EXPRESSION OF MATERNAL AND ZYGOTIC GENES DURING SEA URCHIN EMBRYOGENESIS

Francis Tufaro

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Department of Biology

McGill University

Montreal

For my parents

ABSTRACT

Eggs of many organisms contain a store of mRNA which supports protein synthesis during early embryonic development and various regions of the egg cytoplasm are not identical with respect to developmental potential. I investigated the extent to which sea urchin embryogenesis results from a progression of developmental events directed by the embryo, or an expression of a pre-formed maternal program. By the use of two-dimensional electrophoresis I demonstrated that cellular determination during embryonic development at the 16-cell stage is not accompanied by qualitative changes in the distribution within the embryo of abundantly-synthesized proteins, virtually all of which are coded by sequences present in the egg. Using two-dimensional gel electrophoresis, nucleic acid hybridization and molecular cloning, I demonstrated that there is restricted expression of paternal gene mRNA sequences in interspecies hybrid embryos. In some cases, this is due to a posttranscriptional perturbation of gene expression in the hybrid embryos.

RÉSUMÉ

Chez plusieurs organismes, l'oeuf contient une réserve d'ARNm assurant la synthèse des protéines au cours du développement embryonnaire précoce. De plus, les diverses zones du cytoplasme de l'oeuf diffèrent par leur potentiel de développement.

J'ai tenté de déterminer, chez l'oursin, dans quelle mesure l'embryogènese résulte d'une suite d'événements dirigés par l'embryon ou de l'expression d'un programme maternel préformé.

L'usage de l'électrophorese bidimensionnelle a permis de démonstrer que la détermination cellulaire au stade à 16 cellules a lieu sans changement qualitatif, au sein de l'embryon, dans la distribution de protéines abondamment synthétisées. Celles-ci sont presque toutes codées dans des séquences d'ARN présented dans l'oeuf.

L'usage de l'électrophorèse bidimensionnelle en plus du clonage et de l'hybridisation moléculaires a permis de montrer l'expression restreinte du génome paternal dans des embryons hybrides inter-spécifiques. Dans certains cas, cette expression est restreinte par un processus post-transcription incorrect, affectant la régulation de l'accumulation des ARNm.

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CONTRIBUTION TO ORIGINAL KNOWLEDGE

I improved the procedure for the isolation of the three blastomere types of the 16-cell sea urchin embryo, which enabled me to analyze the patterns of polypeptide synthesis in macromeres, mesomeres and micromeres. Using improved methods for two-dimensional electrophoresis, I was able to resolve about 1000 different newly-synthesized polypeptides. This is the highest resolution of sea urchin proteins which has been achieved to date. I demonstrated that the pattern of polypeptides synthesized in the 16-cell embryos is nearly identical to the pattern of synthesis in the egg, and that cellular determination occurring in early sea urchin development is not accompanied by an asymmetrical distribution of labelled polypeptides, most of which are coded for by sequences present in the egg.

I used high-resolution two-dimensional electrophoresis of labelled polypeptides to demonstrate that there is an underrepresentation of the expression of the paternal genome in interspecies hybrid embryos. I characterized the expression of hundreds of abundant proteins in three different species of sea urchins. I analyzed three hybrid crosses, including a reciprocal pair of crosses at several stages of development. This analysis had not previously been performed on sea urchins but a similar analysis yielded interesting results in amphibians.

I analyzed the representation of paternal and maternal cytoplasmic polysomal-enriched poly(A)-RNA transcripts present in the embryos of SpxLp interspecies hybrid embryos. I examined for the first time the representation in hybrid embryos those transcripts normally present in embryos of the paternal species (Lp).

I demonstrated that restricted expression of the paternal genome is not accompanied by loss of a detectible fraction of the paternal genome and that paternal DNA persists throughout development to the pluteus stage, in a cross between S. purpuratus egg and L. pictus sperm.

Due to the completeness of this analysis, I was able to formulate and test several predictions concerning the expression of the paternal genome in interspecies hybrid embryos and its relation to gene expression in normal embryos.

I prepared a library of cloned cDNA sequences representing transcripts some of which are species-specific and developmentally-regulated.

I characterized the expression of a repeat element in the cytoplasmic RNA of normal and hybrid embryos and demonstrated that there is regulation of the expression of a set of transcripts containing this element in interspecies hybrid embryos.

I characterized the temporally-distinct pattern of developmental expression of a cloned transcript, 16D4, in the embryos of normal and interspecies hybrid embryos. I found evidence that the expression of the gene may be autonomously regulated in hybrid embryos. Transcripts exhibiting this unique pattern of expression during early development have not been previously characterized.

I isolated and characterized a cloned transcript, 15D6, which is underrepresented in the cytoplasm of interspecies hybrid embryos at a time when it is accumulating extensively in the paternal species embryos. I demonstrated that the gene is transcribed in both LpxLp and SpxLp embryos at a stage of development when it normally accumulates in LpxLp embryos. This is the first demonstration that a post-transcriptional processing event might be perturbed in echinoid interspecies hybrid embryos. It is also the first cloned sequence identified which exhibits restricted expression in hybrid embryos. These observations may have important implications for an understanding of gene expression in normal embryonic development.

PREFACE

This thesis is assembled in accordance with the regulations of the Faculty of Graduate Studies and Research of McGill University. It consists of a brief abstract and resume, introduction (and review of the literature), followed by three chapters in a form suitable for publication.

Chapter 2 has been published as a paper by Tufaro and Brandhorst (1979). Dev. Biol. 79: 390-397.

Chapter 3 has been published as a paper by Tufaro and Brandhorst (1982). Dev. Biol. 92: 209-220.

Chapter 4 is being prepared for publication.

The following figures have been published:

Table 2 is a modified version of that which appeared in Tufaro and Brandhorst (1982), and has been published in Molecular Aspects of Early Development. (G.M. Malacinski and W.H. Klein, eds.) Plenum Press, New York. It has also been published in Time, Space, and Pattern in Embryonic Development. (R.A. Raff and W.R. Jeffery, eds.) Alan R. Liss, New York.

Fig.3. has been published in Time, Space, and Pattern in Embryonic Development, and in Developmental Biology, by L. Browder. Saunders College, Philadelphia, and in Molecular Aspects of Early Development.

Fig.9. has been published in Time, Space, and Pattern in Embryonic Development.

For clarity, the discussion of results immediately follows the results section of each chapter, and all cited literature has been combined and placed at the end of this thesis.

All of the results and reviews presented in this thesis are the work of the author.

ACKNOWLEDGEMENTS

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Finally, and most importantly, I thank my wife Donna, for her love, constant encouragement, and unfailing patience.

ABBREVIATIONS

α-	alpha
B-	beta
bp	base pair(s)
cDNA	complementary doexyribonucleic acid
DNAase	deoxyribonuclease
d(T)	deoxythymidilate
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol-bis (B-aminoethyl ether) N,N'-tetraacetic acid
mRNA	messenger ribonucleic acid
MOPS	(3-[N-Morpholino] propanesulfonic acid)
nt	nucleotide(s)
PMSF	phenylmethylsulfonyl fluoride
Pipes	piperazine-N,N'-bis (2-ethanesulfonic acid)
Poly(A)-RNA	ribonucleic acid containing poly(A) tracts
Poly(A)$^+$RNA	ribonucleic acid containing poly(A) tracts
Poly(A)$^-$RNA	ribonucleic acid lacking poly(A) tracts
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RNAase	ribonuclease
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
Tris	tris (hybroxymethyl) aminomethane
u	micro (10^{-9})
U	units
UV	ultraviolet

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MATERIALS

Enzymes and Chemicals

Restriction endonucleases were obtained from: Boehringer Mannheim, PL Biochemicals, Bethesda Research Laboratories and Amersham Corp.

RNAase, DNAase I: Sigma Chemical Company

DNAase I- ultrapure: Worthington Biochemicals

Reverse transcriptase: obtained from Dr. J. Beard, Life Sciences Inc.

S1-nuclease: Boehringer Mannheim, gift of Dr. Forrest Fuller

DNA polymerase- Klenow fragment: PL Biochemicals

Nick-translation kit: Bethesda Research Laboratories

Proteinase K: Sigma Chemical Company

Lysozyme: Sigma Chemical Company

Cloning vectors pBr322, M13-Mp8, M13-Mp9 and DH1 *E. Coli* were a gift of Dr. D.P.S. Verma.

Manganese chloride, ultrapure DMSO and cobalt chloride were a gift of Dr. Forrest Fuller.

All of the chemicals used were of high purity or "Reagent grade".

The following products were obtained from Sigma: glycylglycine, penicillin, streptomycin, dithiothreitol, Tris (Trizma base), PMSF, EGTA, Pipes, Triton X-100, EDTA, ethidium bromide, Hepes, SDS yeast

(Materials-continued)

tRNA, cacodylic acid (sodium salt), tetracycline, ampicillin, chloramphenicol, PVP-40, BSA, mops, acridine orange, salmon sperm DNA, spermidine.

Fisher: Potassium chloride, sucrose, trichloracetic acid, magnesium chloride, sodium chloride, sodium hybroxide, zinc sulfate, sodium acetate, isoamyl alcohol, chloroform, zinc chloride, sodium pyrophosphate, sodium citrate, calcium chloride.

Boehringer Mannheim: dATP, dTTP, dGTP, UTP, α-amanitin.

Pharmacia: Sephadex G-50, G-100, dextran sulfate, Ficoll, blue dextran.

BDH: SDS, diethyl ether, potassium hydroxide, formaldehyde, glycerol, formamide, polyethylene glycol 8000

Bio-Rad: ampholytes, formamide, agarose (low M_r), urea

E. Merck: Rubidium chloride

Dupont: lightning-plus intensifying screens

Whatman: GF/A, DE-81 filters

Schleischer and Schuell: BA-85 nitrocellulose

New England Nuclear: Gene Screen membrane

Malincrodt: phenol crystals

Consolidated alcohols: ethanol

Kodak: B-mercaptoethanol, X-ray film

Difco: yeast extract, agar, bactotryptone

Polaroid: Type 52 and 57 film

Kawecki Berylco Industries: Cesium chloride

Collaborative Research: oligo(dT)-cellulose

(Materials-continued)

Radioactive nucleotides and amino acids were obtained from New England Nuclear or Amersham.

Equipment

The replicating device was constructed by Tony Briza. The dot blot manifold was purchased from BRL. The UV light box was from Fotodyne. The Polaroid pictures were exposed with a Polaroid CU-5 camera.

Biological materials

Strongylocentrotus purpuratus and Lytechinus pictus were obtained from Pacific Biomarine Inc. Strongylocentrotus droebachiensis were collected in New Brunswick.

INTRODUCTION AND REVIEW

The endeavor to discover by experiment the mechanisms by which expression of the genome results in the creation of a differentiated embryo is the central problem in the molecular biology of early development. The egg, formed during oogenesis, is competent to manifest a program of development leading rapidly through ordered cell division to an elaboration of morphological structures made up of differentiated cell types. The process of fertilization activates this program of development. It has been nearly a century since the experiments of Hertwig, Driesch and Wilson on marine embryos clearly demonstrated that mitosis does not result in a qualitative partitioning of nuclear material as previously envisioned by Roux and Weismann (Jenkinson, 1909, for review), and that various regions of the egg cytoplasm are not identical with respect to the developmental potential of blastomeres containing them. With this knowledge, a controversy emerged over the extent to which embryogenesis results from a progression of developmental events directed by the embryo, or an expression of a pre-formed maternal program (See Davidson, 1976; Davidson et al., 1983, for a detailed historical perspective).

It is now known that the eggs of many organisms contain a store of mRNA which supports protein synthesis during early embryonic development (Brandhorst, 1984, for review). While this maternal mRNA accounts for most of the protein synthesis during early development, mRNA is also actively synthesized within a few hours after fertilization and ultimately supplants the maternal mRNA. However, the contribution of embryonic

transcription to the mRNA population during early embryonic development is not easily assessed.

Stored maternal mRNA was first hypothesized and characterized in sea urchin eggs (Brandhorst, 1984, for review), and sea urchin eggs have been the classic system for investigating the nature and role of maternal mRNA. This is partly due to the fact that sea urchin eggs and embryos are among the most completely described cells, both molecularly and physiologically (see Davidson, 1976, 1983, for review). Sea urchin interspecies hybrid embryos have long been used to assess the relative contribution of the maternal and zygotic genome to development (see Davison, 1976; Giudice, 1973, for review). Of the hundreds of interspecies experiments reported in the literature, I will describe several examples in detail in this introductory chapter. I have not restricted my discussion to echinoid experiments, as interspecies hybrid embryos have proven to be a powerful system for the investigation of a variety of diverse and fundamental aspects of gene regulation in many organisms. In chapters 3 and 4, I describe new experiments on gene expression in echinoid hybrid embryos.

Since the establishment of the stored mRNA hypothesis, it has been frequently proposed that maternal mRNAs may act as localized morphogenetic determinants in eggs and embryos (Raff and Showman, 1983; Brandhorst et al., 1984, for reviews). If commitment of a blastomere to a particular cell fate is the result of establishment of a unique population of mRNA in that blastomere, a concurrent change in the pattern of protein synthesis might occur in that blastomere. Sea urchin embryos exhibit an early restriction of developmental potential. The 16-cell embryo is composed of three cell types which have different fates (Okazaki, 1975; Hynes et al.,

1972). In chapter 2, I report on my investigations of protein synthesis in micromeres as an approach to establishing the role of maternal mRNA or newly synthesized RNA to the determination of these cells. In this chapter, I will review several experiments demonstrating localization of morphogenetic information in a variety of organisms in order to illustrate the diverse and elegant nature of this phenomenon.

Oogenesis and Fertilization of Sea Urchins

Mature sea urchin eggs range in size from about 70-120 u in diameter (Giudice, 1973; Hinegardner, 1975). Both meiotic maturation divisions occur within the ovary of sea urchins, unlike most organisms. A gravid female contains, besides several million mature eggs, 0.5-2 x 10^6 vitellogenic oocytes, and about 10^8 previtellogenic oocytes and oogonia (Hough-Evans et al., 1979; Leahy et al., 1978, 1981). The morphology of the sea urchin occyte is typical of most oocytes containing moderate amounts of yolk.

The echinoid egg is surrounded by an outer jelly coat composed of acid mucopolysaccharide and by a glycoprotein vitelline envelope that is closely apposed to the egg surface. After traversing the jelly coat, sperm encounter the vitelline envelope, to which they attach via their acrosomal processes, which are formed on contact with egg jelly (Dave et al., 1964; Decker et al., 1976). The acrosomal process appears to bind to the vitelline envelope by bindin, part of the material released during the acrosomal reaction. To some extent the species-specificity of fertilization is due to an interaction between the sperm bindin and a specific glycoprotein sperm receptor on the vitelline envelope (Summers and Hylander, 1975; Glabe and Vacquier, 1978).

Normally, only a single sperm penetrates the vitelline layer and fuses with the egg plasma membrane. The sperm nucleus enters the egg as a structure having highly condensed chromatin. In one species, Arbacia punctulata, the sperm nucleus vesiculates and partially dissociates; the apical and basal portions remain intact. Chromatin dispersal begins, and membranous vescicles aggregated along the periphery of the chromatin fuse with the remaining apical and basal portions of the original sperm nuclear membrane, forming the male pronuclear envelope. The male and female pronuclei meet and fuse, and the paternal and maternal genetic material become enclosed within a common membrane for the first time. Soon thereafter, the spindle starts to form for the first cell division, which cleaves the egg cytoplasm into two equal blastomeres. The nuclear membrane breaks down immediately thereafter and prophase starts. At metaphase, the chromosomes come to lie in an ordered array at the equator of the egg. The number of chromosomes of the sea urchin has not been accurately determined, although various studies of about ten species indicate that there are 36 to 44 chromosomes per diploid genome (Harvey, 1920; Grimpe, 1930; MacClung, 1939; von Ubisch, 1961; Makino, 1951; Gray, 1921; German, 1964). The first cleavage furrow divides the egg into equal halves along the "animal-vegetal" axis. The "animal pole" later becomes the site of a group of apical cells bearing a tuft of especially long cilia, and the "vegetal pole" later becomes the site of primary mesenchyme ingression and gastrular invagination. At the fourth cleavage, four very small cells, the micromeres, are formed by asymmetric cleavage at the vegetal pole. These are the progenitors of a lineage of cells that gives rise to the larval skeleton and will be considered in greater detail in a later section.

A logarithmic rate of cell division is maintained until there are about 200 cells. Later, when the embryo contains about 400 cells, it secretes a protease called hatching enzyme which dissolves the fertilization membrane and the zygote is free to swim. The hatched embryo, termed a blastula, is organized as a hollow ciliated ball with a prominant ciliary tuft at the animal pole. During the blastula stage, primary mesenchyme cells, descendents of the original micromeres, enter the blastocoel. A thickened, flat plate of cells develops at the vegetal pole and is the site of invagination at the onset of gastrulation. Gastrular invagination leads to the formation of the archenteron, the embryonic gut. The stomadeum, the larval mouth, forms inductively at the point where the archenteron tip makes contact with the inner blastocoelar wall at the animal pole. An internal skeleton is elaborated by primary mesenchyme cells, beginning as a pair of tripartite spicules which are composed of magnesium and calcium carbonates in the proportions of 1:20, as well as an organic matrix (Okazaki, 1970). During the subsequent development to the pluteus stage, the embryo elongates, the archenteron differentiates regionally, and the four skeletally supported arms grow anteriorly (See Fig.4). The free-swimming pluteus larva contains about 1500 cells and begins to feed. Until this stage embryonic development has not been accompanied by growth: until the larvae starts to feed, the mass of protein and RNA in the embryo remain constant. After several weeks of feeding, growth, and extensive morphogenesis, metamorphosis takes place abruptly. Extensive morphogenesis and differentiation takes place after metamorphosis, including gonadal differentiation. A fertile adult forms in several months, depending on the species and the availability of food.

Evolutionary Relationship of Echinoderms

The phylum Echinodermata comprises five classes of marine organisms. These are the Echinoidea (sea urchins), Asteroidea (sea stars), Ophiuroidea (serpent stars), Holothuroidea (sea cucumbers), and the Crinoidea (sea lilies). The sea urchin, upon which the bulk of research has been done, and the sea stars have been used extensively as sources of eggs and sperm for studies of developmental and cell biology. Both sea urchins and sea stars can be maintained in the laboratory. I used several species of sea urchins in my research. Strongylocentrotus droebachiensis is found along the northeastern, northwestern, and arctic coastlines of the United States and Canada, Strongylocentrotus purpuratus, a close relative of S. droebachiensis is an intertidal and subtidal species found along the west coast of North America and Lytechinus pictus grows subtidally from Panama to Southern California (Hinegardner et al., 1981).

The two orders represented by these three species, Temnopleuroidea (Lytechinus) and Echinoidea (Strongylocentrotus), diverged approximately 175 million years ago [MYA] (Durham, 1966). Within the order Echinoidea, the family Strongylocentrotidae is represented in the fossil record from the early Miocene, about 20 MYA. Divergence of the Strongylocentrotidae is not precisely known due to the sparseness of the fossil record, although S. purpuratus and S. droebachiensis were morphologically distinct at least 7 MYA. Single-copy DNA sequence homology measurements are consistent with the fossil record. S. purpuratus shares 67.8% of its single-copy DNA sequence with S. droebachiensis and only 11.5% with L. pictus (Angerer et al., 1976). Interestingly, although about 88% of the single-copy DNA of L. pictus and S. purpuratus has diverged beyond

recognition at a defined, moderately stringent criterion (70°, 1M NaCl), the 12% that does react has been relatively conserved in evolution (Angerer et al., 1976; see also Harpold and Craig, 1978).

The homology of S. purpuratus gastrula polysomal mRNA sequences to the DNA of the other species has also been determined. The homology to S. droebachiensis DNA has not been determined, but when S. purpuratus is compared to S. franciscanus (a species more divergent from S. purpuratus than S. droebachiensis) greater than 90% of the S. purpuratus gastrula polysomal RNA sequences have complements in the S. franciscanus genome. Since this value is higher than the single-copy DNA homology of the two species (77%), structural gene sequences are diverging at a slower rate than the bulk of single-copy sequences. Only 14% of the gastrula polysomal RNA sequences have complements in the L. pictus genome; thus there is little specific conservation of structural gene sequences at this great evolutionary distance. This is not surprising since codon degeneracy and harmless amino acid substitution could allow for extensive divergence.

Only about 30% of L. pictus proteins and 15% of S. droebachiensis proteins can be distinguished from S. purpuratus proteins when analyzed by two-dimensional electrophoresis (Tufaro and Brandhorst, 1982; this thesis, ch. 3). This illustrates the evolutionary constraints on the structure of prevalent proteins during echinoid evolution. The proteins detected on these gels do not represent a high percentage of the complexity of the DNA or RNA populations; consequently less abundant proteins may diverge differently.

Genome Organization

The haploid genome of echinoderms ranges in size from about 0.4 to 4 pg; the largest echinoderm genome is the size of the typical mammalian genome. About 72% of the genome of the sea urchin S. purpuratus consists of a highly ordered pattern of interspersed repeated and single-copy sequences (Graham et al., 1974) which has been shown to be the typical pattern of organization of the DNA of most animal species studied. From about 50 to 80% of the total DNA, with the notable exception of Drosophila (Manning et al., 1975) and certain other insects (Crain et al., 1976), has a "short period" interspersion pattern; that is, short repeat sequences of a few hundred nucleotides are interspersed with single-copy sequences up to several thousand nucleotides long (Britten et al., 1975),

The kinetics of reassociation of sea urchin repetitive and single-copy DNA indicate that there are classes of repetitive DNA ranging from about 20 to 6000 copies per genome (Graham et al., 1974), arranged in long, intermediate and short period spacings, implying the existence of longer and shorter sets of interspersed single-copy sequences. The shorter length is about 1000 nucleotides, and the longer length is probably greater than 3400 nucleotides. As 6% of the genome has been shown to consist of highly-clustered repetitive sequences, 22% of the genome must exist as stretches of almost pure single-copy sequence (Graham et al., 1974). The total sequence complexity of single-copy DNA of S. purpuratus is about 6.1×10^8 nucleotide pairs (Graham et al., 1974).

Several thousand different families of repetitive sequences are represented in sea urchin genomic DNA. The availability of cloned repetitive sequences has made it possible to investigate the

characteristics of individual repeat families, which was not possible by other fractionation methods based on renaturation kinetics. Klein et al. (1978) analyzed the genome of S. purpuratus using 26 cloned repeats. Estimates of sequence divergence of these cloned repeat families were made by comparing the thermal stabilities of heteroduplexes formed with genomic DNA to those of renatured cloned fragments. Most of the families lack any highly divergent relatives and show a nucleotide sequence divergence of 8% or less (Klein et al., 1978). Some families, however, do contain highly divergent members, possibly due to subfamilies of small repeat elements present unequally in different members of a family (Scheller et al., 1981; Posakony et al., 1981). The cloned repeat families are found in closely related species, usually in different frequencies (Moore et al., 1978, 1981), but usually in only a few copies in distant species (Moore et al., 1978).

Gene Expression During Development

Nuclear RNA

Sea urchin nuclear RNA consists of long tracts of interspersed repetitive and single-copy sequences (Smith et al., 1974). Many of these repetitive sequences are probably embedded within the introns which are removed in splicing the primary transcript during the maturation of mRNA. The complexity of heterogeneous nuclear RNA in S. purpuratus gastrulae as measured by RNA-driven reactions with non-repetitive DNA is 1.74×10^8 nucleotides, which is more than ten times the complexity of the polysomal mRNA extracted from embryos at the same stage and is equivalent to 28.5% of the genomic complexity (Hough et al., 1975).

Regulation of Transcript Prevalence

The kinetics of hybridization of cDNA to sea urchin egg poly(A)-RNA indicate that RNA sequences are present in a broad range of abundance (Wilt, 1977; Duncan and Humphreys, 1981). These data imply that about 50 average-sized poly(A)-RNA molecules are present in the egg in an abundance 10 to 100 times higher than rare sequences. Rare sequences are those which are present in a few thousand copies per egg on average, and comprise most of the complexity. A few sequences are present in 1000 fold higher abundance than the rare sequences and are probably mitochondrial in origin (O'Brochta et al., 1981; Wells et al., 1982; see chapters 3,4). Although these more abundant sequences represent little complexity, they comprise about half of the mass of maternal RNA (Wilt, 1977; Duncan and Humphreys, 1981).

The mode of regulation of mRNA complexity and abundance is not clear. Generally, sea urchin development proceeds with a decrease in the number of different (rare) mRNA species represented (Galau et al., 1976). The regulation of this phenomenon appears to be due, at least in part, to regulation of post-transcriptional processes, rather than transcriptional selection, as nuclear RNA complexity and sequence representation remains constant or nearly constant during development (Kleene and Humphreys, 1977) and complex class mRNA sequences characteristic of larval stages or adult tissue are present among nuclear transcripts but absent on polysomes of other stages or tissues (Wold et al., 1978).

Stored Maternal RNA

Fertilization is often considered a "trigger" that switches on the unfertilized egg. Enzyme activities, membrane transport activities and macromolecular synthetic activities are all modified after fertilization and are reviewed elsewhere (Vacquier, 1981; Epel et al., 1975; Giudice, 1973). The most salient molecular event, for this discussion, is a dramatic rise in the rate of protein synthesis upon fertilization of sea urchin eggs. The rate of protein synthesis begins to increase almost immediately at fertilization and reaches a level 15 to 30 fold higher within 2 h (Humphreys, 1969; Regier and Kafatos, 1977; Goustin and Wilt, 1981). While fewer than 1% of the ribosomes of the egg are assembled into polysomes (Humphreys, 1971) about 30% of the egg ribosomes are involved in polysomal structures by the 16-cell stage. The egg contains a large population of mRNA molecules which exist in the cytoplasm in an untranslated state despite the presence of excess ribosomes and all of the necessary components for protein synthesis (Hultin, 1961; Denny and Tyler, 1964). Following fertilization, mRNA is recruited into polysomes at a linear rate for the first hour and at a decreasing rate for the subsequent two hours (Dolecki et al., 1977, Wells et al., 1981). At least 85% of the mRNA molecules on polysomes at 2 h post-fertilization are derived from the maternal population (Humphreys, 1971).

Enucleation Experiments

A variety of evidence has accumulated which suggests that this rise in protein synthesis is independent of mRNA synthesis by the zygote. Brachet et al. (1963) and Denny and Tyler (1964) showed that sea urchin eggs,

enucleated by centrifugation and artificially activated, are able to carry out protein synthesis. Shortly thereafter, a similar conclusion for frog eggs was reached (Smith and Ecker, 1965). The post-fertilization increase in protein synthesis was also demonstrated to occur in sea urchin eggs incubated with Actinomycin D at a concentration sufficient to block 95% of RNA synthesis, and more importantly, to inhibit most synthesis of large, heterogeneous RNA (Gross and Cousineau, 1963; Gross et al., 1964; Greenhouse et al., 1971). These chemically enucleated embryos synthesize DNA, cleave, and undergo limited morphogenesis before arresting at the hatching blastula stage. Protein synthesis continues to occur in the apparent absence of new RNA synthesis. Inhibition of RNA synthesis in embryos of snails (Newrock and Raff, 1975), ascidians (Whittaker, 1977), and mammals (Tasca and Hillman, 1970; Manes, 1973) has yielded results similar to those for sea urchins, confirming that activation of translation of maternal mRNA after fertilization is a widespread phenomenon. In a third approach, interspecies hybrid embryos, (and androgenetic hybrid embryos) of echinoderms exhibit an almost exclusively maternal character in developmental timing and features during early embryogenesis (reviewed by Davidson, 1976).

These experiments, while not entirely convincing due to the low sensitivity of the assays, and the assumptions involved, indicate that protein synthesis occurs on pre-formed RNA synthesized during oogenesis which remains translationally inactive until after fertilization.

Cell-Free Translation

A more direct display of stored, translationally inactive RNA in eggs is provided by results of cell-free translation studies. Ruderman and

Pardue (1977) analyzed the classes of mRNA present in sea urchin eggs by cell-free translation and found translatable poly(A)$^+$ and poly(A)$^-$ non-histone mRNAs as well as poly(A)$^-$ histone mRNAs. It has been estimated that 3 to 4% (by mass) of egg RNA can act as template in heterologous translation systems (eg., E. coli, Sarcoma-180 cells) (Slater and Spiegelman, 1966; Jenkins et al., 1973).

Gross et al. (1973) demonstrated that RNA extracted from 20 to 40s particles of eggs is translated into histones in vitro. Subsequently, Lifton and Kedes (1976) showed that the non-translated, but competent histone mRNAs in sea urchin eggs are identical to histone mRNAs being translated after fertilization. The RNA's have identical electrophoretic mobilities and, in vitro, produce well-defined histones, which have been shown to be characteristic of early development (Childs et al., 1979). The absolute structural equivalence of these mRNAs has not been demonstrated however.

Brandhorst (1981) has shown that the RNA residing in egg polysomes of the unfertilized egg is synthesized in the mature egg, and not during oogenesis; this RNA is relatively unstable, and does not contribute to the post-fertilization rise in protein synthesis. Stored mRNA, synthesized much earlier during oogenesis, is recruited. There appears then, to be a mechanism for selecting mRNA for translation based on the time of its synthesis. The mechanism for the recruitment of stored RNA for translation is at present poorly understood, but is being intensively investigated (See Raff et al., 1983). The predominant hypothesis is that stored mRNAs are "masked" by some factor, probably protein, in the eggs and that this mask is released by some event following fertilization (Spirin, 1966; also see Raff, 1980; Raff and Showman, 1983). Masked mRNA is presumed to be in

the form of translationally inactive RNP particles. Moon et al. (1982) have recently concluded that, under a variety of conditions, RNA in egg RNP particles is not significantly less efficiently translated than RNA purified from these RNP's from later stage embryos.

There are other possible mechanisms of translational restriction in sea urchin eggs (for review, see Brandhorst, 1984). Some mRNA seems to be sequestered in, or associated with membrane vescicles which are abundant in sea urchin eggs. Sea urchin -type histone mRNAs are sequestered in the female pronucleus (Venezky et al., 1981; Showman et al., 1982, 1983); their presence in cytoplasmic particles of eggs is an artifact of cell fractionation. Most embryonic polysomes, unlike most translatable maternal mRNAs of the egg, are associated with the cytoskeleton under certain ionic conditions (Moon et al., 1983). The significance of this is not presently known. The intercellular pH rises after fertilization, and this could affect the translatability of RNA or the activation of some "factor" necessary for translation.

RNA Synthesis and Translation in Eggs

Sea urchin eggs synthesize proteins at a low rate. Two-dimensional electrophoresis of [^{35}S]-methionine-labelled polypeptides of unfertilized and fertilized eggs indicates that virtually all of the 1000 well-resolved polypeptides are synthesized in the same relative amounts in unfertilized and fertilized egg samples (Brandhorst, 1976; Tufaro and Brandhorst, 1979; Bedard and Brandhorst, 1983). The increased rate of protein synthesis after fertilization is clearly not due to the recruitment of a distinct set of RNA coding for qualitatively-distinct

proteins. This is strong evidence that the same coding sequence must exist in at least two different states, distinctive by their translational activity.

Stability of Maternal mRNA

Persistence of Maternal mRNA During Development

Many stored maternal RNA sequences remain present throughout embryonic development of sea urchins. Hough-Evans et al. (1977) showed that rare sequences present in the egg are maintained in embryos for several hours, and decline as development proceeds. The data imply that not all RNA sequences in the egg are utilized as mRNA at any particular stage. A major conclusion of this study, however, is that about half of the maternal single-copy sequence set persists in the polysomal RNA throughout development to the pluteus stage.

The regulation of prevalent and moderately prevalent RNA sequences has been analyzed using libraries of cDNA clones (Lasky et al., 1980; Flytzanis et al., 1982). Several hundred cloned abundant RNA sequences of the egg were analyzed. More than 90% of these are present in a similar abundance in eggs and pluteus stage embryos, while generally declining at the gastrula stage. It may be that maternal mRNAs slowly decay and new synthesis of these same sequences from the embryonic genome begins to counteract this loss after gastrulation (Flytzanis et al., 1982). These data do not eliminate the possibility that some maternal RNA sequences are stable, and persist throughout development to the pluteus stage, or that some disappear early in development, or are extensively regulated throughout development.

Cloned cDNAs representing transcripts which are not only replaced but accumulate extensively during development have been identified (Bruskin et al., 1981, 1982; Crain et al., 1981; Merlino et al., 1982; Flytzanis et al., 1982; Fregien et al., 1983; Tufaro and Brandhorst, 1984; and chapter 4). Most rare and moderately prevalent transcripts present in embryos are not detectible in adult tissues (Galau et al., 1976; Xin et al., 1982). This may be due to a more specific expression of genes in the highly differentiated and more homogeneous adult tissues.

Two-dimensional electrophoretic analyses indicate that most proteins synthesized in eggs are synthesized in similar relative amounts throughout embryonic development to the pluteus stage (Bedard and Brandhorst, 1983). About 20% of the 800 polypeptides analyzed in S. purpuratus and L. pictus undergo some change; 10% undergo changes of at least 10-fold, while 1% undergo changes of more than 100-fold (Bedard and Brandhorst, 1983). Rapidly-cleaving embryos show few changes, while many changes occur between hatching of the blastula and the onset of gastrulation, when the rate of cell division has greatly declined. Polypeptides which become undetectible during development almost always disappear before gastrulation, which suggests that these may be encoded by maternal mRNAs which are not being replaced by embryonic synthesis at this critical developmental stage.

