

ACTIN GENE TRANSCRIPTION DURING SPERMATOGENESIS
IN THE SEA STAR
Pisaster ochraceus (Brandt)

by

John David George Boom

B. Sc., Simon Fraser University, 1974

THESIS SUBMITTED IN PARTIAL FULLFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Biological Sciences

© John David George Boom 1988

SIMON FRASER UNIVERSITY

February, 1988

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.

APPROVAL

Name: John David George Boom

Degree: Master of Science

Title of Thesis:

Actin Gene Transcription During Spermatogenesis in the Sea Star
Pisaster ochraceus

Examining Committee:

Chairman: Dr. A.T. Beckenbach, Associate Professor,
Department of Biological Sciences

Dr. M.J. Smith, Professor, Senior
Supervisor

Dr. D.L. Baillie, Professor

Dr. B.M. Honda, Associate Professor

Dr. K.C. Eastwell, Assistant Professor,
Biological Sciences, SFU, Public Examiner

Date Approved

April 14 - 1988

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Actin Gene Transcription During Spermatogenesis in the Sea Star

Pisaster ochraceus

Author:

(signature)

John David George Boom

(name)

APRIL 14/88

(date)

ABSTRACT

Qualitative and quantitative aspects of actin gene expression in the sea star Pisaster ochraceus were investigated. Total testes RNA was isolated monthly from individual sea stars through two spermatogenic cycles and subjected to Northern blot analysis using an actin coding sequence probe and two actin gene-specific probes previously characterized in P. ochraceus. Three transcript size classes were identified. The largest (3.5 kb) was found to hybridize only with the actin coding probe and only at moderate stringencies. A 2.3 kb transcript encoding the cytoplasmic (Cy) gene class was confirmed by hybridization to a gene specific probe and cDNA sequence comparisons with genomic sequence. The 2.1 kb size class hybridizes with the muscle (M) gene-specific probe. However, testes cDNA isolates representing actin coding sequence differ from those of an existing genomic isolate, suggesting expression of the other gene in this class.

The Cy transcript is the dominant actin transcript, reaching its highest levels early in the cycle. Both the 3.5 kb transcript and M transcript(s) appear at the onset of spermatogenesis. The M transcript maintains low and stable levels throughout the cycle while the 3.5 kb transcript levels increase as the cycle progresses. These patterns sharply contrast those observed for the transcripts of two other important sperm components, beta tubulin and histone H3.

Considerable individual variation in the levels of these transcripts was observed at both ends of the spermatogenic cycle. The variation observed at the onset of spermatogenesis was clearly correlated with spermatogenic state (as judged histologically), but the variation observed in evacuative testes could not be similarly correlated. This observation was extended by comparisons with other populations and has given rise to the hypothesis that unidentified environmental factors and/or disease may complicate patterns of gene expression during spermatogenesis.

Acknowledgements

My thanks are extended to Barry Honda for knowing a problem when he sees one, and to David Baillie for his ability to find them. As my senior supervisor, Mike Smith has taught me the invaluable lesson of resourcefulness in the lab. Together, these gentlemen have been my committee, and their patience and guidance have been my privilege.

My heart goes out to those who dived on my behalf in search of this wily and elusive intertidal creature, and to K. Y. Lee for the histology. The tubulin probe employed in this study was originally cloned by D. W. Cleveland and was the kind gift of Peter Davies.

One quality that defies measurement in this Department resides in interactive human surroundings. These are the things that one comes to take for granted. I am indebted to all for their generosity with their observations, ideas, and criticisms.

Shelter, in its many different forms, has come from those who have tried to make me feel at home. I thank the best of friends in my second family, Rae, Lou, Eric, Eva, and Jen.

And then there's the rest of you, in Bamfield, who actually believed you were rid of me for good...

TABLE OF CONTENTS

Approval.....	ii
Abstract.....	iii
Acknowledgements.....	v
List of Tables.....	viii
List of Figures.....	ix
General Introduction.....	1
Materials and Methods.....	14
Collection of specimens.....	14
Histology.....	14
Extraction of RNA.....	15
Extraction efficiencies.....	17
Northern blot analyses.....	18
Isolation of poly-A ⁺ RNA.....	21
cDNA library construction.....	22
Screening and subcloning.....	25
Restriction digests.....	26
Sequencing.....	26
Results.....	28
1) The spermatogenic cycles sampled.....	28
2) Actin genes expressed during spermatogenesis.....	31
3) Seasonal patterns of actin gene expression at the population level.....	37
4) Actin gene expression as a function of spermatogenic state.....	42

5) Comparisons with Histone H3 and beta tubulin expression patterns.....	50
6) Gene expression immediately after spawning.....	54
Discussion.....	57
1) The spermatogenic cycles.....	57
2) Actin genes expressed in the testes.....	58
3) General transcription patterns during the spermatogenic cycle.....	61
4) Specific gene expression patterns during spermatogenesis.....	65
5) Which transcript encodes acrosomal actin?.....	69
6) What patterns of gene activity are found in spawned testes?.....	73
7) Conclusions and future research.....	76
Appendix A: RNA extraction efficiencies.....	78
References cited.....	89

LIST OF TABLES

Table 1: Probes- Descriptions and origins.....	14
Table 2: Summary of criteria defining spermatogenic state.....	38
Table A.1.: Analysis of RNA extraction efficiencies at checkpoints in the isolation procedure.....	74
Table A.2.: DNA contamination in standard and supernatant RNAs.....	78
Table A.3.: Analysis of supernatants at checkpoint "B".....	78

LIST OF FIGURES

Figure 1: <u>Pisaster ochraceus</u> annual gonadal cycles.....	23
Figure 2: Selected <u>Pisaster</u> testes RNAs probed at increasing stringencies with actin coding sequence..	26
Figure 3: Selected <u>Pisaster</u> testes RNAs analysed using the M, Cy, and B gene-specific probes.....	27
Figure 4: Comparison of testes cDNA sequences with the complete genomic sequence of the cytoplasmic actin gene from <u>Pisaster ochraceus</u>	29
Figure 5: Actin gene transcripts in testes RNAs pooled by month.....	33
Figure 6: Individual variation in actin gene expression during the early part of the spermatogenic cycle....	35
Figure 7: Histological features of the germinal epithelium during the <u>P. ochraceus</u> spermatogenic cycle.....	40
Figure 8: Actin gene transcripts in testes RNAs pooled according to spermatogenic state.....	42
Figure 9: Beta tubulin and histone H3 transcripts in testes RNAs pooled according to spermatogenic state.....	46
Figure 10: Comparison of actin, histone H3, and beta tubulin transcript levels in post-evacuative testes from Woodlands, Bowen Island, and Bamfield.....	48
Figure A.1.: Flow diagram of the RNA isolation procedure....	72
Figure A.2.: Efficiency of Extraction in males vs. females..	75
Figure A.3.: Comparison of isolated and supernatant RNAs from crudely fractionated testes.....	80

GENERAL INTRODUCTION

The existence of multigene families in eukaryotic systems has become a focus of investigation into the genetic control of development. Some of these gene families, such as those encoding the ribosomal RNA (rRNA) genes and the histones, are comprised of several hundred copies of tandemly arranged clusters. More common examples, however, are represented by relatively few dispersed members and encode both enzymatic and structural gene products.

There are several implications of multiple gene copies for regulation in development. The cell can acquire the capacity to produce large amounts of a given gene product within the limited duration of the cell cycle. A second feature is the opportunity to sustain mutational change in "spare" copies without complete forfeiture of the cell's functional requirements. In multicellular organisms, additional gene copies have paved the way for the evolution of tissue-specific expression.

The actin multigene family is one of the best studied exhibiting this last class of differential expression patterns. Found throughout eukaryotes, the protein product is among the most conserved known. During the first 15 years of investigation into actin, research focussed on the biochemical and biophysical aspects of its function in muscle. In this tissue, actin was seen to be organized into long filaments

interacting with other major accessory proteins which facilitate sliding of these filaments past each other to produce muscle contraction. The first demonstration of the presence of actin in nonmuscle cells was in Physarum polycephalum (Hatano and Oosawa, 1966, cited in Korn, 1982). This quickly led to the discovery that actin is a component of all eukaryotic cells and that it is present in very high quantities (10-20% of total soluble protein). Isolation and purification of actin from a variety of nonmuscle cells has since shown that it is present in both filamentous and nonfilamentous (monomeric) forms. It is now known that the controlled polymerization and depolymerization of actin by calcium and magnesium ions, ATP, and a host of associated proteins (reviews: Schliwa, 1982; Weeds, 1982; Craig and Pollard, 1982) provide for diverse cellular functions, including cell motility, cytoskeletal structure, cell-surface mobility, cytoplasmic streaming, cytokinesis, endocytosis, exocytosis, intracellular transport, microvillar movement, and, possibly, chromosomal condensation and mitosis (Hightower and Meagher, 1986).

The extent to which each of the members of a given actin multigene family have diverged to serve the requirements of specific tissues varies considerably in different phyla. In multicellular animals, actin genes have diverged to accommodate muscle function as well as cytoplasmic function. This divergence is seen in its extreme in higher vertebrates, where

actin genes specific to cardiac, smooth, and striated muscle have evolved. In plants, cytoplasmic forms have evolved which are as different from each other as animal cytoplasmic and muscle isoforms (Hightower and Meagher, 1986). Invertebrates typically display several genes encoding cytoplasmic actins and a single gene for muscle actin. Drosophila appears to be an exception, having actin genes specific to various muscles in both larval and adult structures (Fyrberg et al., 1983; Sanchez et al., 1983).

The sea urchin embryo has become a model system in which to study differential expression of an actin gene family. The Strongylocentrotus purpuratus genome contains 8 non-allelic actin genes, of which 6 have been shown to be expressed (Lee et al., 1984). Analysis of the 3' untranslated regions (3' UTRs) of mRNAs has shown expression from 4 of these genes to occur in a developmental stage- and tissue-specific manner (Garcia et al., 1984; Schott et al., 1984). Of these 6, one gene (M) is unique to muscle while the other 5 are distributed among 3 classes of cytoplasmic genes producing 3 transcript size classes (Schott et al., 1984). The CyI class is represented by a single gene encoding a 2.2 kb transcript. CyIIa and CyIIb comprise the CyII class and encode 2.1 and 2.2 kb transcripts, respectively. The CyIII class includes 2 genes which are expressed (CyIIIa and CyIIIb; 1.8 and 2.1 kb, respectively) and a third (CyIIIc), apparently a pseudogene (Lee et al., 1984).

Using the gene-specific 3' UTR probes originally

characterized by Lee et al (1984), Northern analyses have shown a differential accumulation of transcripts from all 6 genes through early development and adult tissue types (Schott et al., 1984). The use of these probes has been extended to the localization of mRNAs to specific cell lineages in the embryo using in situ hybridization (Cox et al., 1984, 1986).

To elucidate control mechanisms responsible for confining expression to certain lineages, eggs have been injected with DNA constructs containing putative 5' control sequences fused to sequences encoding chloramphenicol acetyl transferase (CAT). Such constructs for the CyI and CyIIIa genes suggest that lineage-specific controls for transcription lie in sequences at the 5' ends of these genes (Flytzanis et al., 1987; Katula et al., 1987). These results parallel similar experiments with Xenopus laevis embryos: upstream sequences appear to be responsible for the tissue-specific expression of a cardiac alpha actin gene in early embryos (Wilson et al., 1986).

In spite of our understanding of the evolution, structure, and expression of the actin multigene family in sea urchins and other organisms, that the various member genes actually encode functionally distinct proteins remains to be demonstrated (Lee et al., 1984). However, actin gene expression has not been fully explored in all developmental scenarios, and consequently the possibility that certain actin genes encode functionally distinct proteins can not be completely ruled out. One such scenario is echinoderm spermatogenesis, during which the actin

protein accumulates in a specific state to assume a pivotal role in the fertilization process.

The mechanisms and factors controlling actin polymerization have been the focus of the research of Louis Tilney for almost two decades. Tilney has chosen the Echinoderm sperm as a model system because the actin in mature sperm is entirely in a non-polymerized state before the fertilization, and entirely polymerized in the formation of the acrosomal process during this event (Tilney, 1975).

In the sea urchin, acrosomal actin accumulates during spermiogenesis, after the completion of meiosis. It localizes in a depression in the anterior end of the nucleus, just beneath the acrosome. Termed "periacrosomal material", this localization is not membrane-bound and does not appear to derive directly from the Golgi apparatus, as this organelle lies on the opposite side of the nucleus at the posterior end of the cell. Tilney and coworkers (1975) have isolated this material as an intact "periacrosomal cup" under conditions which normally permit the fractionation of filamentous and nonfilamentous actin. This observation has led to his postulation that actin can exist in a third state which is neither monomeric or polymerized.

At this juncture one can ask whether or not a specialized isoform of actin is transcribed to accommodate the requirements of the acrosomal process. In the studies noted above for S. purpuratus, Schott et al (1984) report the expression of 3

actin genes in testes. The CyI transcript is by far the most prevalent, while the CyIIb and M transcripts are relatively rare. None is reported to be testes-specific. However, the spermatogenic state of the testes was not considered in this analysis, and the question remains as to whether or not a functionally distinct isoform might be expressed at a specific stage of spermatogenesis not covered in this investigation. It is therefore not known whether the specialized state in which actin is found in the periacrosomal cup or the acrosomal process is conferred by unique transcripts, post-translational modifications, or other factors.

This thesis explores the possibility that actin gene(s) are differentially transcribed to encode the actin protein found in the acrosomal process of the asteroid Pisaster ochraceus. Asteroids produce much longer acrosomal processes than do sea urchins ('puny' vs about 25 micrometers; Tilney, 1975), and therefore might be expected to provide a good system for such a study. As in sea urchins, P. ochraceus experiences an annual spermatogenic cycle. Moreover, the most detailed histological and cytological reference for echinoderm spermatogenesis is provided by Walker's studies of the asteroid Asterias vulgaris (Walker 1974, 1975, 1979, 1980, 1984, 1986).

The number of actin genes comprising the P. ochraceus actin multigene family has been shown to be 5, perhaps 6 (Kovesdi and Smith, 1985a). Genomic clones have been characterized which place these genes into 3 classes (Kovesdi and Smith, 1984;

1985a). Of these, the Cy (cytoplasmic) class is represented by one gene and a single 2.3 kb transcript. There appear to be 2 copies of the M (muscle) class generating a single (2.1 kb) transcript size class. While there are 2 genes in the third class (B), no expression from these has yet been reported (Kovesdi, 1984). The patterns of expression in the egg, tube foot, and during early development have been characterized (Kovesdi and Smith, 1985b), but details of actin gene expression in the testes, as in sea urchins, are not known.

This problem is first approached by asking which of the 5 genes known to encode actin in this animal are expressed in testes. A second approach involves the elucidation of the patterns of actin gene expression during the annual spermatogenic cycle. Together, these avenues of investigation have revealed the expression of two transcripts previously identified in P. ochraceus. They display contrasting temporal expression patterns to each other as well as to expression patterns of two other important sperm components, histone and tubulin. The two actin transcripts are discussed in the temporal and cytological context of spermatogenesis in an effort to postulate which might provide for the accumulated acrosomal actin.

MATERIALS AND METHODS

Collection of Specimens: Individuals of Pisaster ochraceus were obtained by SCUBA diving at Woodlands, Indian Arm, Burrard Inlet, Vancouver, British Columbia. This population was sampled monthly from July 1984 to August 1985 and from September 1986 to June 1987. 141 males in total were collected during these periods. Occasional collections were also made from Bowen Island in the Strait of Georgia (April, 1987), and from Barclay Sound (God's Country) on the west coast of Vancouver Island (November, 1986 and May, 1987). They were held without feeding in a closed refrigerated seawater system at 7⁰C for no longer than one week before dissection. More than 2 and usually 8 males were analysed each month (except September 1986, represented by only 1). The total weight of each animal was estimated using a hand-held spring scale (Ohaus Model 8014, 0-2000g). Total gonad weight was obtained by weighing the tissue in pre-tared 50 ml conical tubes (Falcon No. 2098) on a Mettler P1200 balance ($\pm 0.01g$). Using these data, gonad indexes were computed expressing the total gonad weight as a percentage of the total animal weight.

Histology: When gonads could not be sexed visually, squashed preparations were examined on a standard compound microscope. For more detailed microscopy, small fragments of gonad (2-3mm) were fixed in Bouin's fixative, dehydrated in ethanol, and impregnated with Parowax for sectioning. Sections (7-8 microns

in thickness) were stained with haematoxylin-eosin and photographed on a Leitz photomicroscope equipped with a Wild Photoautomat MPS 45 using a blue filter and Kodak Panatomic X black and white film.

Extraction of RNA: All solutions prepared for the manipulation of RNA were filter-sterilized (0.45 microns) and, where necessary, treated with 0.2% diethylpyrocarbonate (DEP), followed by autoclaving. Solutions incompatible with DEP treatment were prepared using DEP-treated double-distilled water. Glass- and plastic-ware was similarly treated, and in most circumstances also silanized in a vacuum dessicator by vaporizing 100% dimethyldichlorosilane.

RNA isolation was carried out following the procedures of Chirgwin et al (1980), with the following modifications. About 5 g of testes from a single male was homogenized in a total volume of 30 mls of homogenization buffer containing 8 M guanidinium hydrochloride, 20 mM sodium acetate (pH 5.5) and 10 mM dithiothreitol (DTT). The tissue was homogenized for 90 s at full speed in a Sorval Omnimixer (Model 17105) fitted with a 50 ml homogenization cannister. The homogenate was transferred to a 50 ml polycarbonate tube and cellular debris pelleted by centrifugation at 10,000 rpm in a Sorval SS-34 rotor at 4 °C. Homogenization buffer was used to bring the volume of the supernatant to 30 mls and 3/4 volume of ice-cold 95% ethanol was added. The preparation was placed at -20 °C for at least

one night (samples could be held at this stage for several months without apparent degradation of RNA). Following centrifugation at 4500 rpm in a Sorval HS-4 rotor at 4 °C for 30 minutes, the pellet was resuspended at room temperature in 15 mls of a freshly prepared concoction containing 7 M urea (BRL Ultrapure), 20 mM ethylenediamine-tetraacetic acid (EDTA), 15 mM ethylene glycol bis-[B-Aminoethyl ether] N,N,N',N' tetraacetic acid (EGTA), 1% sodium dodecyl sulphate (SDS), and 20 ug/ml polyvinyl sulphate (PVS). This suspension was made 0.5 g/ml cesium chloride (CsCl) with the addition of an equal volume of a 1.0 g/ml CsCl solution. The sample was placed in a heat-sealable ultracentrifuge tube, and underlaid with 10 mls of 5.7 M CsCl buffered with 20 mM sodium acetate [pH 5.5] and centrifuged at 36,000 rpm in a Beckman Ti60 rotor for 14-16 hours at 20 °C. The supernatant was removed by aspiration, and the pellet was transferred to a 30 ml Corex tube and washed 3 times with 5 mls of 70% ethanol. A 10 minute spin at 10,000 rpm and 4 °C in a Sorval SS-34 rotor followed each wash. After the final wash the pellets were air dried and dissolved in DEP-treated water. This solution was made either 0.3 M lithium chloride or 0.3 M sodium acetate [pH 5.5] and the RNA was precipitated with the addition of 1.2 or 2.5 volumes, respectively, of ice-cold 95% ethanol and storage at -20 °C. The RNA was recovered by centrifugation at 10,000 rpm and 4 °C for one hour in a Sorval SS-34 rotor. After air drying, the pellet was dissolved in an appropriate volume of DEP-treated

water (usually 1.0 ml or less). Spectrophotometric estimates of concentrations were made by diluting the samples into 0.1 M phosphate buffer (PB; equimolar concentrations of sodium phosphate monobasic and sodium phosphate dibasic [pH 6.8]) and measuring absorbances at 230 nm, 260 nm, 280 nm, and 320 nm. RNAs were routinely stored in DEP-treated water at -80°C .

