the time of the experiment, LR is the RNA transcript length and LP is the initial length of the probe.

Characteristics of the probe used in the experiments:

pPoTA9/650: The initial length of the probe was 680 nt (LP) containing 650 nt complementary to RNA transcript (CP) and some M13 sequence. The tracer length at hybridization was 425 nt long (LT) and the initial specific activity was 1.0×10^9 cpm μg^{-1} (SP).

The RNA transcript length (LR) and the RNA driver length (LD) used in the calculations is the average actin transcript length, 2250 nt.

b) The amount of RNA per embryo is assumed to be constant at about 15 ng. This value was measured for egg RNA (Kovesdi and Smith, 1982). Isotopic dilution experiments were repeated for late gastrula as described in Chapter I, Materials and Methods. Repeat experiments gave values of 14.8 ng (S.D.=2.6;n=2) per embryo. The number of transcript in tube foot is calculated for 15 ng of RNA. The amount of poly(A)$^+$ RNA per embryo is estimated

as the oligo(dT) bound fraction, i.e. 0.8% for tube foot, 3% for egg, 5% for blastula, early gastrula and late gastrula (RNA from the developmental stages were put through the oligo(dT) column only once).

c) Number of cells per embryo are 1 for egg, 600 for blastula (21 h), 4000 for early gastrula (48 h), 16000 for late gastrula (72 h) (Fraser et al., 1981). The late gastrula value is extrapolated from Figure 1 of that paper.

to the probe. Since the length of an average actin transcript is 2250 nt and the probe complexity is 650 nt the true fraction of actin transcript in the egg is $7.1 \times 10^{-6} \times (2250/650) = 2.5 \times 10^{-5}$. Each egg contains 15 ng of total RNA (Chapter I), therefore the mass of actin transcripts per egg is 0.37 pg. This corresponds to 2.9×10^5 molecules per egg. Note that K obs is actually underestimating the rate, as a portion of the probe is M13 sequence and therefore could never react. The rate for a pure 650 nt sequence (K pure) would be higher by the fraction (680/650). However, in that case the true fraction of actin transcript in the RNA would have to be calculated by the equation (K pure/K exp) x (2250/680), in other words using the original 680 nt length. The final result in both cases is the same.

Results of the saturation titration experiments are also listed in Table 3.1. The specific activity of the probe at the time of labeling (SP) was estimated to be 1×10^9 cpm μg^{-1} ($\pm 0.2 \times 10^9$) from comparisons of the experimental and theoretical decay of probe length and initial ^{32}P nucleotide incorporation. The specific activity at the time of the experiment (S) was calculated from this value using the theoretical ^{32}P decay constant. The coding sequence probe was used to titrate increasing amounts of total RNA as described in Materials and Methods. The proportion of probe taken into hybrid form (T/To) was plotted against the (RNA/probe cpm) ratio (R') (Figure 3.6 and Figure 3.7).

Figure 3.6 Titration of transcripts in embryo RNA.

Increasing amounts of total RNA from four stages of early development were hybridized with the ^{32}P-labeled single-stranded actin coding probe pPoTA9/650 and with the 3' non-coding probe pPoTA5/460. The portion of ^{32}P-DNA in hybrid form (T/To) was determined by hydroxyapatite chromatography. R' is the RNA to ^{32}P-DNA mass ratio in units of μg cpm^{-1}. The values shown are corrected for unspecific binding and self-annealing of probe in the absence of RNA and for the extent of probe reactivity.

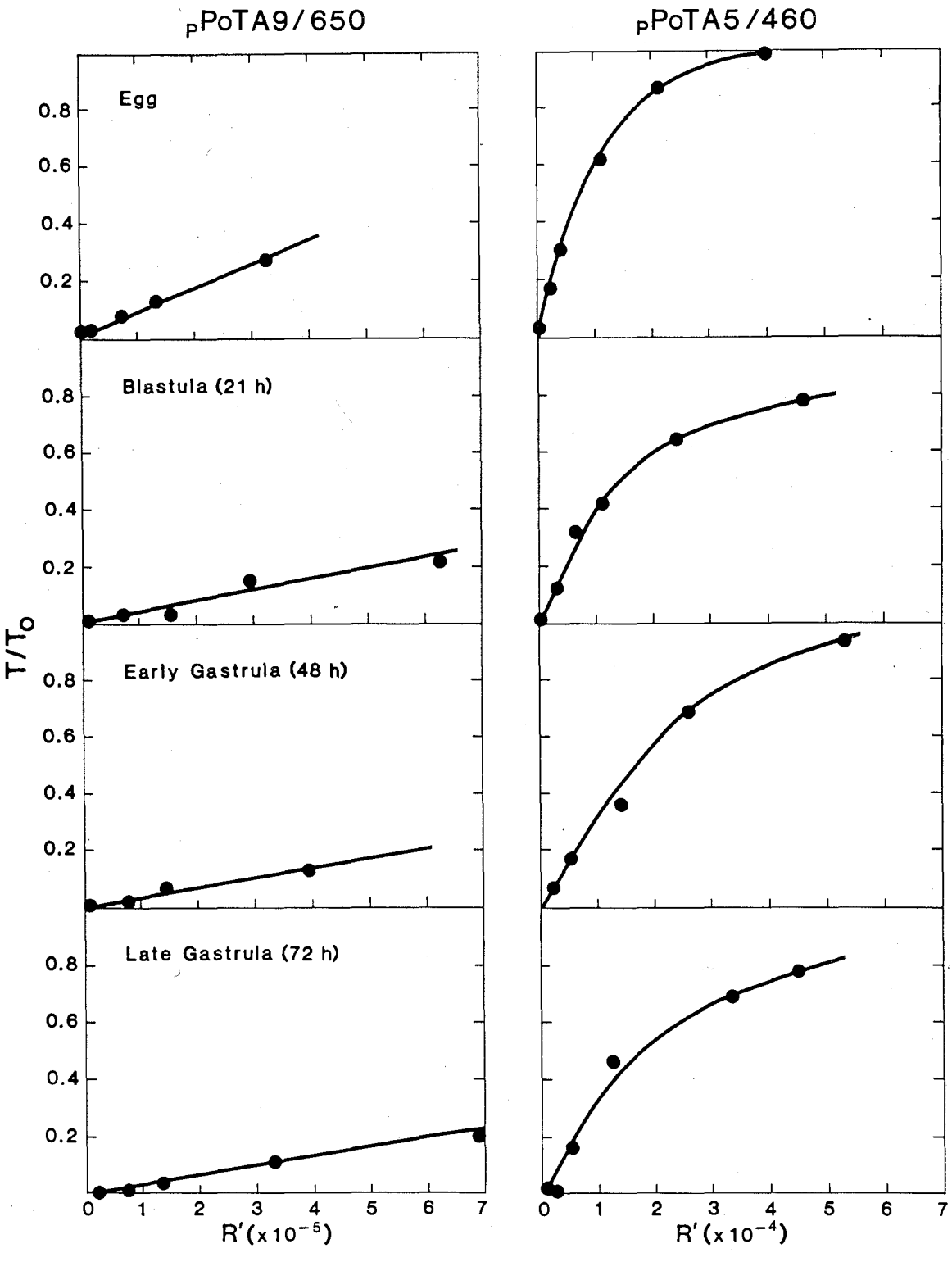
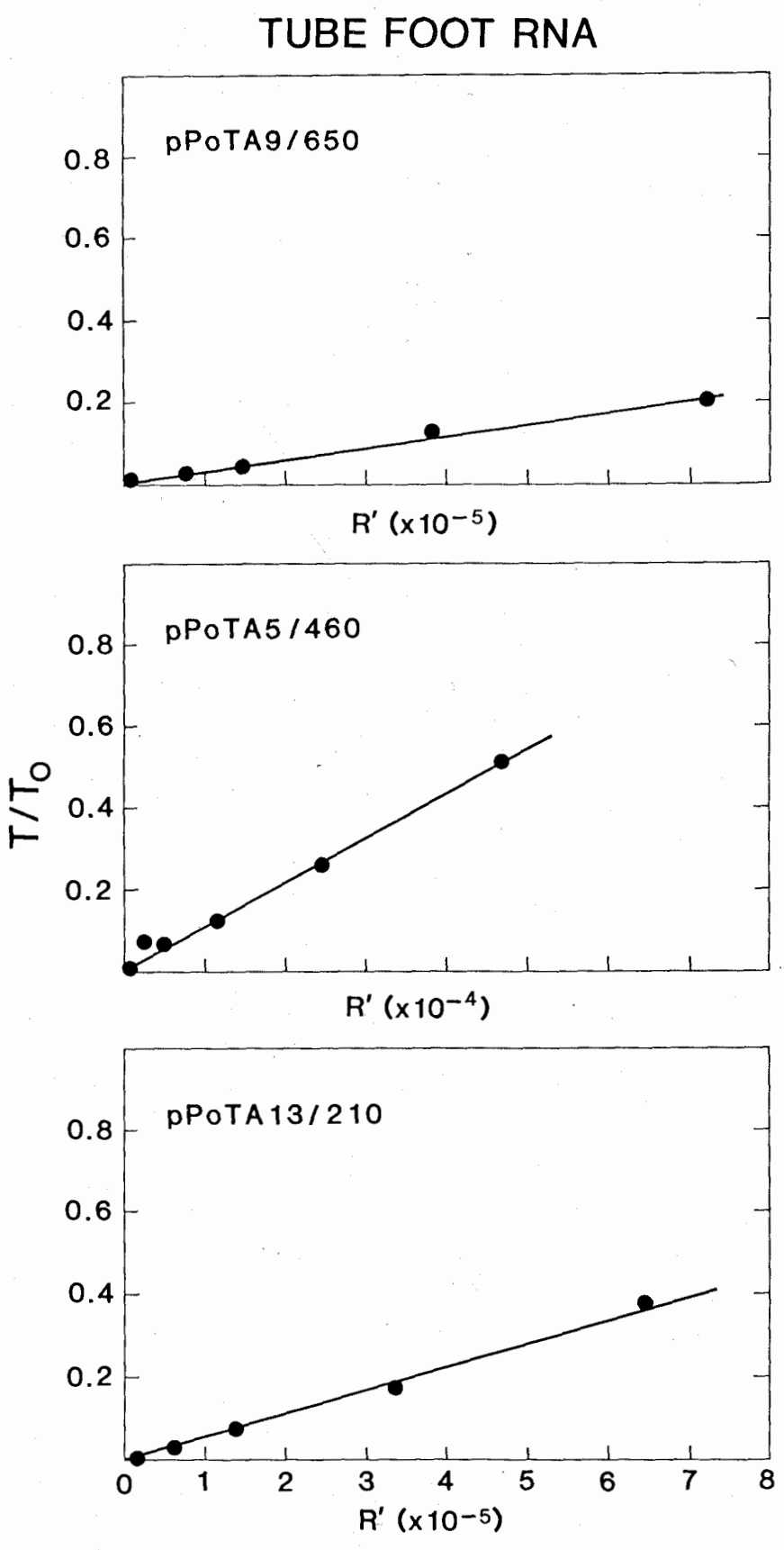

pPoTA9/650
pPoTA5/460
Egg
Blastula (21 h)
Early Gastrula (48 h)
Late Gastrula (72 h)
T/T0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
R'(x10-5)
R'(x10-4)

Figure 3.7 Titration of coding and 3' non-coding actin transcripts in tube foot RNA.

Total tube foot RNA was hybridized to single-stranded actin coding (pPoTA9/650) and 3'non-coding (pPoTA5/460 and pPoTA13/210) probes. The hybridization reactions were processed as described in the legend to Figure 3.6 and the same definitions apply.


TUBE FOOT RNA
pPoTA9/650
0.8
0.6
0.4
0.2
R′ (x10⁻⁵)
pPoTA5/460
T/T₀
0.8
0.6
0.4
0.2
R′ (x10⁻⁴)
pPoTA13/210
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
R′ (x10⁻⁵)

The initial slopes (1/a') derived from these plotes are used to calculate the number of transcripts per embryo.

For example, the slope (1/a') for egg RNA is 8.3×10^3 cpm μg^{-1} from Figure 3.6. Since the specific activity of the probe was 7.5×10^8 cpm μg^{-1} at the time of the experiment the fraction of total egg RNA mass present as probe-specific RNA is $(1/a) = 8.3 \times 10^3 / 7.5 \times 10^8 = 1.1 \times 10^{-5}$. The length of an average actin transcript is 2250 nt and the initial length of the probe is 680 nt. Therefore the true fraction of actin transcript in the egg is $1.1 \times 10^{-5} \times (2250/680) = 3.7 \times 10^{-5}$. Note that in this case the initial probe length instead of the 650 nt complexity was used, as otherwise the transcript fraction would be overestimated by hydroxyapatite chromatography. The number of actin transcripts can be calculated the same way as was described for the RNA driven hybridization. The mass of actin transcripts per egg is 0.56 pg that corresponds to a maternal actin transcript pool of 4.3×10^5 molecules.