Using a variety of elegant techniques, several polypeptides have been identified which are enriched in ectoderm, endoderm or mesenchyme of sea urchin embryos (Bruskin et al., 1982; Bedard and Brandhorst, 1983; Brandhorst et al., 1983; Harkey and Whiteley, 1982). These proteins are developmentally "up-regulated" during embryogenesis. It is likely, then, that tissue-specific proteins are specified by newly-synthesized RNAs

which are not extensively represented in the maternal mRNA population, since there is little evidence for selective activation of translation of maternal mRNA during development (Brandhorst et al., 1983; Bedard and Brandhorst, 1984).

The Information Content of Maternal mRNA

With the advent of recombinant DNA and hybridoma technologies it has become possible to examine whether stored RNA contains information important in controlling embryogenesis (see Brandhorst, 1984; Raff and Showman, 1983, for review). If stored maternal mRNAs are involved in the regulation of morphogenesis, this RNA must have a high information content. The complexity of unfertilized egg RNA for three species has been measured by RNA excess hybridization reactions with single-copy DNA (for Strongylocentrotus purpuratus, Galau et al., 1977; Hough Evans et al., 1977; for Arbacia punctulata and Tripneustes gratilla, Anderson et al., 1976) and by the cDNA kinetic method for Lytechinus pictus (Wilt, 1977). The complexity values obtained in all of these experiments is from 3-3.7 x 10^7 nucleotides, or enough complexity to code for roughly 25,000 different protein species if all of the complexity represents mRNA. About 70-75% of this complexity is present in polysomes in the 16-cell stage embryos (Galau et al., 1976; Hough-Evans et al., 1977); because there are long untranslated mRNA tails, the actual number of proteins encoded is probably about 10,000. The "residual" sequence complexity of RNA not on polysomes represents at least several hundred different repetitive sequence families (Costantini, et al., 1978, 1980). It was found that at least 70% of the mass of egg poly(A)-RNA contains interspersed repeat

sequence transcripts and that both strands of each given repeat sequence are present in egg RNA, though generally in different molecules. If purified maternal poly(A)-RNA is allowed to renature, partial-duplex, multi-molecular structures are formed. Interspersed maternal RNA species persist in the cytoplasm far into embryogenesis (Posakony et al., 1981, Cabrera et al., 1984) and by the gastrula stage, about 15% of the RNA by mass continues to display an interspersed sequence organization. Interestingly, Anderson et al. (1982) found that a major fraction of the cytoplasmic poly(A)-RNA of Xenopus oocytes displays the same interspersed organization. It has been speculated (Davidson et al., 1982) that this fraction represents incompletely processed RNA and that cytoplasmic processing in the embryo could represent a regulative mechanism in the utilization of maternal information. This processing could occur differently at various stages and in different parts of the embryo. The unique structure of this RNA may be recognized by proteins, snRNA's or other "regulatory" molecules which direct it to be sequestered, activated, localized or destroyed (See Thomas et al., 1981). It is intriguing to speculate that the repeat sequences may enable the embryo to regulate functional classes of molecules or allow the embryo to monitor the quantity of a group of transcripts throughout development.

Segregation of Maternal RNA

The possibility that maternal mRNAs are non-uniformly distributed in the egg and segregated during cleavage is an attractive hypothesis currently attracting much attention (see Raff and Showman, 1983; Brandhorst et al., 1983; Jeffrey, 1983). The developmental fate of

specialized cells are determined early in the development of many organisms. Long before the existence of maternal mRNA was established it was established that the egg contains "factors" which are anisotropically distributed in eggs and come to be segregated in various cell lineages. Jenkinson wrote in 1909:

> It is evident that during segmentation at least the nuclei are equipotential, and the hypothesis of self-differentiation in the form originally propounded by Roux can not longer be upheld. It has in fact, been now abandoned by its author. But though in this direction its labours have ended negatively, modern experimental research is yet able to point to a positive achievement of the greatest value and significance. For the same series of investigations has shown that the cytoplasm of the undeveloped ovum is not the homogeneous and isotropic substrate once thought, but heterogeneous, containing various specific organ-forming stuffs which are definitely and necessarily connected with the production of certain parts or organs of the developing animal. - Jenkinson (1909)

Although the existence of these "organ-forming stuffs" has been recognized since the turn of the century, little is known of their molecular nature today. In light of the discovery of a store of mRNA synthesized during oogenesis, it is tempting to speculate that this RNA may represent some or all of the "factors" involved in morphogenetic determination.

There are far more demonstrations of localization per se, than identifications of morphogenetic determinants. A variety of experiments will be considered below.

Anteroposterior-polarity in Dipteran Eggs

Compelling evidence for localized RNA acting as morphological determinants comes from studies on Smittia (see Kalthoff, 1983, for

review). Four types of embryos can be generated by various experimental manipulations; normal embryos, inverted embryos, double encephalons, and double abdomens. Double encephalons and double abdomens develop after centrifugation of embryos (Kaltoff, 1977, 1982; Yajima, 1983), or after UV irradiation of posterior or anterior pole regions of the embryos (Yagima, 1983), or after application of RNase to the anterior pole (Kandler-Sigan and Kaltoff, 1976). Jackle and Kaltoff (1980, 1981) have reported the synthesis, in embryos, of proteins which predict future anterior or posterior development. The anterior indicator protein is synthesized in the anterior end of normal embryos unless they are previously irradiated with UV light. UV irradiation of Smittia embryos causes pyrimidine dimer formation in RNA, while photoreversing light leads to the disappearance of dimers and restores the translatability of mRNA in UV-irradiated embryos (Kaltoff and Jackle, 1982). It has been demonstrated that in vivo generation of pyrimidine dimers involves associated proteins or other photosensitizing components (Kalthoff and Jackle, 1982), and the anterior determinants have been characterized as ribonucleoprotein particles. The posterior determinants might have the same structure since their inactivation by UV light is also photoreversible (Kalthoff and Jackle, 1983).

The Polar Lobe of Ilyanassa

The polar lobe of many annelids and molluscs is a transient protrusion at the vegetal pole of the egg, in which part of the cytoplasm is set apart during cleavage. At the conclusion of the first cleavage, the polar lobe is resorbed into one of the blastomeres (CD) of the two-cell stage.

Lobe-specific information is segregated into the D blastomere, where it will become the dorsal quadrant of the future embryo. The polar lobe can be removed without damage to the embryos. In lobeless Ilyanassa and Bithynia embryos, adult structures such as foot, shell, statocysts, eyes, tentacles and heart are absent (Clements, 1952; Cather and Verdonk, 1974); these structures are derived from, or induced by, mesoderm.

Brandhorst and Newrock (1981), showed that eggs, lobeless embryos, and polar lobes make identical sets of polypeptides during early development. Brandhorst and Newrock (1981) and Collier and McCarthy (1981) investigated the influence of the polar lobe on protein synthesis. Removal of the polar lobe results in quantitative but not qualitative change in protein synthesis by the 29-cell embryo. The synthesis of 98% of the 350 polypeptides detected by Collier and McCarthy was insensitive to actinomycin D (Collier and McCarthy, 1981). There is no evidence for the segregation of a specific set of maternal mRNAs in the polar lobe, although there appears to be temporal regulation of the selection for translation of specific stored mRNAs (Brandhorst and Newrock, 1981).

Ascidian Embryos

Ascidian embryos exhibit determinative cleavage events early in their development, and are excellent for studies of determination (see Whittaker, 1979, for review). Certain genera, including Styela and Boltenia, contain coloured ooplasms which are indicative of the cellular distribution of cytoplasmic regions exhibiting specific morphogenetic potentials (Whittaker, 1980; Conklin, 1905). A complex pattern of ooplasmic segregation occurs during the first 30 min after fertilization

(see Jeffrey, 1983, for details). If eggs are randomly bisected prior to fertilization, normal larvae are obtained from the half containing a nucleus (Reverberi and Ortolani, 1962). If a cut is made after ooplasmic segregation, only nucleated vegetal halves are able to cleave normally (Reverberi, 1937).

Jeffrey (1982, 1983) examined the spatial distribution of histone and actin mRNA sequences in ascidian embryos by in situ hybridization. While histone mRNA was found evenly distributed in the egg and embryo, actin mRNA appears to be localized in the egg and partitioned to specific blastomeres during cleavage.

Whitaker (1973) has studied the regulation of the appearance of tissue-specific enzymes of the tunicate, Ciona, whose embryos exhibit determinative events very early in their development. When cytokinesis and/or nuclear division was arrested at the 2,4,8,16, or 32-cell stages and the enzymes were assayed histochemically, activity was detectible at the proper time in the proper blastomeres (as determined by previous lineage studies) in these arrested embryos. The activity of one enzyme, alkaline phosphatase, was sensitive to the protein synthesis inhibitor puromycin, and insensitive to the RNA synthesis inhibitor Actinomycin D, making it likely that maternal mRNA molecules coding for alkaline phosphatase are localized in the egg and partitioned to the endodermal cells. If maternal RNA is not being segregated, other developmental information, perhaps coding for a regulatory factor, is localized.

Spisula solidissima

In the surf clam Spisula solidissima, first cleavage is unequal and

results in the formation of two unequal blastomeres, termed the AB and CD. The smaller AB gives rise to ciliated ectoderm and specialized muscle cells of the larvae, while the CD gives rise to endoderm, adult muscle, and the shell gland (Meisenheimer, 1901; Wilson, 1925). It is possible to rear AB and CD blastomeres in isolation; to a large extent they are restricted to their normal developmental potential implying that the AB and CD blastomeres are already determined by the two-cell stage (Cheney and Ruderman, 1978). RNA extracted from the AB or CD blastomeres directs the translation of similar polypeptide products *in vitro*; some blastomere-specific polypeptide products are detected indicating that an asymmetric distribution of RNA exists. Since it is unlikely that transcription from a single nucleus during the time between fertilization and first cleavage could account for the accumulation of a sufficient mass of RNA to direct the synthesis of detectible polypeptides *in vitro*, the segregated RNA must have been stored in the egg.

Xenopus

Rare maternal mRNA sequences appear to be localized in eggs of the frog *Xenopus* (Carpenter and Klein, 1982). RNA extracted from three distinct regions of the eggs (animal pole, vegetal pole, and middle) were used to synthesize cDNA. The cDNAs hybridized with excess template RNA with the same kinetics for all three regions. A cDNA probe enriched for vegetal pole sequence was hybridized to the RNA of each fraction. The kinetics of this reaction indicated that certain RNA sequences are enriched at the vegetal end of the animal-vegetal axis. These sequences correspond to rare maternal mRNAs.

The 16-Cell Sea Urchin Embryo

Determination, resulting in restricted developmental potential, occurs very early in sea urchin development. The fourth cleavage gives rise to three types of cells; macromeres, mesomeres, and the vegetal micromeres. Due to size differences, pure populations of the three cell types can be isolated *en masse*. When cultured in isolation, micromeres will differentiate into primary mesenchyme cells (Okazaki, 1975). With the addition of appropriate media, skeletal elements will eventually be deposited resembling in form those normally arising in the embryo (see Harkey, 1983, for review). The four micromeres are clearly different from the other 12 blastomeres of the 16-cell embryo. The pattern of protein synthesis in the 16-cell embryo is nearly identical to that of the egg (Tufaro and Brandhorst, 1979; and this thesis ch. 2); most protein synthesis at this stage is due to translation of stored maternal mRNA (see Brandhorst *et al.*, 1983, for review).

I chose to look at the synthesis of most of the mass of protein synthesized in 16-cell embryos by two-dimensional electrophoresis (O'Farrell, 1975). I determined that micromeres synthesize the same array of proteins, in the same relative proportions, as do mesomeres and macromeres (Tufaro and Brandhorst, 1979; this thesis, chapter 2). The formation of micromeres does not involve a specialization of the pattern of synthesis of detectible proteins, nor the detectible segregation of maternal mRNA. Senger and Gross (1978) reported similar results with a one-dimensional electrophoretic analysis.

Rodgers and Gross (1978) measured the sequence complexity of micromere and mesomere+macromere total RNA using single-copy DNA complementary to

egg RNA as a tracer. Micromere total RNA hybridized 20-32% fewer single-copy DNA molecules than did 16-cell embryo RNA, mesomere+macromere RNA, or gastrula samples. The same results were obtained with RNA from blastomeres or whole embryos developing in the presence of Actinomycin D. These data indicate that there is a non-uniform distribution of RNA sequences between micromeres and other parts of the embryo, and that these sequences were present in maternal RNA. Ernst et al. (1980) extended these results and reported that micromeres lack some non-polysomal maternal RNA sequences found in the cytoplasmic fraction of the whole embryo, and lack the high-complexity nuclear fraction found in other blastomeres (Ernst et al., 1980).

Embryonic Control of Gene Expression

Effective investigation into the role of the embryonic genome in the control of morphogenesis began in 1889 with the first successful interspecific hybrid experiment of Theodor Boveri (1893). Boveri fertilized normal eggs, and enucleated egg fragments of the sea urchin Spherechinus granulatus with sperm of a species of a different genus, Echinus microtuberculatus. The resulting diploid hybrid embryos developed a "hybrid" skeleton, intermediate in the features of each of the parents, while the enucleate merogone hybrid embryos (enucleated eggs fertilized with sperm of a different species) developed a strictly "paternal" skeleton. This interspecies hybrid experiment, though highly criticized and partially qualified in a subsequent paper in 1918, stirred much interest in this system. Since that time hundreds of observations of

echinoid interspecies hybrid embryos have been reported. For a historical review, see Giudice (1973) and Davidson (1976).

Interspecies hybrid embryos have been useful in the study of gene regulation in many organisms, and the development of modern molecular techniques of molecular biology has greatly enhanced the power of this approach. Some of the more elegant experiments will be considered below.

Amphibian Hybrids

The most extensively studied species of amphibian are Xenopus laevis and Xenopus borealis. These may be interbred, and the resulting hybrid embryos and adults have been used to identify a number of biochemical differences between the species, in both protein and nucleic acid components (For review, see DeRobertis and Black, 1979; Vonwyl and Fischberg, 1980). This feature has made amphibians a useful system to study expression of the embryonic genome.

In 1973, Honjo and Reeder reported that hybrid frogs formed between X. laevis and X. borealis preferentially transcribe X. laevis rDNA regardless of which species is the female parent. Repression is nearly complete until the swimming tadpole stage, after which a low level of X. borealis rRNA synthesis is detectible in the total embryo population, and some adult hybrid frogs synthesize substantial amounts of X. borealis DNA. An X. laevis female, heterozygous for an rDNA deletion mutation was crossed with an X. borealis male. Half of the progeny contain only X. borealis rDNA, and rRNA synthesis is delayed in these embryos. In the reverse cross (X. borealis female x X. laevis male heterozygote) X. borealis rRNA synthesis is not delayed. From these four crosses it was concluded that either X. laevis rDNA or X. laevis maternal cytoplasm can each repress

expression of X. borealis rDNA in hybrid embryos. In the presence of X. laevis rDNA, repression can be permanent, while repression by X. laevis cytoplasm is transient and usually reversible.

In 1979 Woodland et al. (1979) investigated histone protein synthesis in interspecies hybrid embryos of X. laevis and X. borealis and detected paternal H1 protein synthesis in the mid-blastula embryo. This is the earliest expression of paternal genes detected to date in amphibia, although histone synthesis may not be representative of the expression of most genes.

Prior to this demonstration of histone expression, expression of lactate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, and malate dehydrogenase was investigated using enzyme activity assays, and had failed to detect paternal activity prior to neurula in diploid or androgenetic haploid hybrid embryos of amphibians (Johnson, 1971; Johnson and Chapman, 1971; Wright and Subtelny, 1971, 1973; Wright, 1975). Whether this reflects a biological phenomenon or a technical limitation of the assays is difficult to assess.

DeRobertis and Black (1979) used high-resolution two-dimensional gel electrophoresis to compare proteins of X. laevis and X. borealis. When the patterns of proteins synthesized by isolated ovaries of the two parental species were compared, at least one-third of the abundantly-synthesized proteins differed in their electrophoretic mobility. Proteins of both parents were detectibly synthesized in interspecific hybrid embryos, with two exceptions. Thus, allelic exclusion observed for rRNA genes does not apply to protein-coding genes in those organisms, at least for the genes analyzed

In 1980 Woodland and Ballantine extended this analysis with a study of

protein synthesis in developing hybrid embryos. In X. laevis eggs x X. borealis sperm hybrid embryos, fourteen borealis-specific proteins were detected. Two appear by the gastrula stage, five in the gastrula, and the rest later in development. Where homologous X. laevis proteins were tentatively identified, the pattern of protein synthesis in androgenetic haploid hybrid embryos (created by fertilizing enucleated X. laevis eggs with X. borealis sperm) was analyzed. X. Laevis proteins appearing after fertilization are the products of translationally-activated stored mRNA, while appearance of X. borealis proteins are due to translation from newly-synthesized mRNA. From this analysis, it was concluded that the two proteins appearing by the gastrulae stage were probably initially coded by stored maternal mRNA.

In an extention of this work, Mohun et al. (1981) analyzed about 75 abundantly-synthesized proteins of early X. laevis and X. borealis embryos by two-dimensional electrophoresis. Six polypeptides specific to X. laevis embryos and a further six specific to X. borealis were identified. Interspecific hybrid embryos (X. laevis female x X. borealis male) synthesize only the maternally-specified set of proteins until gastrulation, after which they produce the full complement of both X. laevis- and X. borealis-specific proteins.

In summary, while nucleolar allelic dominance is a widespread phenomenon, allelic dominance after the gastrula stage is not detected to be prominant in Xenopus. If the absence of paternal-specific polypeptides in hybrid blastula embryos were due to limited allelic dominance of X. laevis over X. borealis, this would infer the existence of stage-specific allelic dominance, limited to post-fertilization embryos prior to gastrulation. A more likely explanation is that maternal mRNA synthesized

during oogenesis directs protein synthesis during early zygotic development, until around gastrulation, after which newly-transcribed mRNA becomes predominant in directing polypeptide synthesis. This is consistent with the known timing of heterogeneous RNA synthesis in Xenopus embryos. Mohun et al. (1981) argues that the use of the reciprocal cross (X. borealis male x X. laevis female) would distinguish these possibilities. I would argue that if allelic dominance were altered in response to putative regulators of gene expression present in the maternal cytoplasm, these alternative mechanisms would not be clearly identified by this strategy.

The isolation of cloned rRNA sequences has facilitated investigation of nucleolar dominance, since the non-transcribed and transcribed spacer segments of ribosomal RNA genes differ greatly between the two species (Brown et al., 1972; Forsheit et al., 1974). It has recently been shown that preferential expression of the X. laevis rDNA is correlated with its hypersensitivity to DNAase I compared to X. borealis rDNA (La Volpe et al., 1983). The significance of this awaits further analysis.

Other amphibians can be crossed and have yielded interesting results. In the axolotl, alcohol dehydrogenase (ADH) is found primarily in larval and adult liver. Starch gel analysis of ADH from adults of the reciprocal crosses of Ambystoma mexicanum x Ambystoma texanum reveals that the maternal form of the enzyme appears at the normal time during development (4 weeks) while the paternal allele is not expressed until three to five weeks later, regardless of which species represents the maternal or paternal parent. In a cross between A. texanum containing the wild-type allele of ADH and an individual of the same species with a variant ADH, both maternal and paternal ADH alleles are expressed simultaneously

(Etkin, 1977). These observations suggest a regulatory incompatability at the transcriptional or post-transcriptional level between the two species.

Drosophila Larvae

It is now well established that the equalization of expression of X-linked genes in XY males and XX females is brought about by hyperactivity of the single X in males. By utilizing the unstable ring chromosome of Drosophila melanogaster, XX/XO mosaic salivary glands can be formed in D. melanogaster females x D. simulans males interspecific hybrid larvae. This genetic system directly compares the activity of the same X chromosome in male and female cells with identical genetic and physiological backgrounds. The X chromosome derived from the D. simulans parent is as extensively dosage compensated in a cell autonomous manner in the interspecific hybrid background as in its own species background (Lakhotier et al., 1981). Apparently, the regulatory sequences involved in dosage compensation of X-linked genes have been specifically conserved during the evolutionary divergence of the two species.

The homologous genes for ADH are expressed in qualitatively different patterns during the development of the Hawaiian Drosophila, D. grimshawi and D. orthofascia. In interspecific hybrid larvae of these species, each parential allele is expressed according to the developmental program characteristic of the species from which it is derived, thus providing strong evidence for a cis-acting control element. The distinct qualitative pattern of expression makes this a unique system in which to study the molecular basis of a gene regulatory difference (Dickinson and Carson, 1979).

Echinoderms

Developmental arrest and paternal genome expression

An enormous amount of research has been carried out on interspecific and intergeneric hybrid embryos of the echinoderm. Some hybrid embryos undergo larval formation followed by metamorphosis, while other crosses result in embryos which arrest early in development, usually in the late blastula stage (For a review of physiology and an historical outline of hybrid experiments, see Giudice, 1973; Davidson, 1976). Arrest at gastrulation is most likely the result of a perturbation of gene expression necessary for gastrula formation, resulting from altered "paternal" gene expression in the presence of "foreign" maternal cytoplasm, or alternatively, aberrant cytoplasmic constitution due to the presence of a "foreign" nucleus. These are not mutually exclusive alternatives. Hybrid embryos that develop into pluteus larvae and undergo metamorphosis do not have catastrophically aberrant expression of genes required for early development, but may exhibit abnormalities as development proceeds.

Loss of paternal DNA or chromosomes has been reported for certain echinoid hybrids and leads to developmental arrest (for review, see Giudice, 1973). This varies among the crosses investigated and may reflect genome incompatibility during chromosome segregation, leading rapidly to aneuploidy.

Many experiments have been conducted to determine when the paternally-derived genome of sea urchins becomes active, and to what extent paternal chromosomes participate in zygotic development (Davidson, 1976, for review). It appears likely that the paternal genome

participates in RNA transcription as early as fertilization (Longo and Kunkle, 1977). Polyspermic zygotes consistently incorporate higher amounts of labelled precursor into RNA than monospermic eggs while monospermic and polyspermic eggs treated with Actinomycin D incorporate the same amounts. Autoradiography also showed incorporation of tritiated uridine into male and female pronuclei.

Histone Gene Expression

The histone genes are members of a moderately repetitive family of tandemly arranged genes coding for the five classes of histone mRNA interspersed with spacer DNA. The DNA sequence of nearly the entire repeating unit is known for several species of sea urchin (Birnstiel et al., 1977; Schaffner et al., 1978, Sures et al., 1978). Histone gene expression during the early stages of sea urchin embryogenesis has been extensively investigated (Mauron et al., 1981, 1982; Childs et al., 1979; Maxson and Wilt, 1982; Lifton and Kedes, 1976; Showman et al., 1982; Venezky et al., 1981; Wells et al., 1981). Histone gene expression in interspecies hybrid embryos has also been investigated successfully.

Easton and Whiteley (1979) demonstrated that H1 histone protein is synthesized using RNA contributed by both parental genomes in the hybrid embryo formed by the fertilization of Dendraster excentricus eggs with S. purpuratus sperm. Stored H1 mRNA (maternal) appears to be involved in H1 synthesis exclusively during pre-gastrular development, with a negligible contribution thereafter. Surprisingly, although both egg-type and newly-synthesized mRNA are included in H1 synthesis during early cleavages, the dominant contribution at this time appears to come from new (paternal type) mRNA. This is in agreement with Infante and Nemer (1967)

who showed that small histone-synthesizing polysomes in early cleaving embryos are abolished upon treatment of embryos with Actinomycin D.

In 1980, Maxson and Egrie used interspecific hybrid embryos of the viable cross S. purpuratus x L. pictus to estimate the contributions of the maternal and paternal genomes to histone mRNA synthesis during early development. Radiolabelled histone mRNA from the two sea urchin species were identified by hybridization to cloned histone genes and shown to be electrophoretically distinguishable. The synthesis of maternal and paternal histone mRNA in these hybrid embryos was detectible as early as the two-cell stage, and appeared to be in polysomes by the 16-cell stage. The relative amounts of the maternal and paternal histone mRNAs synthesized by the zygote were similar. Maxson and Egrie estimate that 0.1 pg/embryo (or about 4×10^5 copies per embryo) can be easily detected by their assay, and suggest that since transcripts of paternally-derived genes are present early in development, stored polymerases and other components carry out transcription of paternal genes at that time. It is clear from these experiments that allelic dominance or exclusion is not affecting the expression of these repetitive genes.

Actin Gene Expression

There are about 8-10 non-allelic actin genes in the genome of S. purpuratus (Scheller et al., 1981) Actin, a prevalent protein in all eukaryotes is involved in mitosis, motility and cytoskeletal architecture. A comparison of members of this multigene family within and among different sea urchin species show that gene number, transcriptional orientation of linked genes, and intron position are the same, while the chromosomal organization of the family appears to vary (Johnson et al.,

1983) Within each species, coding sequence can differ by as much as 30%, while the families appear to similar among species (Johnson et al., 1983). Recently, Crain and Bushman (1983) analyzed actin-coding RNAs in interspecific hybrid embryos of S. purpuratus x L. variegatus (and the reciprocal cross), and S. purpuratus x S. droebachiensis. In L. variegatus x S. purpuratus blastula and prism embryos, transcripts from at least two different paternal alleles are present, while in the reciprocal cross, the same two maternal alleles were expressed in blastula. The S. droebachiensis x S. purpuratus embryos appear to contain transcripts from at least one paternal allele at the blastula stage. This demonstrates that the paternally-derived actin-coding RNA's are transcriptionally active and are apparently processed normally to yield mature mRNA. The timing of expression of these alleles suggests that the expression of the paternal allele is regulated according to the expression characteristic of the paternal species.

DNA Synthesis

Brookbank (1976) measured synthesis of DNA by Feulgen microspectrophotometry in two species and in interspecies hybrid embryos. One parent (Lytechinus variegatus) develops normally at 25°, a temperature lethal for Strongylocentrotus purpuratus. Strongylocentrotus purpuratus develop normally at 10°, a temperature at which Lytechinus embryos are viable, but fail to cleave. It was demonstrated that SpxLv hybrid embryos exhibit a slowing of S phase of the cell cycle at 10° and LvxSp hybrid embryos show a slowing of S phase at 25° These differences manifested themselves between hatching and post-gastrular stages. Brookbank proposes that the slowing of the S phase in SpxLv hybrid embryos

at 10° and in LvxSp hybrid embryos at 25° might be due to accumulation of protein, such as DNA polymerase or associated S phase proteins encoded by the paternal genome following the late blastula stage, which have different temperature optima. These molecules may interfere with DNA synthesis, and in the extreme case may contribute to the lethality of many interspecific crosses.

Rationale of the Experiments

I chose to investigate the segregation of mRNA into different blastomeres of the 16-cell sea urchin embryo. I combined an effective blastomere separation technique with high-resolution two-dimensional electrophoresis to detect patterns of protein synthesis in isolated blastomeres. If qualitative differences had been found, further investigation would have been done to extend this analysis. The results and discussion of this work are contained in chapter 2.

I then decided to exploit the resolution of two-dimensional electrophoresis to assess the extent and timing of zygotic genome expression during embryogenesis. To do this, I chose to analyze the patterns of protein synthesis in interspecies hybrid embryos. The surprising results were that there is an underrepresentation of paternal gene expression in interspecies hybrid embryos. This led to a series of experiments to determine the nature of restricted expression. The results of these investigations are reported and discussed in chapters 3 and 4.

CHAPTER 2

Similarity of Proteins Synthesized in Isolated Blastomeres of Early Sea Urchin Embryos

ABSTRACT

The 16-cell sea urchin embryo has blastomeres of three distinct size classes: micromeres, mesomeres, and macromeres. Each class is already restricted in its developmental fate, micromeres being committed to formation of primary mesenchyme cells. The three classes of blastomeres were isolated in high purity and incubated in [^{35}S] -methionine until the next cleavage. Nearly all the radioactive protein was solubilized and subjected to two-dimensional electrophoresis according to O'Farrell (1975). Of approximately 1000 spots resolved, there are no qualitative differences among the three blastomeres. When embryos were labelled between the first and fourth cleavage and blastomeres then isolated, no qualitative differences in protein synthesis were observed. Moreover, there are very few changes when unfertilized eggs are compared to 16-cell embryos. Thus cellular determination during embryonic development is not accompanied by qualitative changes in the distribution within the embryo of abundantly synthesized proteins, virtually all of which are coded for by sequences present in the egg.

Introduction

The fourth cleavage of the sea urchin egg gives rise to three distinct types of blastomeres having different sizes: micromeres, mesomeres, and macromeres. These blastomeres have different developmental fates in intact embryos and when isolated (Horstadius, 1939). The four micromeres, which form at the vegetal pole, give rise to primary mesenchyme cells of the blastula, which ultimately secrete the spicules of the larval skeleton. Isolated micromeres can be maintained in culture and eventually secrete skeletal spicules resembling in form those of larvae (Okazaki, 1975). Thus, by the 16-cell stage, micromeres are determined to follow a specific developmental pathway in the absence of interaction with other embryonic cells.

It has long been thought that determinative events in early embryos may be brought about by an asymmetric distribution of factors in the egg and their segregation into different blastomeres during cleavage (eg., Wilson, 1927; Davidson and Britten, 1971; Raff, 1977). These factors might be regulatory molecules (or precursors thereof), or they might be structural proteins or the mRNA's coding for them. Several recent reports suggest that mRNA is segregated into different blastomeres of early embryos. In the marine snail Ilyanassa, factors required for the formation of mesodermal organs are localized in the polar lobe, a cytoplasmic extension of one of the first two blastomeres: removal of the lobe results in an embryo most notably devoid of heart, shell and foot (Clement, 1962). Newrock and Raff (1975) observed that lobed and lobeless embryos synthesize proteins having different patterns when

analyzed by discontinuous SDS polyacrylamide gel electrophoresis. These differences, which might be qualitative, persist even when new mRNA synthesis is inhibited. Moen and Namenwirth (1977) have shown that the electrophoretic patterns of proteins synthesized in Xenopus eggs and early embryos are different along the animal-vegetal axis, implying a localization of the maternal mRNA coding for these abundant proteins. The presence of stored maternal mRNA in sea urchin eggs, the translation of which is activated after fertilization, suggests that this class of RNA might include localized morphogenetic determinants.

Rodgers and Gross (1978) have recently provided evidence that different blastomeres of the 16-cell stage sea urchin embryo contain qualitatively distinct populations of RNA. They did not demonstrate that the distinctive RNA molecules are actively translated mRNA. We approached this question by subjecting the proteins synthesized by isolated micromeres, mesomeres, and macromeres, to two dimensional electrophoresis (O'Farrell, 1975).

EXPERIMENTAL PROCEDURES

Preparation of eggs and removal of fertilization membranes

Strongylocentrotus purpuratus were obtained from Pacific Biomarine Inc. For each experiment spawning was induced in a single gravid female by the injection of KCl. The eggs were collected into artificial seawater at 4° and washed by settling three times. Eggs were suspended in 10 mM glycylglycine (pH 9.3), at 10° for 90-120 sec, and then diluted with 40 vol seawater at 4° (Epel et al., 1970). Eggs treated in this manner fail to raise a fertilization membrane upon fertilization. Eggs were routinely incubated with a 50,000-fold dilution of sperm for 1 min, the time required for fertilization to be more than 95% complete. Demembraned eggs tend to clump upon fertilization if maintained in a concentrated suspension. Fertilized eggs (less than 10^6) were therefore diluted into 1 liter of seawater and washed 2-3 times by settling through this volume. For culturing, embryos were suspended in 500 ml seawater containing 50 ug/ml penicillin and streptomycin and allowed to develop at 14°. At this temperature synchrony of early cleavage is optimal, and embryos reached fourth cleavage at about 5 h after fertilization. Treatment of embryos with dithiothreitol had no effect on normal development to pluteus stage.

Separation of blastomeres

At the 16-cell stage, embryos were collected by light centrifugation, and resuspended and washed 3 times in 10 ml seawater lacking calcium and magnesium ions in order to dissociate the hyalin

layer. Brisk aspiration of a 2 ml suspension 5-8 times through a Pasteur pipette dissociated most embryos into single blastomeres. These were layered onto 30 ml linear gradients of 5 to 50% (v/v) isotonic sucrose (1M) in seawater lacking Ca^{2+} and Mg^{2+} ions, and allowed to settle by gravity. After 30-45 min, three bands of cells are visible corresponding to the three types of blastomeres. The approximate positions and identification of these bands are: micromeres in a very narrow band about 1 cm below the surface, mesomeres in a broader band (about 0.5 cm wide) approximately 2 cm below the micromeres, and macromeres in a similar band separated from the bottom of the mesomeres by less than 0.5 cm. Undissociated embryos sediment to the bottom of the gradient (about 6 cm below the surface). The gradients were fractionated by gradually lowering a capillary tube supported by a small jack. The tube was attached to a peristaltic pump and fractions were collected into cold centrifuge tubes. To minimize cross contamination, mesomeres were withdrawn from the top of the band and macromeres from the bottom of the band. The purity of the fractionated blastomeres was estimated by microscopy. In the investigations reported, no larger blastomeres are seen among hundreds of micromeres. Micromeres were never observed among macromeres. There is some cross contamination between mesomeres and macromeres, but it is less than 5%. Occasional smaller cells which might be unusually large micromeres were sometimes observed among mesomeres, but such possible contamination is less than 2%. Fractionated blastomeres were either collected by light centrifugation and frozen or resuspended in seawater for incubation with [^{35}S]methionine.