In some experiments, contaminating DNA was removed from RNA samples by digestion with deoxyribonuclease 1 (DNase 1; RQ 1, Promega) in a buffer containing 75 mM sodium chloride, 50 mM Tris [pH 7.4], and 10 mM magnesium chloride. Crude RNA samples were digested in volumes of 50-100 μl using 4 U of enzyme per 25 μg at 37°C for 3-4 h. The reaction was stopped on ice, and the nucleic acids precipitated out of 0.3 M sodium acetate [pH 5.5] with ethanol.

Extraction Efficiencies: Extraction efficiencies were estimated by the method of isotope dilution. Tritiated E. coli RNA (^3H -RNA) was prepared in liquid culture by incubating wild-type E. coli in the presence of tritiated uridine (5,6- ^3H uridine; Amersham) and isolating the RNA from a Triton X-100 miniprep lysate of these cells (Davis et al., 1980). Aliquots (500,000 dpm, about 20 μg) were then followed through isolations of RNA from 1 male and 7 females by scintillation counting of aliquots taken at the checkpoints indicated in Figure A.1. (see Appendix A). Preparations (4) containing 2 mg of carrier RNA (total ovary RNA isolated by the same procedure)

but no tissue served as controls. Final extraction efficiencies were determined for a total of 7 males and 9 females.

Northern Blot Analyses: RNAs were electrophoresed in formaldehyde-agarose denaturing gels essentially as described by Thomas (1980). Samples (usually 20 ug) were heat denatured at 60 °C for 15 min in 50 ul of buffer containing 1X MOPS (1X MOPS is 0.2 M morpholinopropanesulphonic acid [pH 7.0]; 50 mM sodium acetate; 1.0 mM EDTA), 2.2 M deionized formaldehyde, 50% deionized formamide, and 2 ul of RNA loading buffer (Maniatis et al., 1982) and placed on ice for 5 min before loading. (Samples loaded on gels prepared for transfer to nylon-based membranes[see below] were cooled in an ice-water bath. This appeared to promote concatamerization of transcripts via polymerization of formaldehyde, with the result that higher molecular weight "ladders" appeared on some autoradiographs. So, if you want clearly interpretable autoradiographs, do not over-cool samples prior to electrophoresis). Electrophoresis was carried out in 1X MOPS at 80 V for 5 to 8 hours, or 16 to 24 hours at 40-50 V, with constant recirculation of the electrophoresis buffer.

To visualize the ribosomal RNAs for use as molecular weight markers, single lanes were removed and stained for 30 min in 0.1 M ammonium acetate [pH5.5] containing 0.5 ug/ml ethidium bromide, followed by 1-2 hours destaining in flowing tap water.

In some instances, RNA samples were prepared containing 1.0 ug of ethidium bromide. (This method precluded removal of any lanes and allowed visualization of the ribosomal RNAs as electrophoresis proceeded.)

Two methods were employed for the transfer of electrophoresed RNAs to solid membrane supports. In the first, RNAs were transferred under conditions of high salt to nitrocellulose (Thomas, 1980). Before transfer, the gel was rinsed 3 times (10 min each) in distilled water. The nitrocellulose membrane (Schleicher and Schuell or Gelman Sciences) was first wet in distilled water, then in 20X SSPE (1X SSPE is 0.18 M sodium chloride; 10 mM phosphate buffer [pH 6.8]; 1 mM EDTA [pH 8.0]; pH adjusted to 7.0 with sodium hydroxide). After transferring overnight in 20X SSPE, the membrane was baked immediately (without rinsing) under vacuum at 80 °C for 2 hours.

The second protocol employed nylon-based membranes (GeneScreen; New England Nuclear or BioDyne; Pall Corporation) and transfer under low salt conditions. The gel was rinsed once in distilled water (10 min) and then twice (10 min each) in 25 mM PB. The membrane was soaked in 25 mM PB after being prewet in distilled water. Transfer proceeded overnight in 25 mM PB. After removal from the gel, the membrane was briefly dipped in 25 mM PB, placed on a glass plate (RNA side up) and covered with Saran wrap. This assemblage was exposed for 5 min to UV light (1200 uW/cm^2) emitted from 2 standard germicidal

lamps (General Electric Model G15T8; 15 W) at a distance of 15 cm. This treatment covalently crosslinks the RNA to the membrane, enhances hybridization signals by at least 10-fold, and permits probe removal and rehybridization with minimum loss of RNA from the filter (Khandjian, 1986). The filter was then dried by baking under vacuum at 80 °C.

A summary of the origins and descriptions of the 6 probes used in this study is given in Table 1. In all cases, the

TABLE 1

Probes- Descriptions and Origins

Specificity	Subclone	Description	Reference
Actin Cy, M, and B	pPoTa9/650	From a tubefoot cDNA library, 650bp of 3' coding sequence.	Kovesdi and Smith, 1984
Actin Cy (cytoplasmic)	pPoTa5/460	From a tubefoot cDNA library, 460bp of 3' untranslated region.	Kovesdi and Smith, 1984
Actin M (putative muscle)	pPoTa13/210	From a tubefoot cDNA library, 210bp of 3' untranslated region.	Kovesdi and Smith, 1984
Actin B	lambda PoA137/630	From a genomic library, 630bp of putative 3' untranslated sequence.	Kovesdi and Smith, 1984
Histone H3	pPo/H0.8	From a genomic library, an 860bp EcoRI fragment from the <u>P. ochraceus</u> histone gene cluster.	Howell et al, 1987
Beta Tubulin	pT2/1.2	A 1.2kb Pst1 fragment from the chicken beta tubulin cDNA, containing about 1.0kb of coding sequence.	Valenzuela et al, 1981

inserts of interest were gel purified and radioactively labelled by a Klenow polymerase (Pharmacia) fill-in reaction with alpha-³²P-deoxyribonucleotide triphosphates (³²P-dATP and ³²P-dCTP; Amersham) following limited digestion with Exonuclease III (BRL) (Guo and Wu, 1983). This procedure typically yielded specific activities of 1-5 X 10⁸ cpm/ug.

The filters produced by both transfer procedures were treated identically in the prehybridization/hybridization manipulations. Prehybridization was carried out for 3-8 hours at 62 °C in a buffer containing 5X SSPE, 5X Denhardts (1X Denhardts is 0.02% bovine serum albumin [Pentex Fraction V; Miles Chemical]; 0.02% polyvinylpyrrolidone [PVP-40]; 0.02% Ficoll [M.W. 400,000]), and 0.3% SDS. Hybridization took place overnight at the same temperature in a fresh aliquot (10 mls per 15 cm X 20 cm filter) of the same buffer containing 100-200 ng of probe (1-4 X 10⁷ cpm) per filter. Generally, filters were washed 3 times (1 hour each) in 1X SSPE; 0.3% SDS at 62 °C, and exposed to Kodak X-AR5 and XK-1 films with intensifying screens at -70 °C. Subsequent washes at higher stringencies were performed at 65 °C in 0.2X SSPE; 0.3% SDS.

Isolation of PolyA⁺ RNA: Total testes RNA (5 mg) was adjusted to a concentration of 1 mg/ml with DEP-treated water, and an equal volume of 2X binding buffer was added (1X binding buffer is 0.5 M ammonium acetate; 1 mM EDTA; 0.1% SDS; pH 7.6). This

mixture was heated at 65 °C for 5 minutes prior to loading onto a 1.0 ml oligo-dT cellulose column (Type 2; Collaborative Research) equilibrated with 1X binding buffer. The eluant was reheated at 65 °C for 5 minutes and passed through the column a second time. PolyA⁻ RNA was washed out of the column with 0.5 M ammonium acetate [pH 7.6]. PolyA⁺ RNA was eluted with DEP-treated water, precipitated out of 0.3 M lithium chloride with 95% ethanol, and resuspended in DEP-treated water at a concentration of 1 ug/ul. An equal volume of 2X binding buffer was added and the mixture was again heated at 65 °C for 5 min. This sample was applied to a 0.25 ml oligo-dT cellulose column and the steps outlined above were repeated.

cDNA Library Construction: This cDNA library was constructed according to the procedure of Gubler and Hoffman (1983), with 2 important modifications (communication courtesy of Dr. B. M. Honda). First, 10% of the first-strand reaction was separately incubated with alpha-³²P-deoxycytidine triphosphate (alpha-³²P-dCTP; Amersham) to provide an assay for the progress of the first-strand reaction. Secondly, the first-strand reaction products were not precipitated and purified before commencing with the second-strand reaction.

First strand synthesis was primed by combining 5 ug of polyA⁺ RNA and 2 ug of oligo-dT primers in DEP-treated water to a total volume of 20 ul and heating for 10 min at 65 °C. The first strand was synthesized in a volume of 50 ul using AMV

reverse transcriptase (1.2 U/ul; Life Sciences) in the presence of 50 mM Tris (pH 8.35 at 22 °C), 125 mM potassium chloride, 9 mM magnesium chloride, 2 mM DTT, 2 mM sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$), and 0.8 mM deoxyribonucleotide triphosphates (dNTPs). Immediately upon combining these reaction components, a 5 ul aliquot was transferred to a separate tube containing 10 uCi of alpha $^{32}\text{-dCTP}$ for the assay reaction as noted above. First strand synthesis was allowed to continue for 45 min at 42 °C.

Second strand synthesis was carried out in a volume of 100 ul containing the first strand reaction products (45 ul), 20 ul of 5X second strand buffer (1X second strand buffer is 20 mM N-2-Hydroxyethylpiperazine-N'-ethanesulphonic acid [HEPES]; 20 mM potassium chloride; 4 mM magnesium chloride; pH to 6.9 with potassium hydroxide), 10 mM ammonium sulphate, 50ug/ml BSA (Pentex Fraction V; Miles Chemical), 0.15 mM beta-Nicotinamide adenine dinucleotide (beta-NAD, grade V), 0.2 mM dNTPs, 10 uCi of alpha- $^{32}\text{P-dCTP}$, 50 U of DNA polymerase I, and 1 U of ribonuclease H. After 24 h at 15 °C, the double-stranded cDNA (ds-cDNA) products were extracted with phenol and precipitated out of 2 M ammonium acetate with ethanol.

Methylation was carried out in a 50 ul reaction volume containing 50 mM tris [pH 7.6], 2 mM EDTA [pH 8], 6 mM DTT, 0.125 mM S-Adenosyl-L-Methionine (SAM), and 50 U of EcoR1 methylase (BRL). The reaction was incubated at 37 °C for 20 min and then stopped at 60 °C for 10 min prior to placing on

ice.

The completed methylation reaction was then adjusted to 10 mM magnesium chloride and the fill-in reaction was carried out using 1.0 ul of each (2 mM) dNTP and 2.5 U of DNA Pol 1. After 10 min at room temperature the reaction was stopped with the addition of 10 ul of 0.1 M EDTA. Following the addition of 2 ug of yeast tRNA, the reaction was extracted with phenol/chloroform.

The methylated, blunt-ended dscDNA was passed through a 5 ml Sephadex G-100 column equilibrated with 50 mM Tris [pH 8]; 300 mM sodium chloride; 2 mM EDTA [pH 8]. 300 ul fractions were collected and their cDNA contents estimated by Cerenkov counting. The appropriate fractions were pooled and precipitated with ethanol.

EcoR1 linkers (100-250 pmole; NEB) were kinased (Maniatis et al., 1982) and combined with the dscDNA and 4 ul of 0.5 M Tris (pH 7.6), 4 ul of 0.1 M magnesium chloride, and 20 ul of DEP-treated water, and heated at 45 °C for 10 min. 4 ul of 0.1 M DTT, 4 ul of 10 mM adenosine triphosphate (ATP; BMC), and 3 U of T4 DNA ligase (BRL) were then added and the ligation reaction continued at 15 °C for 48 h. A 1.0 ul aliquot was electrophoresed on a 5% sequencing gel (see below) to confirm that ligation had taken place. The ligase was inactivated with the addition of 40 ul of water and heating at 60 °C for 10 min. Excess linkers were digested with 25 U of EcoR1 (BRL) under the conditions specified by the manufacturer.

The remaining linker fragments were removed and the dscDNA were size-fractionated on a Sepharose 6B column. dscDNA contents were estimated by Cerenkov counting, and aliquots of selected fractions were sized by alkaline agarose gel electrophoresis (Maniatis et al., 1982). A size range of 800 bp to 2000 bp was chosen for subsequent ligation and cloning.

The pooled, precipitated fractions were lyophilized and resuspended in 50 μ l of 10 mM Tris [pH 8]; 1.0 mM EDTA. 1% and 10% (i.e. 0.5 μ l and 5 μ l) were each coprecipitated with 1.0 μ g of EcoR1-digested lambda gt10 phage DNA (a kind gift of Karen Beckenbach in David Baillie's lab) and resuspended in 1.0 μ l of Tris [pH 7.8] and 6 μ l of water. 1.0 μ l of 0.1 M magnesium chloride, 1.0 μ l of 0.1 M DTT, 1.0 μ l of 10 mM ATP, and 0.5 μ l of T4 DNA ligase were then added and ligation was allowed to proceed for 24 h at 14 $^{\circ}$ C.

5 μ l of each ligation reaction was mixed into a 50 μ l aliquot of the packaging extract (prepared by Margaret Stuerzl in this lab) as it thawed. After mixing was complete, the packaging reactions were placed at 37 $^{\circ}$ C for 1 h. To another 50 μ l aliquot of packaging extract was added 2.5 μ l of 0.5 M magnesium chloride and 5 μ l of 100 μ g/ml DNase 1 (Sigma). This was mixed well and left on ice for 10 min. 20 μ l aliquots were removed and mixed into the packaging reactions and incubated an additional 30 min at 37 $^{\circ}$ C. The reactions were finally diluted with 1.0 ml of SM (SM is 0.1 M sodium chloride; 10 mM Tris [pH 8]; 10 mM magnesium sulphate; 0.02% gelatin) and 5 μ l of

chloroform, and centrifuged for 30 s. The supernatant was stored over chloroform at 4 °C. This library was found to contain 20,000 independent recombinants in a background of approximately 30% false recombinants.

Screening and Subcloning: Amplification and screening were carried out by infecting E. coli strains C600 and/or C600hfl (kindly provided by B. M. Honda) grown on standard Luria broth agar (Maniatis et al., 1982). The cells were grown in the presence of 0.2% maltose to increase phage infectivity.

Screening procedures were as described in Maniatis et al., (1982), with probe preparations and hybridization reactions as described above for Northern analyses.

Analyses of positive phage by Southern blot hybridization followed procedures as outlined in Maniatis et al., (1982). Probe preparations and hybridization conditions were as described above for Northern analyses.

Fragments of interest were subcloned into pUC18 or pUC19 and the recombinant plasmids transformed into E. coli JM83. Quantities of recombinant plasmids were isolated following the alkaline lysis miniprep procedure outlined in Maniatis et al., (1982), with minor modifications.

Restriction digests: Restriction enzymes employed in characterizing selected positive phage were purchased from Bethesda Research Labs or Pharmacia and used under the

conditions specified by the manufacturer. Standard agarose gel electrophoresis was used to resolve the fragments and digests of pBR322 with HinfI or lambda phage DNA with EcoRI and HindIII were used as molecular weight standards.

Sequencing: Sequencing was performed with Klenow polymerase and dideoxynucleotides using denatured plasmids as templates (Hattori and Sakaki, 1986). Sequencing reaction products were electrophoresed on 8.3M urea polyacrylamide gels with 0.5X TBE (1X TBE is 89 mM Tris; 89 mM boric acid; 10 mM EDTA; pH 8.3) as the electrophoresis buffer. The dried gels were autoradiographed at room temperature using Kodak XK-1 film.

Preliminary sequence analyses were done using the Delaney 2 sequence analysis program (Delaney Software). The Eyeball Sequence Editing Program (ESEE; developed by E. L. Cabot at this University) was employed for subsequent editing and preparation of sequences for figures.

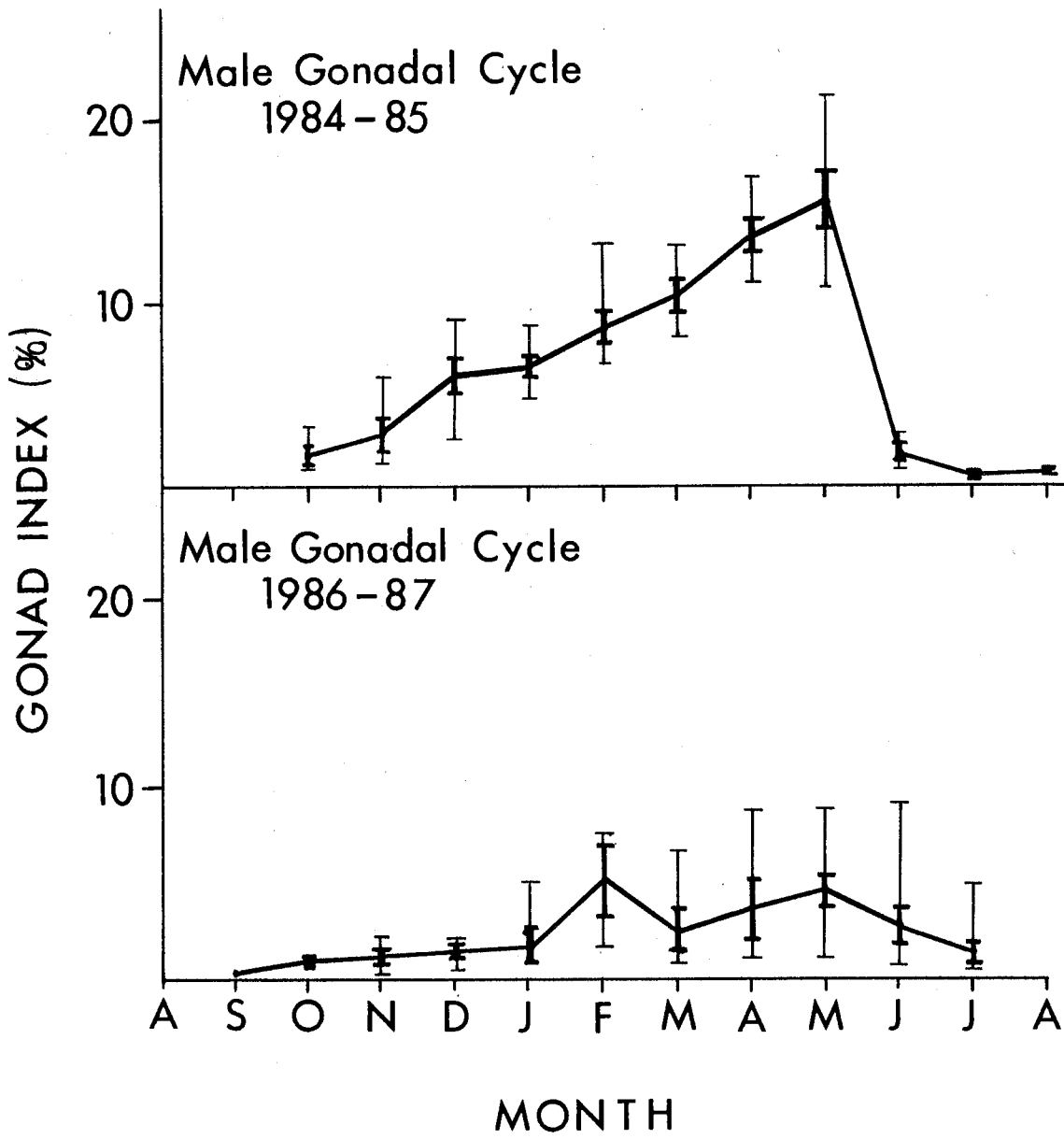
RESULTS

1) The Spermatogenic Cycles Sampled

Echinoderms typically exhibit an annual gametogenic cycle, a period in which the gonads increase in size by at least one order of magnitude. A traditional measure of the reproductive state of a population is made by averaging the gonad indexes of several individuals (male and/or female) at discrete time points throughout the year. Such data have been collected for Pisaster ochraceus on the California coast (Feder, 1957; Greenfield et al., 1958; Farmanfarmaian et al., 1958; Nimitz, 1971, 1976), in the San Juan Islands (Mauzey, 1966), and in the inside waters of the southern coast of British Columbia (Fraser et al., 1981). These data indicate that P. ochraceus initiates its annual cycle in September or October and spawns in May, June, or July.