The time course in the change of actin transcript numbers per embryo is illustrated in Figure 3.8. The data is taken from Table 3.1. The shape of the curve is in very close agreement with that expected from the relative sequence prevalence (Figure 3.3). The absolute number of transcripts vary by a factor of two for the three different experiments, however the deviation between the RNA driven and saturation hybridization measurements with total RNA are much less than that. The average number of transcript estimates are 3.1×10^5 per egg,

Figure 3.8 Number of actin transcripts in sea star development and tube foot.

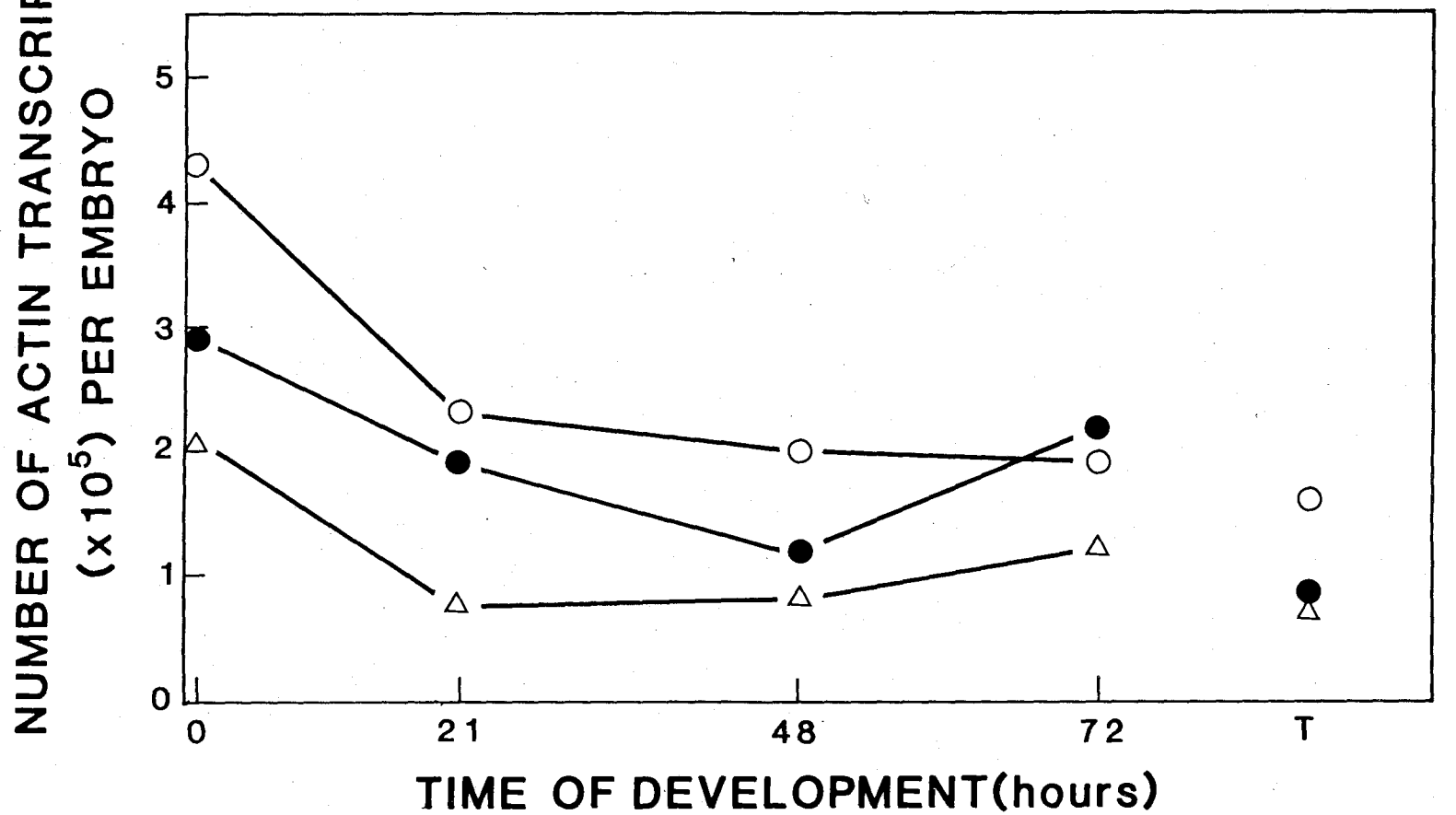
The time course in the change of actin transcript numbers in P. ochraceus early development is illustrated. The number of actin transcripts per 15 ng of tube foot RNA are shown for comparison. Data are taken from Table 3.1.

Symbols:

(•),total RNA driven hybridization;

(Δ),poly(A)$^+$ RNA driven hybridization;

(o),Saturation hybridization with total RNA.


NUMBER OF ACTIN TRANSCRIPTS
(x10^5) PER EMBRYO
0
1
2
3
4
5
0
21
48
72
T
TIME OF DEVELOPMENT(hours)

1.6×10^5 per blastula, 1.3×10^5 per early gastrula and 1.8×10^5 per late gastrula. In tube foot there are approximately 1×10^5 transcripts per 15 ng of total RNA.

Change in the number of actin transcripts per cell during early sea star development is illustrated in Figure 3.9. Data for this figure was taken from the last column of Table 3.1. The average number of transcripts per cell calculated from the three different experiments fit on a straight line up to early gastula stage when plotted on a log-log scale against the number of cells in an embryo.

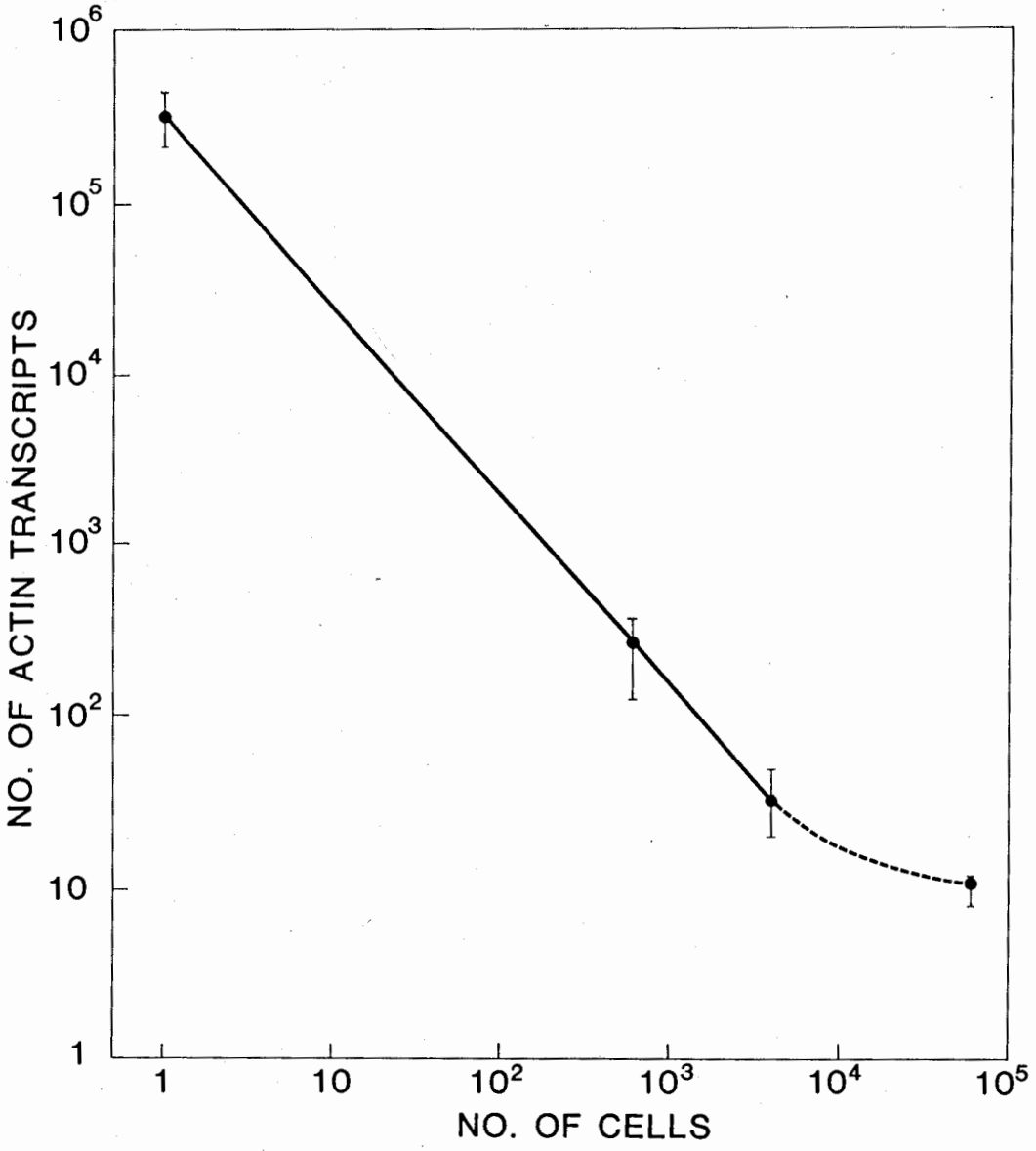
The total amount of RNA per embryo is assumed to be constant at about 15 ng. This value was measured for egg RNA (Chapter I). Isotopic dilution experiments were repeated for late gastrula as described in Chapter I, Materials and Methods. Repeat experiments gave values of 14.8 ng (S.D.=2.6;n=2) per late gastrula embryo.

Number of λPoA145 specific sequences in different developmental stages and in tube foot

Single-stranded M13 probe, containing the 460 nt 3' non-coding region specific to the λPoA145 gene family was used in RNA driven and saturation hybridization reactions in order to estimate the number of homologous transcripts in total and poly(A)$^+$ RNA.

Figure 3.9 Change in the number of actin transcripts per cell during early sea star development.

Data are taken from Table 3.1. The symbol (•) indicates the average of three values, RNA driven hybridization with total and poly(A)$^+$ RNA and saturation hybridization with total RNA. Error bars denote the range of the values. Average values from egg to early gastrula fit on a straight line.


NO. OF ACTIN TRANSCRIPTS
10^6
10^5
10^4
10^3
10^2
10
1
1
10
10^2
10^3
10^4
10^5
NO. OF CELLS

The specific activity was determined by a standard curve (Figure 3.10) titrating the ^{32}P-labeled probe against increasing quantities of unlabeled cold DNA insert as is described in Materials and Methods and Mauron et al. (1981). The initial slope of the curve is 5.3×10^7 cpm μg^{-1} which indicates a specific activity of 1.05×10^8 cpm μg^{-1}. Therefore, the initial specific activity (SP) at the time of labeling could be calculated as 1.2×10^8 cpm μg^{-1}.

All other aspects of the experiments were done as previously described for the coding sequence probe.

An example of RNA driven hybridization experiment with total and poly(A)$^+$ egg RNA is shown in Figure 3.11. The rate constants extracted from these data and the calculated values of the number of transcripts per embryo are listed in Table 3.2. There is very good agreement between values calculated from experiments with total and poly(A)$^+$ RNA. The relative change in transcript abundance is nearly identical with that observed previously (Figure 3.3), but the absolute numerical values are three times higher than expected from the results with the coding probe experiments (Table 3.1 and Figure 3.8). The last column in Table 3.2 shows the ratios between the number of transcripts calculated from these experiments and the maximum expected from the coding probe data of Table 3.1. The expected position of the hybridization curve is also indicated with dashed lines on Figure 3.11, assuming that all the actin transcripts originate from the λPoA145 gene family and,

Figure 3.10 Specific activity determination of the single-stranded 3' probe pPoTA5/460.

The specific activity of the 3' non-coding probe was determined by titration of unlabeled pPoTA5/460 fragment with single-stranded ^{32}P-labeled DNA, as explained in Materials and Methods. The initial slope of the curve is 5.3×10^7 cpm μg^{-1} and the specific activity is 1.05×10^8 cpm μg^{-1}.