Labeling of proteins

A suspension of 200,000 embryos lacking fertilization membranes in 5 ml seawater was incubated at 14° with 100 uCi/ml [^{35}S]-methionine (about 700 Ci/mole) obtained from Amersham. Labeling was halted by immersion in cold seawater lacking calcium and magnesium ions. For labeling separated blastomeres, each type was suspended in 1 ml seawater containing 250 uCi/ml [^{35}S]methionine for 1 h. Labeling was stopped by washing in cold seawater followed by freezing in a dry ice-ethanol bath and storage at -70°.

Two-dimensional polyacrylamide gel electrophoresis

Samples were prepared by a modification of the method of Alton and Lodish (1977). Frozen cells were rapidly resuspended in SDS sample buffer (in which 0.1 M dithiothreitol is substituted for B-mercaptoethanol) and immersed in a boiling water bath for 1 min. Aliquots were withdrawn for determination of radioactivity and protein content and the remainder was frozen and stored at -70°.

The amount of radioactivity in protein was determined by precipitation in hot 15% trichloroacetic acid and collection on Millipore filters. The amount of protein was determined according to Lowry et al., (1951). Samples for isoelectric focusing were prepared by the addition of 10 ul of thawed extract to 9 mg urea (Bio-rad electrophoresis grade) and dilution with 80 ul lysis buffer containing 1.6% pH 5-7 ampholytes and 0.4% pH 3.5-10 ampholytes (Bio-rad). Isoelectric focusing was according to O'Farrell (1975). Samples to be compared contained equal amounts of protein of similar specific activity.

To determine the fraction of radioactive protein entering the isoelectric focusing gels, a paralled gel was sliced into 0.5 cm lengths. Each slice was incubated for 8 h at 70°C in a capped scintillation vial containing 0.5 ml 30% H_2O_2. The dissolved slices were counted in Aquasol-2 (New England Nuclear). More than 85% of the radioactive protein loaded enters the isoelectric focusing gel. The pH range was about 4.5 to 7.2 as measured by a microelectrode (Bio-rad propHiler) inserted into the gels.

Isoelectric focusing gels were equilibrated with four changes of SDS sample buffer over 1 h and subjected to electrophoresis on slab gels consisting of a 10-16% exponential gradient of acrylamide (Brandhorst, 1976; O'Farrell, 1975).

The method used to prepare protein extracts results in the solubilization of virtually all the radioactive protein of the embryos. The molecular weight standards were as previously described (Hutchins and Brandhorst, 1978).

RESULTS

Proteins synthesized by isolated micromeres, mesomeres, and macromeres are shown in Fig. 1. The labeling period of 60 min covered about 90% of the time interval between fourth cleavage and fifth cleavage, which was just beginning. Of the approximately 1000 spots detectable (after various exposure times) no consistent qualitative differences were observed. Occasional differences between blastomeres have sometimes been noted, but these are never reproducible. For example, in Fig. 2 a group of spots is shown in which two spots appear to be restricted to macromeres (Figs. 2A and B); however, these differences are not seen when the same preparations of proteins are electrophoresed again (Fig. 2C), or when blastomeres from different batches of embryos are used (data not shown). These inconsistent differences are apparently due to local distortions in the gels and emphasize the need to base conclusions on the comparison of several gels. There are usually a few consistent differences between the patterns of protein synthesis in early embryos derived from different female adults; for example, compare Figs. 1 and 3. As shown in Fig. 3, nearly all the proteins synthesized by 16-cell embryos of the same specimen shown that the pattern of protein synthesis in both is the same, indicating that the dissociation and separation of blastomeres does not perturb protein synthesis. This also indicates that spots thought to be identical in separated blastomeres are not actually different spots having similar coordinates since they appear as single spots in intact embryos.

To investigate the possibility that a protein synthesized prior to the fourth cleavage is sequestered into a single type of blastomere, embryos were continuously labeled with methionine after the first cleavage until they were dissociated immediately after the fourth cleavage. Again, no consistent differences were observed between blastomeres.

Fig. 1. Two-dimensional gel electrophoresis of proteins synthesized by isolated blastomeres. Blastomeres isolated from 16-cell embryos were incubated with [^{35}S]methionine for 60 min. Extracts containing 7 x 10^5 dpm in protein were layered onto isoelectric focusing gels and subjected to two-dimensional electrophoresis. Autoradiograms were exposed to 10^{10} disintegrations (±10%), which results in the appearance of 995 distinct spots. Approximate pH values for the isoelectric focusing dimension are shown on the horizontal axis and molecular weights (x10^{-3}) are shown on the vertical axis. Spot **1** is never detectible in unfertilized eggs. Spot **R** is shown for reference to the same spot in Fig. 2. **(A)** Micromeres, **(B)** Mesomeres, **(C)** Macromeres.

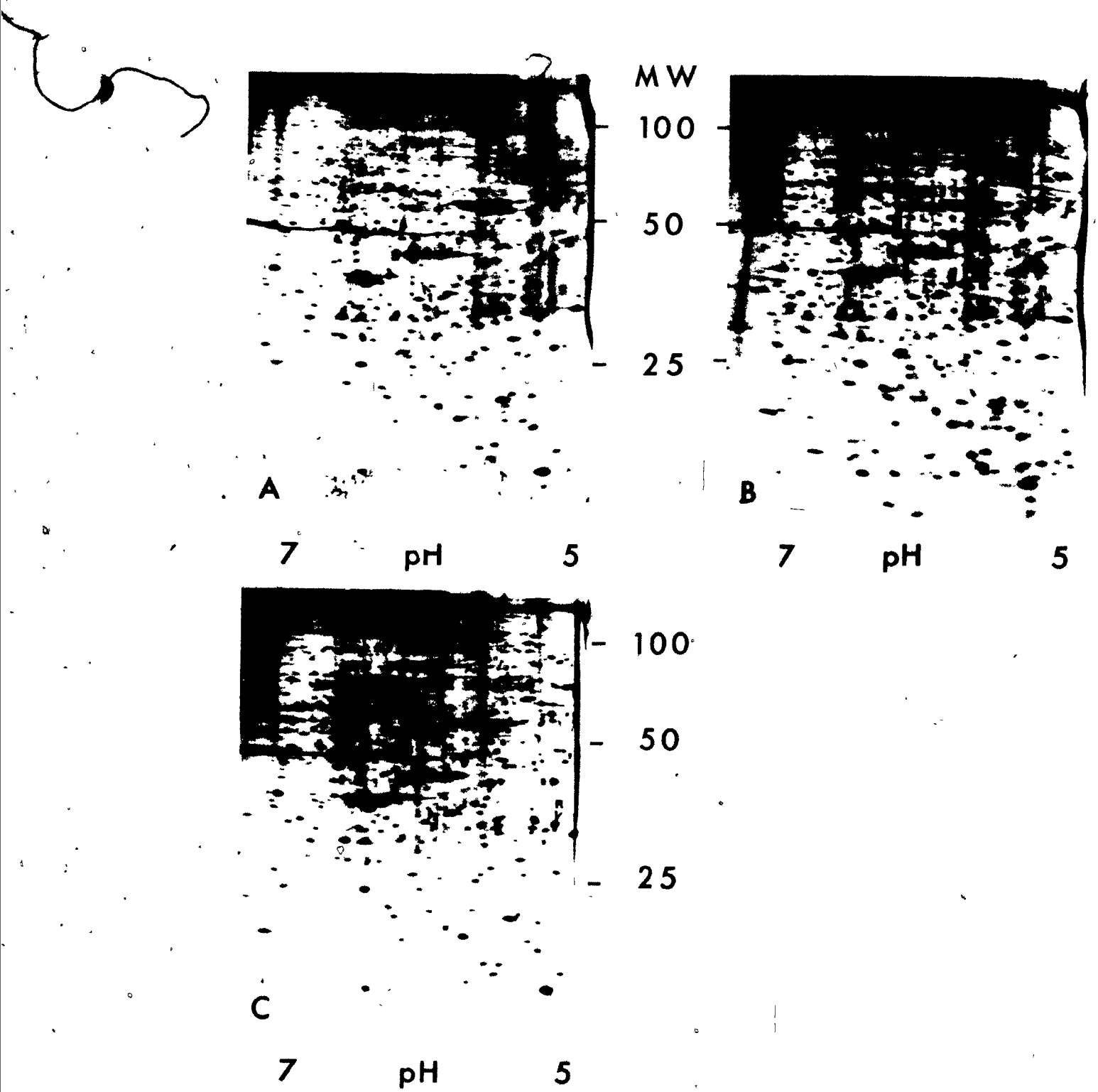
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Fig. 2. Analysis of artifactual differences in electrophoretic pattern. Apparent qualitative differences were noted in Fig. 1 when comparing the area surrounding spot R. Comparison of panel A (macromere pattern) and panel B (mesomere pattern, identical to micromere pattern) suggested that spots S and U were present exclusively in macromeres. Since this had not been seen in previous experiments, macromere proteins from the same extraction were again layered onto isoelectric focusing gels and subjected to two-dimensional electrophoresis. The resulting pattern is shown in panel C, and is indistinguishable from the pattern in B, suggesting that spots S and U are artifacts. A shorter exposure was used in panel C in an attempt to determine if spots U or V were composed of spots migrating close together. It appears that each is a single spot. It can be seen that spots S, U, and W in panel A maintain the same relationship to each other as T, V, and P in panel B, except S, U, and W are shifted toward a higher molecular weight. Shifting S, U, and W downward in panel A so that S comigrates with T yields a pattern identical to panels B and C. (Spot N is most likely obscured in panel A by spot U).

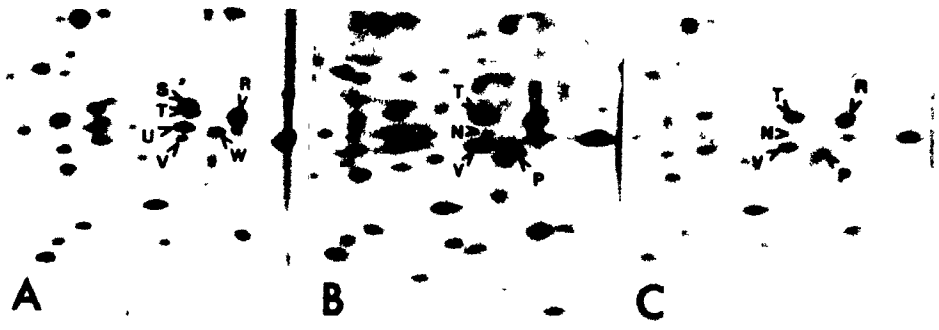
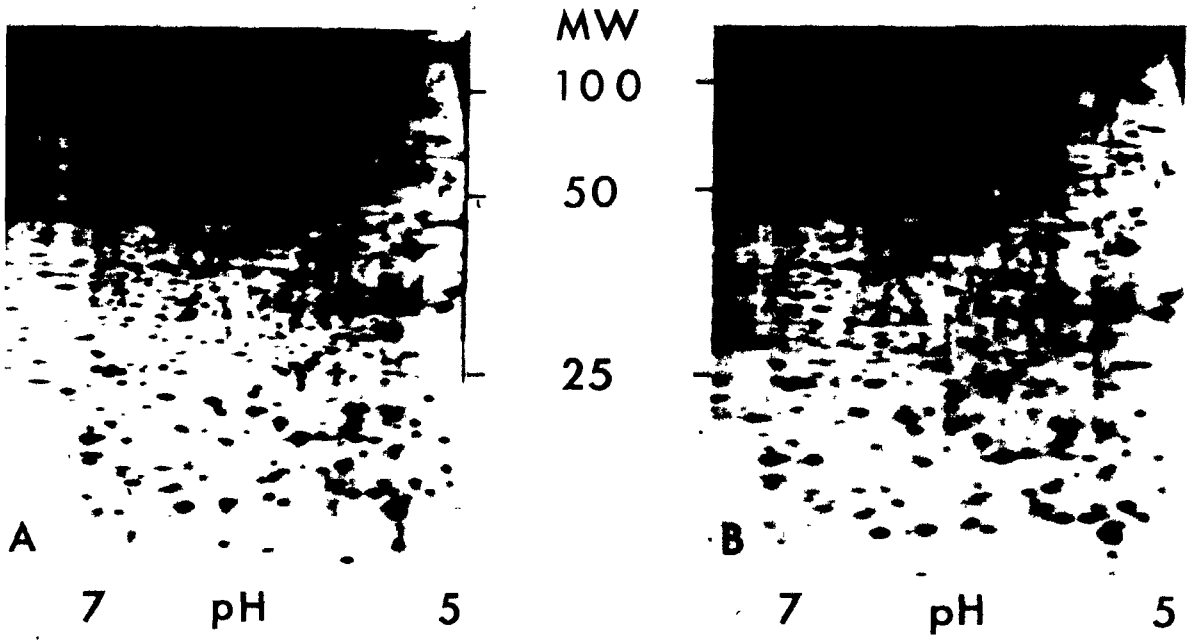

A
B
C

Fig. 3. Two-dimensional electrophoresis of proteins synthesized by unfertilized eggs and 16-cell embryos. About 10,000 unfertilized eggs in 1 ml were incubated with 250 uCi [^{35}S]methionine for 3 h, and 16-cell embryos were incubated under the same conditions for 1 h. Extracts containing 7 x 10^5 dpm in protein were subjected to two-dimensional electrophoresis. Autoradiograms were exposed to 1,2 x 10^{10} disintegrations (±10%). Spot 1 is detected in 16-cell embryos but not in eggs and is shown also in Fig. 1. Spots 2, 3, and 4 are detected in eggs but not in 16-cell embryos. Spot 5 is detected in eggs and embryos of this sea urchin specimen but is absent in other specimens analyzed. (A) Unfertilized eggs, (B) 16-cell embryos.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DISCUSSION

We cannot detect any qualitative differences among the proteins synthesized in different blastomeres of the 16-cell embryos, in spite of their being committed to different developmental fates. We have detected approximately 1000 spots, almost all of which correspond to single polypeptides (O'Farrell, 1975). Galau et al. (1974) have estimated that there are about 15,000 different mRNA sequences present in gastrulae of S. purpuratus; most of the mass of mRNA is accounted for by a much higher number of sequences. Wilt (1977) has shown that polyadenylated egg RNA has a similar sequence complexity and that different sequences are present in a broad range of abundances. Consequently, it is likely that at the 16-cell stage several times as many different mRNA sequences are being translated than have been detected as translation products on two-dimensional gels. We presumably are detecting the translation products of the most abundant sequences, which account for most of the mass of mRNA. Few changes in the pattern of protein synthesis occur between the mature egg and hatching blastula stages (Brandhorst, 1976). Many rapid changes occur, however, during the formation of mesenchyme and the onset of gastrulation, indicating that two-dimensional electrophoretic analyses are sensitive to qualitative regulation of protein synthesis during embryonic development.

We concluded that commitment of individual blastomeres to the formation of mesenchyme is not accompanied by changes in their pattern of protein synthesis relative to other blastomeres. This agrees with

the recent observation that the commitment to vegetalized development of embryos prior to hatching is not accompanied by detectable changes in the patterns of protein synthesis, though the expression of this commitment is accompanied by marked differences compared to normal embryos (Hutchins and Brandhorst, 1979). It remains possible that less extensively labeled proteins or proteins already present in the egg may be segregated into different blastomeres at the fourth cleavage and that this segregation is responsible for developmental determination. Moreover, it is possible that mRNAs coding for distinct populations of proteins are segregated into the different blastomeres but are translated later. Consistent with this possibility is the recent report of Rodgers and Gross (1978) that RNA sequences are segregated into different blastomeres at the 16-cell stage. Specifically, they concluded from nucleic acid hybridization investigations that some sequences of the high-complexity class of total RNA present in eggs are not detectible in micromeres but are detectable in a mixture of mesomeres and macromeres. Since these sequences are unlikely to be abundant enough to be detectable as translation products on two-dimensional gels, since they may not be translated at the 16-cell stage, and since they might not be mRNA at all, there is no discrepancy between our observations and those of Rodgers and Gross (1978). It is possible that the RNA which has been segregated by the 16-cell stage serves a regulatory role, as proposed by Davidson and Britten (1972).

Since we failed to detect qualitative differences between the patterns of protein synthesis of the three types of blastomeres, we were concerned that the similarities might be due to cross contamination of the separated blastomeres. Our method for isolation of blastomeres is

modified from methods described by Hynes and Gross (1970) and Spiegel (1975) and, in our hands, yields fractions of even greater purity than these other methods. Of particular importance is the use of a relatively small number of synchronous embryos. We could not detect any cross contamination between micromere and macromere fractions which have extremely different sizes. The slight possible contamination (unconfirmed) of the mesomere fraction should be much less than the limit of sensitivity to large quantitative differences; large quantitative differences are extremely rare.

A variety of evidence suggests that maternal mRNA is segregated into different blastomeres having different and restricted developmental fates during early cleavage of spiralian eggs (eg., Newrock and Raff, 1975; Donohoo and Kafatos, 1973; Cheney and Ruderman, 1978), tunicate eggs (Whitaker, 1977), and amphibian eggs (Moen and Namenwirth, 1977). It will be interesting to determine whether a common feature of organisms having determinative embryonic development is a qualitatively different pattern of protein synthesis in isolated early blastomeres. Such comparisons may shed light on the relatively regulative development of sea urchin embryos.

CHAPTER 3

Restricted Expression of Paternal Genes in Sea Urchin Interspecies Hybrids

ABSTRACT

We have used two-dimensional electrophoresis to analyze the synthesis of paternal proteins in embryos of three interspecies hybrids which form healthy pluteus larvae: the reciprocal crosses of Strongylocentrotus purpuratus and S. droebachiensis as well as S. purpuratus eggs fertilized with Lytechinus pictus sperm. No proteins specific to the paternal species were detectibly synthesized at the 2-4 cell stage. By hatching blastula stage the synthesis of a few proteins specified by the paternal genome was detected, but these did not increase significantly during later development, and the synthesis of most distinctly paternal proteins was never detected. Radioactive cDNA probes were prepared by reversed transcription of polysome-enriched polyadenylated RNA of S. purpuratus or L. pictus gastrulae. The rate and extent of annealing of these probes to homologous sperm DNA or hybrid embryo DNA indicated that DNA coding for mRNA normally translated in embryos of the paternal species is fully retained in S. purpuratus x L. pictus hybrid gastrulae. Hybridization of these probes to excess cytoplasmic polyadenylated RNA of hybrid embryos indicated substantial underrepresentation of paternal transcripts, particularly those which are normally prevalent. These observations may be explained if much of the mRNA translated into proteins detected on two-dimensional gels is persistent maternal RNA. Alternatively, the synthesis or processing of many paternal mRNA sequences may be impaired in the hybrid embryos.

INTRODUCTION

Interspecies hybrid embryos have long been used to assess the relative contributions of the maternal and embryonic genomes to early development. In echinoid hybrids influence on morphological and biochemical parameters by the paternal genome becomes detectible only at late blastula or later stages in most crosses (for reviews, see Giudice, 1973; Davidson, 1976). It has not been determined if the failure of detection of "paternal" traits in these experiments represents lack of expression or alternatively lack of sufficient sensitivity of the assay employed.

The delayed appearance of paternal traits is usually assumed to be a result of the embryo's reliance during early development on a store of maternal gene products which are synthesized during oogenesis. Following fertilization stored mRNA is recruited into polysomes and accounts for most of the protein synthesis in early embryos (Gross et al., 1964; Humphreys, 1969, 1971). As development proceeds, newly transcribed mRNA makes an increasing contribution to embryonic protein synthesis, until by the gastrula stage most functioning mRNA in the embryo can be accounted for by new synthesis (Brandhorst and Humphreys, 1972; Galau et al., 1977). Early development does not rely solely on stored mRNA, however. It has been shown using cloned probes in a sensitive assay that both maternal and paternal histone mRNAs are associated with polysomes as early as the 16-cell stage in Strongylocentrotus purpuratus x Lytechinus pictus hybrids and transcripts appear in

The cytoplasm by the 2-cell stage (Maxson and Egrie, 1980).

As pointed out by Maxson and Egrie (1980) synthesis of histone mRNA and histones may represent an exception to the general pattern of embryonic gene activation. The timing and extent of expression of the embryonic genome in early stages of sea urchin embryogenesis has remained uncertain. We chose to examine the expression of hundreds of non-histone proteins representing much of the mass of protein synthesized in hybrid embryos by analyzing radioactively labelled proteins by two-dimensional electrophoresis (O'Farrell 1975). We thus restricted our analysis to proteins synthesized in the embryo, whereas previous investigations using protein staining or enzyme assays detected oogenetic products as well. Analysis of three hybrid crosses, S. purpuratus eggs with S. droebachiensis sperm, S. droebachiensis eggs with S. purpuratus sperm, and the interordinal cross S. purpuratus eggs with L. pictus sperm revealed a gross underrepresentation of paternal species specific proteins as late as the pluteus stage of development. The extent of expression of paternal proteins in plutei as revealed by electrophoretic analysis was not greater than in the hatching blastulae. The synthesis of paternal proteins was not detected in early cleavage stage embryos. Nucleic acid hybridization measurements indicate that while the DNA of both species persists throughout development in the S. purpuratus x L. pictus hybrids, there is an underrepresentation of paternal mRNA on polysomes in hybrid gastrula embryos.

MATERIALS AND METHODS

Strongylocentrotus purpuratus and Lytechinus pictus were obtained from Pacific Biomarine, Venice, California. Strongylocentrotus droebachiensis were collected in New Brunswick. Shedding of gametes was stimulated by intracoelomic injection of 0.5 M KCl into gravid adult urchins. Eggs were fertilized in a 0.002% (v/v) suspension of homologous sperm. For the formation of hybrid embryos, eggs were extensively washed in artificial seawater and concentrated to more than 10,000 eggs/ml of seawater. Crosses of S. purpuratus females with S. droebachiensis males (SpxSd) and the reciprocal crosses (SdxSp) were made by fertilization in a 0.005% sperm suspension for 5 min. SpxSd fertilization was 50% complete while SdxSp was more than 95% complete. The other cross of S. purpuratus eggs fertilized with L. pictus sperm (SpxLp) was more than 70% complete within 10 min in a 1% sperm suspension. A cross of L. pictus eggs with S. purpuratus sperm could not be used since less than 1% fertilization occured within 20 min in a 3% sperm suspension. After extensive washing to remove excess sperm, dilute suspensions of embryos were allowed to develop at 14° for LpxLp, SpxLp, SpxSd and SpxSp and at 5-8° for SdxSd and SdxSp. Nearly all cultured embryos developed into well formed plutei. Unfertilized eggs, lacking raised fertilization membranes, were removed by filtration through Nitex mesh. After hatching, cultures were further purified by settling of unfertilized eggs. Unfertilized eggs at no time represented more than 5% of the embryos in cultures used for labelling of protein or isolation of RNA. The presence of these unfertilized eggs, which do not

incorporate much methionine, should not alter the interpretation of the results.

Labelling of proteins, sample preparation, and electrophoresis.

Samples of 12,000 or 20,000 embryos were removed from growing cultures at 2-4 cell, hatching blastula, and pluteus stages and incubated in 1 ml artificial sea water with 50-100 uCi of [^{35}S]-methionine depending on the stage of development. Times of labelling were adjusted for different rates of development of the cultures. At the end of the labelling period, embryos were washed, pelleted, frozen over dry ice-ethanol and stored at -70°. Frozen samples were resuspended in 0.2 ml of 0.01 M Tris (pH 7.4), 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride. RNase and DNase (100 ug/ml) were added and samples were incubated on ice for 30 min. Samples were then lyophilized, and suspended in 50 ul lysis buffer (O'Farrell, 1975) containing 0.2% SDS. Extracts of S. droebachiensis eggs and embryos have a tendency to cause streaking on two-dimensional gels. The sample preparation protocol used reduced this problem below that observed for several other protocols. All samples to be compared were prepared identically. Aliquots of each sample were obtained for determination of radioactivity by precipitation in hot trichloroacetic acid.

Electrophoresis in two dimensions was according to O'Farrell (1975) essentially as described previously (Tufaro and Brandhorst, 1979; chapter 2, this thesis). Samples to be compared contained equal amounts of protein of similar specific activity. Autoradiographs were exposed for varying lengths of time to optimize sensitivity and resolution.

Preparation of polysomes and extraction of RNA

Preparation of RNA was from gastrula stage embryos as previously described (Brandhorst et al., 1979) with some modifications. Briefly, embryos were washed 2-3 times through 1.2 M dextrose, and lysed in 0.4 M KCl, 12 mM $MgCl_2$, 25 mM EGTA, 50 mM Pipes (pH 6.5), containing 0.5% Triton X-100. Polysomes were pelleted through sucrose for 1.5 h at 105,000 x g. After extraction with phenol and chloroform and subsequent precipitation from ethanol, polyadenylated RNA was purified by affinity chromatography on oligo(dT)-cellulose (Brandhorst et al., 1979). The OD_{260}/OD_{280} ratios of RNA were greater than 2.0 in all cases. Gastrulae were labelled for 90 min with [^{3}H]-uridine and the RNA extracted as above. About 48% of the radioactive RNA was in the poly(A)-containing fraction as expected (Brandhorst et al., 1979). RNA prepared in this manner is enriched in polysomal RNA, but can contain contaminating RNA as well.

cDNA preparation

Tritiated DNA complementary to polyadenylated RNA was synthesized using reverse transcriptase (Savage et al., 1978). A mixture containing 50 mM Tris (pH 8.3), 10 mM dithiothreitol, 10 mM $MgCl_2$, 60 mM NaCl, 10 ug/ml actinomycin D, 1 mM each of dATP, dTTP, dGTP and 80 uM [^{3}H]-dCTP (16 Ci/mmol), 10 ug/ml oligo $(dT)_{12-18}$, 5 ug polyadenylated RNA template, and 2-5 units AMV reverse transcriptase (the kind gift of J. W. Beard, Life Sciences, Inc.), was incubated in a 50 ul reaction volume at 42° for 15 min. The reaction was terminated, brought to 0.3 N NaOH, and incubated at 37° for 16 h for hydrolysis of the RNA. The mixture was then neutralized and passed through a Sephadex G-50 column.

Leading radioactive fractions eluting in the void volume were collected by ultracentrifugation at 140,000 x g for 16 h. The pellets were resuspended and stored in buffer at -70°.

DNA-RNA hybridization with cDNA

Trace amounts of [^{3}H]-cDNA were incubated with excess RNA at 70° in 0.6 M NaCl, 1 mM EDTA, 10 mM Hepes (pH 7.8). The reaction mixture was incubated at 100°C for 3 min prior to initiation of the reaction. At appropriate times, 5 ul aliquots containing 1000-2000 cpm of cDNA were removed. The aliquot was quickly diluted into 1 ml of assay buffer containing 0.03 M sodium acetate, 0.03 mM $ZnSO_4$, and 0.01 M NaCl (pH 4.5). The amount of cDNA driven into hybrid molecules was assayed by resistance to S1 nuclease (Boehringer Mannheim Biochemicals). Each sample was incubated for 60 min at 37° with 500 U S1 nuclease and then precipitated in cold 15% TCA for 60 min, collected on Whatman GF/A filters, and counted. In all experiments, the amount of S1-resistant cDNA at the start of the reaction (ie. after reaction was boiled for 3 min to denature the nucleic acids and before incubation at 70°) was subtracted as background. Each reaction was carried out at least three times with slightly different excess amounts of driver RNA. All values were corrected for the increased rate of reaction in 0.6 M NaCl compared to standard conditions and are called EROT. Resulting data were analysed by a computer-aided curve fitting program (Pearson et al., 1977; see appendix III)

Purification of DNA

DNA used to drive cDNA-DNA hybridization reactions was prepared from L. pictus and S. purpuratus sperm by two methods. One procedure

was described previously (Britten et al., 1974) and is a modification of that described by Marmur et al. (1961). Alternatively, DNA was isolated according to Glisin et al. (1974) except the DNA band was retained, suspended in CsCl (p=1.57) containing 0.45 mg/ml ethidium bromide, and centrifuged for 60 h at 100,000 x g. DNA was visualized by UV and removed from the tube. Ethidium bromide was removed by shaking with 1 volume isoamyl alcohol several times. DNA was dialyzed against 0.1 X SSC at 4° and then sonicated to approximately 400 nucleotides average size. DNA from S. purpuratus x L. pictus hybrid embryos was obtained from 90 h pluteus stage embryos essentially as described by Britten et al. (1974).

DNA-cDNA hybridization

Reactions were carried out in 1M NaCl, 0.01 M phosphate buffer, 0.001 M EDTA (Angerer et al., 1976). Driver DNA concentration was 5-6 mg/ml. Samples were boiled for 5 min to denature double stranded DNA and then incubated at 68° for the appropriate times. The amount of cDNA hybridized was assayed by S1 nuclease digestion as described for RNA-cDNA hybridizations. All Cot values were corrected for the increased rate of reaction in 1M NaCl compared to standard conditions (Britten et al., 1974), and are expressed as equivalent Cot or ECOT.

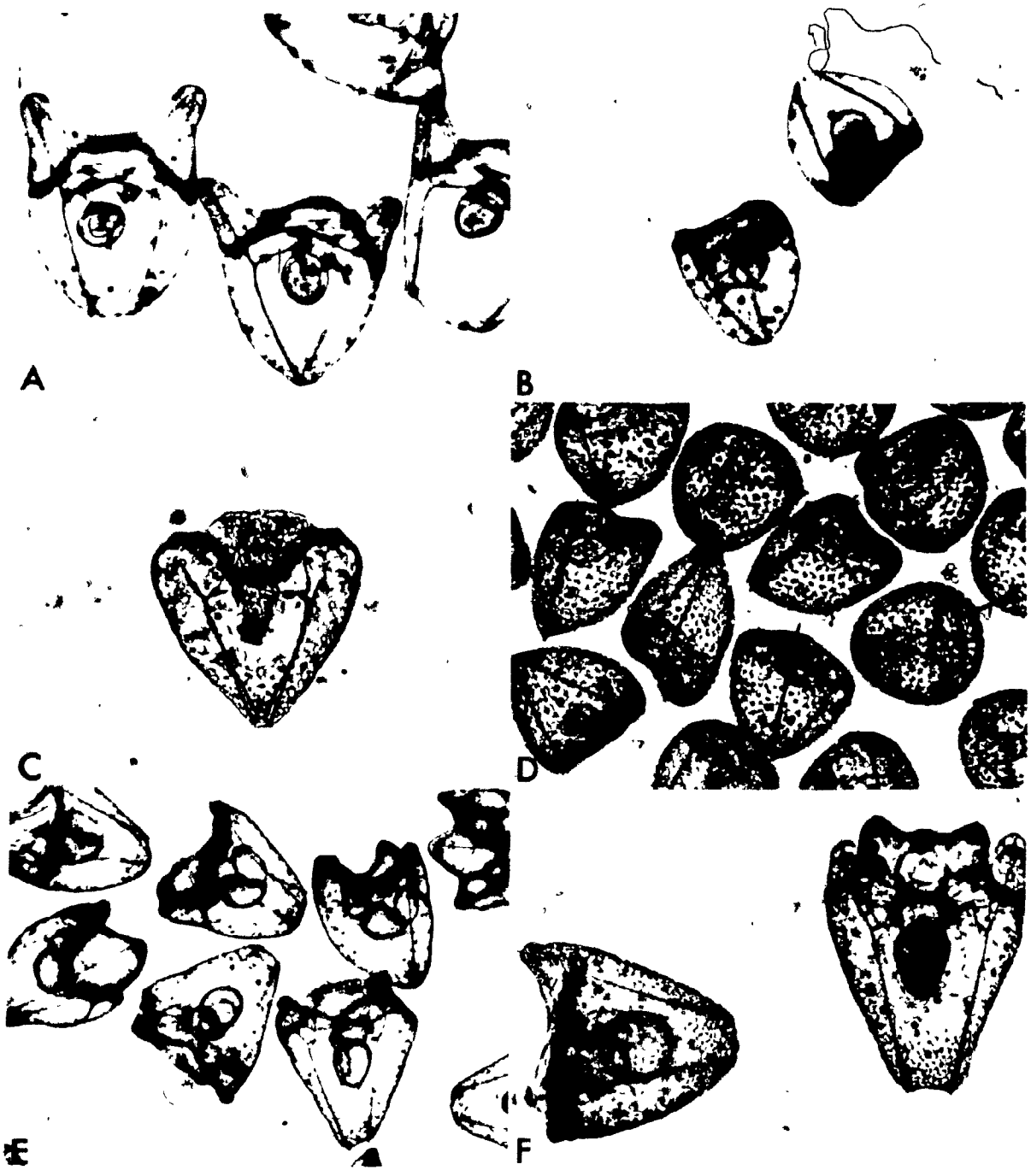
RESULTS

Patterns of protein synthesis

Embryos were analyzed at three stages of development: 2-4 cell, hatching blastula and pluteus. Embryos were incubated with [^{35}S]-methionine at a stage of development defined by morphological characteristics, since the rate of development of different species and interspecific hybrids varied. S. purpuratus and S. droebachiensis were chosen for analysis since these two species form reciprocal hybrids which, unlike many other echinoid hybrids, develop into plutei (Fig. 4C,F). The gross morphological characteristics of the hybrid embryos are very similar to those of the maternal species (Fig. 4D,E). Hybrids formed between S. purpuratus eggs and L. pictus sperm (SpxLp) occur in one direction only, but again the resulting embryos reach the pluteus stage of development and are very similar in appearance to the maternal plutei (Fig. 4A,B).