Gonad index was determined for P. ochraceus males collected from the Woodlands population from fall 1984 through spring 1985, and from fall 1986 through spring 1987. Figure 1 shows a plot of gonad index over time for these two spermatogenic cycles. The heavy error bars indicate ± 1 standard error of the mean, the lighter bars represent the range. The variability within monthly samples is in agreement with similar data reported by others for this species. The 1984-85 cycle reflects a pattern that is consistent with the earlier reports cited above. Gonad index begins to increase late in the year, reaching a maximum followed by a precipitous decline upon

Figure 1: Pisaster ochraceus annual gonadal cycles. The months sampled are indicated on the ordinate axis by the first letter of each month. Gonad indexes are computed expressing the total gonad weight as a percentage of the total animal weight. The heavy error bars indicate ± 1 standard error of the mean, the lighter bars represent the range.



spawning in May and June.

Observations for the 1986-87 cycle do not follow such an obvious pattern. Gonad indexes did not reach previously observed levels, and the frequency of ripe testes appeared to be unusually low. In addition, in the months of February, March, and April (1987) our samples contained very few males compared to females (3:38, 6:22, and 4:29, respectively). The same observation (11:109) was made for a sample collected from a Bowen Island population in April, 1987. However, sex ratios more closely approximating 50:50 were observed in a sample from Barclay Sound (May, 1987) and in samples from the Woodlands population in May, June, and July.

Individual males in spring 1987 differed considerably from those in spring 1985 in several important respects. Animals from January onward in the 84-85 cycle rarely had fewer than the full complement of testes (10) and they were consistently a creamy-white color, releasing sperm wherever damaged by dissection. In contrast, spring 1987 animals rarely contained 10 testes. Individuals frequently displayed both ripe gonads and other testes in a questionable stage of gametogenesis. These other lobes were much smaller and displayed varying intensities of brownish pigmentation. In a few rare cases, small otherwise apparently ripe gonads had reduced pigmented tips.

These 2 gonadal cycles provided the source of RNAs analysed in this study. Since quantification is involved in these

analyses, it was necessary to qualify the RNAs and the method by which they were isolated. These experiments and the results are documented in Appendix A. The following sections are concerned firstly with identifying which actin genes are expressed, and secondly with their patterns of expression in the spermatogenic cycle.

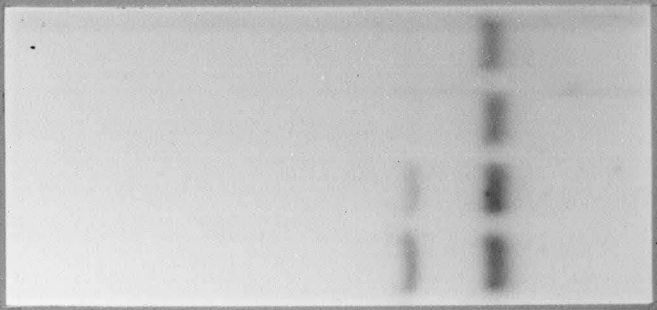
2) Actin Genes Expressed During Spermatogenesis

To assess which of the 5 genes encoding actin is expressed in testes, RNAs from individual sea stars were analysed by Northern blot using the actin Coding probe. This probe is expected to detect all transcripts encoding actin. Shown in Figure 2 are the results of such an experiment comparing 4 testes RNAs at increasing hybridization stringencies. One transcript size class (2.3 kb) is common to all individuals and it appears to retain the probe at high stringencies. In 2 of the individuals, there appears to be a larger transcript (approximately 3.5 kb) which failed to hybridize at higher stringencies.

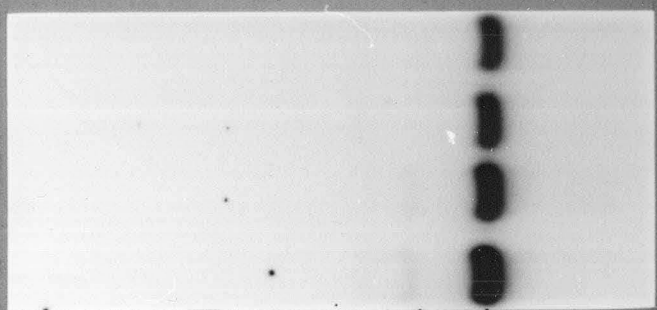
When these same RNAs are probed with the actin gene-specific probes, the result shown in Figure 3 is obtained. These data indicate that the cytoplasmic (Cy) probe (see Table 1, Materials and Methods) hybridizes to a transcript of 2.3 kb while the putative muscle (M) probe detects a 2.1 kb transcript. The M gene transcript is apparent only in those RNAs containing the larger (3.5 kb) transcript (Figures 2 and

Figure 2: Selected Pisaster testes RNAs probed at increasing stringencies with actin coding sequence. Total testes RNA was extracted from the indicated individuals, electrophoresed on formaldehyde agarose gels (20 ug/lane), and transferred to nitrocellulose. The filters were hybridized to actin coding sequence and washed at the following stringencies (from left to right): 55 °C; 1X SSPE, 60 °C; 1X SSPE, 65 °C; 1X SSPE, and 65 °C; 0.2X SSPE.

Nov '84-03

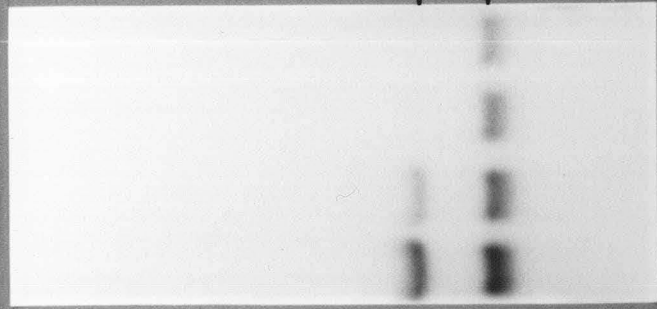


Nov '84-02



-3.5-
-2.3-

Oct '84-04



Oct '84-03

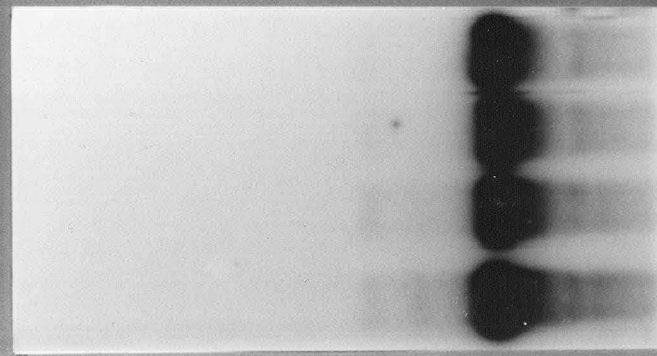
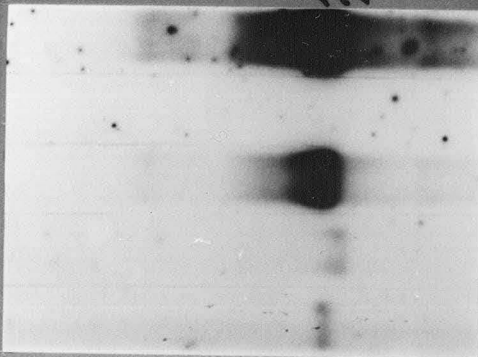
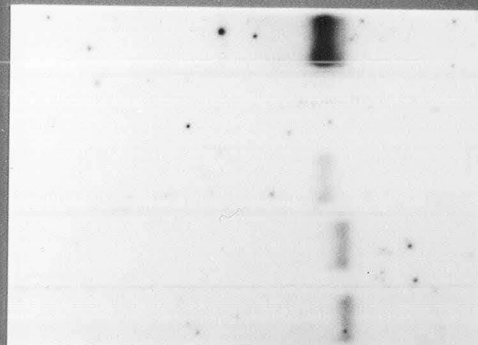


Figure 3: Selected Pisaster testes RNAs analysed using the M, Cy, and B gene-specific probes. Total RNA was extracted from the testes of individual sea stars, electrophoresed on formaldehyde agarose gels (20 ug/lane), and transferred to nitrocellulose. The filters were hybridized to actin coding sequence (Cod) as well as the M (muscle), Cy (cytoplasmic), and B (putative) gene-specific probes (see Table 1, Materials and Methods). Egg and tubefoot (Tf) RNAs are included as controls for the Cy and M gene probes, respectively.

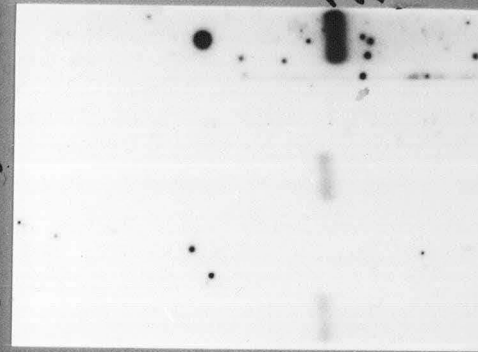
Egg Oct '84-03
Cy M Cy B Cod



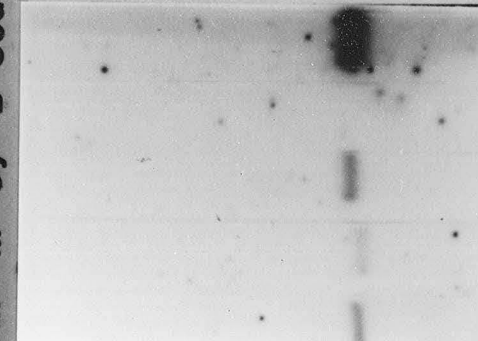
If Oct '84-06
M M Cy B Cod



Egg Sept '84-06
Cy M Cy B Cod



If Nov '84-01
M M Cy B Cod

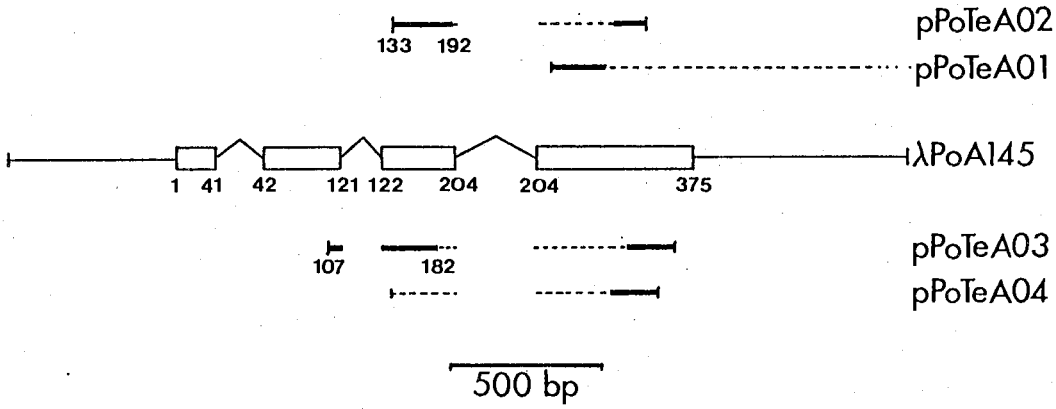


3, above). However, neither the Cy or M gene-specific probes, nor the putative 3'-untranslated sequence represented in the B probe reacted with the larger (3.5 kb) transcript. Other work in this lab has failed to detect transcripts homologous to the B probe (Kovesdi, 1984).

To further characterize the transcripts detected in the above experiments, a lambda gt10 cDNA library was constructed from polyA⁺ RNA which was shown by Northern blot analysis to contain all 3 transcripts. 40,000 phage were screened using the Coding probe on 3 replica plaque lifts, each hybridized at a different stringency. Different stringencies were employed in the hope that a phage representing the 3.5 kb transcript might be cloned. This range of stringencies yielded 14 phage proving to contain inserts hybridizing to actin coding sequence. These were placed into 4 groups based on plaque hybridization stringency, insert size, and restriction patterns produced by digestion of isolated inserts with TaqI and SauIIIa restriction enzymes. The largest insert (designated pPoTeA01, approximately 1.4 kb) was represented by 4 of the 14 positive phage and was shown to contain coding sequence contiguous with (putative) 3'-untranslated sequence: both the Cy and Coding probes hybridized to this insert. The remaining inserts hybridized only to the Coding probe and therefore none could be designated as specifically representing the M gene.

Figure 4 summarizes preliminary sequence analyses of 2 selected subclones of phage inserts (pPoTeA02 and pPoTeA03) and

Figure 4: Comparison of testes cDNA sequences with the complete genomic sequence of the cytoplasmic actin gene from Pisaster ochraceus. The upper panel provides orientation with the complete genomic sequence. Exons are indicated by the open boxes, and their limits are indicated by amino acid number. In the lower panels, genomic sequence of the P. ochraceus cytoplasmic actin gene (derived from lambda PoA145, courtesy David Kowbel) is the upper sequence, the respective cDNA sequences below. Vertical bars represent nucleotide homology. Amino acid differences are boxed.



PoA145 TACGTTGCCATCCAGGCTGTA
 Y V A I Q A V L S L Y A S G R T T G I V 20
 |||
 pPoTeA02 Y V A I Q A V L S L Y A S G R T T G I V 20
 TACGTTGCCATCCAGGCTGTA
 TCGACTCTGGTGACGGTGTG
 F D S G D G V S H T V P I Y E G Y A L P 40
 |||
 F D S G D G V S H T V P I Y E G Y A L P 40
 TTCGACTCTGGTGACGGTGTG
 CACGCCATCCTCCGCTCGGACTTGGCCGGTCTGACTTGACCGACTACCTGATGAAGATC 180
 H A I L R L D L A G R D L T D Y L M K I 60
 |||
 H A I L R L D L A G R D L T D Y L M K I 60
 CACGCCATCCTCCGCTCGGACTTGGCCGGTCTGACTTGACCGACTACCTGATGAAGATC 180

PoA145 GCCCCCCTTAACCCCAAAGCCAACAGAGAAAAGATGACACAGATCATGTTTCGAGACCTTC 60
 A P L N P K A N R E K M T Q I M F E T F 20
 |||
 pPoTeA03 A P L N P K A N R E K M T Q I M F E T F 20
 GCACCATTGAACCCCAAAGCCAACAGGGAATAATGACACAGATAATGTTTGAGACCTTC 60
 AACACACCCGCATGTACGTTGCCATCCAGGCTGTA
 N T P A M Y V A I Q A V L S L Y A S G R 40
 |||
 N T P A M Y V G I Q A V L S L Y A S G R 40
 AACACCCCTGCAATGTATGTTGGTATTCAAGCTGTGTTGCACTCTATGCATCAGGACGT 120
 ACCACCGGAATCGTCTTCGACTCTGGTGACGGTGTG
 T T G I V F D S G D G V S H T V P I Y E 60
 |||
 T T G I V L D S G D G V S H T V P I Y E 60
 ACCACTGGTATTGTCTTGGATTCTGGTGATGGTGTATCACATACTGTACCTATCTATGAG 180
 GGATACGCCCTCCCCACGCCATCCTCCGCTCTGGACTTGGCCGGT 225
 G Y A L P H A I L R L D L A G 75
 |||
 G Y A L P H A I L R L I L L G 75
 GGATATGCATTACCTCATGCTATATTGAGACTGATCTTGTGGGA 225

provides comparisons with the complete genomic sequence of the P. ochraceus Cy actin gene contained within the lambda clone PoA145 (courtesy of D. J. Kowbel, unpublished observations).

Comparison of the amino acid sequence data available for the 2 cDNA subclones with the genomic sequence contained in lambda PoA145 confirmed that these cDNAs contain actin coding sequence. However, the two cDNAs differ markedly in their homology with the genomic sequence at the nucleotide level. The sequence presented for pPoTeA02 is identical to the genomic sequence. In contrast, the pPoTeA03 sequence bears roughly 80% similarity. When similar comparisons are made between partial sequence data for all 4 cDNAs, they appear to fall into 2 classes: pPoTeA01 and pPoTeA02 in one class, pPoTeA03 and pPoTeA04 in the other. Both pPoTeA01 and pPoTeA02 were obtained via screening of the library at high stringency, while pPoTeA03 and pPoTeA04 were derived from the low stringency screen. Thus it appears that the screening strategy has yielded representatives of 2 different transcripts. The first (pPoTeA01 and pPoTeA02) are very likely the product of the lambda PoA145 gene class (Cy). When the partial sequences of pPoTeA03 and pPoTeA04 are compared with sequence data available for a genomic isolate of the M gene (pPoA160; D. Kowbel, unpublished observations), considerable dissimilarity is observed. Thus it is likely that these cDNAs do not originate with pPoA160. Since this isolate represents only one of two M genes believed to exist in the genome (Kovesdi and Smith,

1985), it is possible that it is the other as yet uncharacterized M gene is expressed in the testes. In the absence of complete sequence data or hybridization specificity, it cannot be determined which of the other actin genes encode the pPoTeA03 and pPoTeA04 sequences. The remote possibility also remains that these cDNAs encode portions of the 3.5 kb transcript.