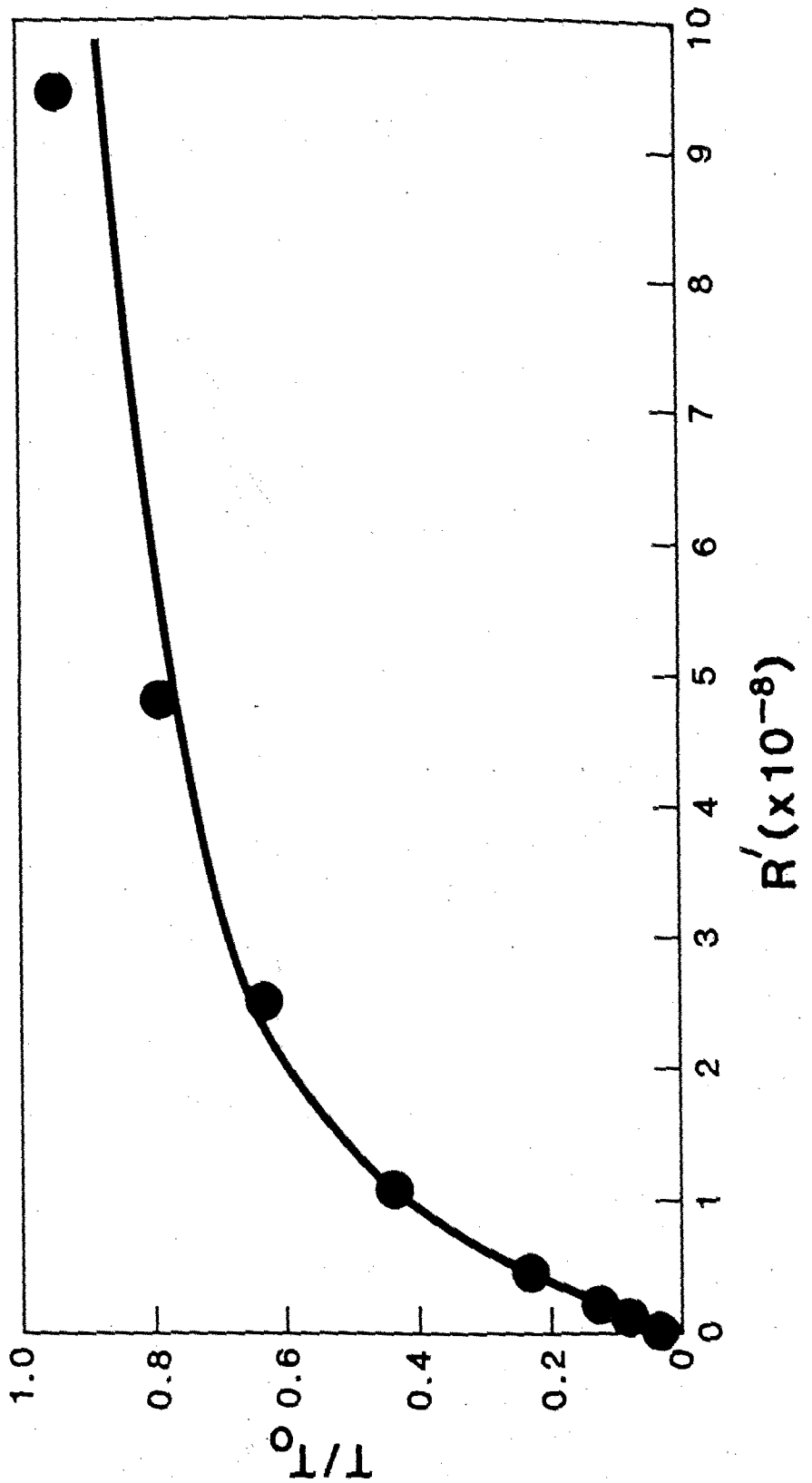

T/T$_o$
1.0
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
7
8
9
10
R′(x10^{-8})

Figure 3.11 RNA driven hybridization to the single-stranded pPoTA5/460 3' non-coding probe.

Total and poly(A)$^+$ RNA from egg was used to drive a ^{32}P-labeled single stranded probe (M13mp8/pPoTA5/460) in reassociation kinetics. Hybrids were analysed by hydroxyapatite chromatography (see Materials and Methods, Chapter I). The solid line represents the best least-square solution for the data assuming pseudo-first-order reaction (see figure legend, Figure 1.2). Dashed line indicates the curve expected if this probe was at the same concentration as actin transcripts. The rate constants obtained from these data fits are listed in Table 3.1.

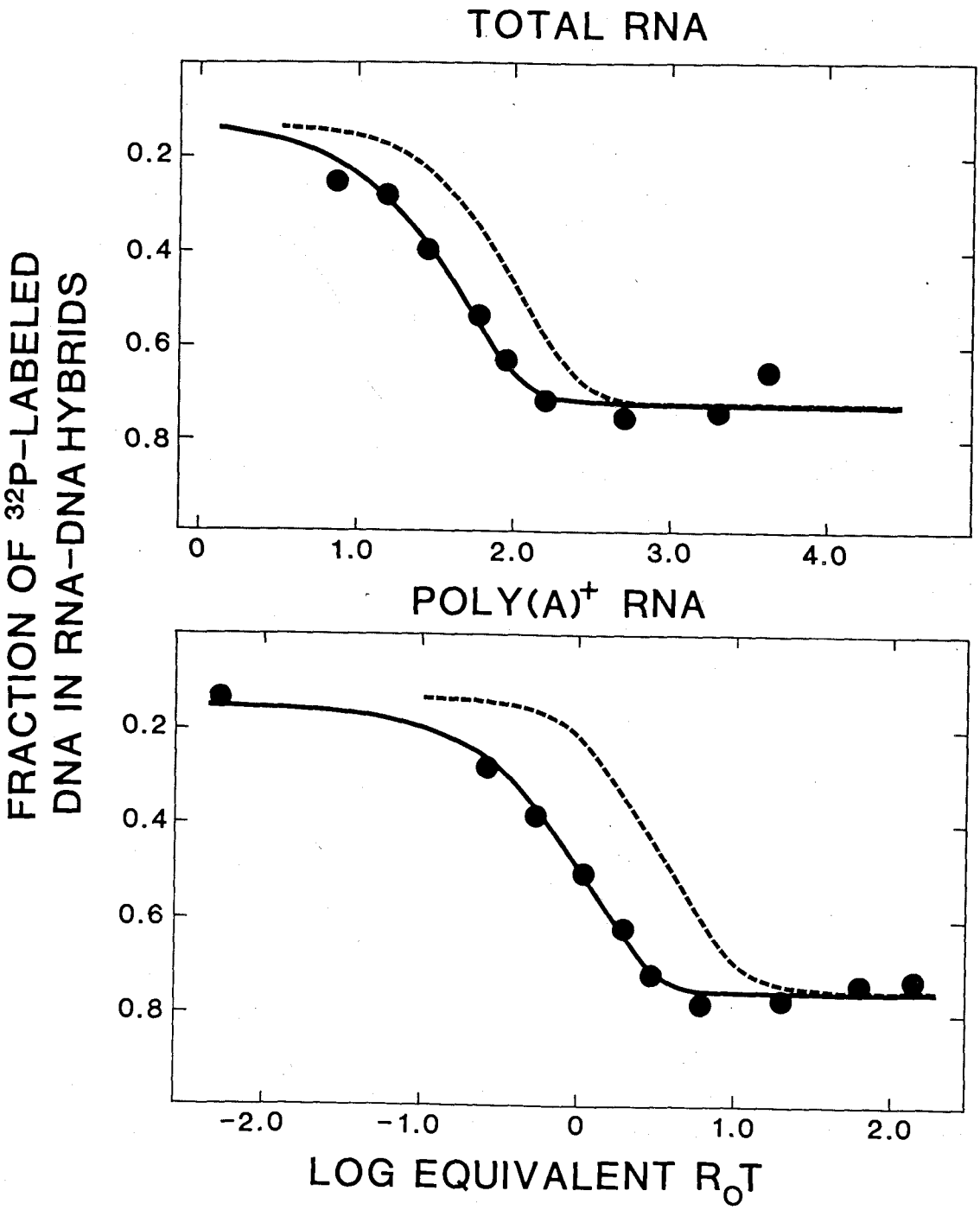

TOTAL RNA
FRACTION OF ^{32}P-LABELED DNA IN RNA-DNA HYBRIDS
0.2
0.4
0.6
0.8
0
1.0
2.0
3.0
4.0
POLY(A)$^+$ RNA
0.2
0.4
0.6
0.8
-2.0
-1.0
0
1.0
2.0
LOG EQUIVALENT R_oT

Table 3.2 Determination of the number of pPoTA5/460 3′ sequences in *Pisaster ochraceus* RNA.[a]

Stage/or Tissue	Hybridization rate K obs(RMS) ($M^{-1}s^{-1}$)(%)	Slope(1/a′) (cpm ug^{-1})	No.transcripts per embryo[b]	No.transcripts / No.transcripts expected[c]
RNA driven hybridization				
Total RNA				
Egg	0.0214 (3.5)		8.2×10^5	2.83
Blastula	0.0156 (2.6)		6.0×10^5	3.16
E.Gastrula	0.0117 (1.8)		4.5×10^5	3.75
L.Gastrula	0.0148 (3.5)		5.7×10^5	2.59
Tube foot	0.00450(1.7)		1.7×10^5	2.21
Poly(A+) RNA				
Egg	0.878 (2.3)		1.0×10^6	4.76
Blastula	0.246 (1.8)		4.7×10^5	6.27
E.Gastrula	0.210 (1.3)		4.1×10^5	5.12
L.Gastrula	0.326 (3.7)		6.5×10^5	5.42
Tube foot	0.234 (1.5)		7.2×10^4	0.99
Saturation hybridization				
Total RNA				
Egg		6.7×10^3	3.2×10^6	7.44
Blastula		3.7×10^3	2.1×10^6	9.13
E.Gastrula		3.1×10^3	1.7×10^6	8.50
L.Gastrula		3.4×10^3	1.9×10^6	10.0
Tube foot		1.1×10^3	5.6×10^5	3.50

Footnotes Table 3.2

a) Details of the calculations are described in footnotes to Table 3.1.

Characteristics of the probe used in the experiments:

pPoTA5/460: The initial length of the probe was 500 nt (LP) containing 460 nt complementary to RNA transcript (CP) and some M13 sequence. The tracer length at hybridization was 475 nt long (LT) and the initial specific activity was 1.2×10^8 cpm μg^{-1} (SP).

b) Assuming full length actin transcripts (2250 nt).

c) The number of transcripts expected are from Table 3.1.

most importantly, that all the transcripts are 2250 nt long. The pPoTA5/460 probe showed some cross reaction with the λPoA160 clone at moderate stringency (Figure 2.8B). However the amount of cross reaction between the two tube foot transcripts was unexpectedly high at the conditions used in these experiments. Therefore no estimates could be made of the number of specific transcripts in tube foot RNA.

Results of the titration experiments for the developmental stages are illustrated in Figure 3.6 and for tube foot in Figure 3.7. The initial slopes and the probe specific activity at the time of the experiments were used to calculate the transcript values listed in Table 3.2. The relative change in transcript abundance shows again the expected developmental time course (Figure 3.3), however the number of transcripts per embryo indicates on the average nine times higher values than the maximum expected from the results with the coding probe experiments (Table 3.1 and Figure 3.8).

Number of λPoA160 specific sequences in different developmental stages and in tube foot

RNA driven and saturation hybridization experiments were repeated with the single-stranded M13 probe containing the 210 nt 3' non-coding region specific to the λPoA160 gene family. RNA driven hybridization experiments indicated that this probe did not react with RNA from any of the developmental stages.

Reactions were carried out which exceeded 100 x Rot(1/2) of that expected for an actin coding probe. These experiments would have detected as few as 0.1% of the total actin transcript population.

Results of the RNA driven hybridization with total and poly(A)$^+$ tube foot RNA are shown in Figure 3.12 and the rate constants listed in Table 3.3. The number of transcripts calculated from this data is 14.3 and 7.5 times higher for total and poly(A)$^+$ RNA respectively than expected from the experiments with the coding sequence probe.

The specific activity of the ^{32}P-labeled probe was determined by a standard curve, as was described previously. The initial slope of the curve is 1 x 10^9 cpm μg^{-1} (data not shown) from which the specific activity at time of the experiment was calculated as 2 x 10^9 cpm μg^{-1} with 2.5 x 10^9 cpm μg^{-1} at labeling. The saturation hybridization with total tube foot RNA is shown in Figure 3.7. Calculations from this slope indicate 2.6 x 10^5 transcript per 15 ng RNA which correlate fairly well with that expected from the coding sequence probe. This is in sharp contrast to the above described results with the RNA driven experiments.

Figure 3.12 RNA driven hybridization to the single-stranded pPoTA13/210 3' non-coding probe.

Total and poly(A)$^+$ RNA from tube foot was used to drive a ^{32}P-labeled single stranded probe (M13mp9/pPoTA13/210) in reassociation kinetics. Hybrids were analysed by hydroxyapatite chromatography (see Materials and Methods, Chapter I). The solid line represents the best least-square solution for the data assuming pseudo-first-order reaction (see figure legend, Figure 1.2). Different symbols indicate independent experiments. Dashed line indicates the curve expected if this probe was at the same concentration as actin transcripts. The rate constants obtained from these data fits are listed in Table 3.1.