To assess the appearance of paternal proteins (ie. those proteins which can be unambiguously identified as being synthesized only in embryos of the paternal species) lysates of embryos labelled with [^{35}S]-methionine were subjected to electrophoresis in two dimensions (O'Farrell, 1975). Fig. 5 is a series of gels of the S. purpuratus x S. droebachiensis reciprocal cross, labelled at the hatching blastula stage. Approximately 500 proteins were resolved which were common to SpxSp and SdxSd embryos at this stage. In addition 80 proteins were unique to SdxSd embryos and 60 proteins were unique to SpxSp embryos based on a comparison of Fig. 5A and 5E; these data are summarized in

Fig. 4. Pluteus stage parental and hybrid embryos. Embryos were removed from culture, incubated in dilute Janus Green for several min and photographed. **(A)** L. pictus (x120), **(B)** S. purpuratus x L. pictus hybrids (x120), (C) S. purpuratus x S. droebachiensis hybrids (x160), **(D)** S. purpuratus (x120), **(E)** S. droebachiensis (x80), **(F)** S. droebachiensis x S. purpuratus hybrids (x120).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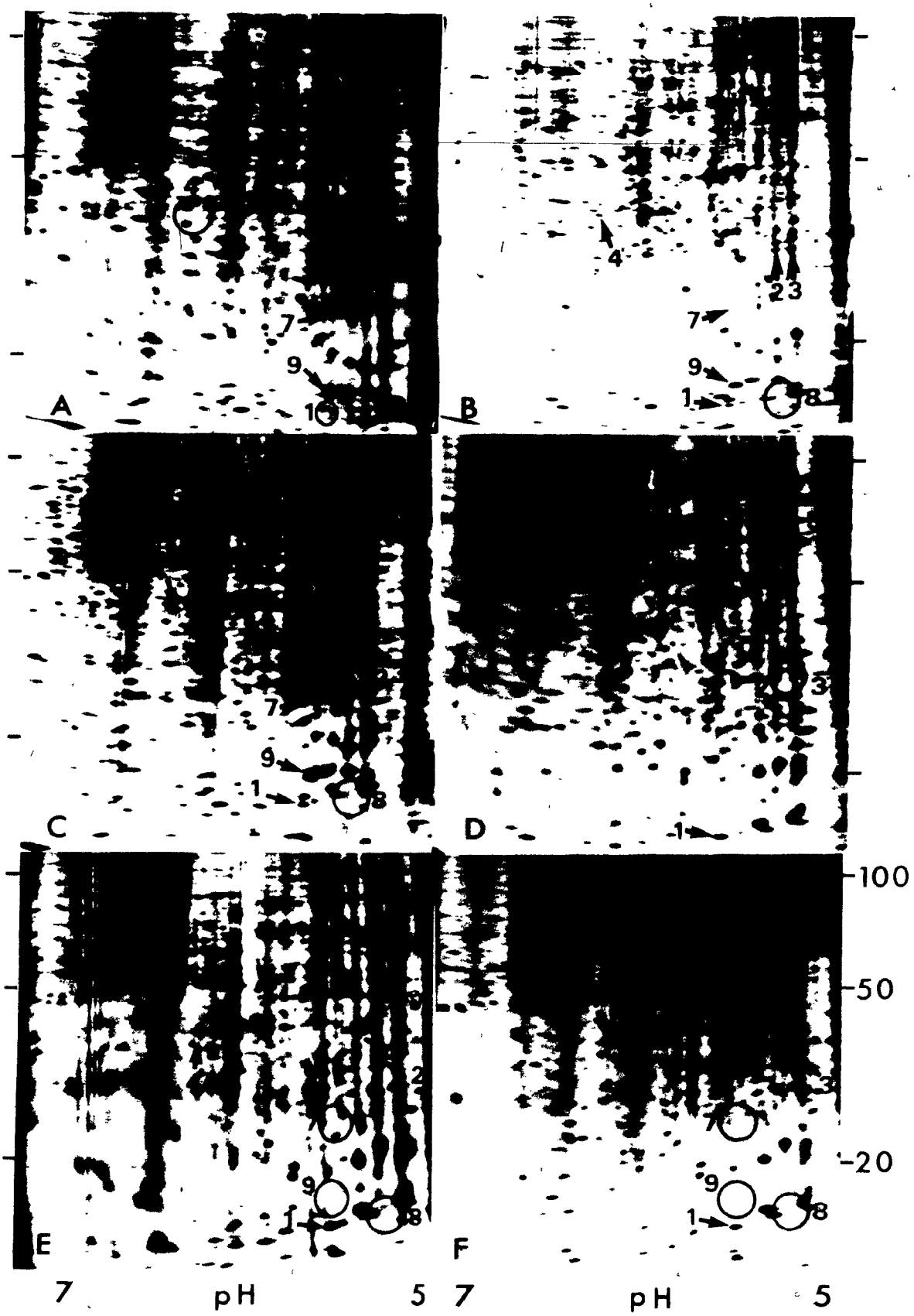
E
F

Fig. 5. Two-dimensional electrophoretic detection of paternal proteins synthesized by hatching blastula stage hybrid embryos. Embryos of S. purpuratus, S. droebachiensis and the reciprocal hybrids formed between them were incubated with [^{35}S]-methionine for 60 min. Extracts containing 2 x 10^6 dpm (±10%) in protein were layered onto isoelectric focusing gels and subjected to two-dimensional electrophoresis. Autoradiograms shown were exposed for 10^{10} distintegrations (±20%) which results in the appearence of 650-800 distinct spots. Approximate pH values for the isoelectric focusing dimension are shown on the horizontal axis and molecular weights are shown on the vertical axis. Arrows point to specific spots while open circles mark corresponding areas on gels in which these spots cannot be detected. Spots 1-5 represent S. droebachiensis specific proteins synthesized in S. purpuratus x S. droebachiensis hybrid embryos. Spots 7, 8 and 9 represent S. purpuratus specific spots synthesized in S. droebachiensis x S. purpuratus hybrid embryos. Consistent numbering is used for all gels. (A) S. purpuratus blastulae, (B) S. purpuratus x S. droebachiensis hybrid blastulae, (C) a mixture of extracts of S. purpuratus and S. droebachiensis blastulae, (D) S. droebachiensis at the 2-4 cell stage, (E) S. droebachiensis blastulae, (F) S. droebachiensis x S. purpuratus hybrid blastulae.


A
B
C
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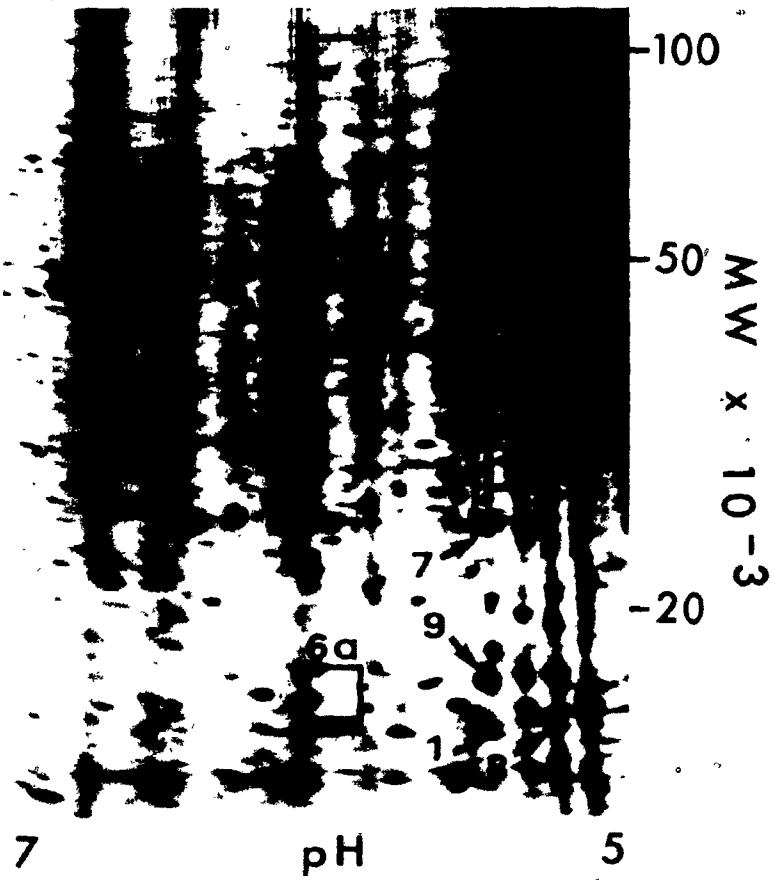
E
F
7
9
10
4
23
1
8
3
100
50
20
7
pH
5

Table 1. In a comparison of Fig. 5A and 5B, 5 S. droebachiensis specific spots were seen to be synthesized in the SpxSd hybrid embryo (Spots 1-5). These proteins were normally synthesized in S. droebachiensis hatching blastulae (Fig. 5E). When labelled proteins of hatching blastulae of SpxSp and SdxSd were mixed and coelectrophoresed, all the species specific spots could be detected (Fig. 5C). Thus the failure to detect more than the 5 paternal spots in Fig. 5B is not due to a loss of resolution of paternal proteins in the presence of maternal egg cytoplasm. Spots labelled 1-4 were reproducible in several experiments on eggs from different females. Spot 5 was not synthesized in embryos from other S. droebachiensis females analyzed and may represent a polymorphism. In an analysis of the reciprocal cross SdxSp at this stage, (Fig. 5E and 5F), no S. purpuratus specific proteins were detectible in the hybrids out of a total of 60 potentially distinct spots at this stage.

Limited expression of the paternal or "embryonic" genome at the hatching blastula stage of development was not surprising in light of experiments showing the apparent ability of embryos to rely on stored mRNA early in development prior to the mesenchyme blastula stage (Gross et al., 1964). We did, however, expect to see extensive synthesis of paternal proteins in hybrid embryos at later stages. Plutei were labelled with [^{35}S]-methionine and the proteins were analyzed by electrophoresis as for hatching stage embryos. Fig. 6 is an enlarged gel summarizing the paternal expression seen at this stage. Of approximately 90 possible paternal specific proteins resolved, only 5 were synthesized in the SpxSd plutei, 4 of which were also synthesized in hatching blastulae. Spot 5 was no longer synthesized in SdxSd

Fig. 6. Two-dimensional electrophoresis of a mixture of proteins synthesized by S. purpuratus and S. droebachiensis pluteus stage embryos. Embryos of S. purpuratus and S. droebachiensis were incubated with [^{35}S]-methionine for 1.5 and 3 h respectively. Extracts containing 1 x 10^6 dpm in protein from each species were mixed and subjected to two-dimensional gel electrophoresis. The autoradiogram shown was exposed for 1.2 x 10^{10} disintegrations and contains approximately 850 detectible proteins. Approximate pH values for the isoelectric focusing dimension are shown on the horizontal axis and molecular weights on the vertical axis. An analysis of proteins was done using a complete series of gels similar to that of Fig. 5. The results are summarized on this gel since it contains the proteins synthesized by both species embryos. Arrows point to specific spots while open squares note the absence of a spot. Spots 1-4 were S. droebachiensis specific proteins synthesized in S. purpuratus x S. droebachiensis hybrid embryos and were first detected at the hatching blastula stage as shown in Fig. 5. Region **6a** corresponds to where a prominent S. droebachiensis protein migrated in one experiment. It was not observed in other experiments using different S. droebachiensis specimens. It appeared as a prominent S. droebachiensis protein synthesized in the corresponding hybrid embryos and is shown in Fig. 7. Spots **7, 8** and **9** are S. purpuratus specific polypeptides synthesized in S. droebachiensis x S. purpuratus hybrid plutei.


100
50
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MW x 10-3
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6a
1
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5

embryos and would not be expected to appear on any gels at this stage. The square labelled 6a indicates the position of a major S. droebachiensis spot which is not observed in all crosses (but see Fig. 7E). Spot 6b is an S. droebachiensis protein which was not detected in SpxSd hybrid plutei in this experiment but was detected in SpxSd hybrid plutei made with different parents (See Fig. 7B). Although the synthesis of many new species of proteins became detectible after hatching of normal embryos, only 7% of the paternal specific proteins were detected in this hybrid. In addition, 3 S. purpuratus spots were synthesized in the SdxSp hybrid (Spots 7,8,9). Spot 8 was newly synthesized after hatching in SpxSp embryos as shown in Fig. 5A. Fig. 7 contains enlarged gel sections showing the appearance of paternal proteins in the pluteus larvae of the SdxSp and SpxSd hybrids. Different batches of eggs were used for Figs. 7 and 8, but the patterns are very reproducible. The appearance of spot 9 was restricted to crosses including S. purpuratus sperm. It may be specified by a male sex chromosome. The appearance of spot 6a similarly required the presence of S. droebachiensis sperm in those crosses in which it appeared. The labelling of spot 9 occurs predominantly in the ectoderm of S. purpuratus embryos (Bruskin et al., 1982).

The patterns of proteins synthesized in the egg and at the 16-cell stage of S. purpuratus vary by only a few spots out of about 1000 analyzed (Tufaro and Brandhorst, 1979); many changes occur after hatching (Brandhorst, 1976; Bédard and Brandhorst, 1982). In an analysis of 2-4 cell hybrid embryos, careful comparison of the gels did not reveal any paternal expression. Some of the paternal proteins which appear in hybrids at the hatching blastula stage or pluteus stage

Fig. 7. High resolution analysis of paternal species specific proteins synthesized in pluteus stage hybrid embryos. Embryos were labelled with [^{35}S]-methionine and protein extracts were subjected to two-dimensional gel electrophoresis as in Fig. 6; different batches of eggs were used in Figs. 6 and 7. Autoradiograms were photographed and enlarged to 1.8 x actual size. Regions containing spots which represent synthesis of paternal proteins in hybrid embryos were cut out and compared. The numbering of spots corresponds to that used in Figs. 5 and 6. Spots 0 and **6b** have not been observed in other experiments. Only a few of the paternal species specific proteins analyzed are shown in this figure. Gel patterns shown in rows **A** and **D** were derived from extracts of the same set of embryos used for Fig. 5, while **B**, **C**, and **E** correspond to a different set.

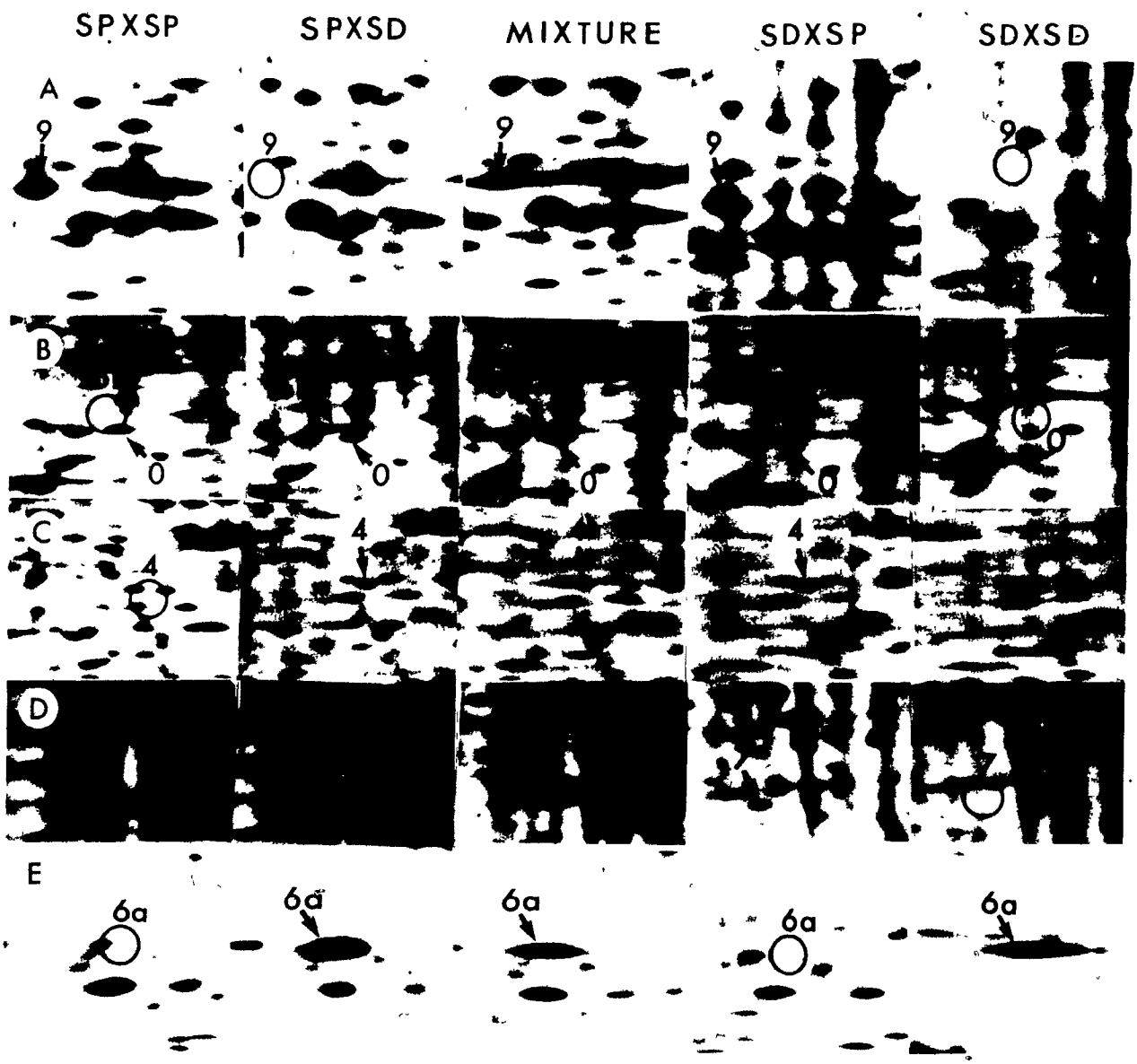

SPXSP
SPXSD
MIXTURE
SDXSP
SDXSD
A
9
B
C
4
D
E
6a

TABLE I

Detection of Synthesis of Paternal Proteins in Hybrid Embryos

Hybrid Cross[a]		Number of	Number of Paternal Spots					
			Cleavage[c]		Hatching[c]		Pluteus[c]	
Egg	Sperm	Common Spots[b]	Obs.[d]	Exp.[e]	Obs.[d]	Exp.[e]	Obs.[d]	Exp.[e]
Sd	Sp	500	0	60	0	60	3	70
Sp	Sd	500	0	80	5	90	5	90
Sp	Lp	275	0	120	1	120	2	130

[a]Species used were S. purpuratus (Sp), S. droebachiensis (Sd), and L. pictus (Lp).

[b]This represents the number of newly synthesized polypeptides common to both species detected on two dimensional gels. It was determined by overlapping autoradiographs of labelled proteins of the two species compared and by confirming the identity of these spots on a gel containing a mixture of the proteins of both species. Numbers are approximate and are limited by the resolution of the gel having the poorest resolution. Only proteins which could be consistently and unambiguously analyzed were counted.

[c]Stage of embryonic development analyzed.

[d]The number of spots distinct to the paternal species observed among proteins synthesized by hybrid embryos of that stage.

[e]The number of paternally distinct spots observed for embryos of the paternal species of that stage and expected if all paternal genes are expressed in hybrids embryos.

Modified from Tufaro and Brandhorst (1982); reproduced with permission of Academic Press, Inc.

were synthesized in the paternal species at the 2-4 cell stage, while others appeared only later in development (Fig. 5D). The latter group of proteins may be translated from mRNA synthesized only during embryonic development.

While only 3-7% of the paternal specific proteins appear in these hybrids, an assessment of the extent of expression of the paternal genome is limited since the majority of proteins comigrate on gels and therefore cannot be detected as being species specific. In fact, all overlapping proteins might represent paternal expression, such that a majority of the paternal proteins might be synthesized. To elaborate on this possibility, we extended our analyses to the hybrid cross of S. purpuratus x L. pictus (SpxLp) in which only 65% of the polypeptides comigrate on two-dimensional gels. In this experiment, paternal representation in the pluteus stage SpxLp hybrid embryo was 2% or less as summarized in Table 1. Thus the maximum paternal expression assuming full expression of all overlapping proteins was only 66% of the total number of paternal proteins detected on gels and is probably far less. The data from electrophetic analyses are summarized in Table 1. In all crosses analyzed, there were examples of proteins which were not synthesized at a particular stage in the hybrid but normally were synthesized at that stage in the maternal homologous crosses. These changes represent only a small number of spots but are reproducible.

Paternal DNA

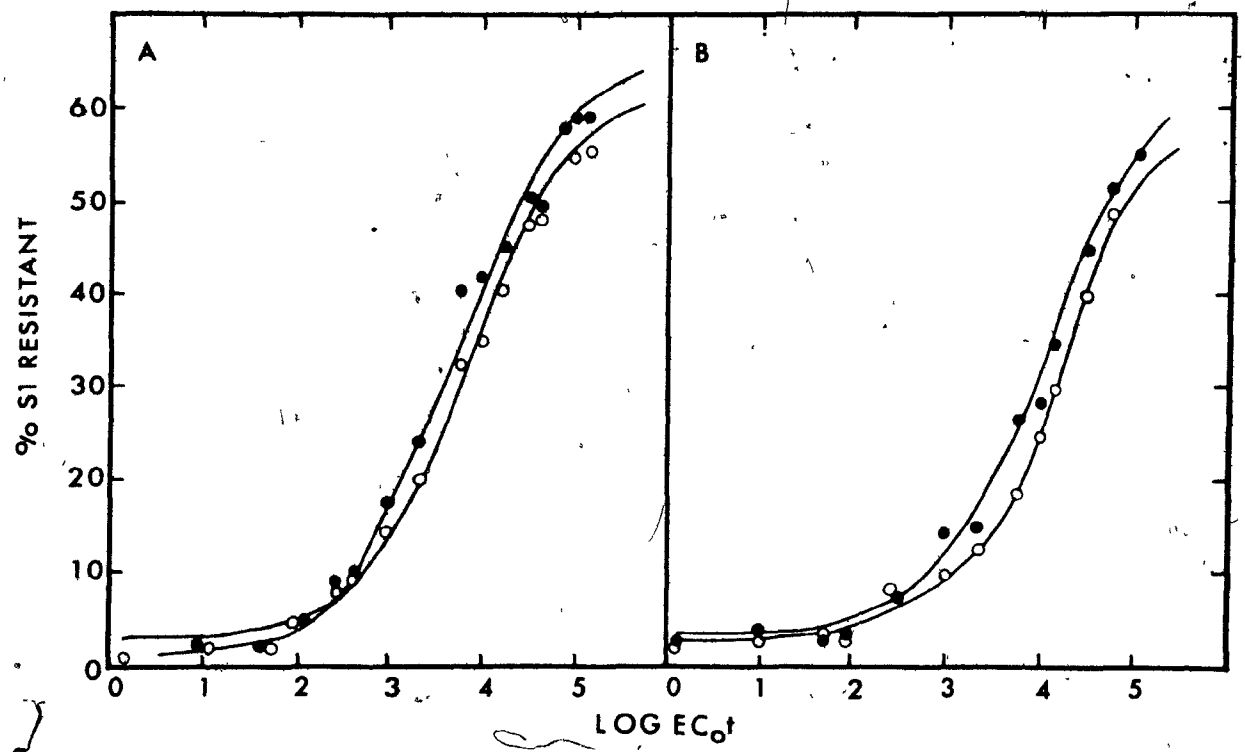
A possible trivial explanation for the restricted expression of the paternal genome in hybrids is that a selective loss of paternal DNA might occur. Loss of paternal DNA or chromosomes has been reported for

certain echinoid hybrids, though the development of those embryos was prematurely arrested (for review, see Giudice, 1973). To investigate this possibility, [^{3}H]-cDNA was synthesized by reversed transcription of polysome-enriched cytoplasmic polyadenylated RNA of L. pictus and S. purpuratus gastrulae. Fig. 8 shows the kinetics of hybridization of each parental cDNA with homologous sperm DNA and DNA prepared from SpxLp hybrid gastrulae. The hybridization reactions were nearly indistinguishable in both kinetics and extent when driven with sperm DNA or hybrid embryo DNA. The sequences reacting represent those coding for proteins normally synthesized in gastrulae of S. purpuratus or L. pictus. Thus the DNA sequences complementary to these probes were present in the DNA of the SpxLp hybrid embryos at the gastrula stage in normal amount and complexity. This analysis should have been quite sensitive to loss of DNA sequences since there is only an 11.5% cross reactivity between the nonrepetitive DNA's of L. pictus and S. purpuratus (Angerer et al., 1976) which compares well to the observed 14% cross reactivity of the L. pictus cDNA probe with S. purpuratus DNA. A similar investigation of the SpxSd and SdxSp hybrids was not attempted because of the extensive (68%) sequence homology between non-repetitive DNA's of S. purpuratus and S. droebachiensis (Angerer et al., 1976).

Paternal species-specific RNA

Finally, we investigated the presence of paternal specific sequences in a polysome-enriched cytoplasmic polyadenylated RNA fraction from the gastrula stage of the SpxLp hybrids. The cDNAs used for the DNA-cDNA hybridization were used as probes to determine the kinetics of

Fig. 8. Reaction of cDNA with excess DNA. The [^{3}H]-cDNA transcribed from polysome-enriched cytoplasmic RNA extracted from S. purpuratus and L. pictus gastrulae was incubated with DNA isolated from sperm or pluteus stage hybrid embryos in $8x10^5$ fold mass excess. **(A)** S. purpuratus cDNA reacted with excess S. purpuratus sperm DNA (●) or S. purpuratus x L. pictus hybrid pluteus stage embryonic DNA (O). **(B)** L. pictus cDNA reacted with excess L. pictus sperm DNA (●) or S. purpuratus x L. pictus hybrid pluteus stage embryonic DNA (O).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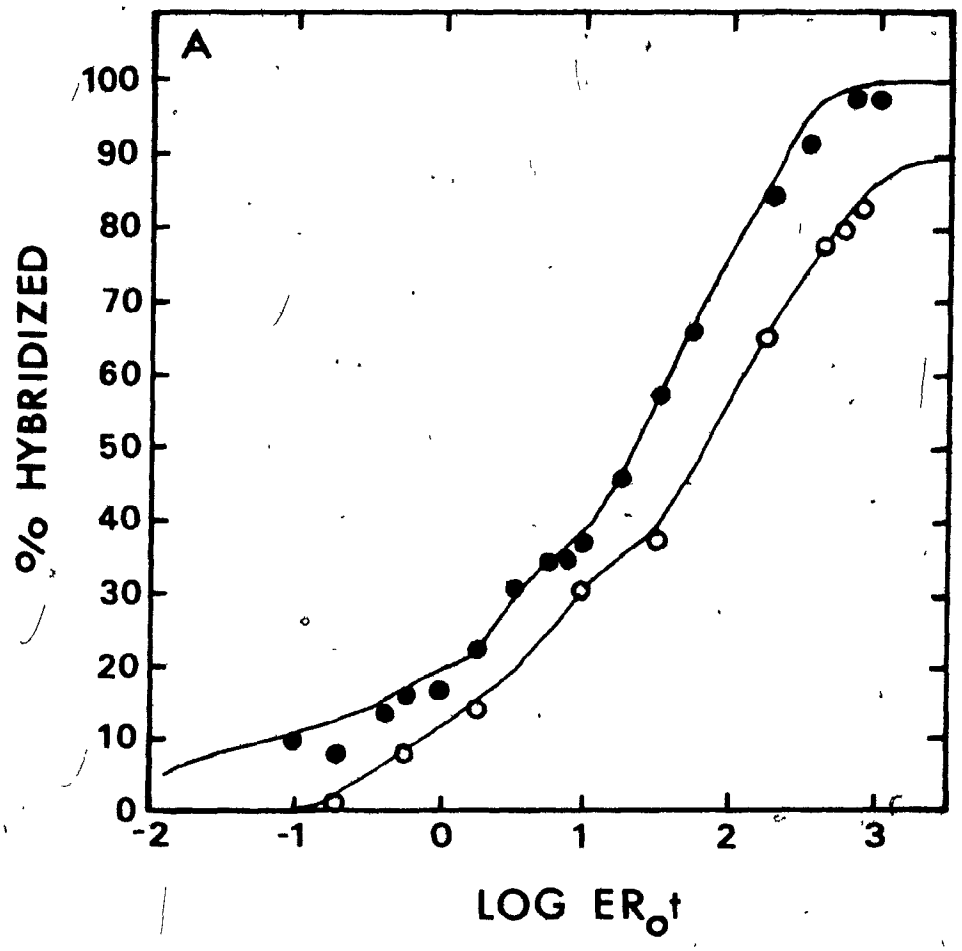
an RNA driven hybridization reaction. To assess the reactivity of the probes these cDNAs were hybridized with their homologous templates as shown in Fig. 9A,B. Sequence complexities and prevalences were estimated from these data using a computer assisted curve fitting program (Pearson et al., 1977) and are summarized in Table 2. When SpxLp hybrid gastrula polyadenylated RNA was used to drive S. purpuratus cDNA into hybrids, the reaction proceeded with slightly altered kinetics and reacted to 90% of the homologous termination value. The 10% of the mass of the probe which did not react by EROT 2500 corresponds to sequences reduced or absent in the RNA of hybrid embryos. Fig. 9B shows the L. pictus cDNA probe driven with SpxLp gastrula polyadenylated RNA. Only 45% of the potentially reactive probe hybridized by EROT 3000, indicating a considerable reduction in the paternal RNA representation of these hybrid embryos. The decreased hybridization is not due to limited reactivity of the probe since the homologous reaction showed that the probe is reactive. RNA driver from hybrid embryos was not degraded since it was able to drive S. purpuratus cDNA into hybrids in a parallel reaction. While this analysis does not establish the complexity of sequences missing, the slow reaction kinetics and protein electrophoresis data indicate that some of the transcripts reduced in hybrid embryos are normally abundant in L. pictus embryos.

Fig. 9. Hybridization of cDNA with excess RNA. The L. pictus and S. purpuratus gastrula stage cDNA probes used in Fig. 8 were incubated with excess homologous (template) RNA or excess polysome-enriched RNA from hybrid gastrulae of the S. purpuratus x L. pictus cross. **(A)** S. purpuratus cDNA reacted with excess S. purpuratus RNA (●) or S. purpuratus x L. pictus hybrid embryo RNA (0). **(B)** L. pictus cDNA reacted with excess L. pictus RNA (●), hybrid embryo RNA (0), or S. purpuratus gastrula RNA (▲). Final reactivity of each probe when driven by excess template RNA was 86% for L. pictus cDNA and 74% for S. purpuratus cDNA. All hybridization plots shown were standardized to 100% probe reactivity.


A
% HYBRIDIZED
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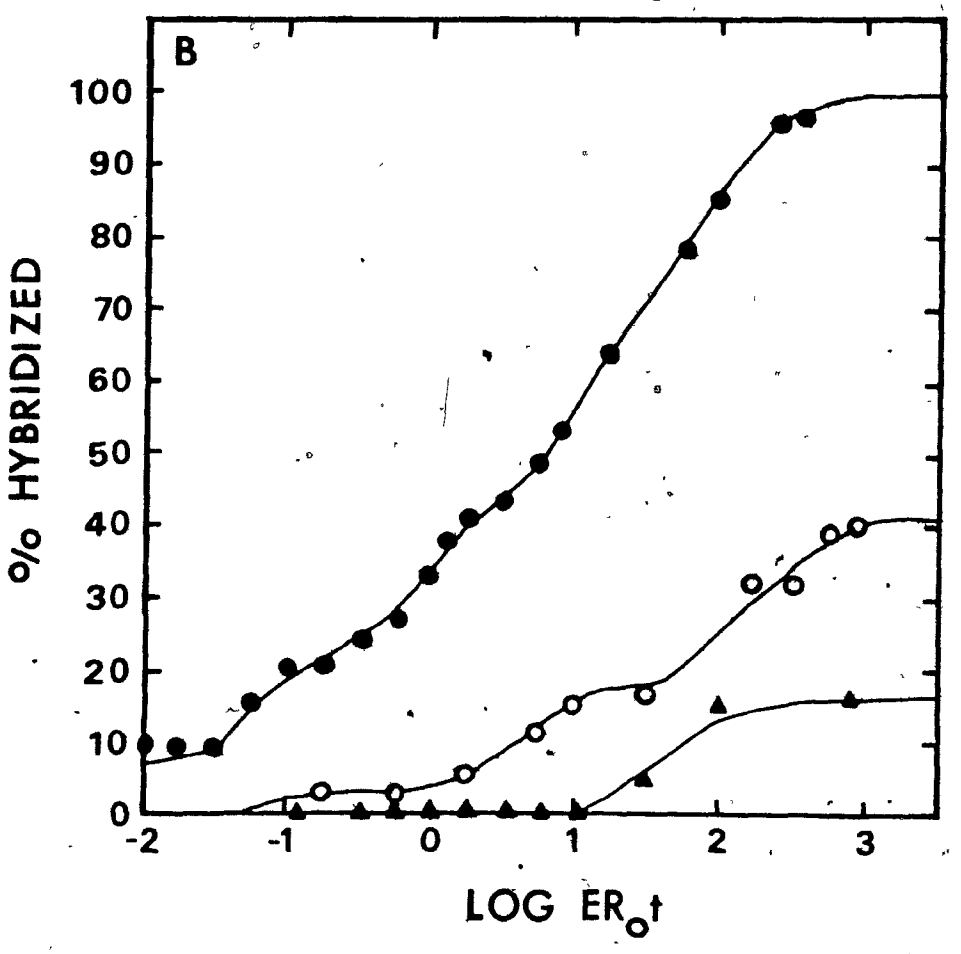
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TABLE 2

Frequency distribution of polyadenylated RNA sequences in *L. pictus* and *S. purpuratus* gastrula stage embryos.