3) Seasonal Patterns of Actin Gene Expression at the Population Level

The Echinoderm gametogenic cycle has been the subject of investigations concerning seasonal fluctuations in carbohydrate (Greenfield et al., 1958), protein and non-protein nitrogen (Vasu and Giese, 1966), lipids (Allen and Giese, 1966), gonadal nutrient reserves (Nimitz, 1971 and 1976), RNA and DNA synthesis (Yamashita and Iwata, 1983; van der Plas, 1983; Davis, 1985; Smith and Walker, 1986), polyamine metabolism (Watts et al., 1987), and steroid metabolism (Schoenmakers and Dieleman, 1981; Voogt and Dieleman, 1984). A major question that has arisen concerns the relative influence of endogenous and exogenous factors over the events of spermatogenesis as they relate to these fluctuations (Walker, 1980; Walker and Larochelle, 1984; Pearse and Eernisse, 1982; Pearse et al., 1986). In general, these studies indicate that these fluctuating conditions in the echinoderm gonad are tied to seasonally changing environmental parameters (e.g. food

availability, temperature, and photoperiod).

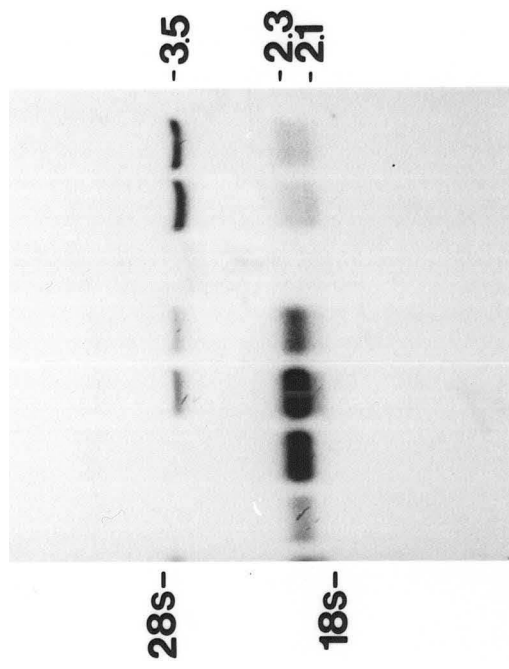
Seasonal patterns of transcription of specific genes in echinoderm spermatogenesis have not been reported. To ascertain whether the expression of actin genes in Pisaster testes bears a relationship to the observed seasonal activities of spermatogenesis, the RNAs isolated from August 1984 to February 1985 were pooled (by month) and analysed by Northern blot using the Coding probe and the Cy and M gene-specific probes, (see Materials and Methods). As shown in Figure 5, broad seasonal patterns are evident for all 3 transcripts.

Figure 5a shows that the coding probe hybridizes to 3 transcripts at moderate stringencies (62°C ; 1X SSPE). The largest (3.5 kb) is not detected at higher hybridization stringencies (65°C ; 0.2X SSPE; see Figure 2, above). Although the levels of this transcript appear to increase with increasing gonad index, it fails to react with any of the other probes employed in this study (Figure 3, above, and Figures 5b and 5c, below).

The Coding probe detects 2 other transcripts, approximately 2.1 kb and 2.3 kb in size. Hybridization with the Cy and M gene probes (Figures 5b and 5c, respectively) demonstrates that these 2 transcripts react specifically with these probes. In addition, the bulk of the actin transcripts present in the peak months (September, October, and November) is contributed by the Cy (2.3 kb) transcript, while the M gene transcript (2.1 kb) appears in October and persists at relatively low and constant

Figure 5: Actin gene transcripts in testes RNAs pooled by month. Testes RNAs were pooled by month (indicated by the first letter of the month), electrophoresed on formaldehyde agarose gels (20 ug/lane), transferred to nitrocellulose, and hybridized to the probes indicated. **a:** August through February RNAs probed with the actin coding sequence. **b** and **c:** The same sequence of pooled RNAs probed with the Cy (cytoplasmic) and M (muscle) gene-specific probes, respectively.

Coding
A S O N D J F



Cy

A S O N D J J F

A S O N D J J F

M



.

.

.



23
21

.

.

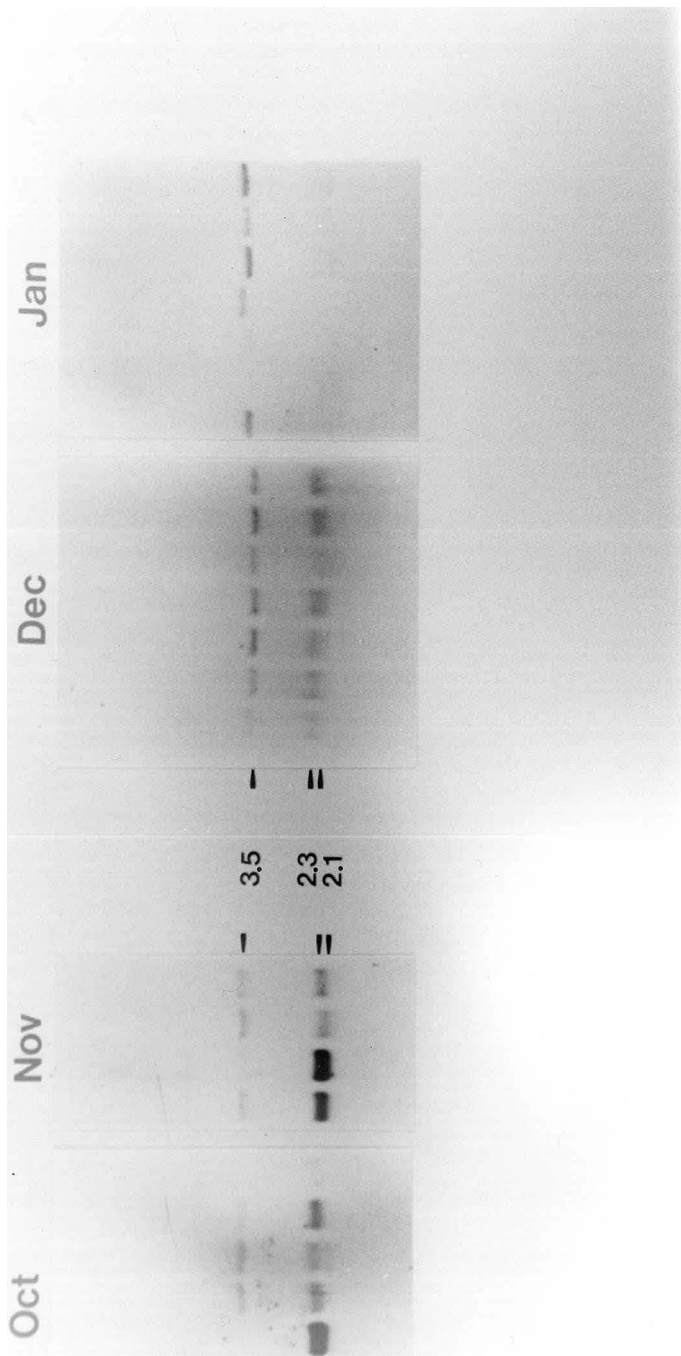
levels.

In the approximately 2/3 of the cycle covered in these experiments, it is apparent that the 3 transcripts are differentially expressed. There are visible inconsistencies, however. Such inconsistencies are manifest in the reduced intensities of hybridization signals in the month of December (longer exposures reveal all 3 transcripts; see below) and the varying intensities of signals produced by the 3.5 kb transcript.

These inconsistencies may have arisen from pooling RNAs from animals in differing spermatogenic states. Such variation had already been implicated in the results shown in Figure 2, wherein it was apparent that 2 individuals displayed the 3.5 kb transcript and 2 did not. Variation among individuals was investigated by comparing individuals from the months of October, November, December, and January (84-85 cycle) by Northern blot analysis using the Coding probe.

The results (Figure 6) show that there was indeed considerable variation in expression patterns for the 3 transcripts in the early part of the spermatogenic cycle. In these blots, the principal indicators of variation are the M gene product and 3.5 kb transcript: In the months of October and November, some individuals express these transcripts and some do not. In later months (December and January) all 3 transcripts are seen in all individuals, suggesting that as the spermatogenic cycle progresses, variation in expression

Figure 6: Individual variation in actin gene expression during the early part of the spermatogenic cycle. Total testes RNA (20 ug/lane) from individual sea stars was electrophoresed on formaldehyde agarose denaturing gels, transferred to nitrocellulose, and hybridized at moderate stringencies (62 °C; 1X SSPE) to actin coding sequence.



patterns between individuals is less pronounced.

The significance of these observations becomes readily apparent when these individuals are compared histologically. The degree of variation in spermatogenic state (as determined histologically) closely parallels that observed in the hybridization data. The following section elaborates on histological patterns in the spermatogenic cycle of P. ochraceus and formulates a set of criteria for the definition of discrete phases of spermatogenesis through which actin gene expression may be followed.

4) Actin Gene Expression as a Function of Spermatogenic State

Echinoderm spermatogenesis has received considerable attention from a histological perspective (reviewed by Chia, 1983). The data gathered from many species has permitted the modelling of a generalized spermatogenic cycle comprised of overlapping phases. Although a detailed cytological description of spermatogenesis in P. ochraceus has not been reported, studies by Nimitz (1971, 1976) and this work indicate that this animal follows the general cycle. The dynamics of cellular activity and histochemistry have been described in considerable detail by Walker (1974, 1975, 1979, 1980, 1984, 1986) for the sea star Asterias vulgaris. The cytological observations and interpretations in this investigation are based largely on these latter studies.

A single Echinoderm testis lobe is comprised of a series of

branching sacs (accini) originating at a common gonoduct. Within these accini one finds the array of germinal and somatic cells participating in sperm differentiation. The distinctive feature of active Echinoderm spermatogenesis is the arrangement of spermatogonia and primary (1°) spermatocytes in long columns (spermatogonial columns) extending luminally from the germinal epithelium (Fig 7c and 7d). Their bases are the sites of mitotic proliferation, while 1° spermatocytes depart the tips to resume meiosis and continue spermiogenesis in the lumen. Fully differentiated spermatozoa accumulate in the center of the lumen. Within a given testis lobe, regional (outer accini versus inner) differences may exist with respect to the relative numbers of cells involved in mitosis, meiosis, and spermiogenesis, but adjacent columns are highly coordinated. During aspermatogenic phases, this extensive organization is replaced by large numbers of motile somatic accessory (SA) cells that phagocytize residual sperm.

The relative numbers of these cells, and their arrangement, as well as histochemical features of the gonad wall have served as criteria in assigning distinct phases in the spermatogenic cycle (Nimitz, 1976; Yamashita and Iwata, 1983; Davis, 1985; Walker 1980, 1984, 1986; Smith and Walker, 1986). Table 2 summarizes the criteria used in this study to pool RNAs from defined spermatogenic states. 5 timepoints in the cycle are defined according to time of year, gonad index, the presence or

TABLE 2

	<u>TIMEPOINTS</u>				
	1	2	3	4	5
	Jul85-07	Oct86-04	Nov84-01	May85-03	May87-05
	Jul85-08	Oct86-05	Nov84-03	May85-04	Jun87-06
	Aug84-03	Oct86-09	Oct86-01	Jul87-03	Jun87-08
	Aug85-03	Sep86-04	Dec84-01	Jun87-03*	Jun87-04*
	Aug85-06	Oct84-06	Dec86-03	Apr85-06	Jun87-07*
GONAD INDEX					
Range (%)	0.3-1.2	0.3-1.5	0.8-2.4	4.9-25.1	0.3-3.1
Mean \pm SE	0.7 \pm 0.2	0.7 \pm 0.3	1.6 \pm 0.4	16.7 \pm 3.8	1.5 \pm 0.5
PHAGOCYTES	+++	+	(+)	(+)	++
SPERMATOCYTES	-	+	+++	+++	+
COLUMNS	-	-	++	+++	+
SPERM	-	(+)	++	++++	(+)

*Individuals with a mixture of both ripe and pigmented gonads.
 (+) indicates the variable presence of the cell type in question.

absence of the various cell types, their organization (i.e. columns present or absent), and the relative abundance of mature spermatozoa.

The first time point (Figure 7a) represents the gonad in a quiescent state. At this stage the gonad index is lowest. The germinal epithelium is reduced, there are no spermatogonial columns and essentially no spermatocytes or sperm. Phagocytic somatic accessory cells prevail in the lumen. The 5 animals pooled for this timepoint were chosen from July and August 1985, at least one month past spawning (May to June 1985).

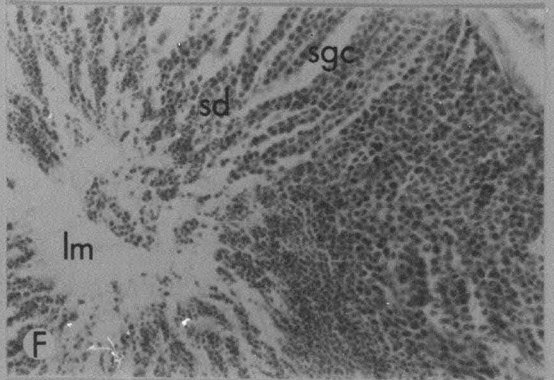
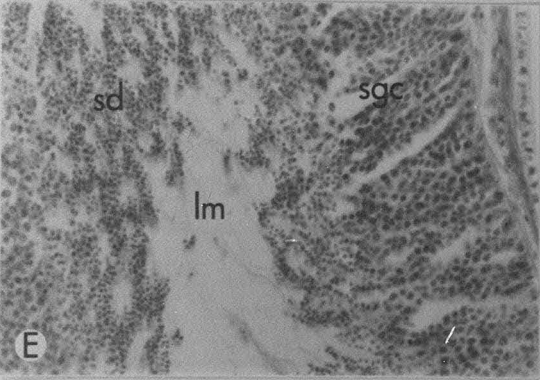
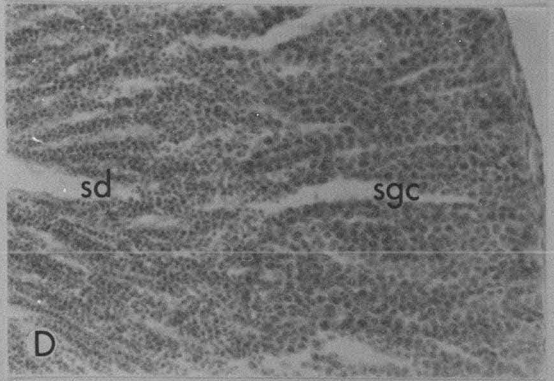
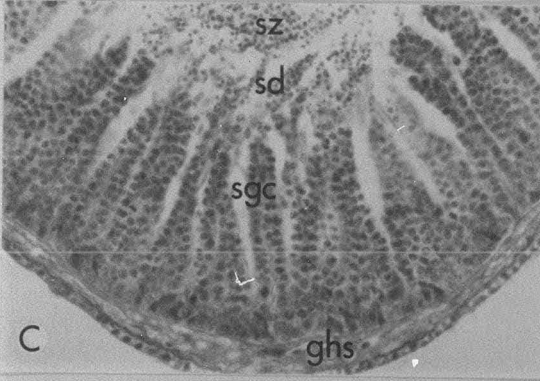
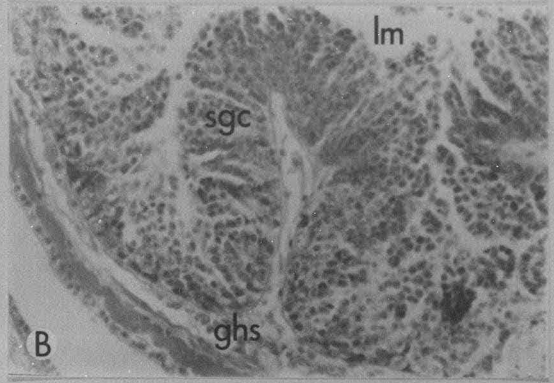
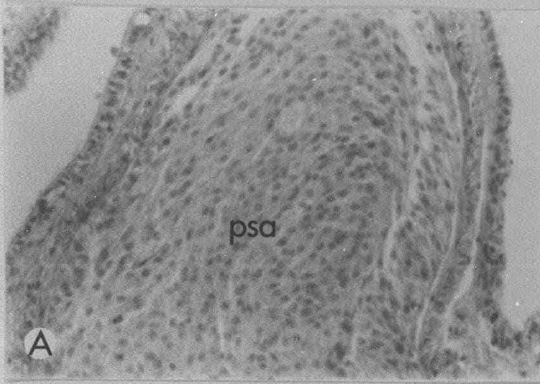
The second timepoint is marked by the first appearance of

mitotic activity (Figure 7b). Spermatogonia and a few spermatocytes are now apparent, but they are not organized into spermatogonial columns. There are few sperm and there has been no significant change in gonad index. September and October animals from both cycles (84/85 and 86/87) were pooled for this timepoint.

Timepoint number 3 manifests a dramatic change in the spermatogenic epithelium (Figure 7c). Large numbers of spermatogonia and spermatocytes are clearly arranged into columns, reflecting intense mitotic activity. Although mature sperm can now be seen in some numbers, gonad index is still relatively low. This class of individuals, chosen from the months of October, November, and December in both cycles, was selected to reflect gene expression patterns primarily concerned with the phase of mitotic proliferation.

The most advanced stages of spermatogenesis are typified by the individuals pooled for timepoint number 4 (Figure 7d). These animals display the highest gonad indexes and the most prevalent cell type is mature spermatozoa. The columnar arrangement of spermatocytes persists but it is presumed, by analogy to the descriptions of Asterias vulgaris by Walker (1980), that mitosis at the bases of these columns has virtually ceased and that now the predominant activities are meiosis and spermiogenesis. Animals in this category were pooled from April through July from both cycles. The fifth and final timepoint is intended to represent the

Figure 7: Histological features of the germinal epithelium during the P. ochraceus spermatogenic cycle. Testes were fixed in Bouin's, embedded in paraplast, sectioned, and stained with haematoxylin-eosin. a) Quiescent. The lumen is filled with phagocytic somatic accessory cells (psa). b) Early mitotic. Spermatogonial columns (sgc) are beginning to form and the lumen (lm) is essentially devoid of cells). The gonadal haemal sinus (ghs) is clearly visible at this stage. c) Peak mitotic. Spermatogonial columns (sgc) are well developed. A region of spermiogenic activity (sd) has developed at their tips and a few mature sperm (sz) can be seen in the lumen (lm). d) Ripe. Testes have increased in size by an order of magnitude compared with (c), so the complete section cannot be included in the picture at this magnification. Spermatogonial columns (sgc) are still well developed, but now the spermiogenic (sd) region predominates and extends leftward of the photograph. Vast numbers of mature sperm fill the lumen. e) and f) Evacuative testes from (e) the Woodlands population in May and (f) the Bamfield population in the same month. In both cases spermatogonial columns (sgc) and spermiogenic (sd) can be seen, but relatively few sperm are present in the lumen (lm). Magnification: X360.