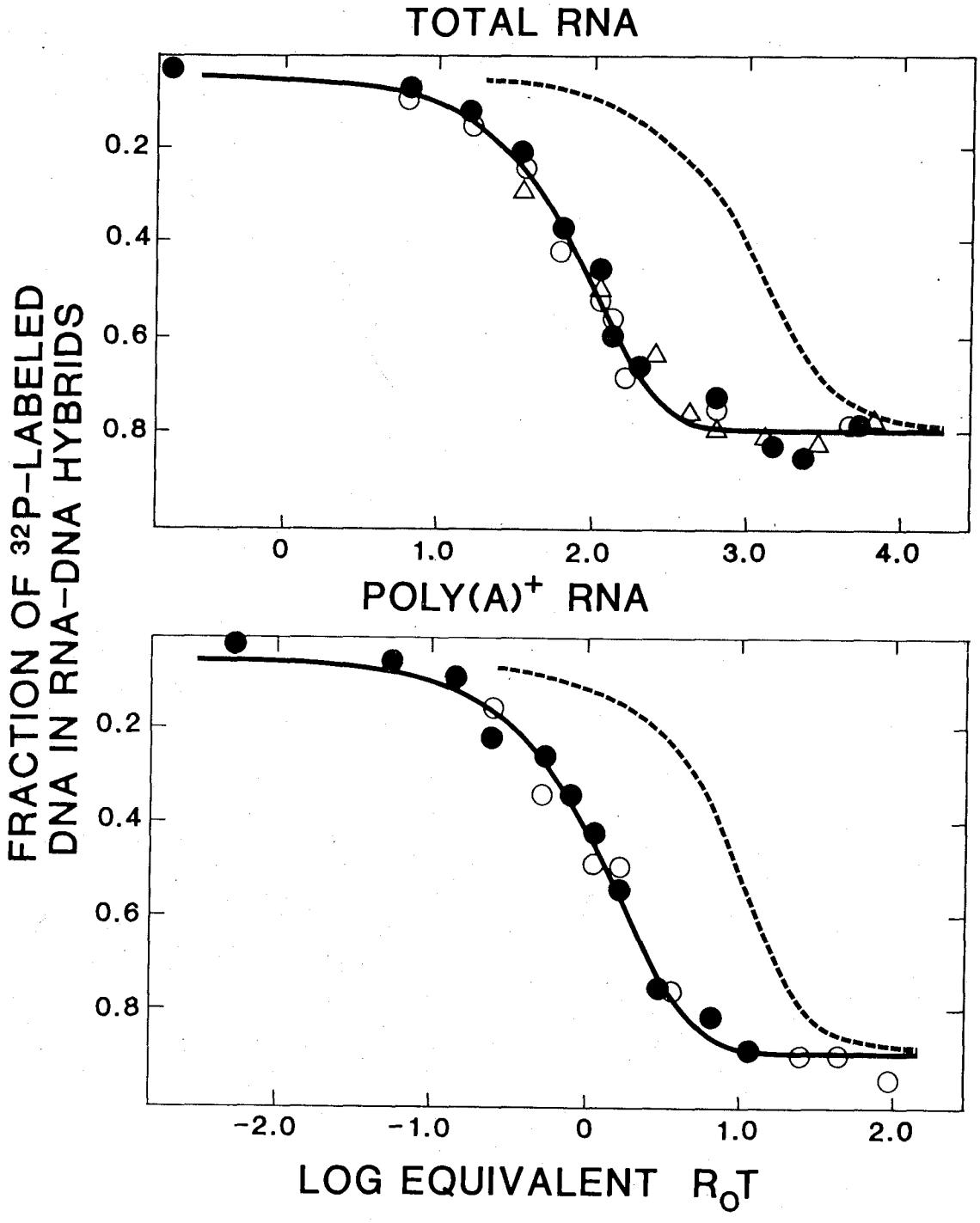

TOTAL RNA
FRACTION OF ^{32}P-LABELED DNA IN RNA-DNA HYBRIDS
0.2
0.4
0.6
0.8
0
1.0
2.0
3.0
4.0
POLY(A)$^+$ RNA
0.2
0.4
0.6
0.8
-2.0
-1.0
0
1.0
2.0
LOG EQUIVALENT R_oT

Table 3.3 Determination of the number of pPoTA13/210 3′ sequences in Pisaster ochraceus RNA.[a]

Stage/or Tissue	Hybridization rate K obs(RMS) ($M^{-1}s^{-1}$)(%)	Slope(1/a′) (cpm ug^{-1})	No.transcripts per embryo[b]	No.transcripts / No.transcripts expected[c]
RNA driven hybridization				
Total RNA				
Egg	No hybridization			
Blastula	No hybridization			
E.Gastrula	No hybridization			
L.Gastrula	No hybridization			
Tube foot	0.00934(3.0)		$1.1x10^6$	14.3
Poly(A+) RNA				
Egg	No hybridization			
Blastula	No hybridization			
E.Gastrula	No hybridization			
L.Gastrula	No hybridization			
Tube foot	0.586 (3.9)[d]		$5.5x10^5$	7.53
Saturation hybridization				
Total RNA				
Tube foot		$5.7x10^3$	$2.6x10^5$	1.63

Footnotes Table 3.3

a) Details of the calculations are described in footnotes to Table 3.1.

Characteristics of the probe used in the experiments:

pPoTA13/210: The initial length of the probe was 230 nt (LP) containing 210 nt complementary to RNA transcript (CP) and some M13 sequence. The tracer length at hybridization was 155 nt long (LT) and the initial specific activity was 2.5×10^9 cpm μg^{-1} (SP).

b) Assuming full length actin transcripts (2250 nt).

c) The number of transcripts expected are from Table 3.1.

d) Saturation hybridization was not done with the embryonic RNAs.

IV. DISCUSSION

Expression of actin transcripts in sea star early development

It was demonstrated in this study that the expression of actin genes in sea stars follows the pattern described for most of the prevalent polyadenylated cytoplasmic RNAs in the sea urchin system (Lasky et al., 1980; Flytzanis et al., 1982). All sea stars examined have a large store of maternal actin transcripts. The number of transcripts in P. ochraceus declines from approximately 3 x 10^5 per egg to half of this value at blastula - early gastrula before it begins to increase again. P. ochraceus embryos start to hatch at 29 h after fertilization and more than 65% of them hatched at 32 h (Fraser et al., 1981). The lowest transcript abundance can be found close to this time period. The apparent lowest transcript concentration at blastula in reality might mean the complete degradation of the maternal RNA between the two stages as was described for S. dröbachiensis (Bushman and Crain, 1983).

The total number of actin transcripts increases after embryo hatching, indicating newly synthesized RNA production. All or most of these new actin sequences originate from the same λPoA145,128 gene family as the maternal RNA transcripts.

The pattern of actin transcript accumulation in P. ochraceus is strikingly different from that observed for sea urchins and a sand dollar (Echinarachnius parma) (Bushman and Crain, 1983). In these systems the abundance of actin-coding RNA is relatively low in early embryos and dramatically increases during development.

The sea urchin studied most extensively is S. purpuratus (Crain et al., 1982; Merlino et al., 1981), where a picture of a very complex actin gene expression pattern is emerging. Shott et al. (1984) demonstrated that at least six of the eight actin genes of this animal are expressed at some stage of development, and each of these genes is regulated differentially. A 2.2 and 1.8 kb transcript of two genes is also expressed in the maternal RNA (Garcia et al., 1984), but the total number of maternal actin transcripts is estimated as less than 3000 (Shott et al., 1984). The presence of the larger transcript in every tissue tested indicates that it is generally expressed and encodes a cytoplasmic or cytoskeletal actin.

The equivalent of this in P. ochraceus system appears to be the 2.3 kb transcript from the λPoA145,128 gene family which is present in all the developmental stages as well as tube foot. It is transcribed from a gene present only once per haploid genome similarily to the CyI gene in S. purpuratus (Lee et al., 1984). This single gene product apparently could account for all the actin transcripts in early embryos. The size of this transcript is very close to that of its equivalent in sea urchins.

Furthermore, the transcript size does not change through development, indicating that the mature sea star egg probably contains fully processed actin transcripts.

The time course of actin transcript accumulation could be explained in more than one way. Due to lack of data for transcript turnover rates, the simplest explanation is that actin transcripts in P. ochraceus embryos are not transcribed at all until hatching stage, or transcribed at a very low rate. In both cases the observed pattern is dominated by the slow degradation of the maternal RNA. In sea urchins most of the prevalent transcripts are very stable (Cabrera et al., 1984). Furthermore the large increase in the number of actin mRNA in S. purpuratus early development was explained by a constant rate of synthesis per cell and very low rate of transcript degradation (Peters and Kleinsmith, 1984).

The decline of the number of actin transcripts in an average cell appears to be correlated with the total number of cells in the embryo until early gastrula stage (Figure 3.9). Following this, the number of transcripts stabilizes at approximately 10 per cell. There is considerable localization occurring during cell differentiation (reviewed by Angerer and Angerer, 1983). Therefore there may be an unequal distribution of transcripts.

Actin gene expression has been extensively analysed in sea urchins (Crain et al., 1981; 1982; Merlino et al., 1981; Durica and Crain, 1982; Flytzanis et al., 1982; Crain and Bushman,

1983; Bushman and Crain, 1983; Garcia et al., 1984; Shott et al., 1984). The main conclusion of these studies is that most sea urchin eggs contain very little actin specific maternal RNA, and the transcript prevalence appears to increase approximately 50-fold from egg to gastrula. The results presented in this chapter demonstrate that the maternal expression of actin genes in sea stars are completely different from that observed in sea urchins.

Actin gene expression in P. ochraceus tube foot

Sea star tube feet are short tubular external projections of the body wall used as sensory, locomotive and anchoring appendages (Fretter and Graham, 1976). Earlier histological studies indicated that at least 50% of the cellular volume of the tube foot is made up of tissue that can be classified as smooth muscle (Nichols, 1961; Kabat-Zinn and Singer, 1981). However a recent ultrastructural examination showed that the retractor muscle of sea star podium is a collection of myoepithelial cells (Wood and Cavey, 1981; Cavey and Wood, 1981). The major abundant protein component of these highly differentiated cells appears to be actin (Kabat-Zinn and Singer, 1981).

The most prevalent actin transcript in P. ochraceus tube foot is 2.2 kb and originates from the λPoA160 gene family. This gene is not expressed in any of the embryonic stages and it

could be analogous to muscle-specific actin. Sea urchins (Shott et al., 1984) and vertebrates (Nudel et al., 1982) appear to contain a single-copy muscle-specific gene. On the other hand in D. melanogaster four out of six actin genes exhibit muscle-specific expression (Fyrberg et al., 1983; Sanchez et al., 1983). Analysis in Chapter II indicated that there might be two genes per haploid genome for the λPoA160 family in P. ochraceus.

The amount of muscle-specific transcripts in tube foot poly(A)$^+$ RNA of S. purpuratus was shown to be considerably lower than the major cytoplasmic gene product (Shott et al., 1984). In P. ochraceus the λPoA160 gene product appears to account for as much as 90% of the actin sequences in total RNA. However when poly(A)$^+$ RNA was used in RNA dot-blots and transfers, the relative amount of reaction with the pPoTA5/460 probe was greatly increased compared to the reaction with the pPoTA13/210 probe (results not shown). This indicates that the ratio of the number of two different tube foot actin transcripts dramatically changes in favor of the λPoA145 gene product in poly(A)$^+$ RNA. The concentration of cytoplasmic transcripts in poly(A)$^+$ RNA is also substantiated by the unexpectedly large number of λPoA145 specific clones in the tube foot cDNA libraries (Chapter II). Hunter and Garrels (1977) comparing vertebrate β- and α-actin transcripts found that the former had considerably shorter poly(A) tails. Because of this, a substantial portion of the β-actins failed in binding to oligo(dT)-cellulose. If echinoderm

muscle-specific actin transcripts have shorter poly(A) tails than cytoplasmic transcripts, experiments using poly(A)$^+$ RNA could have biased results.

Expression and function of 3' non-coding actin sequences

The size of the P. ochraceus actin transcripts is very close to that found in sea urchins, indicating that they might have more than 1000 nt of non-coding sequence. Bushman and Crain (1983) argue for strong selective pressure in the maintenance of these sequences, which could imply some physiological function.

The 3' non-coding probe derived from the λPoA137 gene did not react with any of the RNAs tested. From these negative results it is not possible to determine that these are pseudogenes, or genes which are not transcribed in these specific RNAs. The possibility also exists that the probe was from a non-transcribed or processed region of the gene.

Experiments with the two 3' specific cDNA probes showed surprising results. Most of the numeric values for the number of transcripts far exceeded the maximum expected from the coding probe experiments. Furthermore the wide range of differences between the RNA driven and saturation hybridization results seems to eliminate any simple explanation.

The most obvious explanation would be DNA contamination of the RNA samples. However that should have affected the coding probe experiments and would not be expected to influence the poly(A)$^+$ RNA results. Further, RNase digestion controls did not

show any DNA contamination.

Unspecific hybridization to ribosomal RNA or some other abundant RNA transcripts could have been overlooked as most of the RNA transfers were hybridized at high stringencies. However, when the stringency of hybridization was lowered, there were no obvious unspecific bands. All probes showed some ribosomal background which were in general similar.

The first clue regarding the type of RNA which could cause these discrepencies came from comparisons of saturation and RNA driven hybridization results with the pPoTA13/210 probe (Table 3.3). There are no obvious experimental errors which could account for deviation in an order of magnitude, as the two different experiments gave similar results for the coding sequence probe (Table 3.1). The probe length was approximately 150 nt, which would probably not cause overestimation even with short RNA species in saturation hybridization. Alternatively, RNA driven hybridization results would change substantially if the RNA driver was short. Saturation hybridization indicates 1.63 times more transcripts than the maximum expected from the results with the coding probe experiments (Table 3.3). This would indicate that approximately 60% more sequences react with the 3' non-coding than the actin coding probe. How would these excess sequences affect the RNA driven hybridization results? If somewhat simplistically it is assumed that the extra 60% is derived from homologous fragments in the size range of the probe that is 100-200 nt, then the rate of the RNA driven

hybridization would be determined by the smallest fragments. Substituting this size range for RNA driver length, the number of transcripts can be calculated as 2.3×10^5. This figure is very similar to the results found with saturation hybridization. Therefore, a small highly prevalent transcript (or transcript fragment) homologous to the actin 3' non-coding probes could account for the observed results.