	L. pictus cDNA				*S. purpuratus* cDNA		
	Prevalence				Prevalence		
	Very High	High	Intermediate	Low	High	Intermediate	Low
Fraction of Hybridizable cDNA	16	24	35	25	18	38	44
Rot (Moles l^{-1} sec)							
Observed	0.03	0.90	14.44	114.20	0.93	16.8	110.0
Corrected	0.0048	0.32	5.05	28.55	0.17	6.4	48.4
Complexity nucleotides x 10^{-6}	0.0048	0.320	5.05	28.5	0.170	6.38	48.0
Number of different RNA species[c]	2.4	110	2525	14,250	85	3190	24,000

[a] *L. pictus* cDNA driven by *L. pictus* RNA was best fit to four prevalence classes, while *S. purpuratus* cDNA driven by *S. purpuratus* RNA was fit to three prevalence classes.

[b] Complexity was determined using a globin RNA standard: 1.2 kb complexity, Rot =0.0012.

[c] Based on average size of 2000 nucleotides.

DISCUSSION

Our data indicate that most distinctly paternal proteins are not detectibly synthesized in any of three interspecies hybrids. Of the few that are synthesized nearly all have appeared by the time of hatching indicating that the paternal genome can be transcribed into mRNA before gastrulation. The restriction in appearance of paternal products is not due to loss of DNA coding for mRNA sequences normally translated in the SpxLp cross. The steady state population of cytoplasmic polyadenylated RNA, enriched for polysomal RNA, of the SpxLp gastrulae has a greatly reduced representation of sequences normally found in polysomes of LpxLp gastrulae.

Limited synthesis of paternally coded proteins in echinoid interspecies hybrids has been reported previously by Whiteley et al. (1975). Serological characterization of soluble protein antigens synthesized in hybrids of Dendraster excentricus eggs fertilized by S. purpuratus sperm indicate that there is very little detectible contribution of the paternal genome by gastrula stage, at which stage development is arrested. Only maternal forms of several developmentally regulated enzymes could be detected in the hybrids. DNA-driven nucleic acid hybridization reactions with newly synthesized hybrid embryo polysomal RNA detected no reduction in the paternal mRNA compared to maternal mRNA sequences; Cot values were obtained allowing hybridization of RNA to nonrepetitive DNA (Whiteley et al., 1975). It is not clear whether the contrast with our observation that paternal mRNA is underrepresented is

due to differences in the hybrid crosses utilized or due to differences in the populations of mRNA analyzed (steady state versus pulse labelled). It appears certain, though, that the limited synthesis of paternal proteins in the SpxLp hybrid embryos is due to a correspondingly limited presence of paternal mRNA's in polysomes.

We analyzed about 500 proteins in each of the three crosses enabling us to screen for a large number of distinctly paternal proteins. Our analysis is restricted to proteins having isoelectric points between 4.5 and 7.2 and molecular weights of 10-130 x 10^3 daltons, a range which includes most of the mass of newly synthesized proteins (Brandhorst, 1976) but excludes some major proteins, notably histone. Galau et al. (1974) have estimated that about 15,000 different mRNA sequences are translated in S. purpuratus gastrulae, most of them rare. The newly synthesized polypeptides we detect on two-dimensional gels presumably correspond to the more prevalent mRNA's.

The kinetics of hybridization of the paternal L. pictus cDNA probe to excess SpxLp hybrid RNA indicates that a substantial mass of paternal transcript is present and thus transcribed. The sequence complexity of these paternal transcripts cannot be determined from our data, but the paucity of paternal spots on two-dimensional gels and retarded reaction kinetics for paternal cDNA indicate that much of the mass of normally prevalent transcripts is reduced or absent. In S. purpuratus gastrulae prevalent transcripts account for about half the mass of cytoplasmic RNA (Table 2; see also Lasky et al., 1980; Shepherd and Nemer, 1980) and nearly all of the cytoplasmic sequence complexity is represented in polysomes (Hough-Evans et al., 1977). Hence it is possible that virtually all the sequence complexity and mass of rare mRNA normally

found on polysomes of LpxLp embryos is also transcribed and translated in SpxLp hybrid embryos. Cabrera et al. (1984) have shown that by gastrula stage most or all rare mRNA molecules are newly synthesized.

We have no certain explanation for the observation that the expression of the paternal genome is so restricted in late stage hybrid embryos. One possibility is that newly synthesized proteins appearing on two-dimensional gels even in plutei are the products of translation of persistent, highly stable maternal RNA. Contrary to this proposal is the observation that by gastrula stage 3-4% of the mass of RNA on polysomes is accounted for by newly synthesized embryonic RNA (Brandhorst and Humphreys, 1972; Galau et al., 1977). Thus most or all of the mass of mRNA expected to be in polysomes can be accounted for by transcription of the embryonic genome, but this observation does not exclude the possibility that some maternal transcripts remain in poysomes or continue to be recruited into polysomes in late stage embryos. Cabrera et al (1984) have shown that the accumulation of newly synthesized RNA during embryonic development cannot account for the total mass of some cloned prevalent mRNA species at prism stage. Consequently, some maternal mRNA may persist throughout embryonic development. By gastrula stage many polypeptides are synthesized which were not detectibly synthesized in early embryos (Brandhorst, 1976; Bédard and Brandhorst, 1982). Comparison by two-dimensional electrophoresis of the products of cell-free translation of cytoplasmic RNA with proteins synthesized in vivo indicates that for most proteins there is a close quantitative correlation between the amount of translatable RNA in the embryo and its translation in vivo (Brandhorst et al., 1979; Infante and Heilman, 1980; Bédard and Brandhorst, unpublished obser-

vations). Thus if most of the spots detectible on autoradiograms of two-dimensional separations of proteins synthesized in late stage embryos are products of translation of persistent maternal RNA, those mRNA's would necessarily reside in a form untranslatable in the reticulocyte or wheat germ cell-free systems used in those analyses. A posttranscriptional regulatory event would be required for the stage specific modification and recruitment of those messages into polysomes. While it would be surprising that such a mechanism is operating on such a great scale as late as the pluteus stage, the structure of much of the stored maternal, polyadenylated RNA in eggs and early embryos suggests that it is in a preprocessed, possibly untranslatable, form which might be quite stable and processed into active mRNA during embryonic development (Costantini et al., 1980; Thomas et al., 1981). Other possible explanations for the restricted expression of the paternal genome include impaired synthesis, processing, or transport to polysomes of paternal mRNA. The egg cytoplasm may contain factors which are required for the synthesis or processing of mRNA derived from the genome of the maternal species but which do not allow expression of the paternal genome. We are engaged in an analysis of the metabolism of specific paternal transcripts in embryos of the homologous crosses and in the hybrid embryos (see chapter 4).

It has been shown that in the SpxLp cross paternal H1 histone transcripts are detectible in the cytoplasm as early as the 2-cell stage and that they are translated by the 16-cell stage (Maxson and Egrie, 1980). Substantial synthesis of paternal H1 histone occurs in early embryos of echinoid interspecies hybrids (Easton and Whiteley, 1979). Genes coding for histones synthesized in early embryos are present in

hundreds of copies and their transcripts should therefore accumulate considerably more rapidly than the transcripts of genes expressed in embryos most of which are present in only one or a few copies per haploid genome (Goldberg et al, 1973). Histone mRNA's have some unusual features as well, including absence of poly(A) tracts and lack of a nuclear precursor requiring splicing out of intervening sequences (Hentschel and Birnstiel, 1981), which may allow their expression in interspecies hybrid embryos. It is not surprising then that we failed to detect the synthesis of paternal proteins in early embryos. Indeed, most investigations of echinoid hybrid embryos failed to detect any evidence of paternal genome expression prior to gastrula stage (except for histones), while we can clearly detect the synthesis of distinctly paternal proteins by the time of hatching.

Oocytes isolated from ovaries of hybrid adults of the cross of Xenopus laevis with X. borealis synthesize very nearly both distinguishable parental populations of oocyte proteins (DeRobertis and Black, 1979). We do not yet know whether this contrasting observation indicates profound differences in the degree of paternal genome expression in echinoid versus amphibian interspecies hybrids, or a distinction between gene expression in cells of embryos and mature adults. The transcription of X. borealis ribosomal DNA is repressed by either X. laevis maternal cytoplasm or X. laevis ribosomal DNA in hybrid embryos (Honjo and Reeder, 1973).

The interpretation of many investigations utilizing echinoid interspecies hybrid embryos is dependent on the assumption that the paternal genome is expressed to the same extent, and with the same timing, as the maternal genome in normal embryos. Our observations

indicate that caution should be attached to such interpretations. For example the failure to detect paternal hatching enzyme in S. franciscanus x S. purpuratus hybrids (Barrett and Angelo, 1969) may be due to the restricted expression of the paternal genome rather than the utilization of stored maternal mRNA for the synthesis of hatching enzyme (Showman and Whiteley, 1980). Clarification of the restriction on expression of the paternal genome in interspecies hybrids may have important implications for an understanding of the program of gene expression which normally operates during embryonic development.

CHAPTER 4

Analysis of the Expression of Paternal Genes in Sea Urchin Interspecies Hybrid Embryos: Evidence for Post-transcriptional Regulatory Events

ABSTRACT

A library of cloned cDNA molecules representing L. pictus gastrula embryo cytoplasmic RNA was constructed. The library was screened to select L. pictus species-specific sequences indetectible in SpxLp hybrid embryos. It was also screened to identify transcripts which accumulate during normal embryonic development. My data indicate that most paternal species-specific abundant transcripts do not accumulate to normal levels in the cytoplasm of interspecies hybrid embryos. Of the 20 cloned sequences of this type analyzed, only 2 (16B7, 16C12) accumulate to a level similar to that in embryos of the paternal species, L. pictus. One of these, 16B7, contains a repeat sequence which is expressed in many transcripts which appear in SpxLp hybrid embryos as early as the 64-cell stage. One sequence, 15D6, accumulates in LpxLp plutei to a level 50-fold over that of the L. pictus egg. The 15D6 transcript accumulates in SpxLp hybrid plutei to a level about 2-fold higher than in L. pictus eggs. RNA chain-extension in isolated nuclei indicates that the 15D6 gene is actively transcribed in nuclei isolated from both LpxLp and SpxLp mesenchyme blastula embryos, a stage of development in which the mature transcript is accumulating extensively in LpxLp embryos but not in SpxLp embryos. A third cloned sequence, 16D4, represents a transcript of a conserved gene expressed in a temporally-distinct manner in the two species. SpxLp hybrid embryos accumulate the transcript in an apparently independent and additive manner, although the mass is greater in hybrid embryos than would be expected. There is no apparent restriction of the

expression of this gene in SpxLp embryos. I previously proposed (Tufaro and Brandhorst, 1982), that transcripts up-regulated during development might accumulate normally in SpxLp embryos, and that the underrepresentation of paternal transcripts in SpxLp embryos might be due to persistent maternal RNA which is not normally replaced significantly in normal embryos. The data presented in this chapter excludes this mechanism as the only explanation for the restricted expression of paternal genes in hybrid embryos.

INTRODUCTION

One approach to distinguishing between the expression of the maternal and zygotic genome during development has been to use interspecies hybrid embryos to assess the relative contribution of the paternal, and hence the embryonic genome to development. Echinoid interspecies hybrids have been extensively investigated for nearly a century (for review, see Davidson, 1976; Giudice, 1973). The conclusion which is usually drawn from this large body of literature is that the influence on biochemical and morphological characters by the paternal genome becomes detectible only at the late blastula or later stages in most crosses.

The interpretation of many investigations utilizing echinoid interspecies hybrid embryos is dependent on the assumption that the paternal genome is expressed to the same extent, and with the same timing, as the maternal genome in normal embryos. Recent observations (Tufaro and Brandhorst, 1982; Lee and Whiteley, 1982) indicate that caution should be attached to such interpretations. Analysis of three hybrid crosses revealed an underrepresentation of paternal species-specific proteins as late as the pluteus stage of development (Tufaro and Brandhorst, 1982). Nucleic acid hybridizations with heterologous probes indicate that while the DNA of both species persists throughout development in S. purpuratus x L. pictus (SpxLp) embryos, there is an underrepresentation of paternal mRNA on polysomes in (SpxLp) hybrid gastrula embryos. There are two

possible explanations for these observations. First, much of the mRNA translated into protein detected on two-dimensional gels might be persistent maternal mRNA not normally replenished during embryonic development. Recent observations suggest that certain transcripts may persist throughout embryogenesis (Cabrera et al., 1984). Alternatively, the synthesis, processing, nuclear export, or stabilization of many paternal mRNA transcripts may be impaired in the hybrid embryo.

If the restricted accumulation of paternal transcripts in hybrid embryos is largely the result of persistence of a large mass of maternal transcripts, it might be that the signals for RNA transcription and processing are conserved between the two species and RNA accumulation in interspecies hybrid embryos reflects normal expression of the paternal genome during development. One prediction resulting from this is that transcripts accumulating in normal embryos should also accumulate in hybrid embryos. Conversely, failure of proper transcript accumulation in hybrid embryos would indicate that the mechanisms which regulate accumulation in normal embryos are not functioning.

To test this prediction, a library of cloned cDNA molecules representing L. pictus gastrula embryo cytoplasmic RNA was constructed. The library was screened to select clones for species-specific cytoplasmic transcripts indetectible in hybrid embryos. Most prevalent species-specific transcripts identified accumulate by mass in normal embryos, contrary to the prediction. It is clear from the results of this analysis that the use of highly specific cloned probes to study gene regulation in interspecies hybrid embryos may yield important insights into the regulation of gene expression during embryonic development.

MATERIALS AND METHODS

RNA Isolation

All solutions were autoclaved, if possible, and all glassware was baked before use. Embryos were layered over 1.2 M dextrose (eggs over 4:1 seawater:1.1 M sucrose) and collected. The pellets were washed once with dextrose, then once in lysis buffer (50 mM Pipes (pH 6.5), 400 mM NH_4Cl, 12 mM $MgCl_2$, 25 mM EGTA).

RNA used for cloning was isolated from early gastrulae embryos (22 h at 17°). The embryos for this preparation were lysed by passing them through a 21 gauge needle or by homogenization in a Dounce homogenizer. RNA preparations used for gel blot hybridization experiments were made by lysing embryos in lysis buffer containing 0.5% Triton, using a Dounce homogenizer.

For each sample, the lysates was centrifuged at 11,000 rpm in a Beckman JA-20 rotor for 10 min, and the RNA was precipitated from the supernatant by the addition of an equal volume of 4M LiCl, 8M urea, 0.5 mM EDTA, 20 mM Tris (pH 7.5), on ice for 1-16 h. The RNA was collected and dissolved in 0.1 M sodium acetate (pH 5), 0.5% SDS, 25 mM EGTA and extracted with an equal volume of phenol-chloroform (saturated with acetate buffer, pH 5.0). Extraction was performed at RT and at 50°, and the aqueous phase was precipitated from ethanol at -20° O/N.

Construction of a cDNA Library

cDNA Synthesis

First strand

Avian myeloblastosis virus reverse transcriptase was used to synthesize cDNA from an RNA template under conditions which yield a high proportion of full length copies (Buell et al., 1978). Reactions were carried out in autoclaved, siliconized 1.5 ml test tubes. A standard reaction mixture was 50 mm Tris (pH 8.3) at 42°, 140 mM KCl, 10 mM $MgCl_2$, 30 mM B-mercaptoethanol, 500 uM of each deoxynucleoside triphosphate, and 100 ug of oligo$(dT)_{12-18}$/ml. Actinomycin D was omitted in order to increase second-strand synthesis (Wickens et al., 1978). RNA template was added to a final concentration of 20-40 ug/ml. Reverse transcriptase was added to 1000 U/ml, a level which yields maximum cDNA mass for the amount of RNA used. Reactions were assembled on ice, vortexed gently, centrifuged briefly, and incubated at 42° for 20 min. This short reaction time minimizes transcription from ribosomal RNA.

Second strand

Reversed transcription reactions were stopped by chilling on ice, and centrifuged briefly. RNA-DNA hybrid molecules were denatured by boiling and quickly cooled. Denatured protein was collected into a pellet by brief centrifugation.

The supernatant containing single-stranded cDNA was added to an equal volume of 200 mM Hepes (pH 8.0), and 500 uM of each deoxynucleoside

triphosphate. E. Coli DNA polymerase (15 U) was added and the mixture was incubated for 2 hr at 15°.

Second strand synthesis was stopped by chilling on ice, followed by the addition of 100 ul of 10 mM dGTP, 0.1% SDS, and 25 ug yeast tRNA. The reaction was extracted at room temperature with 0.4 ml of chloroform and the organic phase and interphase were re-extracted with 100-200 ul of 20 mM NaCl.

S1-nuclease Digestion

Fractions which were excluded from Sephadex G-100 were phenol extracted and precipitated from ethanol. The DNA was collected by centifugation at 38,000 RPM in a Beckman type 50 rotor. The DNA pellet was resuspended in 300 mM NaCl, 30 mM NaOAc (pH 4.5), and 3 mM $ZnCl_2$. S1 nuclease was added to 50 U/ml and the reaction was incubated for 10 min at 15°. The reaction was phenol extracted, and the aqueous phase was passed through a 4 ml Sephadex G-50 column. Excluded fractions were pooled, EDTA was added to 20 mM, and the DNA was precipitated from ethanol.

Tailing of the cDNA with Terminal Deoxyribonucleotidyl Transferase

Double-stranded cDNA was tailed at the 3' ends with terminal deoxyribonucleotidyl transferase (Tdt) in a 100 ul reaction containing 140 mM cacodylic acid (free acid, Sigma Biochemicals), 60 mM Tris base adjusted with KOH to pH 7.6, 1 mM cobalt chloride, 0.1 mM dithiothreitol, and 4 uCi [^{32}P]-dCTP (400 Ci/mmol, New England Nuclear). The reaction

was preincubated for 5 min at 37° and chilled on ice. Exactly 30U Tdt was added and the reaction tube was incubated for 2 min at 15°, during which time 20-30 nucleoside residues were added to each end. The reaction was terminated by heating the mixture for 5 min at 60° in 0.5 M NaCl, 10 mM EDTA, extracted with phenol, and precipitated from ethanol twice.

Cesium-purified pBr322 plasmid DNA was cleaved with Pst I and dGMP-tailed with Tdt as described above.

Construction of Recombinant Plasmid DNA

Tailed ds-cDNA was mixed in molar ratios of 1:2 or 1:8 with 250 ng vector/ml in 10 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA. The molecules were annealed at 68° for 5 min, 42° for 2 hr and cooled slowly to room temperature during a 4 hr period. This DNA was used to transform E. coli as described below.

Transformation of E. Coli

A modification of the procedure of Dr. Doug Hanahan was used for all transformations (Hanahan and Meselson, 1980). Briefly, fresh colonies of DH1 bacteria were incubated in SOB broth (20 g/l bacto tryptone, 5 g/l yeast extract, 20 mM $MgCl_2$, 10 mM NaCl (pH 7.0) until the culture reached mid-log phase. The culture was made 15% in sucrose, 5% in DMSO and stored in small aliquots at -85°. About 14-20 hr before transformation, a clump of frozen cells was spread on a rich plate and streaked. Single colonies formed overnight at 37°. A 2 mm colony was picked and dispersed in 1 ml SOB. This was used to innoculate 50 ml

pre-warmed SOB. The culture was incubated at 30° with shaking until it contained 5 x 10^7 cells/ml. The cells were collected in 50 ml Falcon tubes, cooled on ice for 15 min, centrifuged at 2000 RPM for 12 min at 4°, and resuspended in 8 ml cold, autoclaved, millipore-filtered transformation buffer (10 mM $CaCl_2$, 35 mM KOAc (pH 5.9), 12.1 mg/ml rubidium chloride, and 8.9 mg/ml manganese chloride). After 10 min on ice, the cells were pelleted at 2000 RPM for 10 min at 4°, and resuspended gently in 2 ml transformation buffer. Fresh, spectrophotometrically-pure DMSO was added (35 ul), the cells were swirled occasionally for 15 min and an additional 35 ul DMSO was added. The cells were swirled occasionally for 5 min. From 200-210 ml aliquots were dispensed into 15 ml siliconized, autoclaved glass centrifuge tubes, or the entire volume was poured into one 50 ml flask. DNA, suspended in 5-25 ul of TE (10 mM Tris, 1 mM EDTA), was added to the cells. This mixture was placed on ice for 30 min and swirled occasionally to allow the cells to contact DNA. The cells were then subjected to a 42° heat shock for 90-150 sec, and cooled on ice for 2 min. At least four volumes of pre-warmed SOB was added to the cell suspension which was then incubated for 60 min at 37° to allow cells to recover. Aliquots were plated onto LB plates (per liter: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, adjusted to pH 7.6 with NaOH, 20 g agar) containing 10 ug/ml tetracycline. Colonies were allowed to form for several days before being further manipulated. Colonies were picked onto LB plates containing 10 ug/ml tetracycline. The bacteria which grew on tetracycline were tested for their ability to grow on ampicillin (50 ug/ml). Colonies which were tetracycline resistant but ampicillin sensitive were selected for the library.

Screening

Library Storage

Colonies were transferred from agar plates to individual wells of 96-well microtiter dishes. Each well contained 200 ul of L broth supplemented with (g/l) 6.3 K_2HPO_4, 0.45 sodium citrate, 0.09 $MgSO_4$, 0.9 $(NH_4)_2SO_4$, 44 glycerol (adapted from Gergen et al., 1979). Cultures were grown to saturation at 37°. Dishes were sealed and stored at -85°.

Filter replicas

Filter replicas of the library were made by dipping a replicating device in the 96-well dishes and placing it onto a piece of Gene Screen membrane on a large L broth agar plate containng 25 ug/ml tetracycline. Colonies (3mm diameter) formed in 24-30 hr at which time the filter was removed and placed onto an L broth agar plate containing 250 ug/ml chloramphenicol for 24 hr at 37° to amplify the plasmid DNA. Bacterial DNA was immobilized on these filters by a modification of the method of Grunstein and Hogness (1975). To lyse the bacteria, the filters were lifted off the chloramphenicol plates and placed onto a stack of Whatman 3MM filter paper soaked in 0.5 M NaOH for 5-10 min at which time the filter was subjected to suction for 5 min to immobilize the released DNA and to remove the NaOH solution. The filters were then successively placed, for 10 min each, onto stacks of Whatman 3mm soaked in 1) 1 M Tris (pH 7.4), 2) 0.5 M Tris, 0.15 M NaCl, pH 7.4, 3) 20X SET (1X SET = 0.15 M NaCl, 0.03 M Tris, 2 mM EDTA), and 4) 2X SET and were baked for 2 hr at 80-90° in vacuo.

Nucleic Acid Hybridization

Filters were washed in 4X SET, 5X Denhardts (1X Denhardts: 0.02% Ficoll, 0.02% PVP-40, 0.02% BSA), 5 mM EDTA for 1-2 hr at 68°. Colony debris adhering to the filter was wiped off with a gloved hand. The filters were incubated in hybridization solution (4X SET, 5X Denhardt's, 0.1% sodium pyrophosphate, 25 mM phosphate buffer, 50 ug/ml poly (rA), 50% deionized formamide, and 10% dextran sulfate) for 4 hr at 37°.

cDNA probes were made as previously described except that specific activity was optimized at the expense of mass conversion. From 10^7-10^8 cpm of [^{32}P]-cDNA was added to hybridization solution for each assay, which was injected into sealable plastic bags containing 1 or 2 filters, and incubated for 16 h at 37°. Filters were then washed in a 500 ml solution of 2X SET, 0.2% sodium pyrophosphate, 0.5% SDS for 5 min at room temperature. Dextran sulfate sticking to the filters was wiped off and filters were agitated in about 500 ml 2X SET, 0.2% sodium pyrophosphate, 0.1% SDS for 1 h at 68°. Finally, filters were gently agitated in 500 ml 0.1X SET, 0.2% sodium pyrophosphate, 0.1% SDS for 30 min at 68°, placed on Whatman 3mm paper until dry, wrapped in Saran Wrap and exposed for various times (indicated in figure legends) to Kodak X-Omat RP film with 1 Dupont Cronex Lightning-plus intensifying screen at -85°.

Plasmid Preparations

Plasmids containing insert sequences were isolated in ug quantities by several rapid isolation procedures. In one procedure, 2 ml L broth

innoculated with a fresh bacterial colony was incubated for 2 hr at 37° with agitation. From 50-100 ml M9 broth containing 1% glycerol and 2% caseamino acids was innoculated with this starter culture, and incubated at 37° with agitation until A_{550}=0.7-0.9. Chloramphenicol was added to 200 ug/ml and the culture was incubated for 12-18 hr at 37°. The cells were collected in two 50 ml tubes by centrifugation, and resuspended in 550 ul STET (8% sucrose, 5% Triton X-100, 50 mM Tris (pH 8.0), 50 mM EDTA. This solution was distributed evenly to three 1.5 ml test tubes each containing 25 ul of 10 mg/ml lysozyme (Sigma), vortexed, and placed in boiling water for exactly 45 sec. Cell debris and E. coli DNA were removed by centrifugation for 12 min at room temperature, and the plasmid DNA was precipitated from the supernatant by the addition of 200 ul 5M NH_4OAc and 1 ml cold isopropanol. The tubes were placed at -20° for 10 min and centrifuged for 10 min at 4°. The DNA pellets were washed in 70% isopropanol, 0.3% NH_4OAc, dried under vacuum for 30 min, resuspended in 10 mM Tris, 2 mM EDTA (pH 7.4) and stored either at 4° or at -20° in a non-defrosting freezer. The DNA isolated in this manner can be cleaved by most restriction endonucleases without further purification. This DNA is also suitable for dot blot hybridization assays, but contains significant amounts of contaminating bacterial RNA.

Dot Blots

DNA and RNA was dotted onto Gene Screen membrane (New England Nuclear) or nitrocellulose (Schleicher and Schuell, BA-85) by a modification of the method of Kafatos (1975). A 96-well dot blot manifold (Bethesda Research Laboratories) was used for all preparations.

DNA dots

Plasmid DNA was either restricted or acid-depurinated to insure efficient denaturation and binding to filters. Typically, a mixture of 10 ul DNA (see figure legends for concentrations), 80 ul H_2O, 10 ul 3 M HCl was assembled in a 1.5 ml tube on ice, and placed at 22° for 5 min. The DNA was then denatured by adding 10 ul 3M NaOH and incubating the mixture for 15 min at 37°. The DNA was neutralized with NH_4OAc and HCl and aliquots were loaded into wells under low vacuum. The DNA on the filter was rinsed with 1M NH_4OAc before being baked onto the filter for 2 hr at 80-90° in vacuo.

RNA dots

RNA was suspended in 1X SET, heated to 65° for 5 min, quickly cooled and loaded directly into the wells. The filters was rinsed with 1X SET prior to being baked onto the filter for 2 hr at 80-90° in vacuo.

Hybridization Conditions

The procedure for hybridizing radioactive probes to dot blots was similar to the procedure used in colony screening except the filters were not pre-washed, nor was dextran sulfate usually included. Details are contained in the figure legends.

Gel Blot Hybridization

Electrophoresis and transfer of RNA

RNA was suspended in 1X MOPS buffer (20 mM MOPS, 5 mM sodium acetate,

1 mM EDTA (ph 7.0), 6% formaldehyde, 50% deionized formamide), heated to 65° for 10 min, quick cooled and electrophoresed on a 0.8-1.5% agarose gel (Bio-Rad agarose, low M_r) in 1X MOPS buffer containing 6% formaldehyde. Following electrophoresis, RNA was visualized by staining with ethidium bromide or acridine orange and photographed under UV illumination. Sometimes gels were soaked in NaOH to nick the RNA for more efficient transfer of large molecules. Gels were neutralized for 30 min in 25 mM phosphate buffer and blotted to a Gene Screen membrane overnight. Filters were rinsed briefly in 25 mM phosphate buffer, sometimes photographed under UV illumination, and baked for 2-3 hr at 80-90° in vacuo.

Electrophoresis and transfer of DNA

The procedure was essentially that of Southern (1975). DNA was electrophoresed in TAE buffer (0.04 Tris-acetate, 0.002 M EDTA, pH 8.0), denatured in 1.5 M NaCl, 0.5 M NaOH for 45 min, neutralized in 1 M Tris (pH 8.0), 1.5 M NaCl for 20 min, soaked in 25 mM phosphate buffer or 1X SSC for 20 min, and blotted to a Gene Screen membrane. When blotting was complete, the filter was baked for 2-3 hr at 80-90° in vacuo.

Hybridization conditions for blots

Hybridization was carried out at 37°. Filters were placed in plastic bags and incubated with 5X SET, 0.2 M phosphate buffer, 5X Denhardts, 250-500 ugml sheared salmon sperm DNA, 100 ug/ml poly(rA), and 50% formamide. Pre-hybridization continued for 4-16 hr, at which time the solution was removed, and 0.5 ml of fresh solution containing 20-50 ng/ml nick-translated probe (0.5-2 x 10^8 cpm/ug) was added per cm^2 of filter

area. The probe was allowed to hybridize for 16-24 hr . Filters were washed as described above for colony hybridization.

Nick-translation of DNA

Routinely, 200-500 ng of plasmid DNA was nick-translated using a BRL nick-translation kit to a specific activity of 0.5-2 x 10^8 cpm/ug . Reactions were carried out for 0.5-1.5 h at 15°, at which time the DNA was denatured and passed through at Sephadex G-50 column to remove unincorporated nucleotides. DNA was denatured again before being used as a probe.

Sub-cloning into phage M13

All of the procedures used for constructing and identifying cloned sequences were exactly as published previously (Messing et al., 1981). Replicative-form DNA of M13-Mp8 and M13-Mp9 was a kind gift from Dr. D.P.S. Verma.

Recovery of DNA from Gels

Agarose gels

The procedure of Girvitz et al. (1980) was used for isolating restriction fragments from gels. Briefly, the DNA was electrophoresed onto a piece of Whatman 3MM paper backed by a single layer of dialysis membrane. The DNA was eluted off in 0.2 M NaCl, 0.1% SDS and precipitated from ethanol.

Polyacrylamide Gels

The procedure of Maxam and Gilbert (1977) was used to extract DNA from 0.7 mm thick, 0.4% polyacrylamide gels. Briefly, a band containing DNA was cut out, crushed, and the DNA was eluted in 0.5 M ammonium acetate, 1 mM EDTA (pH 8.0) at 37° O/N, and precipitated from ethanol.

Chain-extension assay

Isolation of Nuclei

Sea urchin embryonic nuclei were isolated according to the procedure of Morris and Marzluff (1983). All manipulations were performed at 0-4°. Briefly, embryos were washed with 1) 0.55 M KCl, 2) 2-3 vol 0.25 M Sucrose, 100 mM Tris (pH 8.0), 0.1 mM EDTA; and resuspended in 5-10 vol of Buffer I (0.32 M sucrose, 5 mM $MgCl_2$, 10 mM Tris (pH 8.0), 1 mM EGTA, 1 mM DTT, 1 mM Spermidine). Embryos were homogenized in a Dounce homogenizer. About 2 vol of Buffer II (Buffer I containing 2 M sucrose) was added to the homogenate which was then gently layered onto a 2 ml pad of Buffer II. Nuclei were subjected to centrifugation at 50,000 x g for 40 min, and resuspended in glycerol storage buffer at 3-5 x 10^8 nuclei/ml (25% glycerol, 50 mM Tris (pH 8.0), 1 mM EGTA, 1 mM Spermidine, 1 mM DTT, 5 mM $MgCl_2$, 0.1 mM PMSF), and used immediately or stored frozen in liquid nitrogen.

Chain-extension Reaction

All reactions were assembled on ice. Typically, 200 ul reactions

contained 100 ul reaction buffer (40 mM NaCl, 100 mM potassium acetate, 2.5 mM $MgCl_2$, 0.3 M glycine, 10 mM Tris [8.0]) and 50 uM each ATP, CTP, GTP and 250-300 uCi [^{32}P]-UTP (>3000 Ci/mmol, Amersham). Reactions were initiated by adding 100 ul nuclei in glycerol storage buffer and placing the reaction at 20°. To monitor the reaction, aliquots were spotted onto DE-81 filters, which were then washed extensively in 0.5 M phosphate buffer (pH 6.5) or in 0.3 M ammonium formate followed by 0.3 M ammonium bicarbonate, and counted. After 20-30 min, reactions were frozen or RNA was isolated immediately.

The RNA isolation procedure was adapted from Groudine et al., 1981). Briefly, nuclei were treated with 20 ug/ml DNase I for 5 min at 20°, and 100 ug Proteinase K for 30 min at 42°. The reaction mixture was extracted with phenol-chloroform, and the RNA in the aqueous phase precipitated from 5% cold TCA containing 1.5% sodium pyrophosphate. After 30 min, the precipitate was collected onto a nitrocellulose filter (Schleischer and Schuell, BA85) by gentle suction, and rinsed with 30 ml cold 3% TCA. The filter disk was placed in a glass vial and 1.8 ml DNase buffer (20 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 1 mM $CaCl_2$) and 25 ug DNase I was added. After 30 min at 37°, EDTA was added to 15 mM and SDS to 1%, and the RNA was eluted off of the filter at 65°. After 10 min, the eluate was removed and the filters were incubated for an additional 10 min in 0.5 ml 1% SDS, 10 mM Tris (pH 7.5), 5 mM EDTA at 65°. The eluates were combined and treated with 25 ug/ml Proteinase K at 37° for 30 min, phenol extracted, and the aqueous phase was precipitated from 0.1 M NaCl and 2.5 vol cold ethanol at -20°. The RNA precipitate was collected and resuspended in hybridization solution or stored in 10 mM Tris (pH 7.5), 2 mM EDTA at -85°.