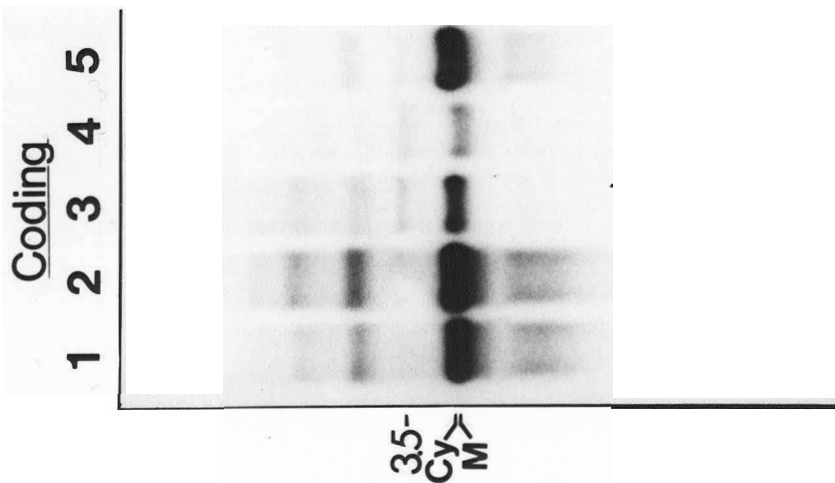
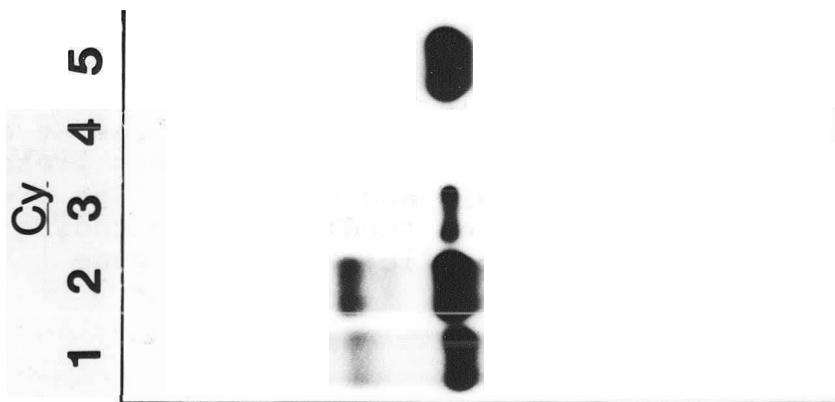
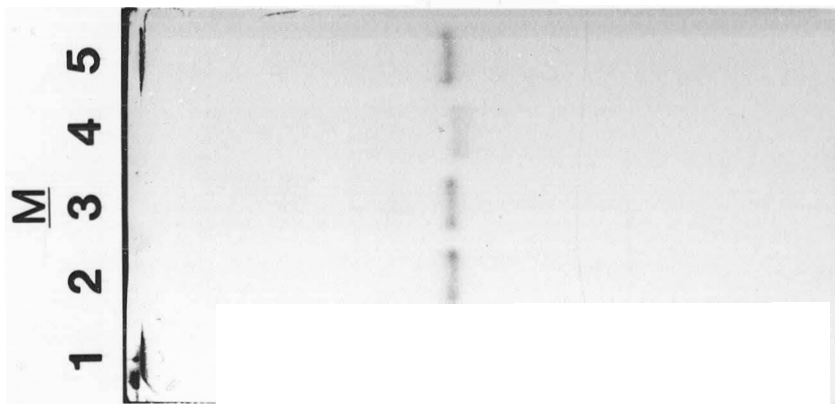


gonad in the evacuative condition (Figure 7e). Remnants of the previous extensive organization of the germinal epithelium remain (i.e. all stages of sperm differentiation, including columns), but gonad indexes are much lower and much fewer sperm are evident. Phagocytes are now more apparent than before. All the animals pooled for this timepoint were taken from May and June in the 86/87 cycle. These testes all had the pigmented phenotype described earlier (Section 1).

These 5 RNA pools were selected to represent 5 distinct spermatogenic states in P. ochraceus testes: quiescent, early mitotic, peak mitotic, peak meiotic/spermiogenic, and evacuative. Figure 8 shows a Northern blot analysis of these 5 timepoints using the actin Coding, Cy, and M gene probes.

Some of the earlier observations made with respect to seasonal patterns are upheld in these experiments. That is, the bulk of actin transcription occurs early in the cycle and most of these transcripts are contributed by the Cy gene. As before, the M gene product appears at the onset of the cycle and persists at relatively low and unchanging levels. It is worth noting here that these blots were performed by the GeneScreen/UV crosslinking method (see Materials and Methods) and this has reduced considerably the signal strength produced by the larger 3.5 kb transcript. However, it is still clearly visible and it seems to increase as the cycle progresses. Timepoint 5, "immediately post-spawned", somewhat surprisingly shows levels of Cy actin transcription essentially equal to the

Figure 8: Actin gene transcripts in testes RNAs pooled according to spermatogenic stage. Each lane is numbered according to spermatogenic stage (see Table 2): 1) quiescent; 2) early mitotic; 3) peak mitotic; 4) ripe; 5) evacuative. Total testes RNA from 5 individuals was pooled for each stage and electrophoresed (20 ug/lane) on formaldehyde agarose gels, transferred to GeneScreen nylon membranes, and cross-linked by exposure to UV light (see Materials and Methods). The probes used are indicated above each panel (Coding= actin coding sequence; Cy= cytoplamic gene-specific; M= muscle gene specific).



maximum early in the cycle. Interestingly, it appears that the levels of the 3.5 kb transcript have fallen to nearly undetectable at this stage.

To this point, expression patterns have been resolved for both the previously described actin transcripts from 2 perspectives: seasonal and spermatogenic state. Both approaches have yielded results in common. The Cy transcript appears at its maximum early in the cycle while the M transcript appears at a constant level for a restricted period. To aid in the explanation of these patterns, they were compared with those of other important sperm components. The next section provides such comparisons with histone H3 and beta tubulin expression patterns through the 5 timepoints.

5) Comparisons With Histone H3 and Beta Tubulin Expression Patterns

The synthesis and accumulation of acrosomal actin represents but one of the major features of echinoderm sperm differentiation. Two other important processes are embodied in nuclear condensation and flagellar development. During nuclear condensation, the chromatin within the haploid spermatid nucleus is repacked into a semicrystalline array, conferring a streamlined phenotype to the mature sperm. Occurring in parallel with nuclear condensation, flagellar development provides the haploid nucleus with its locomotory capabilities.

The highly basic histone proteins, ubiquitous in the

chromatin of all eukaryotic cells, are the prime participants in sperm nuclear condensation (reviewed by Poccia, 1986 and 1987). In some organisms such as the trout, the somatic histones are completely replaced by another set of basic proteins known as protamines (Louie and Dixon, 1972). The renovations to spermatid chromatin in the sea urchin are provided for by variants of a restricted subset of the somatic histones (cited in Poccia et al., 1987). As histone gene expression in P. ochraceus testes has not yet been characterized in such detail, this study provides a preliminary assessment with an investigation of histone H3 expression.

All eukaryotic flagella are composed of equal numbers of alpha and beta tubulin subunits (Alberts et al., 1983). Both polypeptides are represented by multigene families under developmental stage- and tissue-specific regulation. The appearance of a great variety of transcripts has complicated the elucidation of the functional specificity of the various members of these gene families (Cleveland, 1987). In their investigations of the sea urchin Lytechinus pictus, Alexandraki and Ruderman (1981, 1985) identify at least 1 alpha and 2 beta tubulin transcripts in immature spermatogenic testes. There have been no reports analysing tubulin gene expression in P. ochraceus testes. The use of a beta tubulin gene probe serves therefore as a general indicator of tubulin gene expression patterns in this study.

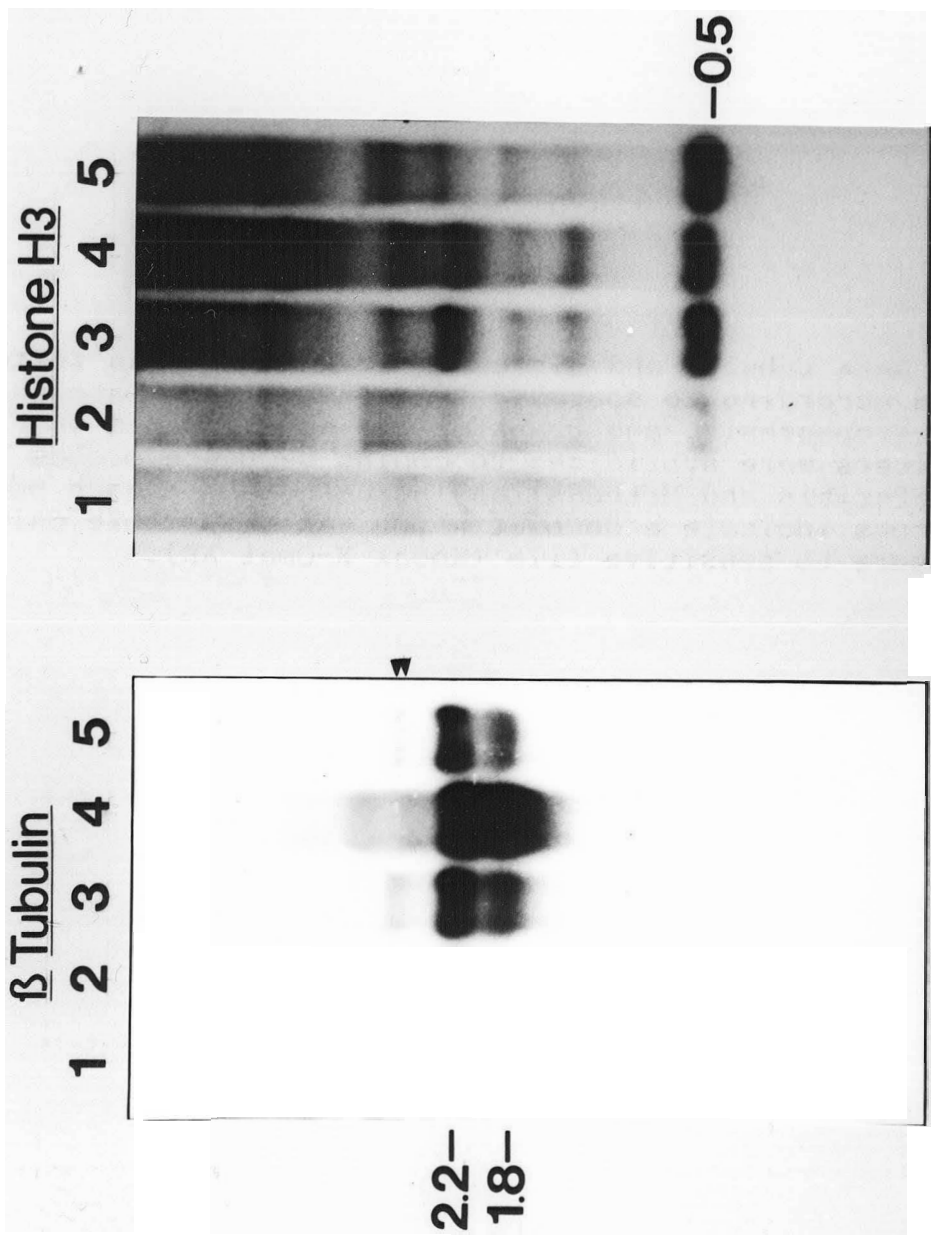
When the 5 timepoint RNAs are subjected to Northern analysis

using probes representing coding regions of beta tubulin and histone H3, the results shown in Figures 9a and 9b, respectively, are obtained. The beta tubulin probe detects 2 major transcript size classes, roughly 1.8 kb and 2.2 kb in size, consistent with observations in sea urchin testes (Alexandraki and Ruderman, 1985). Both are virtually undetectable in the quiescent gonad (lane 1, Figure 9a) and the larger of the 2 is the first to appear (lane 2). Both are at their apparent maximum in the ripe gonad (lane 4) but still abundant in timepoint 5. A variety of other less abundant transcripts are also evident (indicated by arrows in the figure). Whether these represent artifacts of electrophoresis or unprocessed transcripts has not been (see Materials and Methods).

The expression pattern revealed by the H3 probe (Figure 9b) is similar to that of beta tubulin in that the transcript size class (0.5 kb) detected is at a minimum in timepoint 1 and appears at the onset of mitotic proliferation. Unlike beta tubulin, however, the H3 transcript appears to build quickly to a maximum in timepoint 3, thereafter remaining at a relatively constant level. There is no evidence in these data for the appearance of an sperm-specific histone H3 variant. Again, the bands in the higher molecular weight region may reflect artifacts or unprocessed transcripts.

Taken together, the data presented here indicate that the expression patterns of histone H3 and beta tubulin are

Figure 9: Beta tubulin and histone H3 transcripts in testes RNAs pooled according to spermatogenic state. Spermatogenic stage, electrophoresis, and transfer are as described in Figure 8. The filters were hybridized to coding sequence probes (see Table 1, Materials and Methods) as indicated above each panel. The two arrows indicate a doublet which was resolvable only on brief exposure to sensitive film (Kodak X-Omat AR).



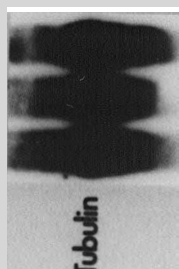
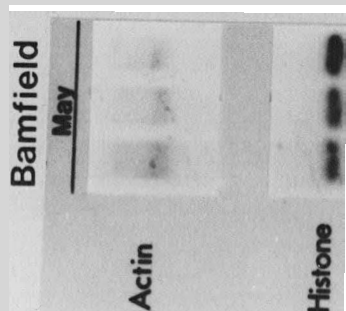
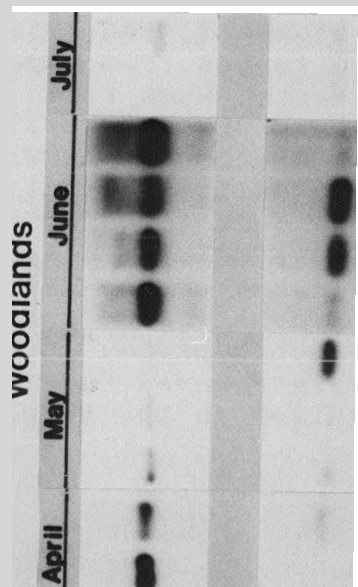
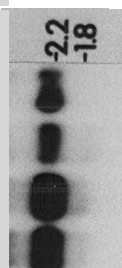
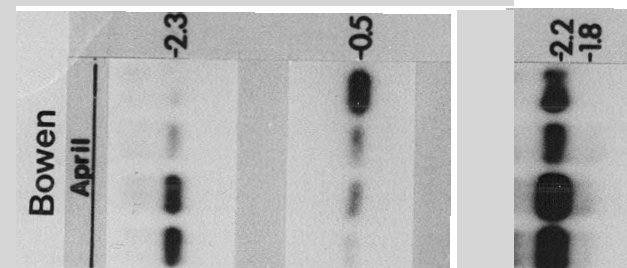
distinctly out of phase with those of the actin transcripts. Actin predominates early in the cycle while H3 and beta tubulin reach their maxima later. A discussion of these observations will be left to the appropriate section: the question that remains is that of timepoint 5.

With the exception of the M actin transcript and the 3.5 kb transcript, there are high levels of the other transcripts investigated in what has been classified as "evacuative" testes. Note again that the RNAs pooled for this timepoint were derived from an atypical spermatogenic cycle and perhaps "questionable" testes. In an effort to resolve whether in fact these gonads truly represent the evacuative condition, comparisons were made with males from the Bamfield population, where it appeared that both males and females had spawned. The next section details these comparisons and attempts to shed light on gene expression patterns during the evacuative phase of spermatogenesis.

6) Gene Expression Immediately After Spawning

The purpose of these experiments is to provide a consensus of criteria defining gene expression patterns in the testes immediately after the majority of differentiated sperm have been evacuated. As outlined above, there is some question as to whether or not the Woodlands males chosen to represent this condition were typical. Comparisons were therefore made with other populations geographically separate from the Woodlands

Figure 10: Comparison of actin, histone H3, and beta tubulin transcript levels in post-evacuative testes from Woodlands, Bowen Island, and Bamfield. Total testes RNAs from individuals from the Woodlands population for the months of April, May, June, June and July were compared by Northern blot with individuals from the Bowen Island and Bamfield populations. After electrophoresis on formaldehyde agarose gels, transfer to GeneScreen, and UV cross-linking, these RNAs were hybridized with the coding sequence probes indicated on the left. The histone H3 probe was used after hybridization to the actin probe, without prior removal of the actin probe. Subsequently, both the actin and histone probes were removed (see Materials and Methods) and the filter rehybridized to the tubulin probe.



population (the Bowen Island and Bamfield populations). The Bamfield sample, taken early in May (1987), consisted of both males and females of low gonad index (1-2%), and was therefore considered to have spawned (see Figure 7f).

Comparisons of actin, histone H3, and beta tubulin expression were made between the 3 populations. The results of Northern blot analyses using coding probes for these 3 genes against 3 Bamfield testes RNAs and RNAs derived from a range of individuals from the Woodlands population are shown in Figure 10. The upper panel represents filters first hybridized to the actin Coding probe and then to the H3 probe, with the result that both signals appear on the same autoradiograph. These probes were removed (see Materials and Methods) and the filters hybridized to the beta tubulin probe (lower panel).

The Bamfield animals are distinguished in these experiments by 2 prominent features: their levels of both tubulin and histone H3 expression are higher and their levels of actin expression lower than in any other animal. Otherwise all individuals investigated, regardless of source population, express varying levels of actin, histone H3, and beta tubulin.

DISCUSSION

1. The Spermatogenic Cycles:

Two very different spermatogenic cycles have been sampled. The 84/85 cycle, which shows initiation in fall and spawning in May or June, conforms well with the other reports cited above. Atypical gonad indexes and gonad morphologies are the hallmarks of the 86/87 cycle. The significance of the difference between the two cycles becomes most apparent when one compares the average mass of total testes isolated per individual male in each cycle: 27.8 grams (84/85; 66 males) versus 6.5 grams (86/87; 74 males).

In consideration of the possibility that samples taken from the 86/87 cycle came from a different age class than those from the 84/85 cycle, the weights of whole animals were compared as averages for each monthly sample. No significant difference could be discerned and all animals were above the weight at which sexual maturity is reported to be achieved (150 g; Mauzey, 1966).

Earlier records from our lab (Fraser et al., 1981; M. J. Smith, unpublished data) concerning the same population suggest that the low male:female ratio in February, March, and April is indeed unusual. Whether this brief departure from the expected sex ratio reflects a sampling artifact or changes in the relative distribution of the sexes in response to environmental factors or reproductive behavior remains a question.