Hybridization with abundant transcripts from repetitive DNA sequences, similar to those of Drosophila transposable elements (Finnegan et al., 1977), would satisfy this criterion.

Another possibilitiy would be reaction with small nuclear RNAs (snRNA) which have a size range of 90 to 220 nt and present 10^5-10^6 copies per HeLa or Novikoff hepatoma cell (Choi and Ro-Choi, 1980). snRNAs are highly conserved and ubiquitous in eukaryotes (reviewed by Busch et al., 1982). These sequences were also shown to be present in comparably high numbers during early Xenopus development (Forbes et al., 1984).

In both cases above, specific fragments would be expected to react with the 3' probe. However, high percent RNA gel transfers hybridized at moderate stringencies failed to turn up any discrete bands which would be expected for the amount of RNA involved.

To test the amount of sequence homology involved in the reactions, RNA driven and saturation hybridizations were carried out using stringent criteria. Increasing the stringency would be expected to reduce the fraction of hybrids significantly if the

sequences are not well matched. However, the results were again negative. Neither RNA driven or saturation hybridization reactions evaluated at 68°C or 70°C showed significant differences from that observed at 60°C for the coding or 3' non-coding probes.

The conclusion from the above analysis is that the reaction must occur with a diverse size range of small RNAs which are homologous to the probes, and probably degradation products of the original transcripts. Since the RNA contains substantially more 3' non-coding fragments than coding sequences the degradation of transcripts appears to be non-random. It is therefore unlikely that the RNA degradation is the result of the RNA extraction procedure.

In a differentiated adult tissue such as tube foot, steady rate of synthesis and degradation of transcripts could be expected. Therefore the steady state quantity of each transcript will be determined by the ratio of synthesis rate and decay constant (reviewed by Davidson, 1976). The synthesis rate is identical for different parts of a transcript. Therefore different steady state levels of the coding and 3' non-coding sequences could be reached if there are differential rates of degradation.

Results of the experiments with the embryonic system using the pPoTA5/460 probe are more difficult to interpret, becouse the saturation hybridization experiments are subject to two technical problems. Firstly, the probe used was close to 500 nt

long which could have caused great overestimation of sequence numbers as explained previously. Using S1-nuclease instead of hydroxyapatite in the evaluation of hybrid formation would resolve this question. Secondly, a systematic error which would cause an underestimate in the specific activity determination could have compounded the the errors. There is a biological problem as well. Early development is a complex dynamic system which contains various amounts of maternal and embryonic transcripts depending on the stage of development (reviewed by Raff, 1980). As it is not a steady state system assumptions cannot be made as to rates of synthesis and degradation of the actin coding and 3' non-coding sequences. Even considering the above difficulties, the high numeric values in RNA driven hybridization indicate the presence of 3' sequences independent of the coding region.

RNA in cells are associated with different proteins to form RNP particles (reviewed by Raff, 1980). The associated proteins can have many different functions, including protection of RNA from nucleases. The most vulnerable place for a transcript might be the 3' non-coding region, as these sequences are neither protected by capping nor shielded by the translational machinery. Peters and Jeffery (1978) reported that there are two high molecular weight proteins bound to the poly(A) tails of sea urchin egg RNAs. Therefore it is possible that the 3' end of mRNAs are protected and degraded slower than the coding sequence.

It is possible that the excess of these 3' sequences in the cell are only a vestige of differential degradation, but on the other hand it could indicate a biological role for the untranslated regions.

Modiano and Pepe (1983) recently demonstrated that the quantitative expression of human globin genes is controlled by both 5' and 3' untranslated regions. The latter was previously shown to be highly conserved in the globin mRNA of primates (Martin et al., 1981). The pPoTA5/460 sequence also appears to be highly conserved between P. ochraceus and P. brevispinus (C. Cuddeford and M.J. Smith unpublished observations). The conservation of specific 3' non-coding sequences in sea urchin actins (Lee et al., 1984), rat and human cardiac actin mRNAs (Mayer et al., 1984), rat and human cytoplasmic β-actin genes (Nudel et al., 1983) and also between chick and rat α-actin genes (Ordahl and Cooper, 1983) argues for some common function. The protection of the 3' end in itself might significantly extend the stability of these prevalent transcripts, allowing for their accumulation without increasing transcription rates (Cabrera et al., 1984; Peters and Kleinsmith, 1984).

Cytoplasmic gene control was demonstrated for casein (Guyett et al., 1979) and vitellogenin (Goldberger, 1981) showing different half lives for the same mRNA in the same cell under diverse circumstances. Evidence exists for a wide range of half lives involving different mRNAs in the same cells (Harpold et al., 1981) and in sea urchin embryos (Cabrera et al., 1984).

Davidson et al. (1982b) also suggested that distinct 5' and/or 3' non-coding sequences might be responsible for the turnover rate difference measured between two embryonically expressed actin transcripts in S. purpuratus.

It is also possible that the 3' non-coding sequences are involved in regulatory functions as observed for procaryotes (reviewed by Gottesman et al., 1982). Feedback control through these 3' sequences or control factors associated with them could influence transcription rates and/or the release rate of stored maternal mRNA.

Apparently not all non-coding sequences are essential, as the D. imbricata has a 300 nt shorter maternal actin transcript than P. ochraceus. This is the case as well in some of the sea urchin species.

There has been no direct demonstration up to the present that the 3' non-coding sequences are involved in any physiological processes. However increasing evidence implies that they have some type of role in regulatory functions.

E. SUMMARY

The objectives of this study were to assess the transcript informational content of the egg RNA of Pisaster ochraceus and to determine the abundance and representation of the different actin gene transcripts at various stages of embryonic development. The following information was gained from this research:

1. Sequence complexity of the mature P. ochraceus oocyte RNA was determined as 4.3×10^7 nucleotides.

2. The percent of RNA mass representing the single-copy informational content of the P. ochraceus maternal RNA was determined as 2-3%.

3. The total RNA content in P. ochraceus early development was established as approximately 15 ng. This RNA content remains constant from egg to late gastrula stage.

4. Actin specific genomic λ clones were isolated and characterized.

5. cDNA libraries were established from P. ochraceus tube foot, egg end late gastrula.

6. Actin specific cDNA clones were isolated and characterized.

7. The number of actin genes in P. ochraceus were estimated to be 5 from dot-blot and Southern transfer analyses.

8. The genomic origin of the two tube foot expressed transcripts were determined using 3' non-coding probes.

9. Restriction fragment polymorphism was demonstrated in all three actin gene families.

10. Three sea star species, P. ochraceus, P. brevispinus, and D. imbricata were kinetically determined to contain 5,5 and 8 actin genes respectively.

11. P. ochraceus tube foot tissue expressed actin transcripts of 2.3 and 2.2 kb with the latter representing as much as 90% of the total amount.

12. 15 ng tube foot total RNA was determined to contain approximately 1 x 10^5 actin transcripts.

13. The eggs of P. ochraceus, P. helianthoides, and E. troschelii expressed a 2.3 kb actin transcript, while the eggs of D. imbricata expressed a 2.0 kb actin transcript.

14. D. imbricata egg RNA was determined to contain approximately 6 times more actin transcripts by mass than P. ochraceus.

15. The relative amount of actin gene expression was assessed in P. ochraceus early development, showing that the amount of transcripts declined by approximately 50% at the blastula stage and increased again in late gastrula.

16. The absolute number of actin transcripts per P. ochraceus egg, 21 h blastula, 48 h early gastrula and 72 h late gastrula were determined as approximately 3.1 x 10^5, 1.6 x 10^5, 1.3 x 10^5 and 1.8 x 10^5 respectively.

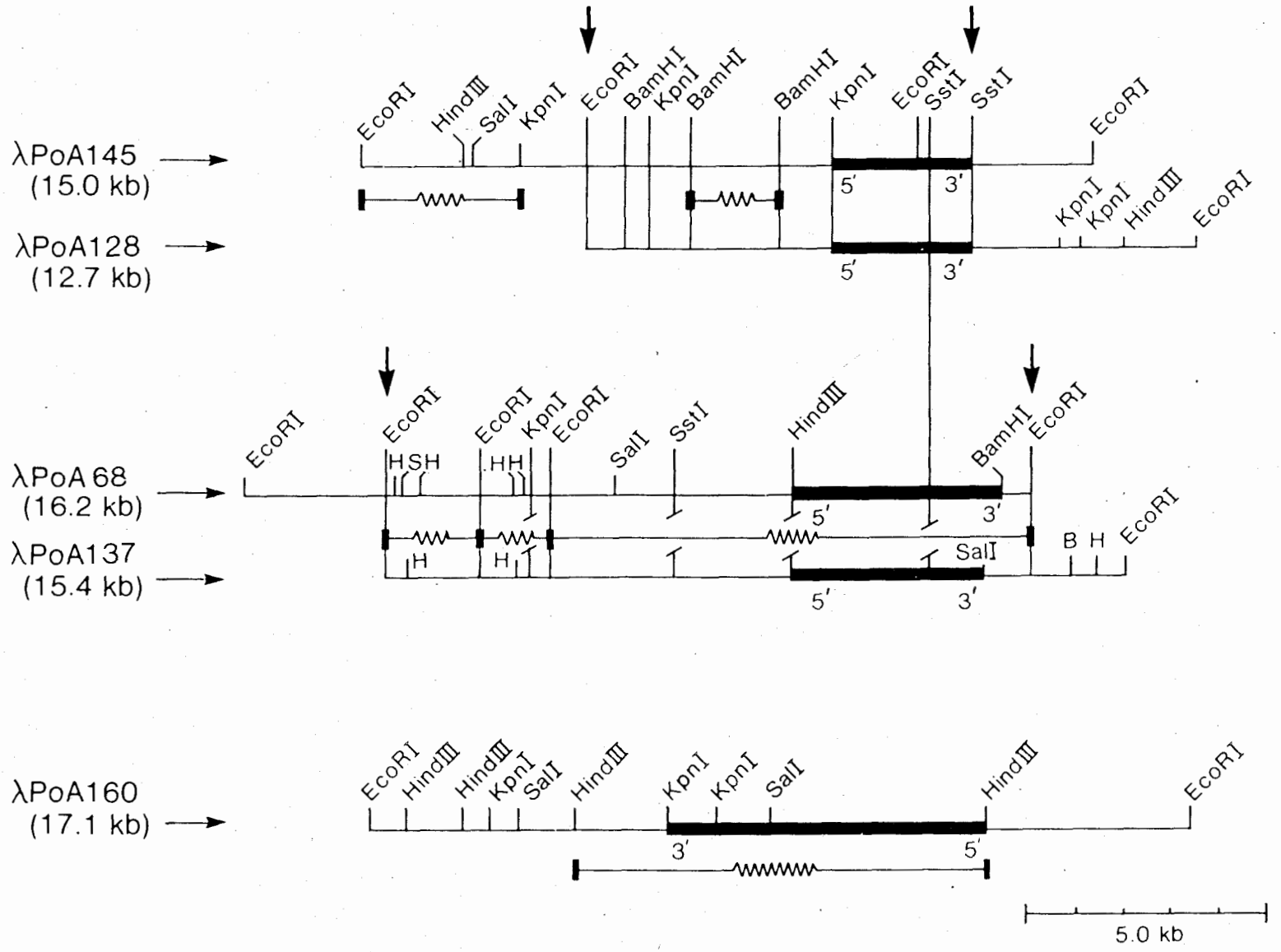
17. Indirect evidence was shown for differential degradation of coding and 3' non-coding regions of actin transcripts in embryonic and tube foot cells.

APPENDIX A

Restriction Enzyme Maps of the Sea Star Actin Genomic Clones

(From Kovesdi *et al.*, 1984)

The four Charon 4 clones λPoA145, λPoA128, λPoA68, λPoA137 are aligned with the SstI site which is found within their actin coding fragments. λPoA145 and λPoA128 are aligned on coincident EcoRI, BamHI, and KpnI sites. λPoA68 and λPoA137 are aligned on coincident EcoRI, KpnI, SstI, and HindIII sites. The solid bars indicate fragments which contain actin coding sequences. The 3' and 5' ends of the actin coding sequence containing fragments were determined by independent hybridization of restriction digests with the 3' actin coding sequence probe pSpG2 from sea urchin and the total actin coding sequence probe pDMA2 from Drosophila melanogaster. Regions common to two clones for which estimates of sequence divergence were made are indicated by heavy vertical arrows. The restriction fragments which contain repetitive sequences are indicated below the restriction maps by horizontal bars with jagged inserts; e.g. the 1.9 kb BamHI limited fragment common to both λPoA145 and λPoA128. To detect repeat sequences sheared genomic DNA, 380 bp long, was labeled with ^{32}P to a specific activity of 7×10^6 cpm μg^{-1} and hybridized to restriction digests of the lambda recombinants. One μg of each of the five clones was totally digested with the following enzyme combinatins: λPoA145 and λPoA128 with EcoRI plus BamHI, or EcoRI plus KpnI; λPoA68 and λPoA137 with EcoRI; and λPoA160 with EcoRI plus HindIII. The Southern transfers of each of these digestions was hybridized with 1×10^6 cpm of the ^{32}P-labeled genomic DNA for 20 h at 68°C in 4X SET. The following notation was used where necessary for restriction enzymes: B, BamHI; H, HindIII; and S, SalI.