Transcription in the Presence of α-amanitin

α-amanitin (1 ug/ml) was added to nuclei and transcription was allowed to proceed as outlined above for 25 min. A parallel control reaction without α-amanitin was run to establish the level of normal transcriptional activity. Aliquots were removed at 3 min intervals and spotted onto DE-81 filters, which were then washed and counted.

Hybridization of RNA to Plasmid Dots

Plasmid DNA was dotted onto nitrocellulose filters as described above for DNA dot blots. Pre-hybridization was carried out for 1 h in hybridization solution containing 10% Dextran sulfate (Wahl et al., 1979). Hybridization of heat denatured, chain-extended RNA probe was allowed to proceed for 72-90 h at 37°. Filters were washed in 2X SET for 15 min at 22°, 3 x in 2X SET, 0.1% SDS for 1 h at 68°, and 1 x in 0.2X SET at 68°, and exposed to Kodak XAR-5 film with intensifying screens at -85°. The autoradiographic signal was quantitated by densitometric scanning (Bio-rad densitometer) and the tracings were integrated using a Hewlett-Packard 3390A integrator. Spots were also cut out and scintillation counted.

RESULTS

Construction of a Gastrula cDNA Library

Cytoplasmic RNA from L. pictus gastrula embryos (LpxLp embryos, lysed in the absence of detergent to reduce mitochondrial RNA contamination) was isolated and poly(A)-RNA transcripts were selected by affinity chromatography on oligo(dT)-cellulose. This fractionation step eliminated most of the mass of ribosomal RNA, and some of the residual mitochondrial transcripts. It is known that the 16S mitochondrial ribosomal transcripts can be retained on oligo(dT)-cellulose (Ojala et al., 1982,; Dworkin and Hershey, 1981; Dworkin et al., 1981). Some mitochondrial transcripts are included in the cDNA library (see below).

Oligo(dT)-primed cDNA synthesis using the poly(A)-containing RNA as template was carried out, the homologous strand was synthesized, and the double-stranded cDNA was tailed with C residues. This tailed cDNA was annealed with Pst I restricted, G-tailed pBr322 and these recombinant plasmids were used to transform bacteria. Colonies forming after several days of growth were picked into individual wells of 96-well microtiter dishes.

1030 colonies were picked for further analysis. About 90% of the original tetracycline-resistant colonies were ampicillin-sensitive, indicating the presence of an insert in the plasmid. This "library" of cDNA sequences was replicated by transferring and growing 2-3 mm colonies directly onto Gene Screen membranes which were placed on agar containing

tetracycline. The colonies were lysed and the DNA was denatured *in situ*, and prepared for colony hybridization.

Screening the cDNA Library by Colony Hybridization

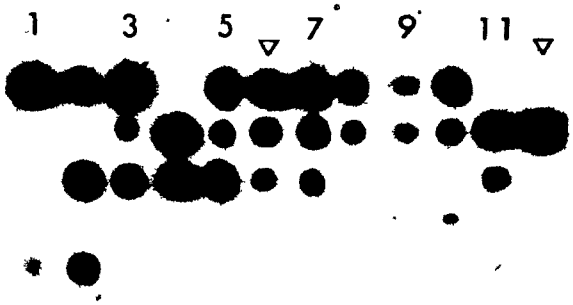
Preliminary Characterization

I first screened the colonies with cDNA transcribed from LpxLp gastrula poly(A)-RNA. It is known that sea urchin and other organisms contain transcripts that are present in the cytoplasm in a range of abundance and this should be reflected in the intensities of hybridization to different colonies. Two filters were incubated with 2.5×10^7 cpm of [^{32}P]-labelled cDNA. The hybridization solutions contained 10% dextran sulfate to increase the rate of hybridization of the single-stranded probe to the DNA on the filters. Incubation was for 36 h and was followed by extensive washing of the filters. Filters were exposed to X-ray film for 1–4 days. Only 15% (157 colonies) of the library gave clear positive signals (compared to the background hybridization to pBr322) in this screen. Clones corresponding to less prevalent transcripts gave very low signals and were not selected for further analysis. The selected colonies were replated on plates 15 and 16.

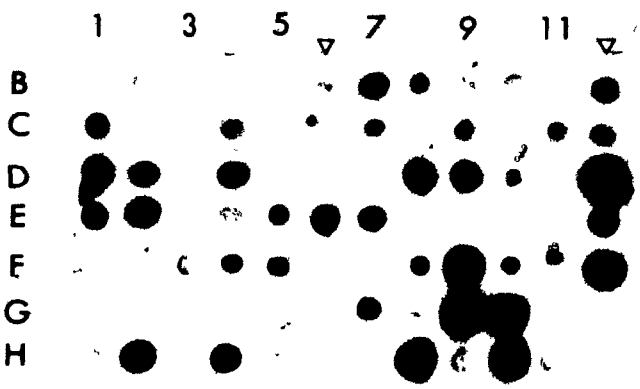
Fig.10 (LL Gast.) shows a typical hybridization of gastrula cDNA to the selected colonies. The hybridization signals indeed represented a range of abundance which were arbitrarily divided into four signal levels. Clone 15A1 is highly abundant and is of mitochondrial origin, 16C12 is abundant and non-mitochondrial, 16H1 is moderately abundant, and 16B3 is barely detectible above background but does contain an insert (henceforth, mitochondrial clones will be designated with an M after the library position number).

Fig.10. Colony filter hybridization analysis of gastrula stage transcript prevalence in L. pictus, S. purpuratus and hybrid embryos. Library dishes (15 and 16) containing 157 colonies of cloned sequences were replicated onto Gene Screen. The filters were then probed with 2.5×10^7 cpm cDNA prepared to RNA of: LL EGG, L. pictus eggs; LL GAST, L. pictus gastrulae; SL GAST, S. purpuratus x L. pictus early gastrulae; SS GAST, S. purpuratus early gastrulae. Library positions are identified by: dish number, row letter, and column number. Positions 15H12 and 16H12 represent ampicillin- and tetracycline-resistant colonies which establish the level of background hybridization. The autoradiographs shown were exposed for 24 h with intensifying screens at -70°.

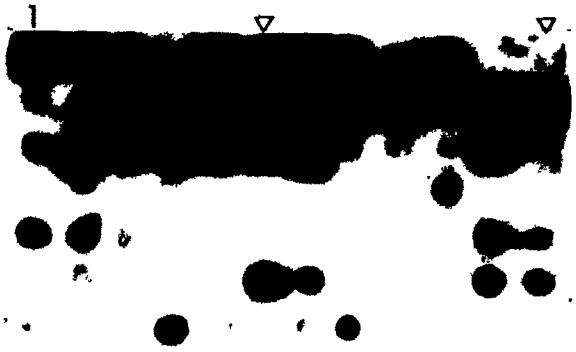
FILTER 15

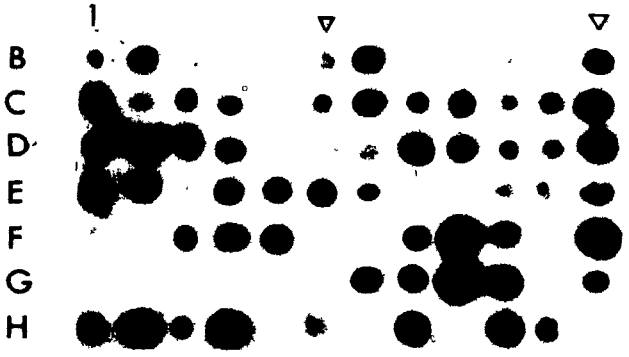


16

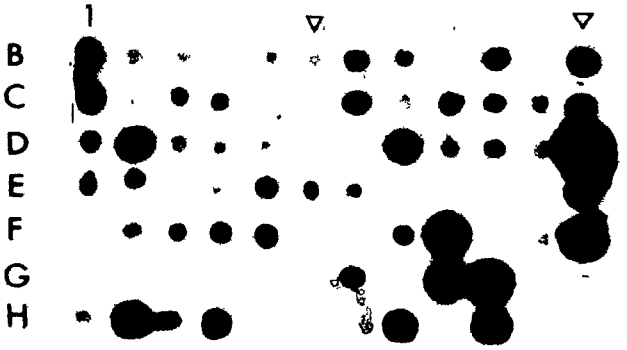


LL EGG

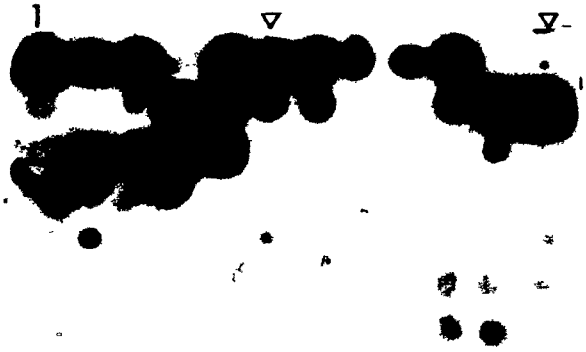


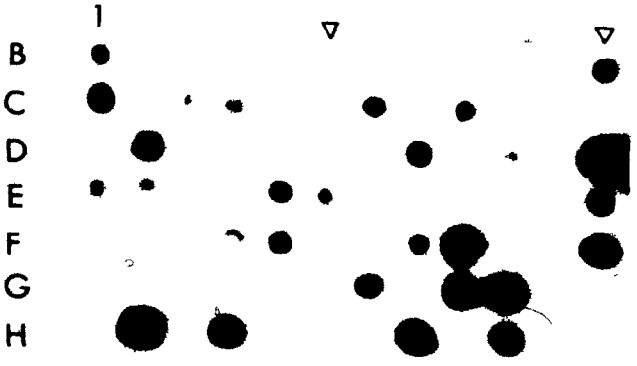


LL GAST.



SL GAST.





SS GAST.

cDNA probes were then prepared using LpxLp egg, LpxLp gastrula, SpxLp hybrid gastrula, and SpxSp gastrula (S. purpuratus) RNA as templates in oligo(dT)-primed reactions. Replicates of filters 15 and 16 were hybridized as above and the results are shown in Fig.10. Mitochondrial DNA was also isolated from LpxLp embryos and nick-translated to high specific activity. This DNA was hybridized to colonies on filters 15 and 16 to identify mitochondrial sequences; 32% of the colonies were shown to contain sequences homologous to mitochondrial DNA. Of the 107 non-mitochondrial clones remaining, 25 or about 24% showed reproducible increases in relative signal intensities between egg and gastrula stages.

Some Species-specific Transcripts Accumulate With Development

Some 23 clones were specific to LpxLp embryos at the gastrula stage of development when compared to SpxSp embryos in the colony screens; 20 of these were developmentally up-regulated to various degrees. The variety of levels of accumulation of specific transcripts with development is well illustrated in Fig.10. While 16B7 accumulation is subtle, 15D6 accumulation is spectacular.

Mitochondrial sequences generally were conserved between species and not developmentally regulated, although exceptions to both characterizations were evident. 15F10M is species-specific while 16F4M, 15B1M, 16E12M and 16C7M are not. 16G8M is developmentally up-regulated and species-specific. Further characterization of some of these sequences is contained in Appendix I.

Most Developmentally-regulated Sequences Do Not Accumulate in SpxLp Hybrid Gastrulae

Few developmentally up-regulated species-specific transcripts

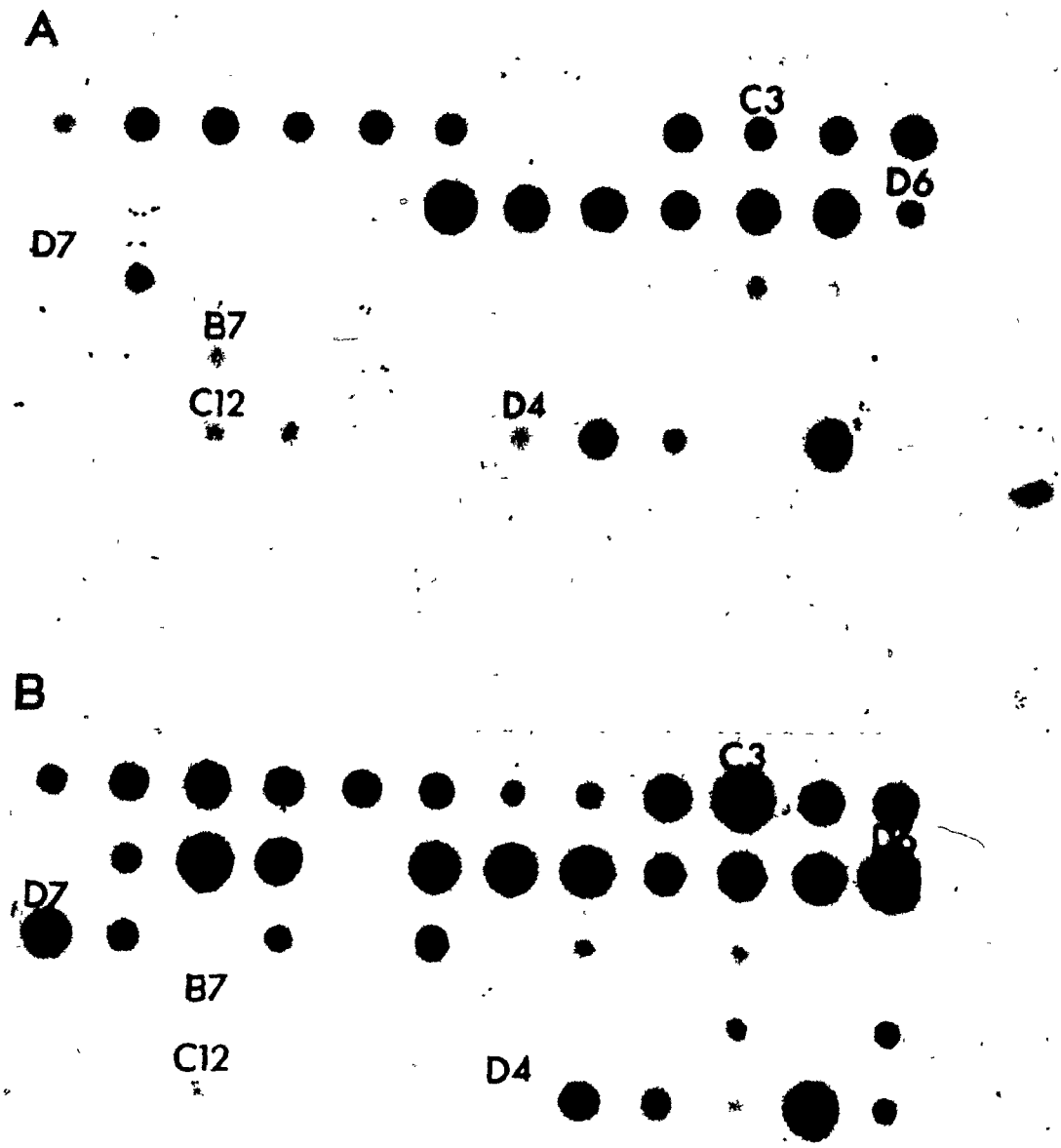
accumulate in SpxLp hybrid gastrulae. 15C9, 15D6, and 15D7 are especially notable; these clones have been shown to contain different insert sequences. The striking increase of these sequences in the cytoplasmic RNA fraction of LpxLp gastrulae with development makes it unlikely that the lack of SpxLp hybrid expression seen is due to artefacts or limitations of the screening procedure.

16B7 and 16C12 appeared to accumulate to near normal levels in SpxLp hybrid embryos. The many sequences which appeared to remain at a constant prevalence in LpxLp RNA samples between egg and gastrula stages represent maternal sequences which may or may not be replaced during development. The lack of information about the transcription of these particular sequences did not allow me to conclude whether expression of these sequences in SpxLp hybrid embryos was normal or abnormal in relation to the expression in LpxLp embryos. One of these sequences (16D4) was selected for further investigation on the basis of its behaviour in plasmid screens (see below).

Screening of isolated plasmids

Plasmids were isolated from colonies containing sequences which were still of interest after the colony screens (principally non-mitochondrial). The plasmid DNA isolated from DH1 bacteria for each clone was isolated from single bacterial colonies which were then picked and amplified. Fig.11 shows a sample of plasmid DNA dots hybridized with cDNA transcribed from cytoplasmic poly(A)-RNA from LpxLp eggs and LpxLp plutei. While different dotted clones do not contain the same mass of insert sequence, the dots to be compared between each filter do contain

Fig.11. Plasmid DNA dot blot hybridization analysis of egg and pluteus stage transcript prevalence. Plasmid DNA from a subset of colonies analyzed in fig.10 was isolated, linearized, denatured, and from 1-5 ug of each preparation was dotted onto Gene Screen. An equal mass of each plasmid was spotted on each of the two filters, whereas the mass of different plasmid samples were not necessarily identical. The plasmid DNAs were then probed with 1 x 10^6 cpm [^{32}P]-cDNA prepared to RNA of: A, L. pictus egg; B, L. pictus plutei; and detected by autoradiography. The autoradiographs shown were exposed for 8 h with intensifying screen at -70°. Alphanumeric symbols indicate: C3, clone 15C3; D6, clone 15D6; D7, clone 15D7; B7, clone 16B7; C12, clone 16C12; D4, clone 16D4.


A
C3
D6
D7
B7
C12
D4
B
C3
D7
B7
C12
D4

the same mass. The most highly developmentally up-regulated sequences identified in the colony screen again are shown to accumulate extensively during embryogenesis; for example, 15D6 and 15D7 accumulate extensively by the pluteus stage. 15C3 does not accumulate as extensively in the pluteus stage embryo (Fig.11B), or gastrula stage embryo (not shown) as it appears to in colony hybridizations. The amount of developmental up-regulation of 15C3 transcripts apparent in the colony screen (Fig.10) is artefactual and is probably due to contamination from the mitochondrial colony adjacent to 15C3 in the library. 16C12 and 16B7 did not appear to accumulate as dramatically in this screen as in the colony screens. 16D4 transcripts appear to decrease in plutei compared to eggs, but appeared to be present in similar amounts in eggs and gastrulae. This pattern of 16D4 expression was unique among the cDNA clones analyzed.

16B7 and 16D4 cDNA Clones Include Repetitive Sequences

Sheared LpxLp gastrula DNA was nick-translated and hybridized to plasmid DNA dotted on filters. This was performed principally by Ronald Conlon. It was found that while most of the signals were low (particularly for 15D6), the signal for 16D4 was significantly higher than most and 16B7 was hundreds of times higher than 16D4. The 16B7 cloned insert thus includes a highly repetitive sequence of the type which have been shown to exist in the genome of most organisms, including sea urchins (see Davidson and Posakony 1982, for review). Hybridization of nick-translated 16B7 DNA to restricted sea urchin genomic DNA blotted onto nitrocellulose filters confirmed the presence of a highly repetitive sequence element. The structure of the 16B7 cDNA clone is being further analyzed by Ronald Conlon.

Identification and Behaviour of Cloned Transcripts

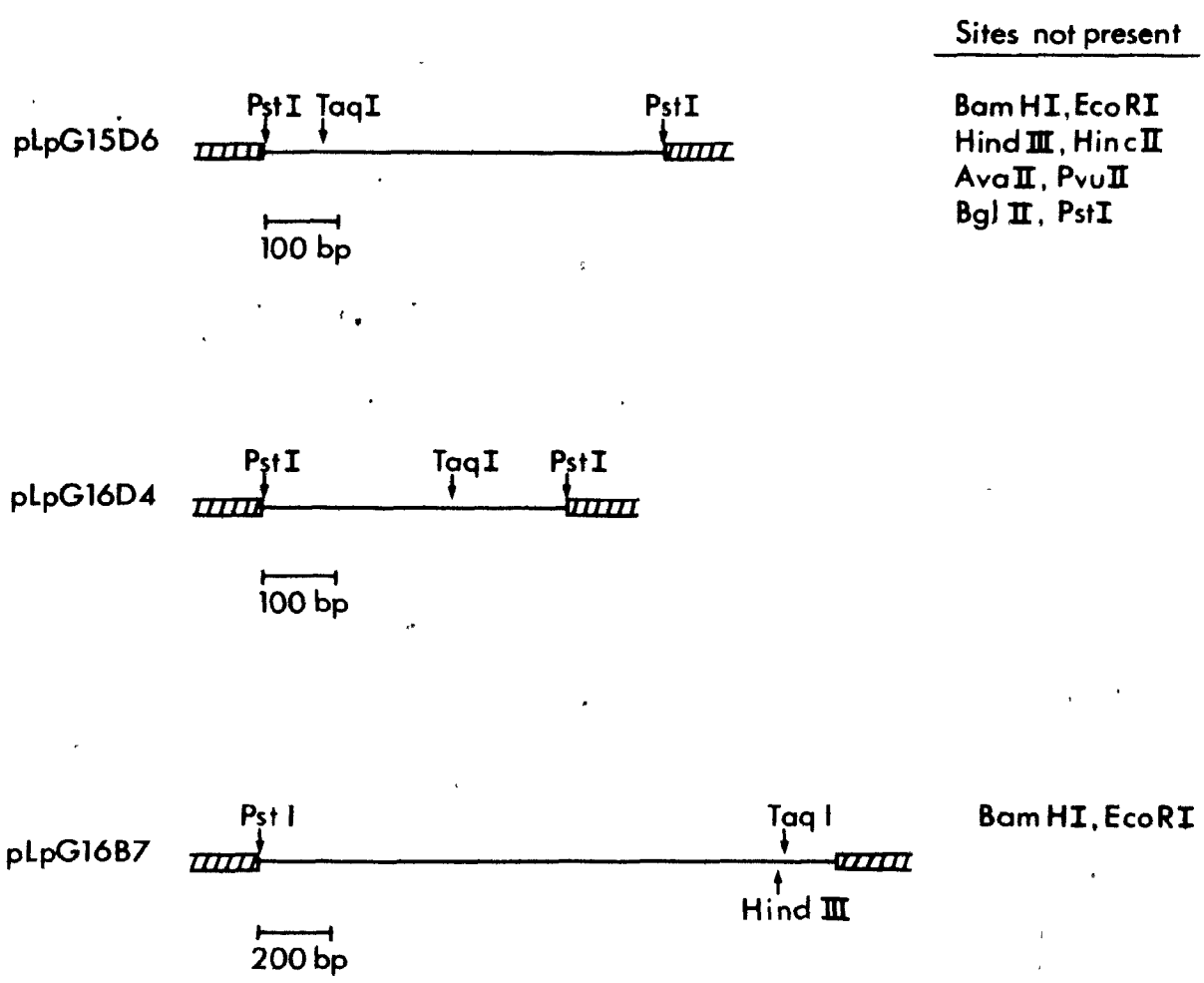
Cytoplasmic RNA was prepared from a developmental series of LpxLp, SpxLp hybrid and SpxSp embryos. To minimize degradation during preparation and to minimize loss of transcripts which may be more prevalent in one fraction than another, this RNA was not fractionated into poly A-containing and -lacking fractions. RNA samples were subjected to electrophoresis in agarose gels containing formaldehyde. RNA was visualized by ethidium bromide or acridine orange staining and photographed. RNA was transferred from the gels onto Gene Screen membranes by standard blotting procedures. Sometimes, the Gene Screen blot was visualized by UV light and photographed to establish the efficiency of transfer and fidelity of blotting across gel lanes (see Appendix 2). Gel blots were also prepared from RNA isolated from different cultures to minimize the chance of artefactual patterns of transcript expression. It should be noted that significant differences in transcript size or relative prevalence were never observed. Nick-translated recombinant plasmid DNAs were hybridized to the RNA filter blots. Specific activities of 0.5-2.5 x 10^8 cpm/ug were routinely obtained for these probes.

Fig.12 illustrates preliminary restriction maps of the three clones analyzed. 16B7 has the largest insert identified in the library to date. These maps facilitated sub-cloning in later experiments.

16B7 Hybridizes to Many Transcripts

This repeat-containing transcript hybridizes to a smear of bands on gel blots ranging in size from less than 1 kb to greater than 30 kb

Fig.12. Preliminary restriction maps of pLpG15D6, pLpG16D4, and pLpG16B7. Plasmids were cleaved with restriction enzymes, resolved on agarose or polyacrylamide gels and detected by staining with ethidium bromide. DNA in agarose gels was blotted onto Gene Screen and hybridized to nick-translated restriction fragments to detect small fragments obscured by bacterial RNA contaminating the plasmid preparations. Markers used were lambda phage cleaved with Hind III, pBr322 cleaved with various enzymes, and the 123 bp ladder obtained from Bethesda Research Laboratories. Restriction sites which were searched for and not detected are listed.

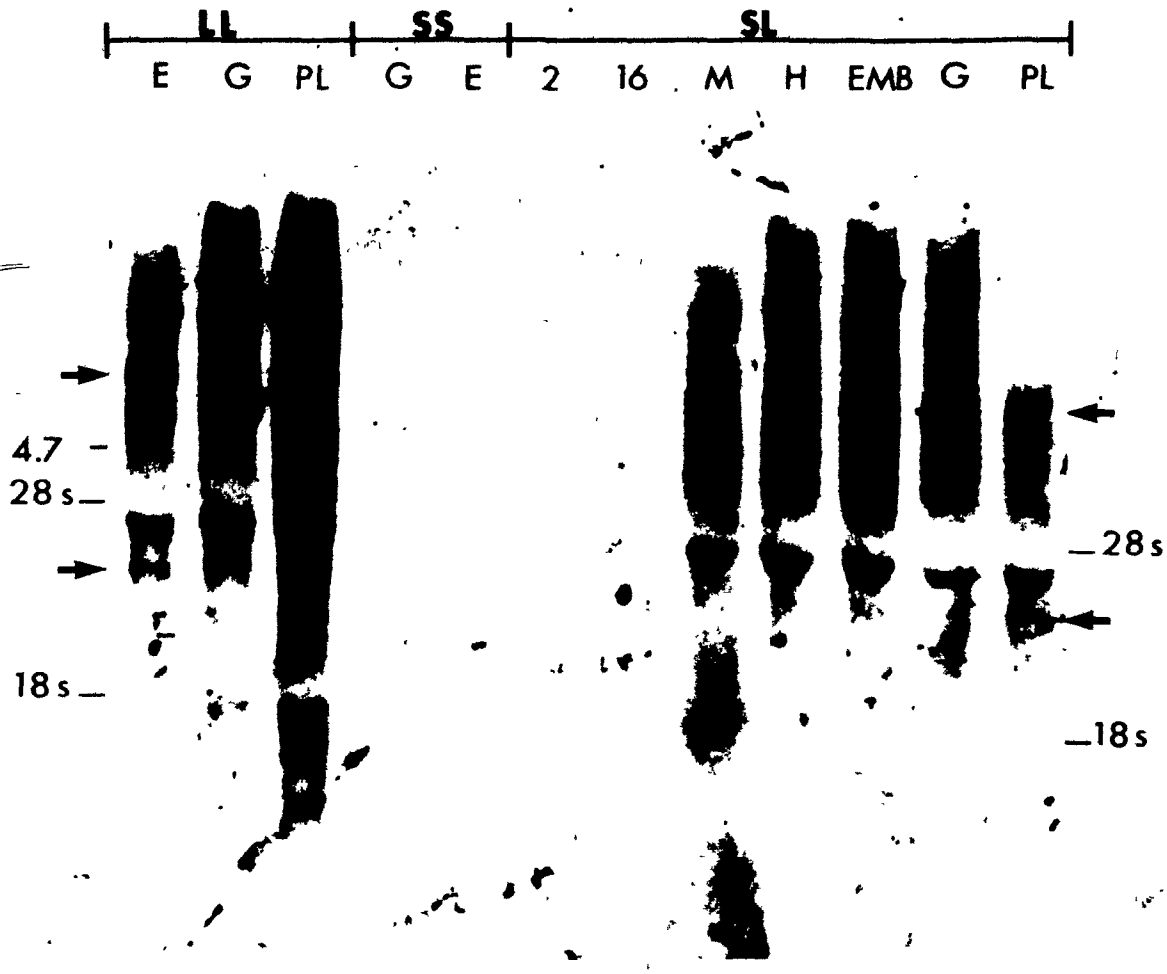

Sites not present
pLpG15D6
PstI TaqI
PstI
100 bp
BamHI, EcoRI
HindIII, HincII
AvaII, PvuII
BglII, PstI
pLpG16D4
PstI
TaqI
PstI
100 bp
pLpG16B7
Pst I
Taq I
Hind III
200 bp
BamHI, EcoRI

(Fig.13). This pattern of expression does not change significantly during development. Single-copy fragments of 16B7 have not yet been identified; repetitive sequence elements are distributed over at least the internal 1200 nucleotides of the insert (R. Conlon, personal communication). The transcripts sharing sequences with 16B7 are highly enriched in nuclei of embryos (R. Conlon, personal communication), and the cytoplasmic transcripts observed in Fig.13 might be the result of artefactual nuclear leakage.

This striking pattern of transcript expression appears in SpxLp hybrid embryos as early as the morula (64-100 cell) stage of development (Fig.13). The discreet band (upper arrow) does not appear at this stage but does appear by the hatching blastula stage. It is interesting that the entire smear appears at the same time as if the regulation of these sequences were coordinated in some fashion. The transcripts are not detectible in SpxSp egg or embryo RNA, thus confirming the species-specific expression of these transcripts. The SpxLp hybrid RNA samples were made from the same batch of S. purpuratus eggs. While I cannot rule out the possibility that the similar transcript pattern observed for SpxLp and LpxLp RNA samples are made up of entirely different transcripts as a result of abnormal expression of the L. pictus genome in the "hybrid environment", this seems unlikely, particularly because of the appearance of the discreet bands having identical mobilities. The overall prevalence of the transcript smear does not reach the LpxLp pluteus level at any stage of SpxLp development analyzed. Since few prevalent bands are resolvable, the smear may represent the expression of many relatively rare transcripts. I conclude from this data that the genes representing 16B7 are active in SpxLp hybrid embryos at an early stage of development.

Fig.13. RNA transfer blot hybridization analysis of 16B7 transcript representation in L. pictus, S. purpuratus, and hybrid embryos. Total cytoplasmic RNA was prepared, and after denaturation, 10 ug samples in each lane were separated on agarose gels containing formaldehyde. After transfer to Gene Screen, the RNA was probed with nick translated 16B7 plasmid DNA, and detected by autoradiography. LL lanes correspond to RNA of L. pictus embryos of various stages, while SS and SL lanes correspond to S. purpuratus embryos and S. purpuratus x L. pictus hybrid embryos, respectively. The positions of size markers are shown. Arrows indicate discrete bands and are discussed in Results.

Stages: E, egg; 2-16, blastomere numbers; M, morula (64-100 cells); H, hatching blastula; EMB, early mesenchyme blastula; G, gastrula; PL, pluteus.


LL
SS
SL
E G PL G E 2 16 M H EMB G PL
4.7
28 s
18 s
28 s
18 s

There is not a gross underrepresentation of these transcripts by mass in hybrid embryos. These transcripts accumulate in the SpxLp cytoplasm and remain almost constant in mass during development of L. pictus embryos. These data confirm the results of the colony hybridization screen.

16D4 Transcripts Accumulate in Early Cleavage and Degrade by the Gastrula Stage

16D4 contains a 420 bp insert representing a 900 nt transcript. The single transcript has detectibly increased in mass by the 8-cell stage in LpxLp embryos (Fig.14A). The transcript accumulates during early development reaching a peak in hatching or mesenchyme blastulae and then rapidly disappears from the cytoplasm of LpxLp gastrulae (Fig.14A,B,C,). The changes in prevalence of 16D4 transcripts are plotted in Fig.15. It declines to a level consistently lower in gastrulae than in the eggs, and is ten-fold lower in plutei than in gastrulae.

Surprisingly, this sequence is not species-specific and has a counterpart in SpxSp embryos, which accumulate a transcript unresolvable in size from the LpxLp transcript. The pattern of expression is somewhat different however. There is no detectible accumulation by the 16-cell stage, and the transcript declines in prevalence earlier than it does in LpxLp embryos. The rapid and dramatic decline in transcript prevalence is precisely conserved between the species but the timing is different. Pluteus and gastrula stage levels were confirmed by RNA dot blots.

Three blots are included in Fig.14 to illustrate the reproducibility of this pattern in different RNA preparations on different gels. While the exposures are not equalized, the pattern of expression is reproducible. While it was difficult to obtain RNA samples from the same

Fig.14. RNA transfer blot hybridization analysis of 16D4 transcript representation in L. pictus, S. purpuratus and hybrid embryos. Total, cytoplasmic RNA was prepared and, after denaturation, 10 ug samples in each lane were separated on agarose gels containing formaldehyde. After transfer to Gene Screen, the RNA was probed with nick translated 16D4 plasmid DNA, and detected by autoradiography. LL lanes correspond to RNA of L. pictus embryos of various stages, while SS and SL lanes correspond to S. purpuratus embryos and S. purpuratus x L. pictus hybrid embryos, respectively. A, B, and C represent different transfer blots. The developmental series of LL RNA within each blot was prepared by fertilizing the eggs of a single L. pictus with the sperm of a single L. pictus. The series of SS and SL RNA samples were prepared by fertilizing the eggs of a single S. purpuratus with the sperm of a single S. purpuratus or the L. pictus used to create LL embryos, respectively. LL RNA samples in A and C are from the same preparations. Filter B was washed in an identical manner to filters A and C and was then treated with 1000 U S1-nuclease for 30 min at 37° before exposing. This reduced non-specific filter background while it preserved the relative signal intensities. The RNA on filter C was previously probed with nick-translated 15D6 and is shown in fig.15.