The unusual morphologies of testes taken from most of the

individuals in the 86/87 cycle are clearly correlated with the reduced spermatogenic productivity of this population in this cycle. Particularly interesting are the inconsistencies in spermatogenic states within individuals during this cycle. These are surprising in view of the fact that the circulatory system believed to supply nutrients to the gonads interconnects the gonads with other organ systems, including the hepatic caecae, the putative nutrient storage organs (Walker, 1974). Such a system of interconnections might be presumed to promote synchrony in gonadal development via an equal distribution of nutritive and hormonal requirements among the ten testes. Gonads in such varying states are also common in the Atlantic asteroid Asterias vulgaris. In this species, a ciliated protozoan parasite is abundant and can be seen actively ingesting sperm. There is a strong possibility that this parasitism leads to the reduced pigmented phenotype and thus may reflect disease specific to males (Bang, 1982). However, these parasites have not yet been identified in P. ochraceus. Alternatively, these observations may reflect considerable asynchrony in the initiation and completion of spermatogenesis, or males skipping a gametogenic cycle, as has been suggested by Davis (1985) in her investigations of Patiria miniata.

2. Actin Genes Expressed in the Testes:

On the basis of hybridization to previously characterized coding and gene-specific probes, P. ochraceus testes express 3

transcripts related to actin. The largest of these (3.5 kb) is detected only by the coding probe and only at moderate stringencies. The transcripts distinguished by the gene-specific probes (Cy and M) appear to be the same size as transcripts detected by these probes in other tissues and developmental stages (Kovesdi and Smith, 1985). A cDNA representing the Cy transcript (pPoTeA01) has been partially characterized and is tentatively designated as a product of the lambda PoA145/128 gene class. Sequence comparisons (Figure 4) imply that the cDNA clone pPoTeA02 also derives from this gene.

A cDNA specifically representing the putative Muscle (M) gene transcript was not recovered in an additional screen of the same cDNA library using both the M gene-specific and coding probes. It can therefore not be determined whether the M gene transcript detected here is a product of one or both loci thought to encode the PoA160 gene class (Kovesdi and Smith, 1985). It appears that both the cDNA clones pPoTeA03 and pPoTeA04 are not products of the Cy gene (Figure 4). However, preliminary sequence comparisons of these clones with a partial sequence of a genomic clone representing one of the 2 genes in the PoA160 (M) class suggests that these clones are not derived from this gene either. 17 genomic isolates representing the M gene class were originally recovered from a genomic library (Kovesdi, 1985), but as yet not all of these have been characterized. It is possible that pPoTeA03 and pPoTeA04 represent transcripts produced by the other member of the M

gene class.

The identity of the 3.5 kb transcript and its relationship to actin coding sequence remain a mystery. No insert having its "hybridization phenotype" (i.e. only to coding sequence, only at moderate stringencies) has been isolated. On the level of Northern blot analyses, however, a couple of features have emerged to provide a preliminary characterization of this transcript: 1) Based on its failure to hybridize to coding sequence at high stringency, or any of our gene-specific probes, this transcript does not encode actin; 2) It is tentatively testes-specific, not having been detected in ovaries, tubefeet, hepatic caecae, or any stage of early development; and 3) Its level of expression appears to be differentially regulated during spermatogenesis. These facts, together with the possibility that it encodes a protein sharing a domain in common with actin, make this transcript worthy of further study. The formal possibility remains that the 2 cDNA clones pPoTeA03 and pPoTeA04 represent this transcript, but this suggestion initially requires a demonstration of hybridization specificity of these 2 clones (or fragments thereof) to the 3.5 kb transcript at high stringency.

The results presented here are consistent with those reported for the sea urchin (Schott et al., 1984). Sea urchin testes contain a single major cytoplasmic transcript (CyI actin) and two minor transcripts, CyIIb (cytoplasmic) and M (muscle). In spite of the diversity of sea urchin actin

transcripts, all but one of which encode a cytoplasmic product, none have been reported to be testis-specific. This investigation has also failed to confirm the expression of a testes-specific actin. One of the participating genes has been identified and it is the P. ochraceus PoA128/145 (Cy) gene class. Hybridization data have been presented which demonstrate that the PoA160 (M) class is transcribed, but which of the two genes are expressed has not been discerned. The possibility remains that these loci may give rise to testes-specific transcripts by way of alternate splicing, as has been suggested in the mouse (Waters et al., 1985). Such alterations would have to be sufficiently minor so as not to substantially distinguish the lengths of the alternative products, and it is therefore unlikely that such mechanisms prevail. Post-translational modification(s), on the other hand, could provide for the unique features of acrosomal actin but this is currently not known.

3. General Transcription Patterns During the Spermatogenic Cycle:

Most of the interest in gene expression in spermatogenesis in the last two decades has been concerned with the relative levels of transcription in haploid and diploid stages of sperm differentiation. By examining testes and/or separated spermatogenic cells that have been exposed to ³H-Uridine, these studies have shown the bulk of transcriptional activity to

occur in diploid cells (mouse, Geremia, 1977; Drosophila, Gould-Somero and Holland, 1974). Whether the same is true for echinoderms has not been confirmed. While experiments similar to those noted above were beyond the scope of this thesis, data have been collected which reflect upon this subject.

The nature of this data involves estimates of the total amount of RNA available per gram of testis, and fluctuations in this parameter in relation to spermatogenic state. Before interpretations of such data can be made, it is first necessary to question possible changes in RNA extraction efficiency in relation to the biochemical state of the tissue. The experiments outlined in Appendix A address this problem via isotope dilution assays and the recovery and analysis of RNA normally discarded as a supernatant in routine extractions. Extraction efficiencies are generally low (10-20%) for testis involved in sperm production and accumulation. It appears, however, that efficiencies are much greater (at least 60%) for testes chosen to represent the recently spawned condition. This latter conclusion is drawn from the observation that little to no additional RNA from these testes could be recovered from the supernatants normally discarded.

In view of the observation that extraction efficiencies do not appear to vary significantly with spermatogenic state (disregarding, for the moment, evacuative testes), this parameter becomes a constant. Thus the mass of RNA extracted per gram of testes constitutes a measure that can be compared

with a measure of spermatogenic state, for example gonad index. Such a comparison was made using data derived from the 1984/85 cycle. The highest values of total mass RNA per gram of testes were found for animals of lowest gonad index, and were almost exclusively restricted to September, October, and November animals. The converse relationship was seen for animals of high gonad index. Furthermore, the high values were at least tenfold higher than the lowest.

When the same strategy is applied to the testes pooled for the timepoint RNAs, a similar relationship is revealed. Uncorrected (for extraction efficiency) values for mass RNA per gram of testes appear to increase slightly from timepoint 1 (quiescent) through timepoint 3 (mitotic) (1.0 ± 0.2 , 1.5 ± 0.6 , and 1.5 ± 0.5 mg/g; mean \pm SE, respectively), but the magnitude of the increase is not significant. The significant trend is seen in timepoint 4 (ripe) wherein these levels drop tenfold to 0.13 ± 0.03 mg/g. In the absence of correction for extraction efficiency, the value for evacuative (timepoint 5) is roughly the same as those for the first three timepoints (1.4 ± 0.16 mg/g). However, corrections can be made for extraction efficiency. As noted above, the extraction efficiency at timepoint 5 was high (>60%). therefore the correction factor for this timepoint was set at 1.5 while the others were multiplied by factors of 5 to 10. When these corrections are applied, the following estimates of total RNA per gram of testes are arrived at for timepoints 1 through 5: 5.0 ± 1.0 ;

7.5 \pm 3.0; 7.5 \pm 2.5; 1.3 \pm 0.3; 2.1 \pm 0.24 mg/g. This pattern is reminiscent of that noted above in general correlations in the 84/85 cycle: at least 5-fold higher RNA concentrations are seen in early spermatogenic testes. In addition, after correction for extraction efficiency, it appears that both ripe and (putatively) evacuative testes contain roughly equivalent concentrations of RNA. If cell volume is considered in these calculations (i.e. number of spermatogonia and spermatocytes per gram of early spermatogenic testes versus number of sperm and spermatids in ripe testes), these values compare well with those obtained by Louie and Dixon (1972a), who measured the relative amounts of RNA and DNA in purified cell types from trout testes. They find approximately 50 fold less RNA in sperm and spermatids than in diploid cells, reflecting the depletion of cytoplasm through differentiation.

Nimitz (1971, 1976) has quantified the relative numbers of diploid and haploid cells through the Pisaster spermatogenic cycle. She has observed the largest number of spermatogonia and spermatocytes in the first half of the cycle while spermatids and spermatozoa predominate later. Together with her observations, the above manipulations of "incidental data" invite the hypothesis that the general pattern of RNA transcription during Pisaster spermatogenesis is consistent with that seen in other organisms, namely that most transcriptional activity is associated with diploid cells. This feature is of some importance to the discussions of

specific gene expression patterns which follow.

4. Specific Gene Expression Patterns During Spermatogenesis:

This study has investigated the patterns of actin gene expression during spermatogenesis from three perspectives. The first addresses the question of whether or not actin gene expression is in any way related to seasonal parameters. The second approach attempts to show correlations of actin gene expression patterns with histological features reflecting spermatogenic states. A third and final approach involves the comparison of the patterns observed for actin with patterns of expression of beta tubulin and histone H3, two other major components in sperm differentiation.

The first two approaches, concerned strictly with actin transcripts, have yielded results in common. Seasonal patterns of expression are reflected in patterns ascribed to defined spermatogenic states. It appears that the bulk of actin gene transcripts arise from the cytoplasmic gene (PoA128/145 gene class) and that this transcript, although present year round, shows its highest level of expression at the onset of spermatogenesis and (tentatively) immediately after spawning. This pattern is contrasted by that of the M gene transcript(s), which appear only at the onset of spermatogenesis and maintain consistently low levels throughout the remainder of the cycle.

How do these two divergent patterns of actin gene expression compare with those of other important sperm components? While

the expression patterns of histone H3 and beta tubulin differ somewhat between them, they are essentially the converse of actin. Transcripts for both of these proteins are seen in abundance when actin transcripts are at their lowest prevalence.

However, these two gene products differ significantly: Histone H3 has a defined role in the cell while tubulin performs multiple tasks. In view of the fact that the function of histone proteins involves the packaging of DNA into chromatin, and is therefore tied to DNA replication, one can infer that their expression is simply correlated with mitosis and meiosis I. The pattern shown in this study for histone H3 is consistent with this notion. Histone H3 transcripts are barely detectable in the quiescent gonad, but accumulate rapidly to a maximum in actively mitotic testes. Their abundance in ripe gonads raises the question as to whether they are present as stored transcripts or haploid transcription products. In their study of gene expression during spermatogenesis in the flounder, Kennedy et al (1985) report the majority of H3 expression in diploid cells. They do, however, show the expression of an H3 variant in early post-meiotic cells, but its levels are much lower. Similarly, Dixon and coworkers (Ingles et al., 1966) have identified a class of highly basic proteins (protamines) in trout testis. These replace the somatic histones during nuclear condensation. Their (Iatrou et al., 1978) work has shown that protamine mRNAs

are transcribed in primary spermatocytes and stabilized for later translation when the protein product is required. The presence of an H3 variant or highly basic sperm-specific proteins has not been demonstrated here and therefore no conclusions can be drawn regarding the transcriptional activity of histone genes in specific stages of spermatogenesis.

Whether these high levels of histone H3 transcripts in spawned testes represents residual stored H3 mRNA or continued gene activation, they are probably associated with cellular proliferation. Walker (1980) has reported mitotic activity at the base of spermatogonial columns to be lowest immediately before spawning, while the predominant activity involves the resumption of meiosis (in primary spermatocytes) and spermiogenesis. This results in a progressive shortening of the spermatogonial columns as more and more germ cells enter prophase of meiosis I. At the time of spawning, somatic accessory cells are believed to phagocytose germ cells at the base of the columns while the entry of primary spermatocytes into meiosis and spermiogenesis at the tips of the columns proceeds at an accelerated rate. This accelerated burst of differentiative activity could account for at least part of the high levels of H3 seen. It is equally likely that new types of cells are now proliferating to deal with the "clean-up" (e.g. phagocytic somatic accessory cells).

The maximum beta tubulin expression is highest in ripe testes, suggesting haploid expression. This is not without

precedent. Haploid expression of a testis-specific alpha tubulin has been demonstrated in the mouse (Distel et al., 1984) and of both alpha and beta tubulin in flounder testes (Kennedy et al., 1980).

In view of the numbers of sperm produced, the observed levels of beta tubulin in ripe testes are probably largely devoted to sperm flagellar construction. However, there are at least four different types of flagellated cells, including sperm, in an asteroid testis (Walker, 1979). The perivisceral lining of the gonad is populated by flagellated collar cells, and another flagellated cell has been observed in the genital coelomic (perihæmal) sinus. Somatic accessory cells appear to be flagellated in their motile, phagocytic form. In addition, primary spermatocytes appear to develop a flagellum that is lost when these cells depart the spermatogonial column and develop a second during spermiogenesis. Furthermore, each developing germ cell in the spermatogonial columns extends a long cytoplasmic process to the basal lamina of the germinal epithelium: these contain an array of microtubules extending their full length. To these demands on tubulin expression we can add those of mitosis, meiosis, and other cytoplasmic functions. The proportion and activities of these cells is undoubtedly changing with spermatogenic state, suggesting a potential for complex expression patterns. Indeed, several transcripts hybridizing to beta tubulin have been seen in this work and in sea urchins (Alexandraki and Ruderman, 1981).

Consideration of these other cell types is certainly pertinent to tubulin expression patterns in evacuative testes. Here beta tubulin levels are equal to those in ripe testes. This is discussed along with actin and histone in the context of the spawned condition (Section 6).

5. Which Transcript Encodes Acrosomal Actin?

There appear two actin transcript classes (Cy and M), each regulated differently, which could in principle encode the acrosomal actin localized in the mature sperm. The Cy transcript shows a burst of expression at a point in the cycle when spermatogenic cells are predominantly diploid. This peak in Cy expression is at least tenfold higher than the levels seen for the M transcript(s) at any time during spermatogenesis. To judge which of these transcripts might provide for acrosomal actin, one can first determine which of these two patterns and levels of expression are sufficient to provide the amount of actin required to construct the acrosomal process.

Tilney and Inuoe (1982) have estimated the total amount of actin in the acrosomal process of Thyone sperm. Their calculations are based on the average number of actin microfilaments comprising the process, its length, and the fact that there are about 370 actin monomers per micrometer of filament length. In the absence of data on Pisaster, estimates of filament number and length in the acrosomal process of

Asterias amurensis (Dan and Hagiwara, 1967; Tilney et al., 1973) are used in these calculations. The sperm of this asteroid produces an acrosomal process 20 μm in length, containing about 75 microfilaments. Thus, the process contains 1500 μm of microfilaments or $(X370) 5.5 \times 10^5$ actin monomers.

If we assume that the period of differentiation of a single sperm is in the order of two weeks (Chia, 1983) then, given an estimate of translation rate, one can approximate the minimum steady state number of transcripts required per cell to provide for acrosomal actin. A spacing between ribosomes on polysomes has been estimated to be about 135 nt and their rate of translation estimated to be 1.8 codons per second at 15 $^{\circ}\text{C}$ during translation in the sea urchin (Davidson, 1986). Using a value of 10 for the number of ribosomes on the actin polysome, and assuming that each ribosome translates a complete polypeptide, one arrives at a steady state requirement of 10 transcripts per cell. This value is consistent with the measurements of actin transcripts per cell in the late gastrula (8-14; Kovesdi and Smith, 1985).

Thus, without invoking cell type-specific expression, it appears that an "average" number of transcripts is sufficient to accommodate the requirements of acrosomal actin. The hybridization signal strength and expression pattern of the M gene transcript suggest that this transcript has the potential of fulfilling this role.

However, note that this line of reasoning assumes the steady

state level of M actin transcripts to be equal in all stages of sperm differentiation. This means that late spermatids contain as many transcripts as primary spermatocytes, and that therefore these haploid cells must be actively transcribing actin. Considerable evidence exists demonstrating that post-meiotic transcription is a rare event. There have been no reports of the haploid expression of actin. Nevertheless, in the absence of direct evidence that the M gene transcript(s) is/are specific to muscle or not in fact a transcription product of post-meiotic cells, this transcript remains as a candidate for the source of acrosomal actin. This hypothesis may be further tested by Northern analysis of spermatid RNA with the M gene-specific probe.

If 10 actin transcripts present in all differentiating cell types suffices to fill the order, then ten times that number of transcripts present in one-tenth the number of cells should also be sufficient. This alternative distribution fits the levels and patterns of expression of the Cy transcript. Cy levels are highest when the proportion of diploid cells is greatest. The fact that Cy levels are lowest when the proportion of haploid (i.e. spermiogenic) cells is greatest precludes the notion that these mRNAs might be stabilized for later translation. Therefore, the protein product must be synthesized immediately.

If the protein product is synthesized immediately following translation, for what purpose? Korn (1982) has succinctly

summarized the biology of actin: "The temporal and spatial regulation of actin polymerization is central to the structural and motile activities of nonmuscle cells." Perhaps the large burst of actin protein synthesized provides for the cytoskeletal requirements of the large numbers of proliferating cells at this time in the cycle. Subsequently (i.e. post-meiotically), these cells decrease in size as spermiogenesis proceeds. The ordered depolymerization of existing cytoskeletal actin and its localization beneath the acrosome could be an alternative source of acrosomal actin. This model is consistent with Louis Tilney's (1976) proposal that acrosomal actin is initially homogeneously distributed in the cytoplasm to become localized in the periacrosomal cup through interactions with other protein factors. This hypothesis, attractive in its simplicity and consistency with the mechanisms of actin function, must withstand tests of the half-life of the actin protein and the identification of post-translational modifications and/or protein factors that might effect its stabilization in the periacrosomal material.

None of the preceding discussion has considered the possibility that the required actin is derived from somatic cells. The most likely cells to participate in such a transfer of gene products are the somatic accessory (SA) cells. These cells are comparable to the mammalian Sertoli cells in that they provide a "scaffolding" for the arrangement of differentiating spermatogenic cells and an endocrine function

(discussed by Walker, 1980). However, the close association of spermatogenic and Sertoli cells is not seen between SA cells and spermatocytes. Furthermore, there has been no clear demonstration of the transfer of products from the SA cell to the germline.

6. What Patterns of Gene Activity are Found in Evacuative Testes?

Considerable attention in this study has been directed to an evaluation of "normal" and "abnormal" post-evacuative testes. Gene expression data presented for this state of the gonad derives from a population wherein several serious abnormalities were observed. With respect to the Woodlands males, most of these observations have been noted in Section 1; they will be briefly reviewed here. Small testes lobes having the pigmented phenotype were observed in virtually all males from January through July, 1987. Most individuals displayed this type of gonad as well as "typical" ripening testes, but rarely was the full complement of testes seen in any one individual. Perhaps the most striking condition in this population was that females appeared to be developing normally (as judged by external morphology): if the pigmented testes represented the evacuative condition, then those testes had not only spawned out of synchrony with other testes in the same individuals, they had spawned out of synchrony with the females of the population. This feature is not expected of organisms that reproduce by

broadcast fertilization.