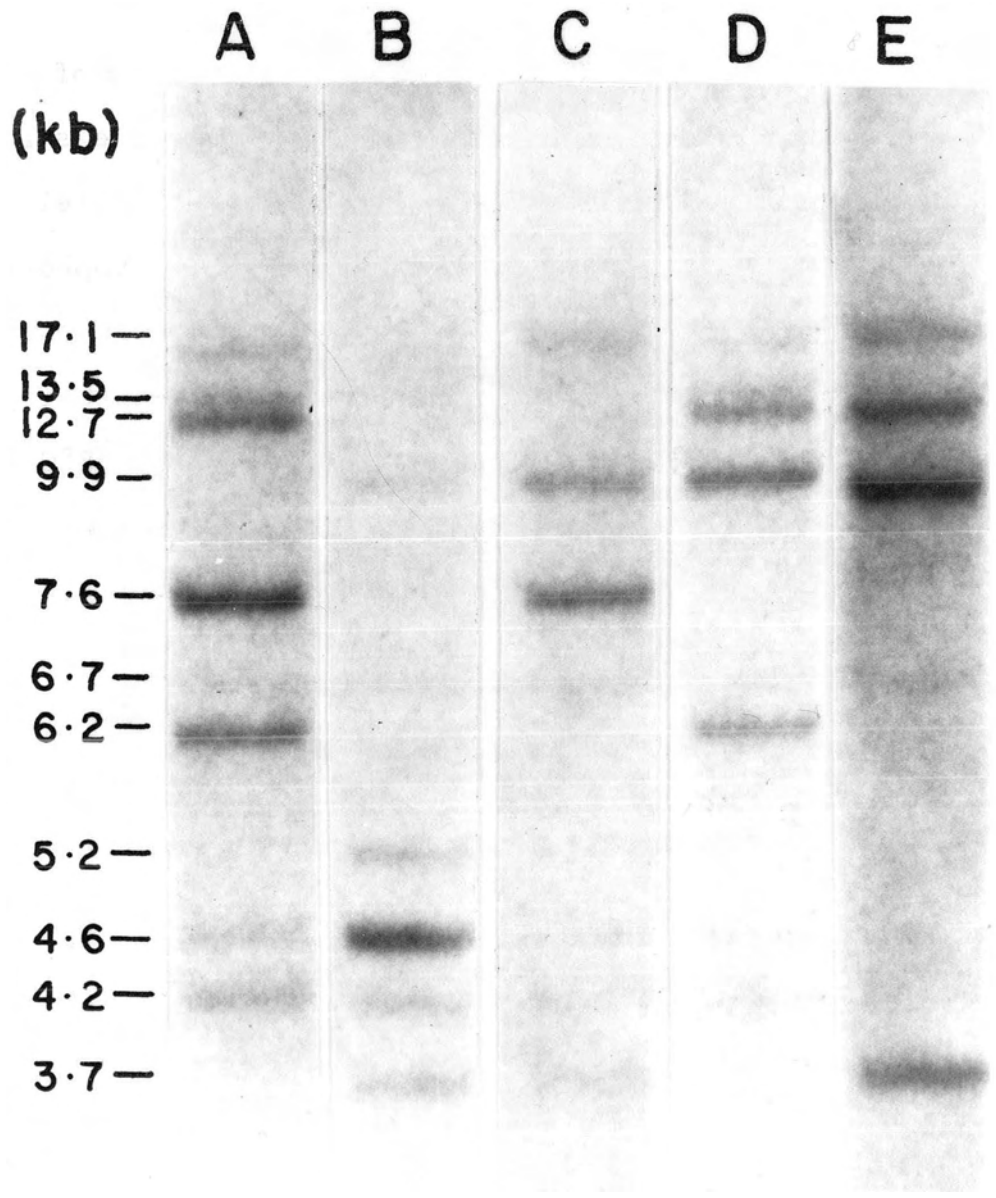

λPoA145
(15.0 kb)
λPoA128
(12.7 kb)
λPoA 68
(16.2 kb)
λPoA137
(15.4 kb)
λPoA160
(17.1 kb)
EcoRI
HindIII
SalI
KpnI
BamHI
SstI
H
B
5′
3′
5.0 kb

APPENDIX B

Southern Blot Hybridization of EcoRI-Digested Genomic DNA from Individual Sea Stars with an Actin cDNA Probe

(From Kovesdi *et al.*, 1984)

Genomic DNA from five individual sea stars was totally digested with EcoRI. Each DNA sample was incubated with a two-fold unit excess of EcoRI μg^{-1} of DNA for two hours at 37°C in 50 mM NaCl, 100 mM Tris pH 7.4, and 6 mM magnesium chloride. The completeness of reaction was judged by the appearance of characteristic EcoRI bands visible in ethidium bromide stained agarose gels under U.V. transillumination and by parallel digestions of lambda DNA. The digested DNA was electrophoresed in 0.7% agarose gels and transferred to nitrocellulose paper. The filters were hybridized simultaneously with ^{32}P-labeled insert fragment from the tube foot plasmid pPoTA9 at 68°C in 5X SET overnight. The pPoTA9 insert fragment (1050 bp) was removed from the vector by PstI digestion and preparative gel electrophoresis. The isolated fragment was digested with AvaI and the two resulting fragments (590 bp and 460 bp) were end-filled with the Klenow fragment and ^{32}P-dNTPs. The probe specific activity was 1.2×10^8 cpm μg^{-1}. After hybridization, the filters were washed sequentially in 4X SET 0.2% SDS, 2X SET 0.2% SDS, 1X SET 0.2% SDS at 68°C with two one hour changes per salt condition. The filters were washed finally for 4 h with 0.2X SET 0.2% SDS at 68°C, dried and exposed to x-ray film with intensifying screens. The following amounts of EcoRI digested genomic DNA were applied to the original electrophoretic gel: A, 4.8 μg; B, 2.9 μg; C, 5.4 μg; D, 3.1 μg; E, 5.2 μg.


A
B
C
D
E
(kb)
17·1
13·5
12·7
9·9
7·6
6·7
6·2
5·2
4·6
4·2
3·7

REFERENCES

Angerer, R.C. and Angerer, L.M. (1983). RNA localization in sea urchin embryos. In "Time, space, and pattern in embryonic development." (Jeffery, W.R. and Raff, R.A., eds.) pp.101-129. Alan R. Lis, Inc., New York.

Aviv, H., and Leder, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Nat. Acad. Sci. U.S.A. 69:1408-1412.

Benton, W.D. and Davis, R.W. (1977). Screening λgt recombinant clones by hybridization to single plaques in situ. Science 196:180-182.

Blattner, F.R., Williams, B.G., Blechl, A.E., Denniston-Thompson, K., Faber, H.E., Furlong, L.A., Grunwald, D.J., Kiefer, D.O., Moore, D.D., Sheldon, E.L., Smithies, O. (1977). Charon phages: Safer derivatives of bacteriophage lambda for DNA cloning. Science 196:161-169.

Blin, N. and Stafford, D.W. (1976). A general method for isolation of high molecular weight DNA from eukaryotes. Nucleic Acids Res. 3:2303-2308.

Bolivar, F., Rodrigues, R.L., Greene, P.J., Betlach, M.C. Heyneker, H.L., Boyer, H.W. (1977). Contruction and characterization of new cloning vehicles. Gene 2:95-113.

Boylan, E.S., LaMarca, M.J., Schuetz, A.W. (1973). RNA synthesis in the ovary and oocytes of the starfish. Dev. Biol. 31:136-146.

Brandhorst, B.P. (1976). Two-dimensional gel patterns of protein synthesis before and after fertilization of sea urchin eggs. Dev. Biol. 52:310-317.

Brandhorst, B.P., (1980). Simultaneous synthesis, translation, and storage of mRNA including histone mRNA in sea urchin eggs. Dev. Biol. 79:139-148.

Brandhorst, B.P., Bedard, P., Tufaro, F. (1983). Patterns of protein metabolism and the role of maternal RNA in sea urchin embryos. In "Time, space, and pattern in embryonic development." (Jeffery, W.R. and Raff, R.A., eds.) pp.29-47. Alan R. Lis, Inc., New York.

Britten, R.J., Graham, D.E., and Neufeld, B.R. (1974). Analysis of repeating DNA sequences by reassociation. In "Methods in enzymolology." (Grossman, L. and Moldave, K., eds.) Vol. 29E:363-418. Academic Press, New York.

Brosius, J., Palmer, M.L., Poindexter, J.K., Noller, H.F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 75:4801-4805.

Brosius, J., Dull, T.J., Noller, H.F. (1980). Complete nucleotide sequence of a 23S ribosomal RNA gene from Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 77:201-204.

Busch, H., Reddy, R., Rothblum, L., Choi, Y.C. (1982). SnRNAs, SnRNPs, and RNA processing. Ann. Rev. Biochem. 51:617-654.

Bushman, F.D. and Crain, W.R. Jr. (1983). Conserved pattern of embryonic actin gene expression in several sea urchins and a sand dollar. Dev. Biol. 98:429-436.

Cabrera, C.V., Jacobs, H.T., Posakony, J.W., Grula, J.W. Roberts, J.W., Britten, R.J., Davidson, E.H. (1983). Transcripts of three mitochondrial genes in the RNA of sea urchin eggs and embryos. Dev. Biol. 97:500-505.

Cabrera, C.V., Lee, J.J., Ellison, J.W., Britten, R.J., Davidson, E.H. (1984). Regulation of cytoplasmic mRNA prevalence in sea urchin embryos. J. Mol. Biol. 174:85-111.

Casadaban, M.J. and Cohen, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. J. Mol. Biol. 138:179-207.

Cavey, M.J. and Wood, R.L. (1981). Specializations for excitation-contraction coupling in the podial retractor cells of the starfish Stylasterias forreri. Cell Tissue Res. 218:475-485.

Chamberlin, M.E., Galau, G.A., Britten, R.J., and Davidson, E.H. (1978). Studies on nucleic acid reassociation kinetics: V. Effects of disparity in tracer and driver fragment lengths. Nucleic Acids Res. 5:2073-1094.

Chan, H., Ruyechan, W.T., and Wetmur, J.R. (1976). In vitro iodination of low complexity nucleic acids without chain scission. Biochem. 15:5487-5490.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochem. 18:5294-5299.

Choi, Y.C. and Ro-Choi, T. (1980). Basic characteristics of different classes of cellular RNA's: A directory. In "Cell biology a comprehensive treatise." Vol. 3:609-667. Academic Press, New York.

Clarke, L. and Carbon, J. (1976). A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire E. coli genome. Cell 9:91-99.

Commerford, S.L. (1971). Iodination of nucleic acids in vitro. Biochem. 10:1993-1999.

Constantini, F.D., Scheller, R.H., Britten, R.J. and Davidson, E.H. (1978). Repetitive sequence transcripts in the mature sea urchin oocyte. Cell 15:173-187.

Constantini, F.D., Britten, R.J., and Davidson, E.H. (1980). Message sequences and short repetitive sequences are interspersed in sea urchin egg poly(A)$^+$ RNAs. Nature 287:111-117.

Cooper, A.D. and Crain, W.R., Jr. (1982). Complete nucleotide sequence of a sea urchin actin gene. Nucleic Acids Res. 13:4081-1092.

Crain, W.R. Jr., Durica, D.S., Van Doren, K. (1981). Actin gene expression in developing sea urchin embryos. Mol. Cell. Biol. 1:711-720.

Crain, W.R. Jr., Durica, D.S., Cooper, A.D., Van Doren, K., Bushman, F.D. (1982). Structure and developmental expression of actin genes in the sea urchin. In "Muscle development: Molecular and cellular control." pp.97-105. Cold Spring Harbour Laboratory, New York.

Crain, W.R. Jr. and Bushman, F.D. (1983). Transcripts of paternal and maternal actin gene alleles are present in interspecific sea urchin embryo hybrids. Dev. Biol. 100:190-196.

Crouse, G.F., Frischauf, A., Lehrach, H. (1983). An integrated and simplified approach to cloning into plasmids and single-stranded phages. In "Methods in Enzymology." (Wu, R., Grossman, L., Moldave, K., eds.) Part C, Vol. 101:78-89.

Czihak, G., ed. (1975). "The sea urchin embryo." Springer-Verlag, Berlin.

Davidson, E.H. (1976). "Gene activity in early development." 2nd Ed. Academic Press, New York.

Davidson, E.H., Hough-Evans, B.R., Britten, R.J. (1982a). Molecular biology of the sea urchin embryo. Science. 217:17-26.