Stages: E, Egg; 2-16, blastomere number; B, blastula; G, gastrula; M, morula (64-100 cells); P, pluteus.

Dashed lines indicate blank lanes. Arrows serve as lane markers to indicate positions of low or indetectible signal. A 900 nt size marker is shown in C. (A) 36 h exposure, (B) 168 h exposure, (C) 72 h exposure.

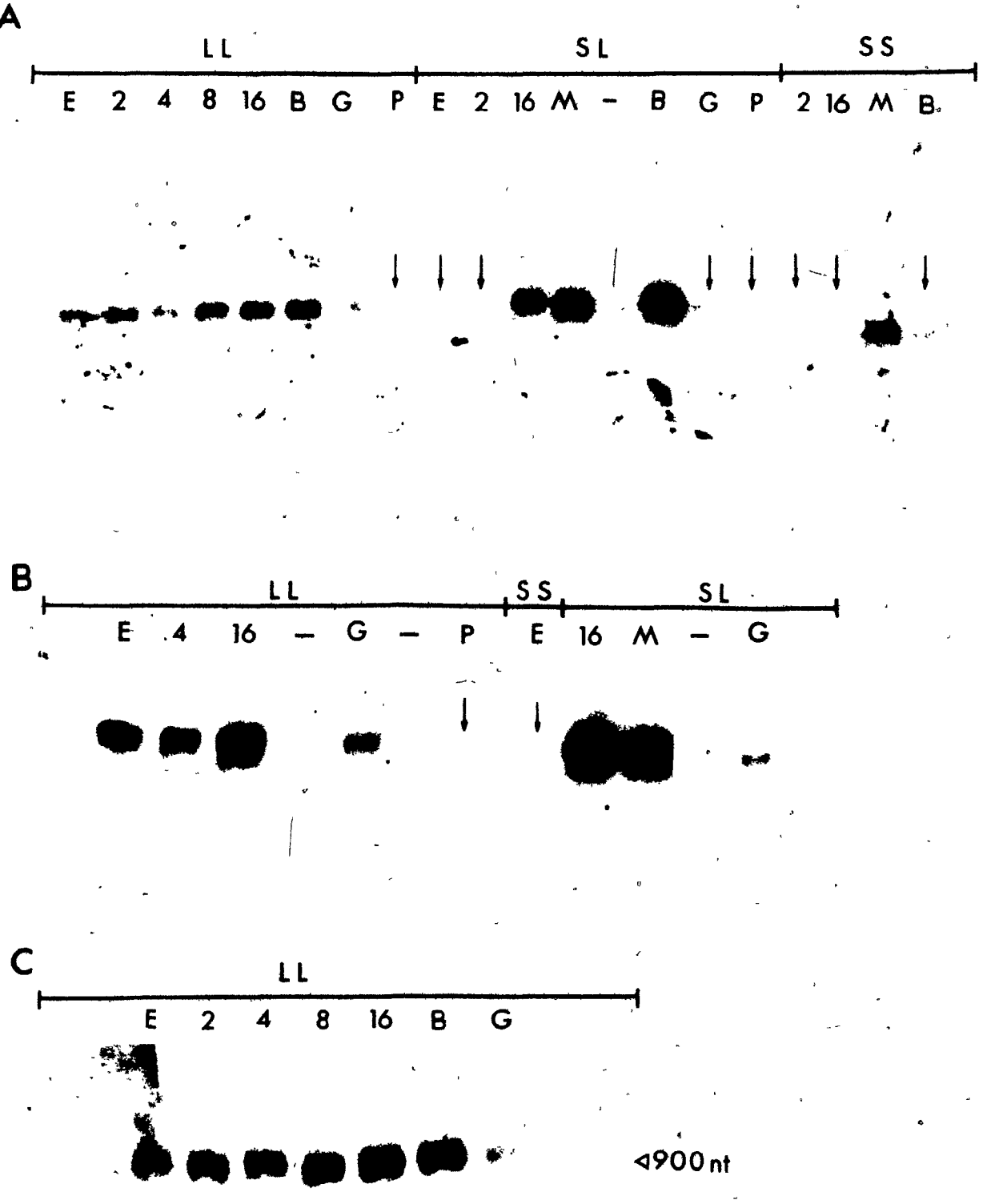
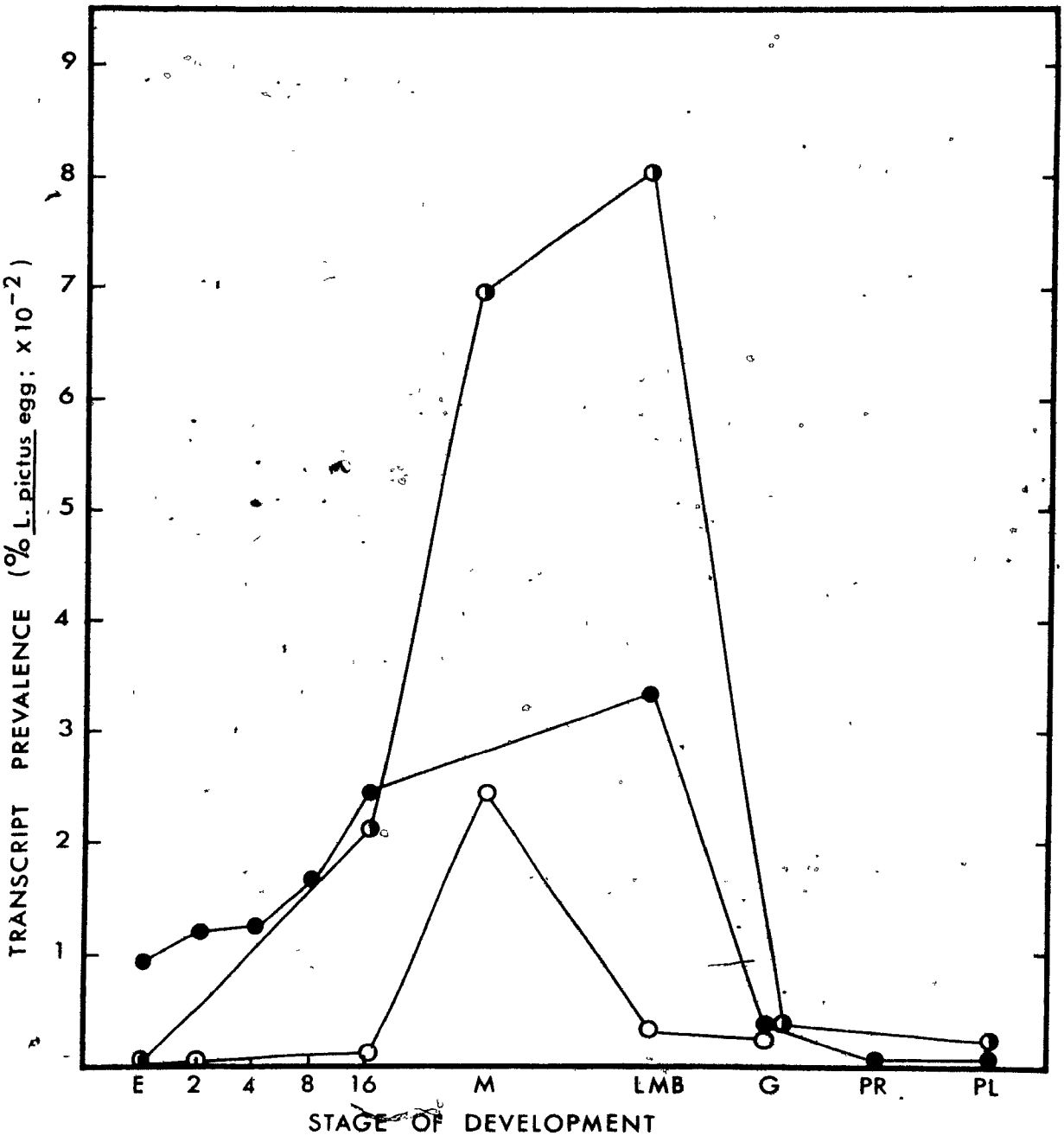

A
LL
SL
SS
E 2 4 8 16 B G P E 2 16 M – B G P 2 16 M B
B
LL
SS
SL
E 4 16 – G – P E 16 M – G
C
LL
E 2 4 8 16 B G
◁900 nt

Fig.15. Quantitative analysis of 16D4 transcript representation in L. pictus, S. purpuratus and hybrid embryos. The autoradiographic signal on each film was scanned by reflectance using a Bio-rad densitometer and integrated using a Hewlett-Packard 3390A integrator. Exposures in the linear range of the film were compared and numerical data were corrected for the different times of exposure. The stages of development are plotted for clarity and do not represent a linear time scale. The egg to 16-cell stage represents 3 h, 45 min of development while LMB to PR represent 25 h of development at 16°.
Stages:E, Egg; 2-16, blastomere number; M, morula (64-100 cells); LMB, late mesenchyme blastula; G, gastrula; PR, prism; PL, pluteus.

Symbols: (●) LpxLp; (○) SpxSp; (◑) SpxLp interspecies hybrids.


TRANSCRIPT PREVALENCE (% L. pictus egg; x 10⁻²)
9
8
7
6
5
4
3
2
1
E
2
4
8
16
M
LMB
G
PR
PL
STAGE OF DEVELOPMENT

batch of embryos for all stages evaluated, closely spaced developmental time-points were always obtained from the same batch of sea urchins.

The expression of 16D4 in SpxLp hybrid embryos is also shown in Fig.14A. S. purpuratus eggs start off with a lower maternal level of 16D4 transcript than L. pictus eggs. The SpxLp hybrid embryo quickly overcomes this deficiency so that the 16-cell embryo contains at least as much transcript, by mass, as the LpxLp embryo, and is clearly higher than the SpxSp embryo at this stage. The timing of expression in the principally maternal cytoplasm appears to be that of the paternal genome, whether or not the actual transcription is from one or both parental complements. Eventually, the SpxLp expression "overshoots" the level of either parental complement, and is greater than the sum of the two. The rapid decrease in transcript prevalence in gastrula embryos is similar to that seen in the paternal species.

The Expression of 15D6 Cytoplasmic Transcript is Restricted in SpxLp Hybrid Embryos

Figs.16 and 17 show the RNA gel blots and quantitative data respectively for 15D6 cytoplasmic transcripts in LpxLp, SpxLp hybrid and SpxSp embryos. 15D6 which has an insert of 560 bp, is homologous to two small transcripts in L. pictus eggs and either one or two (unresolvable) transcripts in later stages of developments. The smaller egg transcript is 900 nt, and is unresolvable from the 16D4 transcript in Fig.14C (Fig.14C is the second usage of the same gel blot shown in Fig.16). The larger egg band is about 1100 nucleotides and may represent a form of the lower band differing in the extent of polyadenylation (Tansey and Ruderman, 1983). The larger faint transcript is approximately 1700 nt and

Fig.16. RNA transfer blot hybridization analysis of 15D6 transcript representation in L. pictus, S. purpuratus, and hybrid embryos. Total cytoplasmic RNA was prepared, and after denaturation, 10 ug samples in each lane were separated on agarose gels containing formaldehyde. After transfer to Gene Screen, the RNA was probed with nick translated 15D6 plasmid DNA, and detected by autoradiography. LL lanes correspond to RNA of L. pictus embryos of various stages, while SS and SL lanes correspond to S. purpuratus embryos and S. purpuratus x L. pictus hybrid embryos, respectively.

Stages: E, egg; 2-16, blastomere numbers; MB, mesenchyme blastula; G, gastrula; PR, prism; PL, pluteus. The positions of size markers are shown.

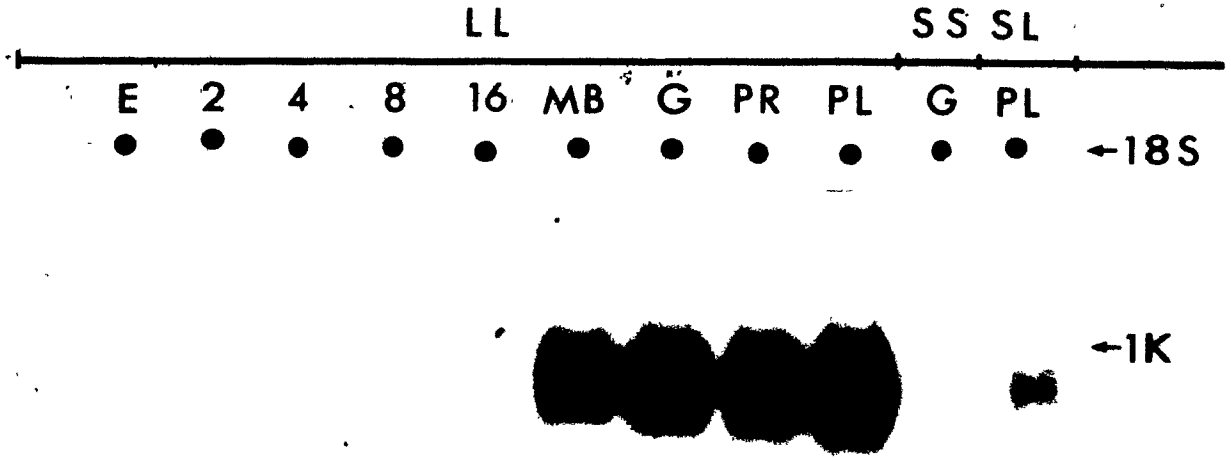
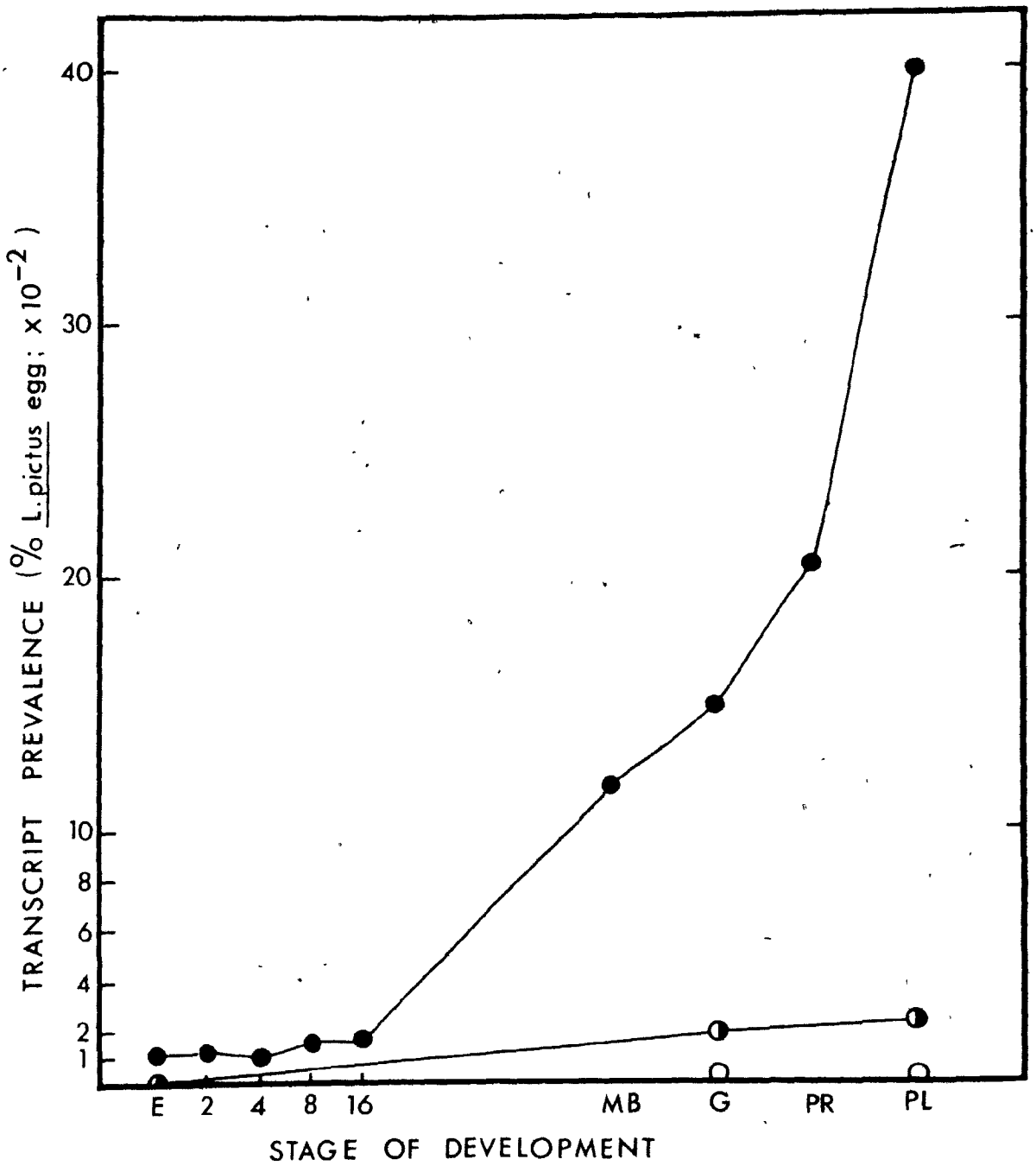

LL
SS SL
E 2 4 8 16 MB G PR PL G PL
←18S
←1K

Fig.17. Quantitative analysis of 15D6 transcript representation in *L. pictus*, *S. purpuratus* and hybrid embryos. RNA gel blots were exposed to film for various times. See legend to Fig.15.

Stages: E, Egg; 2-16, blastomere number; M, morula (64-100 cells); LMB, late mesenchyme blastula; G, gastrula; PR, prism; PL, pluteus.

Symbols: (●) LpxLp; (○) SpxSp; (◑) SpxLp interspecies hybrid.


40
30
20
10
8
6
4
2
1
TRANSCRIPT PREVALENCE (% L.pictus egg; x10⁻²)
E
2
4
8
16
MB
G
PR
PL
STAGE OF DEVELOPMENT

may represent aggregation of the smaller transcripts or a nuclear precursor which has leaked into the cytoplasm.

15D6 represents a highly species-specific transcript as can be seen in the SpxSp gastrula lane (Fig.16). The transcript is not detectible in SpxSp plutei either, as determined by RNA dot blots (not shown). The SpxLp hybrid pluteus lane shows a low level of expression in these embryos, approximately equalling that reached in the LpxLp cleavage stage samples. This level was also low in SpxLp hybrid gastrulae as determined by dot blots. The profound difference in expression in SpxLp hybrid embryos compared to LpxLp embryos for this very developmentally up-regulated transcript confirms that some prevalent transcripts do exist which are normally accumulated in LpxLp embryos during development but which are grossly underrepresented in the RNA of SpxLp hybrid embryos. While the expression is limited in hybrids, the size of the transcript in SpxLp hybrid embryos appears to be identical to the normal transcript in LpxLp embryos. The 1700 nt larger band is also clearly evident at darker exposures of the blot.

There are several possible explanations for this phenomenon. The DNA complement may have been lost from a majority of the embryos in culture, even though the embryos develop into plutei. Secondly, the transcription of the 15D6 gene may not be activated correctly, even though other paternal genes (eg., 16B7) are known to be active. Thirdly, the gene could be actively transcribed but the transcripts may not accumulate in the cytoplasm in SpxLp hybrid embryos. I decided to distinguish among these possibilities by assaying the level of transcription of the gene by a chain-extension assay in isolated nuclei.

Chain-extension Assays

15D6 Homologues are Transcribed in SpxLp Hybrid Nuclei

L. pictus and *S. purpuratus* eggs were fertilized with the sperm of the *L. pictus* male, and SpxLp and SpxSp embryos were collected from the same *S. purpuratus* female. LpxLp, SpxLp, and SpxSp mesenchyme blastulae were collected and nuclei were isolated by centrifugation through cold sucrose. *L. pictus* embryos were expected to be actively transcribing the 15D6 gene at this stage, because the cytoplasmic transcript is actively accumulating in mass. The purified nuclei were incubated for 20 min at 20°. The nuclei incorporated [^{32}P]-UTP into macromolecules in a linear fashion for this period of time (See Fig.18). By 30 min, recovery of labelled product was the same or less than at 20 min. The RNA from 50-100 x 10^8 nuclei per sample was isolated and extensively purified by multiple rounds of DNAase and Proteinase K digestions, TCA precipitation and TCA washes to remove unincorporated nucleotides, binding and elution from nitrocellulose filters, phenol extraction and ethanol precipitation. This method, modified from Groudine et al.(1981) greatly reduces filter background. From 1-2 x 10^8 cpm/5 x 10^7 nuclei were obtained, and from 1-2 x 10^7 cpm was used for each hybridization assay.

Isolated plasmid DNA from clones 15D6, pSpecI (gift from Dr. William Klein), 16B7 (in pBr322 and one strand in M13-Mp9) and 16D4 were denatured and spotted onto nitrocellulose filters. Nuclear RNA was diluted into hybridization solution containing 10% dextran sulfate. Duplicate filters were incubated for 72 h at which time they were removed from the radioactive probe and washed extensively at 68°C in 0.15 M NaCl buffer. Exposure times varied from 2h to 3 days and are indicated in the figure legends.

Fig.18. Quantitative analysis of RNA chain-extended in the presence of α-amanitin. Nuclei were incubated with 100 uCi [^{32}P]-UTP in the presence or absence of α-amanitin. Samples of the reaction were removed at various time points and incorporation of nucleotides into macromolecules was assayed by spotting onto DE-81 filters (see Materials and Methods). The presence of this low dose of α-amanitin inhibited 97% of the incorporation of [^{32}P]-UTP after 25 min of incubation.

Symbols: (■) control; (○) + 1 ug/ml α-amanitin.

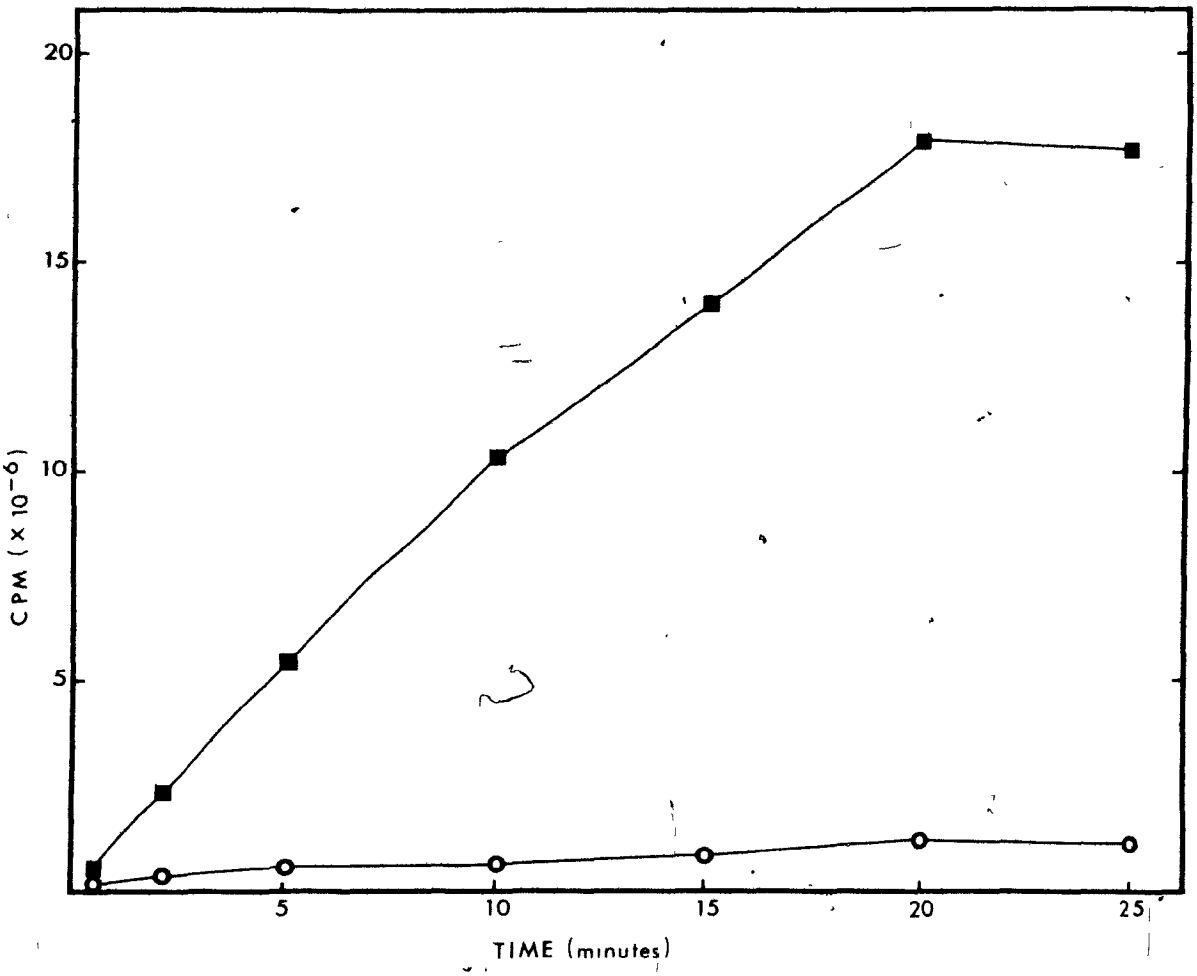

20
15
10
5
CPM (X 10⁻⁶)
5
10
15
20
25
TIME (minutes)

Fig.19A shows an 18 hr and a 2 hr exposure of a set of filters. pBr322 background was slightly higher for SpxLp hybrids, but this was partly due to non-specific binding of radioactivity to the nitrocellulose in the region around the pBr322 control. A variety of exposures were scanned by densitometry, and quantitated. The 15D6 sequence is transcribed in SpxLp hybrid embryos at levels approximately 80% of the normal LpxLp embryo level, a slight reduction which is insignificant compared to the differences in cytoplasmic transcript prevalence.

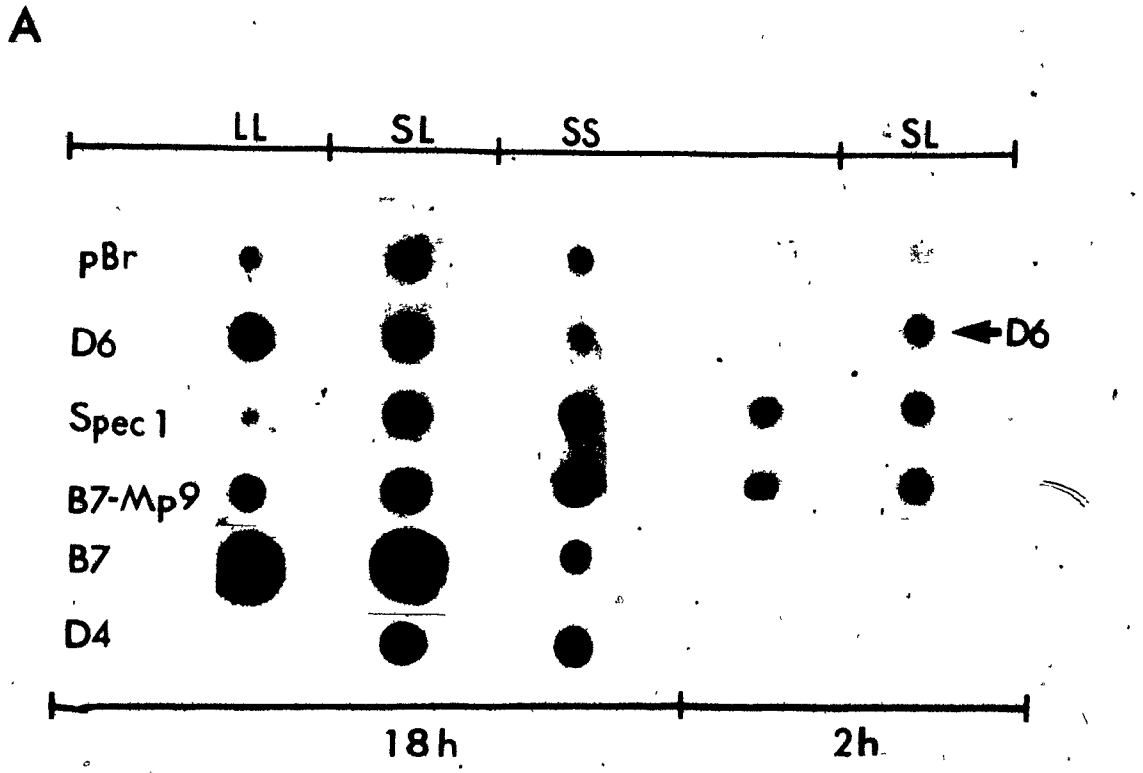
16B7-pBr is equally transcribed in SpxLp hybrid embryos and LpxLp embryos, but reduced in SpxSp embryos. This is to be expected from gel blot analyses of transcript prevalence. The extensive transcription suggests that many genes are active at this stage of development. The 16B7-Mp9 single-stranded insert lights up equally well when probed with RNA synthesized by LpxLp, SpxLp, and SpxSp nuclei. There is apparently a related repetitive sequence in S. purpuratus which is actively transcribed but does not accumulate in the cytoplasm, or alternatively, only one strand is principally expressed in SpxSp embryos, and it is represented by the cloned single-strand. LpxLp and SpxLp hybrid embryos transcribe both strands, but predominantly the strand opposite to that represented by B7-Mp9; SpxSp embryos may be transcribing only the strand represented by B7-Mp9.

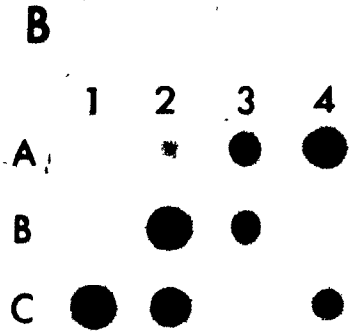
16D4 genes are transcribed in SpxLp and SpxSp embryos. LpxLp data was not obtained for this clone. pSpec1 is transcribed with similar activity in SpxSp and SpxLp hybrid embryos and represents normal transcription from a "maternal" gene (Bruskin et al., 1981).

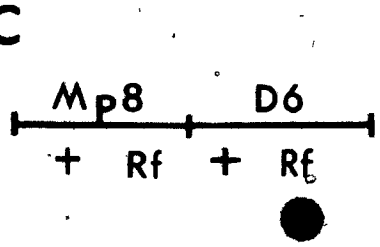
Fig.19. DNA dot blot hybridization analysis of transcription using chain-extended RNA probes. (A) Chain-extended RNA was isolated from nuclei as described in Methods, denatured, and hybridized to isolated plasmid or phage DNA dots contained on nitrocellulose filters, and detected by autoradiography. Alphanumeric symbols identify the DNA dotted onto each filter as follows: pBr, pBr322 plasmid DNA; D6, 15D6 plasmid DNA; Specl, pSpecl plasmid DNA (gift from Dr. B. Klein); B7-Mp9, single-stranded DNA isolated from M13-Mp9 containing the PstI/Hind III restriction fragment of clone 16B7; B7, 16B7 plasmid DNA; D4, 16D4 DNA. Source of probe: LL, LpxLp mesenchyme blastula nuclei; SS, SpxSp mesenchyme blastula nuclei; SL, SpxLp hybrid embryo nuclei. The symbols 18h and 2h refer to exposure times of the filters shown. The signal for 15D6 represents 0.006% of the total input CPM.

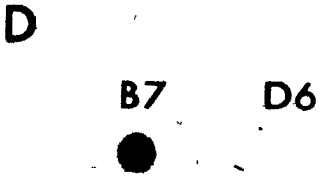
(B) Clone 15D6 was cleaved with PstI and subcloned into vector M13-Mp9. Single-stranded DNA was isolated from 10 "plaques" and spotted onto Gene Screen. The PstI insert fragment isolated from 15D6 was nick-translated and hybridized to the DNA on the filters and detected by autoradiography. DNA dots: A1, M13-Mp8 DNA; A2, M13-B7 DNA; A3, A4, B2, B3, C1, C2, C4, contain DNA from 15D6 subclones.

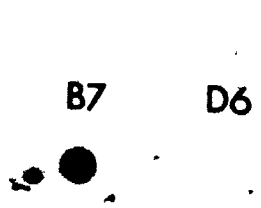
(Legend continued on following page)


A
LL
SL
SS
SL
pBr
D6
Spec 1
B7-Mp9
B7
D4
D6
18h
2h


B
1
2
3
4
A
B
C


C
Mp8
D6
+
Rf
+
Rf


D
B7
D6


E
B7
D6

(continued from previous page)

(C) Single- and double-stranded DNA isolated from clones C1 and A1 (control) was denatured and spotted onto a nitrocellulose filter. About 2 x 10^7 cpm SpxLp hybrid mesenchyme blastula chain-extended RNA was hybridized to the DNA and detected by autoradiography.
DNA dots: Mp8, clone A1 (+) and RF form DNA; D6, clone C1 (+) and RF form DNA.

(D) LpxLp gastrula RNA end-labelled with polynucleotide kinase was hybridized to DNA dotted on Gene Screen and detected by autoradiography. A 24 h exposure is shown. D6 was not detectible after a 96 h exposure (not shown). DNA dots: B7, clone A1; D6, clone C1 (described in (B).