A comparison of transcript levels in these animals with those presumed to this point to represent evacuative testes (the Bamfield animals) provides some interesting observations. In these "normal" testes are found the highest levels of beta tubulin transcripts and the lowest of actin. Actin transcript levels are roughly the same as for ripe testes, suggesting that the transition from ripe to evacuative has elicited no change in actin expression. Likewise, the levels of histone H3 levels have not changed significantly. The extraordinarily high tubulin levels might be explained by a final post-evacuative burst of differentiation of sperm or the rapid proliferation of the accessory cells which predominate in the post-evacuative gonad, as noted above.

While these interpretations are plausible, it is interesting to note that histologically these "normal" testes bear very little difference to the "abnormal" gonads of the Woodlands population. Both types of testes show all stages of sperm differentiation, including spermatogonial columns, but little to no sperm. The only difference appears to be a somewhat higher incidence of phagocytic cells in "abnormal" testes.

An interpretation of this observation might be assisted by first reviewing the chronology of gene expression through the spermatogenic cycle. Actin is the first transcript to reach maximum levels, followed by H3, and then immediately by beta tubulin. Given that the cycle begins with a predominance of

diploid cells and arrives at a predominance of haploid cells and that the relative proportions of these cells is reflected in the composition of the spermatogonial columns, one can hypothesize that this chronology of expression is also reflected in the columns. That is, the bulk of actin transcripts would be restricted to actively proliferating spermatogonia at the base of the columns while the increased levels of the other two transcripts would parallel the increasing length of the columns and entry into spermiogenesis. As mature sperm accumulate in the lumen, mitotic proliferation and hence actin transcription, ceases. For purposes of this discussion, this model is taken to represent the normal course of events as the testes approach the evacuative phase.

At the point of spawning there are (hypothetically) two different categories of testes to consider. The three Bamfield animals have been termed "normal": they are assumed to have accumulated large stores of sperm which have been evacuated en masse in the natural course of events. In support of this interpretation it can be seen that these testes express levels of actin and histone transcripts which differ little from those seen in maximally ripe testes, and they contain essentially no mature sperm.

Pigmented ("abnormal") testes also contain few sperm. Perhaps these gonads experienced no sperm accumulation during the cycle. There is a strong possibility that parasitic activity is responsible for this. Although such a parasite

remains to be indentified in P. ochraceus, this condition has been positively documented for Asterias vulgaris males on the Atlantic coast (see above). If the mature products of spermatogenesis are being continuously removed by such a parasite, one might visualize a gonad which is "locked into" earlier stages of the cycle. The consequence of this hypothesis leads to an explanation of the high levels of actin transcript observed in these testes: mitotic proliferation at the base of the columns continues at maximum with the corresponding activation of actin transcription.

7. Conclusions and Future Research:

This is the first study investigating gene expression during spermatogenesis in Echinoderms. With respect to actin gene expression in Pisaster ochraceus, this work has extended our knowledge to another basic, and very important, tissue in this organism. Apparently the same (or at least very similar) actin transcripts encode this protein in testes as in the other tissues and developmental stages. There is no evidence in this study to refute the hypothesis that tissue-specific actin gene expression results from lineage-specificity rather than requirements for functionally distinct proteins.

The contrasting expression patterns of the Cy and M gene transcripts reflect the dynamics of the protracted period of spermatogenesis. That the Cy transcript is most abundant at a time when diploid germ cells predominate is strong evidence for

the hypothesis that this transcript is maximally expressed in these cells. This possibility is enhanced with discovery that RNA from isolated spermatogonial columns is at least 5-fold enriched for actin and H3 transcripts over RNA from total testes in Asterias vulgaris. Questions of this nature will best be answered with the continued analysis of transcripts from separated cell types in combination with in situ hybridizations.

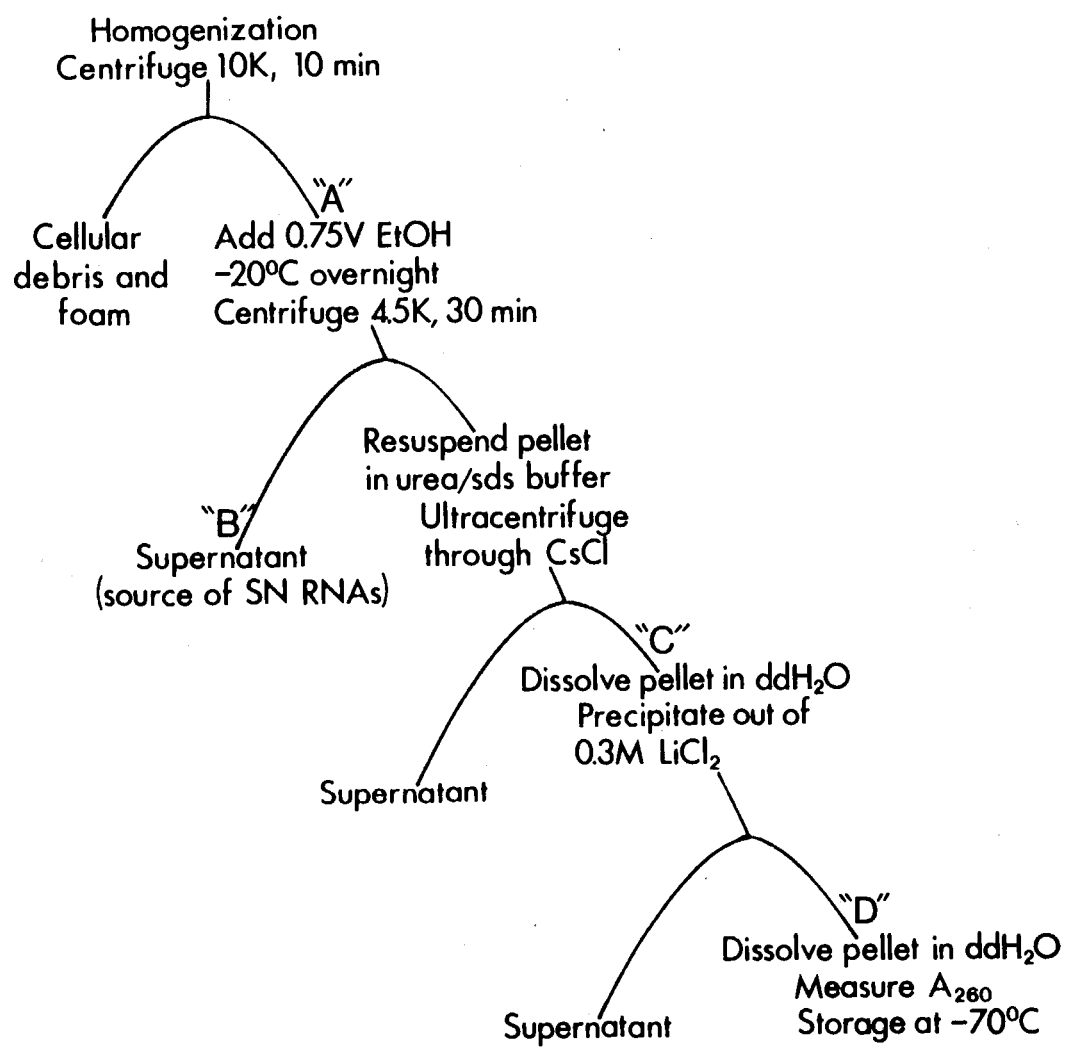
APPENDIX A

RNA Extraction Efficiencies

A routine procedure for the isolation of RNA was developed as a modification of that of Chirgwin et al (1979), and is summarized in Figure A.1. The first phase of the process (to checkpoint "B" in the figure) involves the differential precipitation of RNA out of the cleared guanidine homogenate with the addition of an appropriate volume of ethanol. This precipitated RNA is then resuspended in a urea/SDS buffer and purified through a cesium chloride cushion. The resulting pellet is washed with 70% ethanol, precipitated, and dissolved in water.

This method is likely to selectively lose small RNA molecules because they pellet less efficiently through solutions of increased viscosity (the guanidinium homogenate) and density (the cesium cushion) (Davidson, 1969). Seasonal changes in the biochemical composition of various organs, including the testes, of sea stars have been reported (e.g. Nimitz, 1976; Allen and Giese, 1966; Walker, 1980; Smith and Walker, 1986). With respect to testes, the most obvious changes are manifest as a result of the large numbers of sperm produced (Walker, 1980; Davis, 1985; Smith and Walker, 1986). There was indeed a dramatic increase in the viscosity of homogenates derived from ripe tissue, and abundant DNA was readily apparent when these were subsequently combined with

Figure A.1.: Flow diagram of the RNA isolation procedure. The letters "A", "B", "C", and "D" refer to checkpoints at which the loss of RNA was evaluated via isotope dilution procedures (see Table A.1. and Materials and Methods).



ethanol. These biochemical changes and the nature of the RNA isolation procedure suggested a necessity to determine overall extraction efficiencies and where in the procedure losses were taking place. This was explored relative to sex, gonad index, and the amount of tissue processed. The objective was to discern whether there were qualitative or quantitative losses of RNA, or whether levels of DNA contamination might exist in the isolated RNAs as a function of increasing gonad index.

Two approaches were taken to address these problems. The first involved an analysis of the extraction procedure using the method of "isotope dilution". Quantification of tritium-labelled E. coli RNA (^3H -RNA) in aliquots selected at discrete checkpoints provided an assessment of losses through different parts of the process (Table A.1.; see Materials and Methods). Isolations of RNA from the gonads of 8 animals (7 females and 1 male, all from September, all of low gonad index) were followed in this fashion. Controls were provided by processing of ^3H -RNA with 2 mg of carrier RNA (see Materials and Methods). These data revealed that the greatest losses of RNA (50-75%) were confined to the first phase of the procedure. Losses incurred during purification through cesium chloride appeared to be minimal. Both the control and sample prep data were similar in this regard.

Net RNA extraction efficiencies were evaluated in this manner for an additional 6 males and 2 females of low gonad index (October, 1986). A summary of the combined data for all

16 animals is given in Figure A.2. The distribution of data points in Figure A.2.a. shows that extraction efficiencies for females were always greater (28-44%) than those for males (6% to 23%). Control efficiencies approximated those of the males (see Table A.1.). Figure A.2.b. shows that for both males and females, extraction efficiency varied with amount of gonad processed. The optimal mass for extraction appeared to be somewhat lower for males than females.

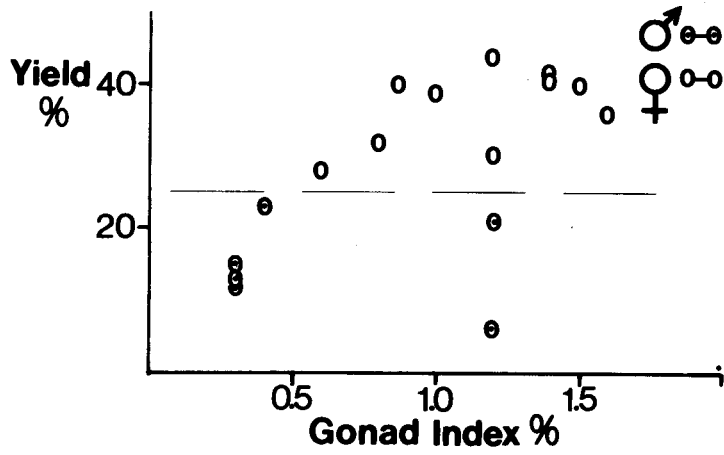
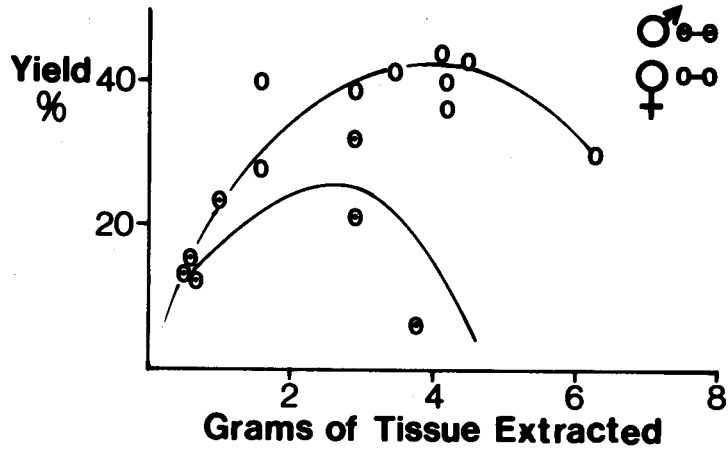
TABLE A.1.

Analysis of RNA Extraction Efficiencies at Checkpoints in the Isolation Procedure				
Sample	Percent Recovery at Checkpoints			
	"A"	"B"	"C"	"D"
Control 01	78	26	28	28
Control 02	83	25	28	27
Control 03	88	25	29	25
Control 04	<u>93</u>	<u>23</u>	<u>30</u>	<u>25</u>
MEAN	85*	25	29	26
September 01	70	(female) 48	32	44
September 02	86	(female) 45	28	42
September 03	58	(female) 52	23	30
September 04	99	(male) 32	17	15
September 05	91	(female) 46	33	40
September 06	86	(female) 49	30	40
September 07	85	(female) 45	30	39
September 08	69	(female) 45	31	41

*Since there was no loss to pellet or foam in the control samples, this value is used as a quench factor in evaluating recoveries at checkpoint "A" in the experimental samples.

Values at checkpoint "B" have been corrected for quench factors, while values at checkpoints "C" and "D" have not.

Figure A.2.: Efficiency of RNA extraction in males versus females in relation to a) mass of tissue processed, and b) gonad index. Extraction efficiencies were determined by isotope dilution (see Table A.1. and Materials and Methods).



The extraction efficiencies observed in these experiments were derived from animals of low gonad index. The viscosities of the guanidine homogenates of these testes were minimal by comparison to homogenates of ripe testes, a parameter which strongly influences extraction efficiency. It was therefore assumed that extraction efficiencies from ripe testes would be lower than those observed here, and that a more direct approach was necessary to qualify isolated RNAs for subsequent analyses.

On the premise that most of the RNA that is lost in this procedure remains in the differential precipitation supernatant (checkpoint "B", Figure A.1.; Table A.1.), the nucleic acids in this supernatant were analysed. Four classes of tissue from May (1986) animals were processed. The first was total (TOT) ripe testes. The second consisted of ripe gonad that had been minced with scissors and washed in 3 changes of Millipore filtered seawater to remove sperm (SPN). The third was comprised primarily of the released sperm that had been concentrated by centrifugation (CELLS). The fourth category included pigmented testes of the type described above (TAN).

"Standard" (ST) RNAs were isolated from these tissue fractions by the procedure as outlined above, and the supernatants of the differential precipitations were retained. The nucleic acids (SN) remaining in the supernatants were recovered by precipitation with the addition of 2 volumes of ethanol. The resulting pellets were resuspended by homogenization in the urea/SDS lysis buffer (see Materials and

Methods) and purified through cesium chloride. In order to estimate the relative quantities of DNA and RNA in these samples, equal quantities (about 30ug) of ST and SN nucleic acids derived from the 4 tissue categories from a single individual (May87-04) were subjected to DNase 1 digestion (see Materials and Methods). The amount of enzyme used (4U) and the duration of digestion (3.5h) were chosen such that DNA contents of up to 50% would be removed. Ribonuclease A (RNase A) was employed to digest the RNA present in the samples. Parallel degradation of RNA by DNase 1 or RNase A was estimated by following ³H-RNA through each digest. The reactions were terminated by precipitation and, following 2 washes with 70% ethanol, the products were resuspended in 0.1M PB. An aliquot was retained for scintillation counting and the nucleic acid contents of the remainder was measured by spectrophotometry.

The results (Table A.2.) indicate spectrophotometrically detectable contamination of RNA by DNA occurs only in SN RNA samples: the mass total of nucleic acid in ST samples was not substantially altered by DNase 1 treatment. Furthermore, these levels of contamination were substantial (50%) only in samples derived from sources heavily enriched for DNA (i.e. CELLS). Taken together, these results imply that contamination of RNAs isolated by the methods employed in this study are minimal. This does not rule out DNA contamination completely, as high molecular weight hybridization signals reducible by

TABLE A.2.

DNA Contamination in Standard and Supernatant RNAs		
Sample	Standard (ST) %RNA*	Supernatant (SN) %RNA*
CELLS	n/a	50
SPN	100	81
TOT	100	100
TAN	100	100

*As determined by spectrophotometry after DNase I digestion and precipitation.

In all cases, precipitation after RNase A digestion resulted in the recovery of 25% of the input ³H-RNA. This can be attributed to the use of excessive amounts of RNase A which, as an abundant protein in the sample, results in the precipitation of digested ³H-RNA.

TABLE A.3.

Analysis of Supernatants at Checkpoint "B"				
Sample	Total Tissue (g)	Total Yield (mg)		ST
		Standard(ST)	Supernatant(SN)	ST + SN (EE %)
May87-02	7.9	0.60	6.42	9
May87-03	8.5	0.72	6.96	10
May87-04	8.2	0.66	6.39	10
May87-06	5.6	0.39	6.45	6
May87-07	5.4	0.44	6.27	7
May87-08	5.1	0.78	6.54	12
May87-03T*	2.8	4.50	0.06	98
May87-08T*	4.1	6.90	1.10	86

*Pigmented (TAN) testes.

DNase 1 treatment have been seen in some autoradiographs (another project, data not shown). The important result here is that quantities loaded on Northern blots are sufficiently consistent to permit the interpretations presented in this study.

Table A.3. summarizes a comparison of the yields of RNA derived from the pellet (ST) and supernatant (SN) from the combined tissue categories (TOT + SPN + CELLS) for 6 males. Similar results for pigmented testes (May87-03T and May87-04T) are shown at the bottom of the table. The extraction efficiency (EE) of the standard isolation method is calculated by assuming that the total amount of RNA (100%) available is the sum of ST and SN RNAs. Therefore EE is expressed as the percentage of ST RNA relative to ST + SN. For all ripe testes, the range of values arrived at (roughly 6% to 12%) is consistent with the values obtained by isotope dilution. If RNAs were contaminated to the level of 50% by DNA, as is suggested in Table A.2., the EE values given for those RNAs would double but the overall range for EE would not change significantly. It is interesting to note that EE is much greater in the case of pigmented (TAN) testes: while these testes contained substantial quantities of RNA, very little could be retrieved from supernatant preparations from these tissues, which contain very few mature sperm.

To this point, an analysis of the extraction procedure by isotope dilution and by retrieval of "lost" RNA has

Figure A.3.: Comparison of isolated and supernatant RNAs from crudely fractionated testes. Ripe testes (Total) were crudely fractionated (see text) into sperm and spermatids (Cells) and that which remained ("Spawned"). From these fractions RNA was isolated by the standard procedure (ST) used in this study (see Figure A.1.), and by recovery of RNA normally discarded as a supernatant in this procedure (SN). These RNAs were electrophoresed (20 ug/lane) on formaldehyde agarose gels, transferred to nitrocellulose, and hybridized with actin coding sequence at moderate stringency (62 °C; 1X SSPE).