Davidson, E.H., Thomas, T.L., Scheller, R.H., and Britten, R.J. (1982b). The sea urchin actin genes and a speculation on the evolutionary significance of small gene families. In "Genome evolution." (Dover, G. A. and Flavell R. B., eds.)

pp.177-191, Academic Press, New York.

Davis, R.W., Botstein, D., Roth, J.R. (1980). "A manual for genetic engineering: Advanced bacterial genetics." Cold Spring Harbor Laboratory, New York.

Deeley, R.G., Gordon, J.I., Burns, A.T.H., Mullinix, K.P., Binastein, M., and Goldberger, R.F. (1977). Primary activation of the vitellogenin gene in the rooster. J. Biol. Chem. 252:8310-8319.

Denhardt, D.T. (1966). A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23:641-646.

Dische, Z. (1955). Color reactions of nucleic acid components. In "The nucleic acids." (Chargaff, E., and Davidson, J.N., eds.) Vol. 1:285-306. Academic Press, New York.

Dolecki, G.J., Duncan, R.F. and Humphreys, T. (1977). Complete turnover of poly(A) on maternal mRNA of sea urchin embryos. Cell, 11:339-344.

Drury, M.F. (1948). Identification and estimation of pentoses in the presence of glucose. Arch. Biochem. 19:450-466.

Duncan, R. and Humphreys, T. (1981). Multiple oligo(A) tracts associated with inactive sea urchin maternal mRNA sequences. Dev. Biol. 88:211-219.

Duncan, R. and Humphreys, T. (1983). Oligo(U) sequences present in sea urchin maternal RNA decrease following fertilization. Dev. Biol. 96:258-262.

Durica, D.S., Schloss, J.A., Crain, Jr., W.R. (1980). Organization of actin gene sequences in the sea urchin: Molecular cloning of an intron-containing DNA sequence coding for a cytoplasmic actin. Proc. Natl. Acad. Sci. U.S.A. 77:5683-5687.

Durica, D.S. and Crain, W.R. Jr. (1982). Analysis of actin synthesis in early sea urchin development. Dev. Biol. 92:428-439.

Efstratiadis, A., Posakony, J.W., Maniatis, T., Lawn, R.M., O'Connell, C., Spritz, R.A., DeRiell, J.K., Forget, B.G., Weissman, S.M., Slightom, J.L., Blechl, A.E., Smithies, O., Baralle, F.E., Shoulders, C.C., Proudfoot, N.J. (1980) The structure and evolution of the human β-globin gene family. Cell 21:653-668.

Engel, J.N., Gunning, P.W., Kedes, L. (1981). Isolation and characterization of human actin genes. Proc. Natl. Acad. Sci. U.S.A. 78:4674-4678.

Engels, W. R. (1981). Estimating genetic divergence and genetic variability with restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 78:6329-6333.

Epel, D. (1967). Protein synthesis in sea urchin eggs: A "late" response to fertilization. Proc. Natl. Acad. Sci. U.S.A. 57:899-906.

Epel, D. (1980). Experimental analysis of the role of intracellular calcium in the activation of sea urchin egg at fertilization. In "The cell surface: Mediator of developmental processes." (Subtelny, S. and Wessells, N.K., eds.) pp.169-185. Academic Press, New York.

Ernst, S.G., Hough-Evans, B.R., Britten, R.J., and Davidson, E.H. (1980). Limited complexity of the RNA in micromeres of 16-cell sea urchin embryos. Dev. Biol. 79:119-127.

Files, J.G., Carr, S., Hirsh, D. (1983). Actin gene family of Caenorhabditis elegans. J. Mol. Biol. 164:355-375.

Finnegan, D.J., Rubin, G.M., Young, M.W., Hogness, D.S. (1977). Repeated gene families in Drosophila melanogaster. Cold Spring Harbor Symp. Quant. Biol. 42:1053-1063.

Firtel, R. A. (1981). Multigene families encoding actin and tubulin. Cell 24:6-7.

Fleck, A., and Begg, D. (1965). The estimation of ribonucleic acid using ultraviolet absorption measurements. Biochem. Biophys. Acta 108:333-339.

Flytzanis, C.N., Brandhorst, B.P., Britten, R.J. and Davidson, E.H. (1982). Developmental patterns of cytoplasmic transcript prevalence in sea urchin embryos. Dev. Biol. 91:27-35.

Forbes, D.J., Kirschner, M.W., Caput, D., Dahlberg, J.E., Lund, E. (19849. Differential expression of multiple U1 small nuclear RNAs in oocytes and embryos of Xenopus laevis. Cell 38:681-689.

Fornwald, J.A., Kuncio, G., Peng, I., Ordahl, C.P. (1982). The complete nucleotide sequence of the chick a-actin gene and its evolutionary relationship to the actin gene family. Nucleic Acids Res. 10:3861-3876.

Fraser, A., Gomez, J., Hartwick, E.B., and Smith, M.J. (1981). Observations on the reproduction and development of Pisaster ochraceus (Brandt). Canad. J. Zool. 59:1700-1707.

Fretter, V. and Graham, A. (1976). "A functional anatomy of invertebrates." pp.403. Academic Press, New York.

Fyrberg, E.A., Kindle, K.L., Davidson, N. and Sodja, A. (1980).

The actin genes of Drosophila: a dispersed multigene family. Cell 19:365-378.

Fyrberg, E.A., Bond, B.J., Hershey, N.D., Mixter, K.S., Davidson, N. (1981). The actin genes of Drosophila: Protein coding regions are highly conserved but intron positions are not. Cell 24:107-116.

Fyrberg, E.A., Mahaffey, J.W., Bond, B.J., Davidson, N. (1983). Transcripts of the six Drosophila actin genes accumulate in a stage- and tissue-specific manner. Cell 33:115-123.

Galau, G.A., Britten, R.J., and Davidson, E.H. (1974). A measurement of the sequence complexity of polysomal messenger RNA in sea urchin embryos. Cell 2:9-11.

Galau, G.A., Klein, W.H., Davis, M.M., Wold, B.J., Britten, R.J., and Davidson, E.H. (1976). Structural gene sets active in embryos and adult tissues of the sea urchin. Cell 7:487-505.

Garcia, R., Paz-Aliaga, B., Ernst, S.G., Crain, W.R. Jr. (1984). Three sea urchin actin genes show different patterns of expression: Muscle specific, embryo specific, and constitutive. Mol. Cell. Biol. 4:840-845.

Glisin, V., Crkvejaov, R., Byus,C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. Biochemistry 13:2633-2637.

Goldberger, R. (1981). Regulation of gene expression in avian liver. In "The role of RNA in development and reproduction." (Niu, M. and Chuang, H.H., eds.) p.215-231. Science Press, Beijing.

Gottesman, M., Oppenheim, A., Court, D. (1982). Retroregulation: Control of gene expression from sites distal to the gene. Cell 29:727-728.

Guo, L. and Wu, R. (1983). Exonuclease III: Use for DNA sequence analysis and in specific deletions of nucleotides. In "Methods in Enzymology." (Wu, R., Grossman, L., Moldave, K., eds.) Part B, Vol. 100:60-96.

Guyette, W.A., Matusik, R.J., Rosen, J.M. (1979). Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression. Cell 17:1013-1023.

Harpold, M.M., Wilson, M.C., Darnell, J.E. Jr. (1981). Chinese hamster polyadenylated messenger ribonucleic acid: Relationship to non-polyadenylated sequences and relative conservation during messenger ribonucleic acid processing. Mol. Cell. Biol. 1:188-198.

Higuchi, R., Paddoc, G.V., Wall, R. and Salser, W. (1976). A general method for cloning eukaryotic structural gene sequences. Proc. Natl. Acad. Sci. U.S.A. 73:3146-3150.

Hinegardner, R.T. (1967). Echinoderms. In "Methods in developmental biology." (Trinkaus, J.P. ed.) pp.136-155. Thomas, Y. Crowele Co., New York.

Hinegardner, R. (1974). Cellular DNA content of the Echinodermata. Comp. Biochem. Physiol. 49B:219-126.

Hoopes, B.C. and McClure, W.R. (1981). Studies on the selectivity of DNA precipitation by spermine. Nucleic Acids Res. 9:5493-5504.

Hough, B.R., Smith, M.J., Britten, R.J., and Davidson, E.H. (1975). Sequence complexity of heterogeneous nuclear RNA in sea urchin embryos. Cell 5:291-199.

Hough-Evans, B.R., Wold, B.J., Ernst, S.G., Britten, R.J., and Davidson, E.H. (1977). Appearance and persitance of maternal RNA sequences in sea urchin development. Dev. Biol. 60:258-177.

Hough-Evans, B.R., Jacobs-Lorena, M., Cummings, M.R., Britten, R.J., and Davidson, E.H. (1980). Complexity of RNA in eggs of Drosophila melanogaster and Musca domestica. Genetics 95:81-94.

Houk, M.S., and Epel, D. (1974) Protein synthesis during hormonally induced meiotic maturation and fertilization in starfish oocytes. Dev. Biol. 40:298-310.

Humphreys, T. (1969). Efficiency of translation of messenger RNA before and after fertilization of sea urchin eggs. Dev. Biol. 20:435-458.

Humphreys, T. (1971). Measurements of messenger RNA entering polysomes upon fertilization of sea urchin eggs. Dev. Biol. 26:201-208.

Hunter, T. and Garrels, J.I. (1977). Characterization of the mRNAs for α-, β-, γ-actin. Cell 12:767-781.

Jeffery, W.R. (1977). Polyadenylation of maternal and newly-synthesized RNA during starfish oocyte maturation. Dev. Biol. 57:98-108.

Jeffery, W.R. (1983). Messenger RNA localization and cytoskeletal domains in ascidian embryos. In "Time, space, and pattern in embryonic development." (Jeffery, W.R. and Raff, R.A., eds.) pp.241-259. Alan R. Lis, Inc., New York.

Jeffery, W.R. and Raff, R.A., ed. (1983). "Time, space and pattern in embryonic development." Alan R. Liss, Inc., New

York.

Jefferys, A.J. (1981). Recent studies of gene evolution using recombinant DNA. In "Genetic Engineering." (Williamson, R., ed.) Vol. 2:1-48. Academic Press, New York.

Kabat-Zinn, R. and Singer, R. H. (1981). Sea urchin tube feet: Unique structures that allow a cytological and molecular approach to the study of actin and its gene expression. J. Cell. Biol. 89:109-114.

Kafatos, F.C., Jones, C.W., Efstratiadis, A. (1979). Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. 7:1541-1552.

Kanatani, H. (1969). Induction of spawning and oocyte maturation by L-methyl adenine in starfishes. Exp. Cell Res. 57:333-337.

Kanatani, H. (1975). Maturation-inducing substances in asteroid and echinoid oocytes. Amer. Zool. 15:493-505.

Khalili, K., Salas, C., Weinmann, R. (1983). Isolation and characterization of human actin genes cloned in phage lambda vectors. Gene 21:9-17.

Kindle, K.L. and Firtel, R.A. (1978). Identification and analysis of Dictyostelium actin genes, a family of moderately repeated genes. Cell 15:763-778.

Kiper, M. (1979). Gene numbers as measured by single-copy saturation with mRNA are routinely overestimates. Nature 278:279-180.

Kovesdi, I., and Smith, M.J. (1982). Sequence complexity in the maternal RNA of the starfish Pisaster ochraceus (Brandt). Dev. Biol. 89,56-63.

Kovesdi, I., Preugschat, F., Sturzel, M., and Smith, M.J. (1984). Actin genes from the sea star Pisaster ochraceus. Biochim. Biophys. Acta 782,76-86.

Lasky, L.A., Lev, Z., Xin, J., Britten, R.J., and Davidson, E.H. (1980). Messenger RNA prevalence in sea urchin embryos measured with cloned cDNAs. Proc. Nat. Acad. Sci. U.S.A. 77:5317-5321.

Lee, J.J., Shott, R.J., Rose III, S.J., Thomas, T.L., Britten, R.J., Davidson, E.H. (1984). Sea urchin actin gene subtypes. J. Mol. Biol. 172:149-176.

Maniatis, T., Jeffrey, A., Kleid, D.G. (1975). Nucleotide sequence of the rightward operator of phage λ Proc. Natl. Acad. Sci. U.S.A. 72:1184-1188.

Maniatis, T., Hardison, R.C., Lacy, E., Lauer, J., O'Connel, C., Quon, D., Kim, G.K. and Efstratiadis, A. (1978). The isolation of structural genes from libraries eukaryotic DNA. Cell 15:687-701.

Maniatis, T., Fritsch, E.F., Sambrook, J. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York.

Martin, S.L., Zimmer, E.A., Davidson, W.S., Wilson, A.C. (1981). The untranslated regions of β-Globin mRNA evolve at a functional rate in higher primates. Cell 25:737-741.

Martindale, M.Q. and Brandhorst, B.P. (1984). Translational changes induced by 1-methyladenine in anucleate starfish oocytes. Dev. Biol. 101:512-515.

Mauron, A., Levy, S., Childs, G., Kedes, L. (1981). Monocistronic transcription is the physiological mechanism of sea urchin embryonic histone gene expression. Mol. Cell. Biol. 1:661-671.

Mauron, A., Kedes, L., Hough-Evans, B.R., Davidson, E.H. (1982). Accumulation of individual histone mRNAs during Embryogenesis of the sea urchin Strongylocentrotus purpuratus. Dev. Biol. 94:425-434.

Maxam, A.M. and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. In "Methods in Enzymology." (Colowick, S.P., and Kaplan, N.O., eds.) Vol. 65:499-560.

Mayer, Y., Czosnek, H., Zeelon, P.E., Yaffe, D., Nudel, U. (1984). Expression of the genes coding for the skeletal muscle and cardiac actins in the heart. Nucleic Acids Res. 12:1087-1100.

Merlino, G.T., Water, R.D., Moore, G.P., Kleinsmith, L.J. (1981). Change in expression of the actin gene family during early sea urchin development. Dev. Biol. 85:505-508.

Messing, J. and Vieira, J. (1982). A new pair of M13 vectors for selecting either strand of double-digest restriction fragments. Gene 19:269-276.

Messing, J. (1983). New M13 vectors for cloning. In "Methods in Enzymology." (Wu, R., Grossman, L., Moldave, K., eds.) Part C, Vol. 101:20-77.

Modiano, G. and Pepe, G. (1983). The quantitative expression of δ and β human globin genes is controlled by both 5' and 3' untranslated regions. Mol. Biol. Med. 1:157-159.

Munro, H.N., and Fleck, A. (1966). The determination of nucleic

acids. In "Methods of biochemical analysis." (Glick, D., ed.). Vol. 14:113-176. Interscience, New York.

Murre, C., Reiss, C.S., Bernabeu, C., Chen, L.B., Burakoff, S.J., Seidman, J.G. (1984). Construction, expression and recognition of an H-2 molecule lacking its carboxyl terminus. Nature 307:432-436.

McDonnell, M.W., Simon, M.N., Studier, F.W. (1977). Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. 110:119-146.

McKeown, M., Taylor, W.C., Kindle, K., Firtel, R.A., Bender, W. and Davidson, N. (1978). Multiple, heterogeneous actin genes in Dictyostelium. Cell 15:789-800.

McKeown, M. and Firtel, R.A. (1981). Differential expression and 5' end mapping of actin genes in Dictyostelium. Cell 24:799-807.

McMaster, G.K. and Carmichael, G.G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. U.S.A. 74:4835-4838.

Nemer, M. (1975). Developmental changes in the synthesis of sea urchin embryo messenger RNA containing and lacking polyadenylic acid. Cell 6:559-570.

Ng, R. and Abelson, J. (1980). Isolation and sequence of the gene for actin in Sacchromyces cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 77:3912-3916.

Nichols, D. (1961). A comparative histological study of the tube feet of two regular echinoids. Q.J. Microsc. Sci. 102:157-180.

Noll, H. (1967). Characterization of macromolecules by constant velocity sedimentation. Nature 215:360-363.

Nudel, U., Katcoff, D., Zakut, R., Shani, M., Carmon, Y., Finer, M., Czosnek, H., Ginsburg, I., Yaffe, D. (1982). Isolation and characterization of rat skeletal muscle and cytoplasmic actin genes. Proc. Natl. Acad. Sci. U.S.A. 79:2763-2767.

Nudel, U., Zakut, R., Shani, M., Neuman, S., Levy, Z. Yaffe, D. (1983). The nucleotide sequence of the rat cytoplasmic β-actin gene. Nucleic Acids Research 11:1759-1771.

Ohama, T., Hori, H., Osawa, S. (1983). The nucleotide sequences of 5S rRNAs from a sea-cucumber, a starfish and a sea-urchin. Nucleic Acids Research 11:5181-5184.

Ordahl, C.P. and Cooper, T.A. (1983). Strong homology in promotor and 3'-untranslated regions of chick and rat α-actin genes. Nature 303:348-349.

Overbeek, P.A., Merlino, G.T., Peters, N.K., Cohn, V.H., Moore, G.P., Kleinsmith, L.J. (1981). Characterization of five members of the actin gene family in the sea urchin. Biochim. Biophys. Acta. 656:195-205.

Patient, R. (1984). DNA hybridization - beware. Nature 308:15-16.

Paul, C.R.C. (1977). Evolution of primitive echinoderms. In "Patterns of evolution." pp.123-158. (Hallam, A.,ed.) Elsevier, Amsterdam.

Pearson, W., Davidson, E.H., and Britten, R.J. (1977). Program for least squares analyses of reassociation hybridization data. Nucleic Acids Res. 4:1727-1737.

Peters, C. and Jeffery, W.R. (1978). Postfertilization poly(A) protein complex formation on sea urchin maternal messenger RNA. Differentiation 12:91-97.

Peters, N.K. and Kleinsmith, L.J. (1984). Kinetic model for the study of gene expression in the developing sea urchin. Dev. Biol. 102:133-137.

Raff, R.A. (1980). Masked messenger RNA and the regulation of protein synthesis in eggs and embryos. In "Cell biology a comprehensive treatise." (Prescott, D.M. and Goldstein, L. eds.) Vol. 4:107-136. Academic Press, New York.

Raff, R.A. (1983). Localization and temporal control of expression of maternal histone mRNA in sea urchin embryos. In "Time, space, and pattern in embryonic development." (Jeffery, W.R. and Raff, R.A., eds.) pp.65-86. Alan R. Lis, Inc., New York.

Raff, R.A., Anstrom, J.A., Huffman, C.J., Leaf, D.S., Loo, J., Showman, R.M. (1984). Origin of a gene regulatory mechanism in the evolution of echinoderms. Nature 310:312-314.

Rodgers, W.H., and Gross, P.R. (1978). Inhomogeneous distribution of egg RNA sequences in early embryos. Cell 14:279-188.

Rosenthal, E.T., Hunt T., Ruderman, J.V. (1980). Selective translation of mRNA controls the pattern of protein synthesis during early development of the surf clam, Spisula solidissima. Cell 20:487-494.

Rosenthal, E.T., Brandhorst, B.P., Ruderman, J.V. (1982). Translationally mediated changes in patterns of protein

synthesis during maturation of starfish oocytes. Dev. Biol. 91:215-220.

Roychoudhury, R. and Wu, R. (1980). Terminal transferase-catalyzed addition of nucleotides to the 3' termini of DNA. In "Methods in enzymology." (Colowick, S.P. and Kaplan, N.O. eds.) Vol. 65:43-62. Academic Press, New York.

Sanchez, F., Tobin, S.L., Rdest, U., Zulauf, E., McCarthy, B.J. (1983). Two Drosophila actin genes in detail. J. Mol. Biol. 163:533-551.

Sanger, F., Air, G.M., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchison III, C.A., Slocomber, P.M., Smith, M. (1977a). Nucleotide sequence of bacteriophage ΦX174 DNA. Nature 265:687-695.

Sanger, F., Nicklen, S., Coulson, A.R. (1977b). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74:5463-5467.

Scheller, R.H., Costantini, F.D., Kozlowski, M.R., Britten, R.J., Davidson, E.H. (1978). Specific representation of cloned repetitive DNA sequences in sea urchin RNAs. Cell. 15:189-203.

Scheller, R.H., McAllister, L.B., Crain, W.R., Durica, D.S., Posakony, J.W., Britten, R.J., and Davidson, E.H. (1981). Organization and expression of multiple actin genes in sea urchin. Mol. Cell. Biol. 1:609-628.

Schroeder, T.E. and Stricker, S.A. (1983). Morphological changes during maturation of starfish oocytes: Surface ultrastructure and cortical actin. Dev. Biol. 98:373-384.

Schuler, M.A. and Keller, E.B. (1981). The chromosomal arrangement of two linked actin genes in the sea urchin S. purpuratus. Nucleic Acids Res. 9:591-604.

Schuler, M.A., McOsker, P., Keller, E.B. (1983). DNA sequence of two linked actin genes of sea urchin. Mol. Cell. Biol. 3:448-456.

Schwartz, R.J. and Rothblum, K. (1980). Regulation of muscle differentiation: Isolation and purification of chick actin messenger ribonucleic acid and quantitation with complementary deoxyribonucleic acid probes. Biochem. 19:2506-2514.

Shah, D.M., Hightower, R.C., Meagher, R.B. (1982). Complete nucleotide sequence of a soybean actin gene. Proc. Natl. Acad. Sci. U.S.A. 79:1022-1026.

Shott, R.J., Lee, J.J., Britten, R.J., Davidson, E.H. (1984).

Differential expression of the actin gene family of Strongylocentrotus purpuratus. Dev. Biol. 101:295-306.

Smith, G.E., and Summers, M.D. (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. 109:123-129.

Smith, M.J., and Boal, R. (1978). DNA sequence organization in the common Pacific starfish Pisaster ochraceus. Canad. J. Biochem. 56:1048-1054.

Smith, M.J., Lui, A., Gibson, K.K., and Etzkorn, J.K. (1980). DNA sequence organization in the starfish Dermasterias imbricata. Canad. J. Biochem. 58:352-360.

Smith, M.J., Nicholson, R., Stuerzl, M., and Lui, A. (1982). Single copy DNA homology in sea stars. J. Molec. Evol. 18:92-101.

Southern, E. M., (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Spencer, W.K., Wright, C.W. (1966). Asterozoans. In "Treatise on invertebrate paleontology." (Moore, R.C.,ed.) Part U. Echinodermata 3. Vol. 1:4-103. The Geological Society of America Inc. and the University of Kansas Press.

Studier, F.W. (1965). Sedimentation studies of the size and shape of DNA. J. Mol. Biol. 11:373-390.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. U.S.A. 77:5201-5205.

Thomas, P.S. (1983). Hybridization of denatured RNA transferred or dotted to nitrocellulose paper. In "Methods in Enzymology." (Wu, R., Grossman, L., Modave, K., eds.) Part B, Vol. 100:255-266.

Thomas, T.L., Posakony, J.W., Anderson, D.M., Britten, R.J. and Davidson, E.H. (1981). Molecular structure of maternal RNA. Chromosoma 84:319-335.

Tobin, S.L., Zulauf, E., Sanchez, F., Craig, E.A., McCarthy, B.J. (1980). Multiple actin-related sequences in the Drosophila melanogaster genome. Cell 19:121-131.

Tufaro, F. and Brandhorst, B.P., (1982). Restricted expression of paternal genes in sea urchin interspecies hybrids. Dev. Biol. 92:209-220.

Vanderkerckhove, J. and Weber, K. (1978a). At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the

amino-terminal tryptic peptide. J. Mol. Biol. 126:783-802.

Vanderkerckhove, J. and Weber, K. (1978b). Actin amino-acid sequences. Comparison of actins from calf thymus, bovine brain, and SV40-transformed mouse 3TC cells with rabbit skeletal muscle actin. Eur. J. Biochem. 90:451-462.

Vandekerckhove, J.and Weber, K. (1980). Vegetative Dictyostelium cells containing 17 actin genes express a single major actin. Nature 284: 475-477.

Vanderkerckhove, J., Franke, W.W., Weber, K. (1981). Diversity of expression of non-muscle actin in amphibia. J. Mol. Biol. 152:413-426.

Van Ness, J., Maxwell, I.H., and Hahn, W.E. (1979). Complex population of nonpolyadenylated messenger RNA in mouse brain. Cell 18:1341-1349.

Wartell, R.M. and Reznikoff, W.S. (1980). Cloning DNA restriction endonuclease fragments with protruding, single-stranded ends. Gene 9:307-319.

Watson, J.D. (1977). "Molecular biology of the gene." 3rd Ed. W.A. Benjamin, Inc., California.

Wetmur, J.G. (1976). Hybridization and renaturation kinetics of nucleic acids. In "Methods in Enzymology." (Grossman, L., and Moldave, K., eds.) Vol. 29E:337-361

Whitely, A.H. (1949). The phosphorous compounds of sea urchin eggs and the uptake of radio-phosphate upon fertilization. Amer. Nat. 83:249-167.

Wilt, F.H., and Wessells, N.K. eds. (1967). "Methods in developmental biology." Thomas Y. Crowell, New York.

Wilt, F.H. (1973). Polyadenlylation of maternal RNA of sea urchin eggs after fertilization. Proc. Nat. Acad. Sci. U.S.A. 70:2345-1349.

Wilt, F.H. (1977). The dynamics of maternal polyA-containing mRNA in fertilized sea urchin eggs. Cell 11:673-681.

Woo, S.L.C., Rosen, J.M., Llarakos, C.D., Choi, C., Busch, H., Means, A.R., O'Malley, B.W. (1975). Physical and chemical characterization of purified ovalbumin messenger RNA. J. Biol. Chem. 250:7027-7039.

Wood, R.L., Cavey, M.J. (1981). Ultrastructure of the coelomic lining in the podium of the starfish Stylasterias forreri. Cell Tissue Res. 218:449-473.

Zulauf, E., Sanchez, F., Tobin, Rdest, U., McCarthy, B.J. (1981). Developmental expression of a Drosophila actin

gene encoding actin I. Nature 292:556-558.