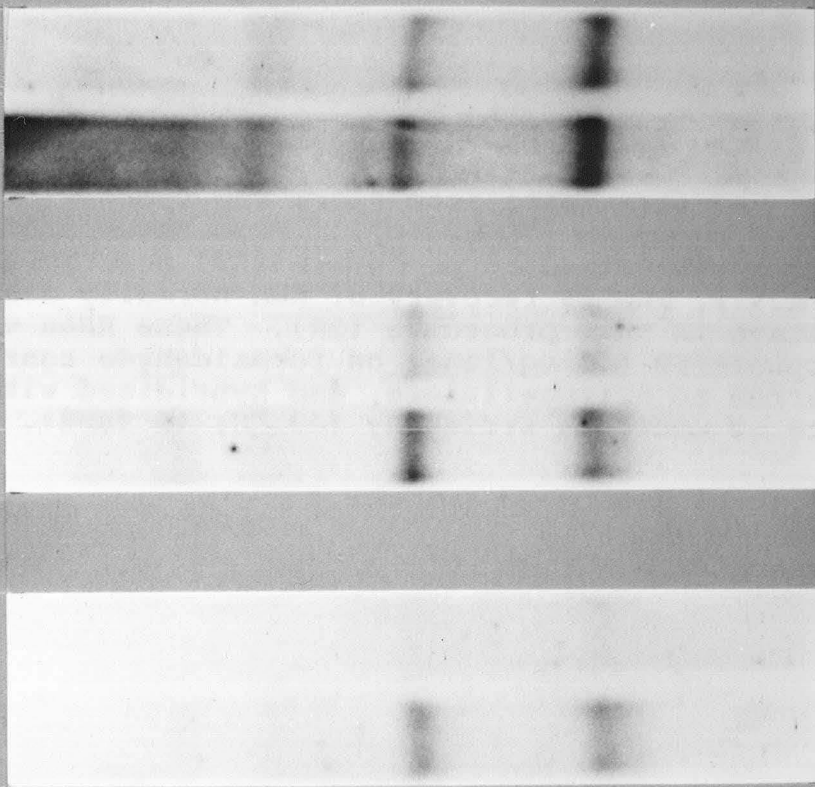
Total
ST SN

Spawned
ST SN

Cells
ST SN

3.5-

2.3-



demonstrated a substantial loss of RNA. To address the potential problem of qualitative loss (i.e. selective loss of an RNA size class of interest), ST and SN RNAs from categories of tissue (TOT, SP, and CELLS) were compared by Northern blot analysis using the actin coding probe (Figure A.3.). In none of the 3 categories do SN RNAs appear to be enriched for the transcripts detected by this probe. In addition, comparisons within lanes suggest that there is no enrichment for the smaller (actin) transcript(s) relative to the larger (3.5 kb) transcript in the SN RNAs. If an RNA size class containing actin transcripts was being selectively lost to the differential precipitation supernatant, one would expect the ratio of hybridization signals of actin:3.5 kb to be increased in the SN RNA lanes.

In summary, it appears that the RNAs isolated by the methods in this study contain a reliable representation of the transcripts present in the testes, regardless of spermatogenic state. While it is likely that they contain some DNA contamination, it is minimal and should not affect interpretations of the magnitudes and patterns of gene expression presented here.

REFERENCES

- Akhurst, R. J., F. J. Calzone, J. J. Lee, R. J. Britten, and E. H. Davidson. 1987. Structure and organization of the CyIII actin gene subfamily of the sea urchin, Strongylocentrotus purpuratus. J. Mol. Biol. 194: 193-203.
- Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1983. The Molecular Biology of the Cell. Garland Publishing Co., New York, New York.
- Allen, W. V. and A. C. Giese. 1966. An in vitro study of lipogenesis in the sea star, Pisaster ochraceus. Comp. Biochem. Physiol. 17: 23-38.
- Alexandraki, D. and J. V. Ruderman. 1981. Sequence heterogeneity, multiplicity, and genomic organization of alpha and beta tubulin genes in sea urchins. Mol. Cell. Biol. 1(12): 1125-1137.
- Alexandraki, D. and J. V. Ruderman. 1985. Expression of alpha and beta tubulin genes during the development of sea urchin embryos. Dev. Biol. 109: 436-451.
- Bang, F. B. 1982. Disease processes in seastars: A Metchnikovian challenge. Biol. Bull. 162: 135-148.
- Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294-5299.
- Chia, F.-S. 1983. Echinodermata. In: Reproductive Biology of Invertebrates, Volume II: Spermatozoa and Sperm Function. K. G. Adiyodi and R. G. Adiyodi, Eds. John Wiley and Sons.
- Cleveland, D. W. 1987. The multitubulin hypothesis revisited: What have we learned? J. Cell Biol. 104: 381-383.
- Cox, K. H., L. M. Angerer, J. J. Lee, E. H. Davidson, and R. C. Angerer. 1986. Cell lineage-specific programs of expression of multiple actin genes during sea urchin embryogenesis. J. Mol. Biol. 188: 159-172.
- Craig, S. W. and T. D. Pollard. 1982. Actin-binding proteins. TIBS 7(3): 88-92.

- Crain, W. R., D. S. Durica, and K. van Doren. 1981. Actin gene expression in developing sea urchin embryos. *Mol. Cell. Biol.* 1: 711-720.
- Dan, J. C. and Y. Hagiwara. 1967. Studies on the acrosome IX. Course of acrosome reaction in the starfish. *J. Ultrastruc. Res.* 18: 562-579.
- Davidson, E. H. 1986. **Gene Activity in Early Development.** Academic Press, Inc.
- Davidson, J. N. 1969. **The Biochemistry of the Nucleic Acids.** Methuen and Co. Ltd., London.
- Davis, K. K. 1985. DNA synthesis and the annual spermatogenic cycle in individuals of the sea star Patiria miniata. *Biol. Bull.* 169: 313-327.
- Davis, R. W., D. Botstein and J. R. Roth. 1980. **A Manual for Genetic Engineering: Advanced Bacterial Genetics.** Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- Distel, R. J., K. C. Kleene, and N. B. Hecht. 1984. Haploid expression of a mouse testis alpha tubulin gene. *Science* 224: 68-70.
- Erickson, R. P., J. M. Kramer, J. Rittenhouse, and A. Salkeld. 1980. Quantitation of mRNAs during mouse spermatogenesis: Protamine-like histone and phosphoglycerate kinase-2 mRNAs increase after meiosis. *Proc. Natl. Acad. Sci. USA* 77(10): 6086-6090.
- Farmanfarmanian, A., A. C. Giese, R. A. Boolootian and J. Bennett. 1958. Annual reproductive cycles in four species of west coast starfishes. *J. Exp. Zool.* 138: 355-367.
- Feder, H. 1957. Natural history studies on the starfish Pisaster ochraceus (Brandt, 1835) in the Monterey Bay area. Doctoral dissertation, Stanford University. Publication No. 20,446.
- Flytzanis, C. N., R. J. Britten, and E. H. Britten. 1987. Ontogenetic activation of a fusion gene introduced into the sea urchin egg. *Proc. Nat. Acad. Sci. U.S.A.* 84: 151-155.
- Fraser, A., J. Gomez, E. B. Hartwick, and M. J. Smith. 1981. Observations on the reproduction and development of Pisaster ochraceus (Brandt). *Can. J. Zool.* 59: 1700-1707.

- Fyrberg, E. A., J. W. Mahaffey, B. J. Bond, and N. Davidson. 1983. Transcripts of the six Drosophila actin genes accumulate in a stage- and tissue-specific manner. *Cell* 33: 115-123.
- Garcia, R., B. Paz-Aliaga, S. G. Ernst, and W. R. Crain, Jr. 1984. Three sea urchin actin genes show different patterns of expression: muscle specific, embryo specific, and constitutive. *Mol. Cell. Biol.* 4: 840- 845.
- Geremia, R., C. Boitani, M. Conti, and V. Monesi. 1977. RNA synthesis in spermatocytes and spermatids and preservation of meiotic RNA during spermiogenesis in the mouse. *Cell Diff.* 5: 343-355.
- Gould-Somero, M. and L. Holland. 1974. The timing of RNA synthesis for spermiogenesis in organ cultures of Drosophila melanogaster testes. *Wilhelm Roux' Archiv* 174: 133-148.
- Greenfield, L., A. C. Giese, A. Farmanfarmanian, and R. A. Boolootian. 1958. Cyclic biochemical changes in several echinoderms. *J. Exp. Zool.* 139: 507-524.
- Gubler, U. and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25: 263-269.
- Guo, L-H. and R. Wu. 1983. Exonuclease III: Use for DNA sequence analysis and specific deletions of nucleotides. *Methods in Enzymology* 100: 60-95.
- Hattori, M. and Y. Sakaki. 1986. Dideoxysequencing method using denatured plasmid templates. *Analytical Biochem.* 152: 232-238.
- Hightower, R. C. and R. B. Meagher. 1986. The molecular evolution of actin. *Genetics* 114: 315-332.
- Holland, N. D. 1967. Gametogenesis during the annual reproductive cycle in a cidaroid sea urchin (Stylocidaris affinis). *Biol. Bull.* 133: 578-590.
- Howell, A. M., J. Hewitt, B. Ydenberg, M. J. Smith, and B. M. Honda. 1987. Organization and unusual expression of histone genes in the sea star Pisaster ochraceus. *J. Mol. Evol.* 25: 29-36.
- Iatrou, K. A. Spira and G. H. Dixon. 1978. Protamine messenger RNA; Evidence for early synthesis and accumulation during spermatogenesis in rainbow trout. *Dev. Biol.* 64: 82-98.

- Ingles, C. J., J. R. Trevithick, and G. H. Dixon. 1966. Biochem. Biophys. Res. Commun. 22: 627.
- Katula, K. S., B. R. Hough-Evans, R. J. Britten, and E. H. Davidson. 1987. Ontogenetic expression of a CyI actin fusion gene injected into sea urchin eggs. Development 101: 437-447.
- Kennedy, B. P., L. W. Crim, and P. L. Davies. 1985. Expression of histone and tubulin genes during spermatogenesis. Evidence of post-meiotic transcription. Exp. Cell Res. 158: 445-460.
- Khandjian, E. W. 1986. UV crosslinking of RNA to nylon membranes enhances hybridization signals. Molec. Biol. Rep. 11: 107-115.
- Korn, E. D. 1982. Actin polymerization and its regulation by proteins from nonmuscle cells. Physiological Reviews 62: 672-737.
- Kovesdi, I. 1984. Molecular studies of gene expression in development of the sea star Pisaster ochraceus (Brandt). Doctoral dissertation, Simon Fraser University, Burnaby, British Columbia, Canada.
- Kovesdi, I., F. Preugschat, M. Stuerzl, and M. J. Smith. 1984. Actin genes from the sea star Pisaster ochraceus. Biochim. Biophys. Acta 782: 74-86.
- Kovesdi, I. and M. J. Smith. 1985a. Actin gene number in the sea star Pisaster ochraceus. Can. J. Biochem. Cell Biol. 63: 1145-1151.
- Kovesdi, I. and M. J. Smith. 1985b. Quantitative assessment of actin transcript number in eggs, embryos, and tube feet of the sea star Pisaster ochraceus. Mol. Cell. Biol. 5: 3001-3008.
- Laskey, R. A. and A. D. Mills. 1977. Enhanced autoradiographic detection of ^{32}P and ^{125}I using intensifying screens and hypersensitive film. FEBS Lett. 82: 314-316.
- Lee, J. J., R. J. Shott, S. J. Rose III, T. L. Thomas, R. J. Britten, and E. H. Davidson. 1984. Sea urchin actin gene subtypes: Gene number, linkage, and evolution. J. Mol. Biol. 172: 149-176.
- Longo, F. J. and E. Anderson. 1969. Sperm differentiation in the sea urchins Arbacia punctulata and Strongylocentotus purpuratus. J. Ultrastruc. Res. 27: 486-509.

- Louie, A. J. and G. H. Dixon. 1972a. Trout testis cells:
I. Characterization by deoxyribonucleic acid and protein analysis of cells separated by velocity sedimentation. *J. Biol. Chem.* 247(17): 5490-5497.
- Louie, A. J. and G. H. Dixon. 1972b. Trout testis cells:
II. Synthesis and phosphorylation of histones and protamines in different cell types. *J. Biol. Chem.* 247(17): 5498-5505.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. **Molecular Cloning: A Laboratory Manual.** Cold Spring Harbor Laboratory.
- Mauzey, K. P. 1966. Feeding behavior and reproductive cycles in Pisaster ochraceus. *Biol. Bull.* 131: 127-144.
- Nimitz, Sister M. A. (O. P.). 1971. Histochemical study of gut nutrient reserves in relation to reproduction and nutrition in the sea stars, Pisaster ochraceus and Patiria miniata. *Biol. Bull.* 140: 461-481.
- Nimitz, Sister M. A. (O. P.). 1976. Histochemical changes in gonadal nutrient reserves correlated with nutrition in the sea stars, Pisaster ochraceus and Patiria miniata. *Biol. Bull.* 151: 357-369.
- Pearse, J. S. and D. J. Eernisse. 1982. Photoperiodic regulation of gametogenesis and gonadal growth in the sea star Pisaster ochraceus. *Mar. Biol.* 67: 121-125.
- Pearse, J. S., V. B. Pearse, and K. K. Davis. 1986. Photoperiodic regulation of gametogenesis and growth in the sea urchin Strongylocentrotus purpuratus. *J. Exp. Zool.* 237: 107-118.
- Poccia, D. L. 1986. Remodelling of nucleoproteins during gametogenesis. *Int. Rev. Cytol.* 105: 1-65.
- Poccia, D. L., M. V. Simpson, and G. R. Green. 1987. Transitions in histone variants during sea urchin spermatogenesis. *Dev. Biol.* 121: 445-453.
- Poccia, D. L. 1987. Regulation of chromatin condensation and decondensation in sea urchin pronuclei. In: **Molecular Regulation of Nuclear Events in Mitosis and Meiosis.** Robert A. Schlegel, Margaret S. Halleck, and Potu N. Rao, Eds. Academic Press, Inc.
- Roosen-Runge, E. C. 1977. **Spermatogenesis in animals.** Cambridge University Press, London.

- Sanchez, F., S. L. Tobin, U. Rdest, E. Zulauf, and B. J. McCarthy. 1983. Two Drosophila actin genes in detail: gene structure, protein structure and transcription during development. *J. Mol. Biol.* 163: 533-551.
- Schliwa, M. 1981. Proteins associated with cytoplasmic actin. *Cell* 25: 587-590.
- Schoenmakers, H. J. N. and S. J. Dieleman. 1981. Progesterone and estrone levels in the ovaries, pyloric ceca, and perivisceral fluid during the annual reproductive cycle of starfish, Asterias rubens. *General and Comparative Endocrinology* 43: 63-70.
- Schott, R. J., J. J. Lee, R. J. Britten, and E. H. Davidson. 1984. Differential expression of the actin gene family of Strongylocentrotus purpuratus. *Dev. Biol.* 101: 295-306..
- Smith, F. F. and C. W. Walker. 1986. Biochemical changes in the composition of the testes during spermatogenesis in the sea star Asterias vulgaris. *J. Exp. Zool.* 237: 351-364.
- Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Nat. Acad. Sci.* 77: 5201.
- Tilney, L. G. 1975. The Role of actin in non-muscle cell motility. Pp. 339-388 in **Molecules and Cell Movement**, S. Inoue and R. E. Stephens, eds. Raven Press, N. Y.
- Tilney, L. G. and S. Inoue. 1982. Acrosomal reaction of Thyone sperm. II. The kinetics and possible mechanism of acrosomal process elongation. *J. Cell. Biol.* 93: 820-827.
- Valenzuela, P., M. Quiroga, J. Zaldivar, W. J. Rutter, M. W. Kirschner, and D. W. Cleveland. 1981. Nucleotide and corresponding amino acid sequences encoded by alpha and beta tubulin mRNAs. *Nature* 289: 650-655.
- van der Plas, A. J., F. Brands, and P. A. Voogt. 1983. An autoradiographic investigation of the proliferative activity of pyloric caeca and gonads of Asterias rubens. *J. Morph.* 177: 51-58.
- Vasu, B. S. and A. C. Giese. 1966. Protein and non-protein nitrogen in the body fluid of Pisaster ochraceus (Echinodermata) in relation to nutrition and reproduction. *Comp. Biochem. Physiol.* 19: 351-361.

- Voogt, P. A. and S. J. Dieleman. 1984. Progesterone and oestrone levels in the gonads and pyloric caeca of the male sea star Asterias rubens: A comparison with the corresponding levels in the female sea star. *Comp. Biochem. Physiol.* 79a(4): 635-639.
- Walker, C. W. 1974. Studies on the reproductive systems of sea-stars. I. The morphology and histology of the gonad of Asterias vulgaris. *Biol. Bull.* 147: 661-677.
- Walker, C. W. 1975. Studies on the reproductive systems of sea-stars. II. The morphology and histology of the gonoduct of Asterias vulgaris. *Biol. Bull.* 148: 461-471.
- Walker, C. W. 1979. Ultrastructure of the somatic portion of the gonads in Asteroids, with emphasis on flagellated-collar cells and nutrient transport. *J. Morphol.* 162: 127-162.
- Walker, C. W. 1980. Spermatogenic columns, somatic cells, and the microenvironment of germinal cells in the testes of asteroids. *J. Morphol.* 166: 81-107.
- Walker, C. W. and D. Larochelle. 1984. Interactions between germinal and somatic accessory (SA) cells of the spermatogenic epithelium of Asterias vulgaris in vivo and in vitro. Pp. 41-52 in: **Advances in Invertebrate Reproduction**, vol. 3, W. Engels et al., eds. Elsevier, Amsterdam.
- Walker, C. W. 1986. Isolation and culture of spermatogenic epithelia from sea star testes for use in studies of mitosis in spermatogonia and meiosis I in primary spermatocytes. Pp. 1-17 in: **Techniques in the Life Sciences: In vitro Invertebrate Hormones and Genes**. Elsevier Scientific Publishers Ireland Ltd.
- Waters, S. H., R. J. Distel, and N. B. Hecht. 1985. Mouse testes contain two size classes of actin mRNA that are differentially expressed during spermatogenesis. *Mol. Cell. Biol.* 5(7): 1649-1654.
- Watts, S. A., K. J. Lee, G. A. Hines, and C. W. Walker. 1987. Determination of polyamines in digestive and reproductive tissues of adult Asterias vulgaris (Echinodermata: Asteroidea). *Comp. Biochem. Physiol.* 88b(1): 309-312.
- Weeds, A. 1982. Actin-binding proteins- regulators of cell architecture and motility. *Nature* 296: 811-816.
- Wilson, C., G. S. Cross, and H. R. Woodland. 1986. Tissue-specific expression of actin genes injected into Xenopus embryos. *Cell* 47: 589-599.

Vierira, J. and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19: 259-268.

Yamashita, M. and F. Iwata. 1983. A quantitative analysis of the annual testicular cycle of the brittle star Amphiopholis kochii by means of autoradiographic investigation. *Biol. Bull.* 164: 327-340.