(E) Chain-extended RNA isolated from SpxLp mesenchyme blastula nuclei, and used in the hybridization of filter (C), was hybridized to a duplicate of filter (D), and detected by autoradiography. A 24 h exposure is shown. No D6 signal was detected at longer exposure times. This indicates that the chain-extended RNA isolated from SpxLp embryos is synthesized from the same strand as the RNA from LpxLp gastrula used in (D) above.

15D6 Nuclear Transcripts Result From Asymmetric Transcription of the Gene

To eliminate the possibility that this dot blot assay was measuring transcription from artefactually initiated sites of nicked DNA resulting from nuclease activity during nuclear isolation, several experiments were performed. 15D6 in pBr322 was digested with Pst I, the insert was isolated from an agarose gel, annealed and ligated to M13-Mp8 replicative form DNA . Recombinant molecules were transfected into JM103 bacteria. Single-stranded DNA, isolated from "plaques" was dotted onto nitrocellulose and probed with nick-translated [^{32}P]-labelled 15D6 insert (both strands). Fig.19B shows an array of these dots. Dot A1 contains cesium-purified MP8 (+) strand DNA, spot A2 contains B7-Mp9 used in Fig.19A., and the remaining 10 dots represent various candidates for 15D6-Mp8 subclones. A4, B2, and C1 elicited strong signals. These were checked for complementarity to each other and were found to represent the same strand of 15D6 insert.

Clone C1 was grown in culture, and (+) strand and RF DNA was isolated. These were dotted onto filters in a 1:2 mass ratio to insure that equal masses of the (+) and (-) strand were dotted. Fig.19C shows this filter. The left two dots represent controls for (+) and (-) strand vector, while the right dots contain 15D6 (+) and (-) strand in equal masses. This filter was incubated with SpxLp chain-extended nuclear RNA for 72 h. The filters were washed and exposed for 18 hours. The strong signal due to hybridization to the opposite strand of the RF form confirms that the 15D6 transcripts are overwhelmingly synthesized from only one strand of the gene.

In another experiment, kinase-labelled cytoplasmic RNA of LpxLp

gastrula was hybridized to M13 sub-clones as shown in Fig.19D. This probe has the same "sense" as the nuclear RNA and 15D6-Mp8 did not light up, but B7-Mp9 did. Chain-extended SpxLp hybrid nuclear RNA was then incubated with an identical filter for 72 h (see Fig.19E). There again was no hybridization to 15D6-Mp8. I conclude that the transcript synthesized in SpxLp hybrid nuclei is from the same strand as that which is in the cytoplasm of LpxLp gastrulae. The same RNA which was hybridized to the filter shown in 19F was hybridized to the filter shown in 19C. The strong hybridization elicited by this RNA with little filter background shows that the nuclear RNA is stable in hybridization reactions for at least 4 days and probably much longer.

The Transcription of 15D6 and 16B7 Is Sensitive to α-amanitin

RNA polymerase II, which is responsible for the synthesis of mRNA, is sensitive to low levels of α-amanitin while polymerase I and III are not. Nuclei were incubated in the presence of 1 ug/ml α-amanitin (a low dose) and 100 uCi [^{32}P]-UTP. Samples of the reaction were taken at various time points and incorporation of nucleotides into macromolecules was assayed by spotting onto DE-81 filters. The results are shown in Fig.18. The α-amanitin inhibited 97% of the incorporation of [^{32}P]-UTP at 25 min. The lesser inhibition at earlier time points may indicate that there is a short time lag before α-amanitin becomes effective. The extensive polymerase II activity for isolated sea urchin nuclei has been reported previously (Morris and Marzluff, 1983). When this chain-extended RNA synthesized in the presence of low α-amanitin was hybridized with 15D6 and 16B7 plasmid DNA dotted onto nitrocellulose, the signal intensities were decreased by greater than 95% (R. Conlon, personal communication). Thus

the transcription of these genes is carried out by polymerase II in the isolated nuclei.

The data obtained from a variety of experiments indicate the existence of prevalent transcripts which are greatly reduced in SpxLp hybrid embryo cytoplasm at stages of development when they are normally accumulating in LpxLp embryos; in the case of 15D6, this restricted accumulation is primarily the result of post-transcriptional events. It is possible that transcription is occuring at the stage analyzed and subsequently is turned off in hybrids. Preliminary data obtained by chain-extension analysis in hybrid nuclei of later stages indicate that the 15D6 gene is transcribed. The relative incorporation into a cloned tRNA gene is the same in all isolated nuclei of a particular stage (LpxLp, SpxLp, SpxSp), indicating that there are no gross alterations in relative activities of polymerase II and III in hybrid nuclei.

These results obviate the need for establishing the presence of a full complement of 15D6 DNA sequences in SpxLp hybrid embryos, since the rate of transcription is unlikely to occur without the gene being retained in most cells normally transcribing the gene. DNA dot blot hybridization experiments indicate that S. purpuratus DNA has little homology with 15D6 sequences, accounting for the failure to detect 15D6 transcripts in the cytoplasm of SpxSp embryos.

DISCUSSION

In a previous paper, I analyzed the kinetics of hybridization of a paternal L. pictus gastrula cDNA probe to excess SpxLp mRNA (Tufaro and Brandhorst, 1982). The data indicated that a substantial mass of paternal mRNA is present and thus transcribed in SpxLp embryos. The retarded reaction kinetics for paternal cDNA, and the paucity of paternal species-specific spots on two-dimensional gels indicated that much of the mass of normally prevalent paternal mRNAs is reduced or absent in SpxLp hybrid RNA. In a similar analysis of the echinoid cross D. excentricus x S. purpuratus, Lee and Whiteley (1982) reported that paternal transcripts made in the hybrid embryos may be underrepresented in the more abundant classes of RNA normally present. In light of these data, it was decided to analyze cloned sequences representing prevalent and moderately-prevalent transcripts. These transcripts are also most easily analyzed in a quantitative sense by blotting methods. Lasky et al. (1980) hybridized cDNA probes representing gastrula RNA to gastrula stage cDNA cloned sequences from S. purpuratus and found that about 80% of the gastrula clones represent transcripts present in fewer than 10 copies/cell, and about 55% of these are present in fewer than 3 copies/cell. I analyzed 1050 clones of which about 15% reacted strongly with cDNA probes. These represent the more abundant transcripts present in the gastrula cytoplasm.

Only 23 of the 107 non-mitochondrial cloned sequences analyzed by colony hybridization were detected as species-specific. It has been reported that the sequence divergence of gastrula polysomal RNA sequence between L. pictus and S. purpuratus is about 86%, using hybridization conditions of similar stringency. The gastrula mRNA population contains transcripts of about 14,000 genes active in S. purpuratus gastrulae, and thus represents a large sample of structural genes (Galau et al., 1974) Although structural genes sequences do not appear to be more highly conserved than single-copy DNA sequences in general (Angerer et al., 1976), a small fraction of non-repetitive sequences appears to be highly conserved (Harpold and Craig, 1978). In S. purpuratus gastrulae, prevalent transcripts (including those of mitochondria) account for about half of the mass of cytoplasmic RNA (Lasky et al., 1980; Shepherd and Nemer, 1980; this thesis ch.3) and nearly all of the cytoplasmic sequence complexity is represented in polysomes (Hough-Evans et al., 1977). Hence, it is possible that the highly conserved structural sequence fraction detected by Harpold and Craig (1978), while representing a small fraction of the complexity, may comprise most of the more prevalent transcripts. The failure to detect a large proportion of species-specific proteins (Tufaro and Brandhorst, 1982), most of which are coded by more prevalent transcripts (Bedard and Brandhorst, 1983), probably reflects the divergence of the non-coding region of transcripts or third-position wobble which can occur without altering amino acid composition. In addition, the apparent conservation of abundant sequences may be due to selection pressure to maintain amino acid sequences and/or some nucleotide sequence-specific functions. Prevalent proteins may frequently serve structural rather than enzymatic functions and may be evolutionarily

conserved because of their complex interactions with other proteins.

Interestingly, 80% of the developmentally-regulated cloned L. pictus sequences analyzed were species-specific. This indicates that the majority of prevalent transcripts having conserved sequences are not developmentally-regulated. Hence, they may serve functions which are needed at all times by all echinoid embryos during development, such as those required for macromolecular synthesis, cleavage or membrane biogenesis.

The cloned sequence 16D4 is an example of a developmentally-regulated transcript which has been highly conserved between the two species. Although its nucleotide sequence has not been determined, high stringency hybridization conditions indicate that its sequence is highly conserved and that the mature cytoplasmic transcript is identical in size in the two species, within the resolution of the agarose gel system used. The transcript is present in maternal RNA, increases during the period of rapid cleavage and declines by the gastrula stage. The temporal pattern of expression is not precisely the same between these species. While limited divergence of the transcripts expressed in the two species could account for the lower apparent mass in SpxSp gastrulae, this seem unlikely since the transcript is detected easily in SpxSp morulae, although a higher level of expression in SpxSp morulae than in LpxLp morulae could be occurring. If the S. purpuratus transcript is less detectible due to sequence divergence, the conclusion that the 16D4 gene is actively expressed in hybrids is even more convincing.

The pattern of expression of the L. pictus 16D4 gene suggests that the mechanism for regulating this gene enters with the sperm genome. The autonomous timing of expression of a paternal actin gene in echinoid

hybrid embryos has recently been reported (Crain and Bushman, 1983). This apparent phenomenon is not limited to echinoid hybrids, as Drosophila interspecies hybrid larvae express each parental structural allele for ADH according to the developmental program characteristic of the species from which it is derived. In this system, there is strong evidence for a cis-acting control element (Dickinson and Carson, 1979) Evidence for such cis-acting regulators has also been obtained in studies of strain-specific quantitative differences in the pattern of expression of enzymes and other proteins in mice, such as B-glucuronidase (Paigen, 1964), B-galactosidase (Paigen, 1976), aryl-sulfatase (Daniel, 1976) and H2 antigen (Boubelik et al., 1975). The correlation of the appearance of 16D4 transcript with early rapid cleavage and pre-gastrular development, and the strong conservation of the gene suggest that it may have an important function in early embryonic development. This is currently being characterized more extensively.

Clone 16B7 was initially detected as being species-specific, containing a highly repetitive sequence element, and expressed in SpxLp hybrid embryos. The RNA gel blot pattern indicates that the 16B7 repeat sequence element is included in many relatively rare transcripts, many of which are expressed to an apparently normal extent in SpxLp hybrid embryos. The transcript pattern in hybrid embryos is very similar to that of L. pictus embryos, but the structural equivalence of these transcripts between LpxLp and SpxLp embryos has not been directly demonstrated. It is likely that many of the transcripts containing the 16B7 repeat are expressed in both hybrid and normal embryos. The isolation of unique sequences contiguous to this repeat sequence should allow a partial determination of structural equivalence, and this is currently being done.

The mass of 16B7 transcripts in SpxLp early hybrid embryos is reduced compared to LpxLp early embryos, since the S. purpuratus egg lacks the normal complement of stored 16B7 RNA. The rapid accumulation of 16B7 transcripts in SpxLp hybrid embryos probably reflects the rapid decay of 16B7 maternal transcripts and replacement by new transcription in paternal embryos.

It has been convincingly demonstrated that there are sets of diverse transcripts in the egg that share homologous repeat sequences, and several examples belonging to such sets have been cloned and their structures analyzed (Posakony et al., 1983). The presence of interspersed repeats in maternal RNA is directly reminiscent of the structure of nuclear RNA, and it has been suggested that a major fraction of the stored maternal mRNAs may not be fully processed (Davidson et al., 1983; ch.1, this thesis). It has been proposed that cytoplasmic processing during development could represent a mechanism for regulating the utilization of maternal information. Maternal transcripts might include sequences, such as common repeats, which are recognized by proteins, snRNAs or other RNAs (Davidson and Britten, 1979) which regulate their utilization by controlling turnover rates, processing events, spatial localization, or other interactions. In light of this, the expression of 16B7 genes in SpxLp embryos may represent a transcriptional response of the paternal genome to the absence of maternal sequence in an effort to achieve an appropriate level of transcript in the embryo. It is tempting to speculate that cytoplasmic prevalence of maternal RNA is regulated by the level of maternal RNA itself.

The majority of the species-specific cloned transcripts analyzed which are up-regulated are underrepresented in SpxLp embryos. One cloned

sequence, 15D6, has been analyzed in some detail. Restriction of expression could be due to 1) loss of the paternal gene, 2) absence of appropriate maternal factors required for transcription, 3) alteration of the gene prohibiting proper transcription or processing, 4) the absence in the foreign cytoplasm of factors required in order to process, transport or otherwise stabilize the transcript, 5) autoregulation of transcript prevalence by accumulation of translation products unable to associate with maternal structural proteins. Hybridization of 15D6 to genomic DNA on filters demonstrated that the DNA, at least in part, was retained in hybrid embryos, but is not detectible in S. purpuratus. Hybridization of a 15D6 probe to DNA cleaved with restriction enzymes is a more sensitive assay, but restriction site polymorphisms between individuals make these data difficult to interpret. RNA chain-extension analysis in isolated nuclei allowed me to investigate the retention of the gene while at the same time demonstrate that the gene is actively transcribed.

RNA chain-extension in isolated nuclei has been carried out for a wide variety of genes and organisms to investigate transcription. Isolated nuclei are readily permeable to ribonucleoside triphosphates and to a variety of other small molecules, as well as to some proteins and nucleic acids (Manley et al., 1979). Isolated nuclei are capable of synthesizing significant masses of RNA having high specific radioactivities (Marzluff et al., 1973; Wu and Zubay, 1974; Manley et al., 1979), and also of retaining the strand-specificity of transcription observed in vivo (Vennstrom and Phillipson, 1977). Levy et al. (1978) demonstrated that nuclei isolated from sea urchin oocytes synthesize discrete species of RNA containing sequences complementary to cloned histone DNA. It has been shown for Ad-2 transcripts synthesized in nuclei from infected HeLa cells

that *in vitro*-synthesized RNA is of high molecular weight and that a large percentage of these molecules contain discrete 5' and 3' termini which correspond to the termini of viral nuclear RNA *in vivo* (Manley *et al.*, 1979). The primary transcription unit of the mouse B-globin gene was established by analysis of RNA extended in isolated nuclei (Hofer and Darnell, 1981). Available evidence suggests that the majority of RNA synthesis in isolated nuclei represents elongation of already initiated RNA chains (Evans *et al.*, 1977) and that RNA processing in isolated nuclei is inefficient even for already completed RNA chains (Blanchard *et al.*, 1978). It has been demonstrated that isolated sea urchin nuclei synthesize RNA at a rate comparable to other animal cell nuclei under conditions similar to those utilized in this investigation (Morris *et al.*, 1983). The relative incorporation of nucleotides into a particular transcript by chain extension in isolated nuclei is considered to be a measure of the fraction of RNA polymerase molecules engaged in transcribing that gene *in vivo*. In some instances, estimates of transcription rates *in vivo* confirm this (eg. Derman *et al.*, 1981). In addition, transcriptional regulation of ovalbumin and conalbumin genes in isolated oviduct (McKnight and Palmiter, 1979) and hemoglobin switching in chick embryos (Groudine *et al.*, 1981) has been convincingly demonstrated by chain-extension assays. I thus regard this chain-extension assay as an appropriate measure of relative transcriptional activity *in vivo*.

I measured transcripts synthesized in isolated LpxLp and SpxLp nuclei which were at least partly homologous to the 15D6 insert sequence by dot-blot hybridization. The structural equivalence of the transcripts from each type of nucleus cannot be demonstrated by this approach, however. The fact that transcription occurred using polymerase II from

one strand of the gene having the same sense as that coding for the cytoplasmic transcript which accumulates in LpxLp embryos makes it likely that transcription was occurring *in vivo* prior to the isolation of the nuclei rather than as the result of some non-specific gene activation due to DNA nicking or damage to the chromatin. Quantitation of the assay indicates that the 15D6 gene is nearly as actively transcribed per nucleus in the SpxLp hybrid as in LpxLp embryos of the same stage, Since there is only one half the number of gene copies in hybrid nuclei, the transcription rate suggests that some dosage compensation has occurred. The continuous accumulation of 15D6 transcripts in LpxLp embryos suggests that the gene is maintained throughout embryonic development in a configuration which allows active transcription; chain extension assays are consistent with this interpretation, but not all stages have been analyzed.

Hybridization of nuclear RNA to fractionated genomic DNA has indicated that most single-copy RNA sequences are shared by all nuclei (Kleene and Humphreys, 1977). Morover, tissue- or stage-specific mRNAs can be detected among the nuclear RNA sequences of other stages and tissues (Wold *et al.*, 1978; Shepherd and Nemer, 1980). This suggests that absolute transcriptional control is not the mechanism regulating gene expression, and that post-transcriptional regulatory events may predominate. Genes coding for abundant tissue- or stage-specific genes might be transcriptionally modulated during development, but there are too few of those to detect by the saturation-hybridization techniques frequently used. The similar relative rates of transcription of the 15D6 gene in isolated nuclei from cells which accumulate different masses of cytoplasmic transcripts is strong evidence that a post-transcriptional regulatory event is being perturbed in the hybrid embryos.

In recent years, several processing steps leading to the formation of mature mRNA have been identified. Transcription units for mRNAs are generally larger than the mature mRNA products. The sequence of events leading to the formation of stable mRNA include initiation, capping of the 5' end of the transcript, cleavage and the addition of adenylic acid residues at sites in the primary transcript to create 3' poly(A) tracts, and the splicing out of introns (for review, see Darnell, 1982; Breathnach and Chambon, 1981). Gene regulation may be exercised at each of these processing steps, in transport of mRNA from the nucleus, and in the stabilization and efficiency of translation of the mRNA. Not all nuclear RNA sequences are destined to become cytoplasmic mRNA. In sea urchins, the sequence complexity of hnRNA is about 10 times greater than the complexity of cytoplasmic RNA (Wold et al., 1978).

Post-transcriptional mechanisms of regulation of particular transcripts have been demonstrated in a wide variety of systems. Nuclear processing of mRNA precursors in differentiating multicellular Dictyostelium discoideum aggregates is markedly slower than in growing amoebae (Mangiarotti et al., 1983). In this system, mRNAs encoded by different genes display different times of transit to the cytoplasm and different efficiencies of nuclear processing. Kantor et al. (1980) provided evidence that a deficiency in B-globin production in certain patients with B-thalassemia may occur because of a mutation which affects RNA processing and, consequently, cytoplasmic transcript levels. Esumi et al. (1982) demonstrated that in analbuminemic rats, which lack serum albumin and albumin mRNA transcripts in the cytoplasm of the liver, there are clearly albumin mRNA precursors in the nuclei of liver cells at almost normal levels, and concluded that the rats have a mutation affecting

albumin mRNA maturation. The cytoplasmic amounts of tubulin mRNAs in Chinese hamster ovary cells or diploid human fibroblasts are regulated post-transcriptionally by levels of unpolymerized tubulin subunits (Cleveland and Havercroft, 1983). A similar feedback regulatory mechanism may restrict the accumulation of paternally-specified subunits of cellular structures in hybrid embryos. The prevention of the accumulation of aberrant structural proteins in some echinoid hybrid embryos may permit those embryos to develop through larval stages, rather than suffering developmental arrest.

It is likely that transcript-specific intracellular mechanisms result in different stabilities of different mRNAs, depending on secondary structure, protein binding, terminal sequence elements, localization, or sequestration. Cabrera et al. (1984) have shown that sea urchin mRNAs can have very different rate constants for decay. In hybrid embryos, the 15D6 gene may be inefficiently or abnormally processed, inefficiently transported to the cytoplasm, or rapidly degraded due to a failure of as yet unidentified mechanisms. Establishment of the structure of the 15D6 transcription unit and its processing pathway, may enable us to evaluate which step in gene expression is aberrant in the hybrid embryos. This in turn may lead to a better understanding of the mechanisms regulating transcript prevalence in normal sea urchin embryos and other eukaryotic cells.

CONCLUSION

Sea urchin eggs have been the classic system for investigating the nature and role of maternal mRNA. In this thesis, I investigated the extent to which embryogenesis results from a progression of developmental events directed by the embryo or an expression of a pre-formed maternal program.

Since the establishment of the stored mRNA hypothesis, it has been proposed frequently that maternal mRNAs may act as localized morphogenetic determinants in eggs and embryos (Raff and Showman, 1983; Brandhorst et al., 1984). I approached this question by subjecting the proteins synthesized by isolated micromeres, mesomeres, and macromeres to two-dimensinoal electrophoresis. Of approximately 1000 spots resolved, there are no qualitative differences among the three blastomere types. When embryos were labelled between the first and fourth cleavage, and blastomeres then isolated, no qualitative difference in protein synthesis were observed. Moreover, there are very few changes when unfertilized eggs are compared to 16-cell embryos. Thus cellular determination during embryonic development is not accompanied by qualitative changes in the distribution within the embryo of abundantly synthesized proteins, virtually all of which are coded for by sequences present in the egg. It remains possible that less extensively labelled proteins or proteins already present in the egg and not detectible by my methods may be segregated into different blastomeres at the fourth cleavage and that this segregation is responsible for developmental determination. Moreover, it is possible that mRNAs coding for distinct populations of proteins are

segregated into the different blastomeres at the 16-cell stage. It is also possible that the RNA which has been segregated by the 16-cell stage serves a regulatory role, as proposed by Davidson and Britten (1972).

There has been much interest in establishing when the zygotic genome of sea urchins becomes actively expressed, and to what extent it participates in embryonic development (Davidson, 1976, for review). Interspecies hybrid embryos have been used to assess the relative contributions of the maternal and embryonic genomes to early development. I have used two-dimensional electrophoresis to analyze the synthesis of paternal proteins in embryos of three interspecies hybrids which form healthy pluteus larvae: the reciprocal crosses of S. purpuratus and S. droebachiensis as well as S. purpuratus eggs fertilized with L. pictus sperm. The synthesis of most distinctly paternal proteins was never detected at the stages of development analyzed. Nucleic acid hybridization analysis revealed that DNA coding for mRNA normally translated in embryos of the paternal species is fully retained in S. purpuratus x L. pictus hybrid gastrulae. Nucleic acid hybridization experiments revealed that there is a substantial underrepresentation of paternal transcripts, particularly those which are normally prevalent in the cytoplasmic polyadenylated RNA of hybrid embryos.

There are two possible explanations for these observations. First, much of the mRNA translated into protein detected on two-dimensional gels might be persistent maternal mRNA not normally replenished during embryonic development. Recent observations suggest that certain transcripts may persist throughout embryogenesis (Cabrera et al., 1984). Alternatively, the synthesis, processing, nuclear export, or stabilization of many paternal mRNA transcripts may be impaired in the hybrid embryo.

If the restricted accumulation of paternal transcripts in hybrid embryos is largely the result of persistence of a large mass of maternal transcripts, it might be that the signals for RNA transcription and processing are conserved between the two species and RNA accumulation in interspecies hybrid embryos reflects normal expression of the paternal genome during development. One prediction resulting from this is that transcripts accumulating in normal embryos should also accumulate in hybrid embryos. Conversely, failure of proper transcript accumulation in hybrid embryos would indicate that the mechanisms which regulate RNA accumulation in normal embryos and are aberrant in hybrid embryos.

To test this prediction, a library of cloned cDNA molecules representing L. pictus gastrula cytoplasmic RNA was constructed. The library was screened to select clones for species-specific cytoplasmic transcripts indetectible in hybrid embryos. Most prevalent species-specific transcripts identified accumulate by mass in normal embryos, contrary to the prediction. One cloned sequence, 15D6, has been analyzed in some detail. Restriction of expression could be due to 1) loss of the paternal gene, 2) absence of appropriate maternal factors required for transcription, 3) alteration of the gene prohibiting proper transcription or procesing, 4) absence in the foreign cytoplasm of factors required in order to process, transport or otherwise stabilize the transcript, 5) autoregulation of transcript prevalence by accumulation of translation products unable to associate with maternal structural proteins. RNA chain-extension analysis in isolated nuclei allowed me to investigate the retention of the gene while at the same time demonstrate that the gene is actively transcribed.

I measured transcripts synthesized in isolated LpxLp and SpxLp nuclei which were at least partly homologous to the 15D6 insert sequence by dot-blot hybridization. Transcription occurred using RNA polymerase II from one strand of the gene having the same sense as that coding for the cytoplasmic transcript which accumulated in LpxLp embryos. These data indicate that the 15D6 gene is nearly as actively transcribed per nucleus in the SpxLp hybrid as in LpxLp embryos of the same stage. The continuous accumulation of 15D6 transcripts in LpxLp embryos suggests that the gene is maintained throughout embryonic development in a configuration which allows active transcription. An alternative possibility is that the L. pictus genome contains several copies of the 15D6 gene and that transcription from other 15D6 genes occurs in hybrid nuclei but does not produce the cytoplasmic transcript. If the 15D6 transcript is derived from one of several transcribed homologous genes, a reduction in its relative transcriptional activity might not have been detectible in nuclei isolated from hybrid embryos. DNA dot-blot hybridization of nick-translated genomic DNA to 15D6 plasmid DNA indicate that the 15D6 sequence is present no more than a few copies per haploid genome, but may be part of a multigene family. Southern blot hybridization of genomic DNA with 15D6 probes will allow an estimation of gene copy number.

Establishment of the structure of the 15D6 gene transcription unit and its processing pathway may enable us to evaluate which step in gene expression is aberrant in the hybrid embryos. This in turn may lead to a better understanding of the mechanisms regulating transcript prevalence in normal sea urchin embryos and other eukaryotic cells. A collection of cDNA clones have been identified corresponding to transcripts having

interesting developmental histories. The investigation of these will hopefully provide more information about the regulation of gene expression in sea urchin embryos and its relationship to important developmental events.

APPENDIX I

MITOCHONDRIAL TRANSCRIPTS

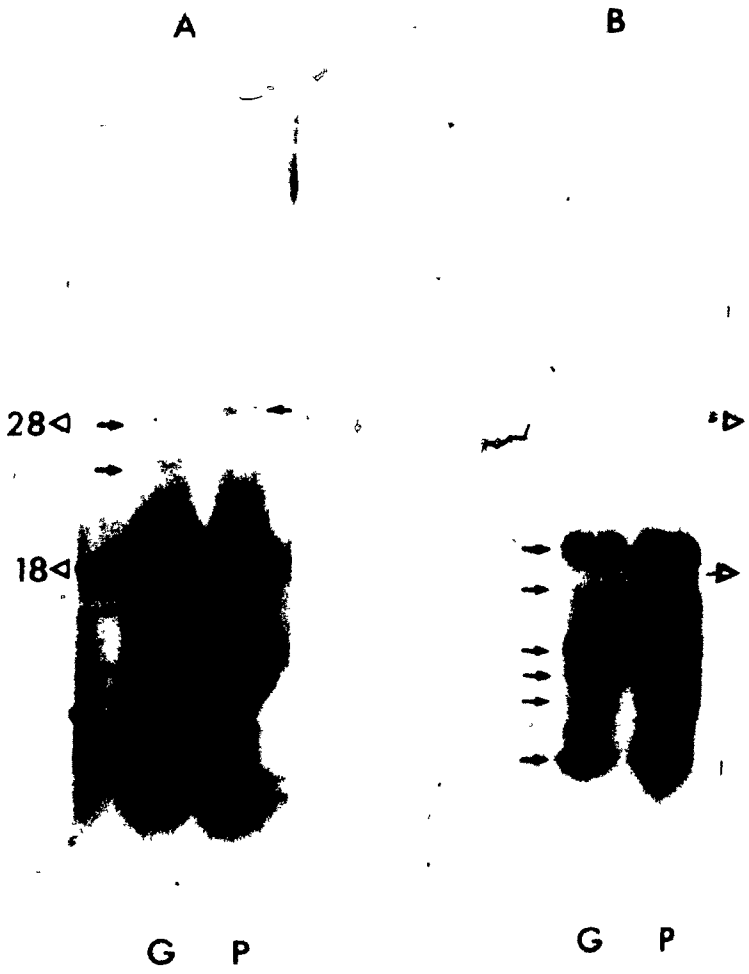
Results from colony hybridizations (Fig.10.) suggested that some of the mitochondrial cDNA clones represented transcripts which are developmentally-regulated. Several were also detected as being strongly species-specific. Several were chosen for further characterization. Fig. 20A and 20B show the analysis of transcript expression for two different cloned sequences. In Fig.20A, large transcripts (about 28s) are detected in gastrula and pluteus RNA samples when probed with 16G7 plasmid DNA, which contains an insert which is relatively conserved between L. pictus and S. purpuratus. The transcripts detected for each developmental stage are different in size, however. In Fig.20B, the pattern of expression of species-specific 16F10 transcripts does not include large transcripts. Arrows indicate the positions of discrete transcripts, which can be seen to differ for each probe.

Fig.20C shows a typical dot blot hybridization analysis. Since 15D2 is present in all stages and species analyzed, it was used to insure that dot blots contained RNA which was available for hybridization. This was an effective control for the efficiency of dot blotting.

Fig.20. RNA transfer blot hybridization analysis of 16G7 and 16F10 mitochondrial transcript representation in *L. pictus* embryos. Total cytoplasmic RNA was prepared, and after denaturation, 10 ug samples in each lane were separated on agarose gels containing formaldehyde. After transfer to Gene Screen, the RNA was probed with nick translated (A) 16G7 or (B) 16F10 plasmid DNA, and detected by autoradiography. Arrows indicate discrete bands and are discussed in Appendix I. The positions of 18s and 28s RNA are indicated.

(C) autoradiograph of an RNA dot blot probed with nick-translated 15D2 DNA. LL, SL, and SS refer to RNA isolated from LpxLp, SpxLp and SpxSp embryos, respectively.

Stages: E, egg; G, gastrula; P, pluteus.


A
B
28
18
G
P
G
P
C
E
G
P
G
G
LL
SL
SS

APPENDIX II

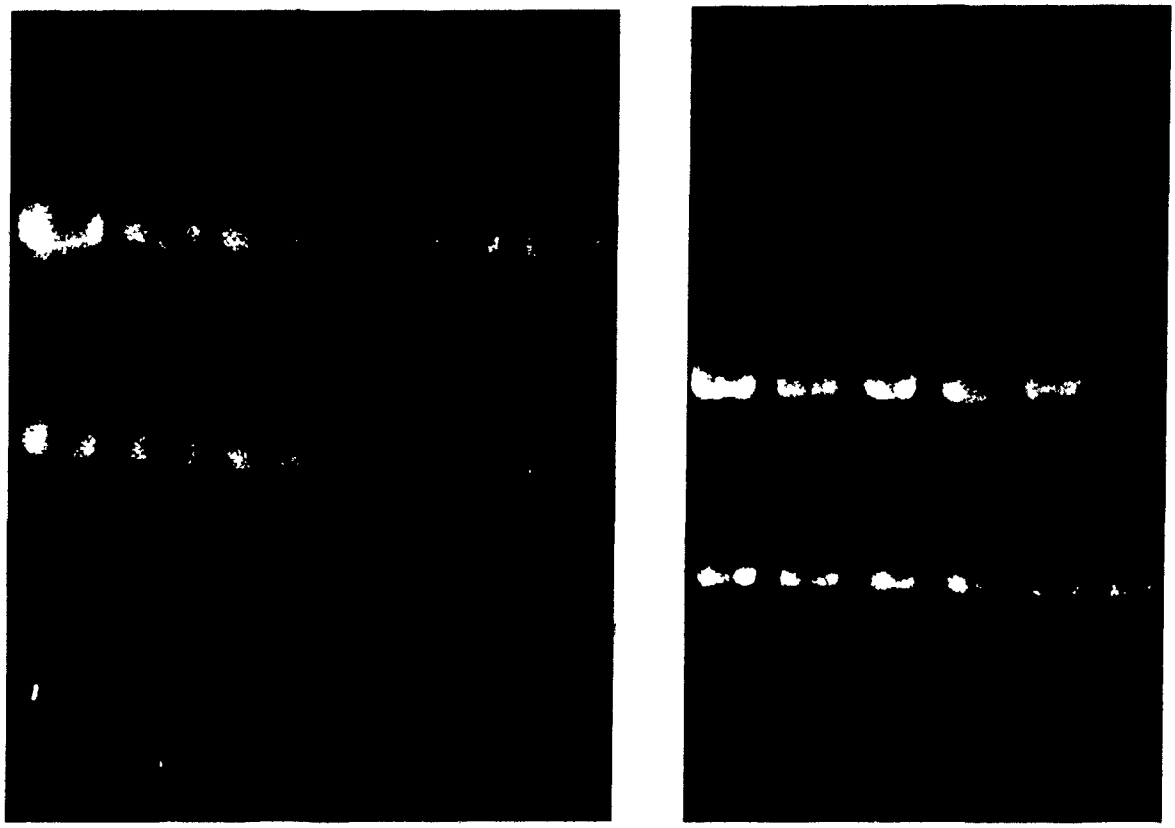
EFFICIENCY OF BLOTTING RNA

The quantitation of specific RNA transcripts in a population of RNA depends in large part on the efficiency of blotting RNA out of a gel and onto a filter. Gene Screen was used in this analysis since it has superior RNA binding capability (F. Fuller, personal communication). All of the RNA blots shown in this thesis were photographed under UV illumination to allow semi-quantitation of the efficiency of blotting RNA from each lane of the gel (see Fig.21). This insured that the differential signals detected on autoradiographs were an accurate measurement of a particular transcript in a given mass of RNA applied to the gel.

Fig.21. Analysis of the efficiency of blotting RNA from an agarose gel onto Gene Screen. The two prominent bands represent 18s and 28s RNA. (A) photograph of ethidium bromide pattern of RNA electrophoresed in an agarose gel containing formaldehyde.
(B) photograph of ethidium bromide pattern of RNA blotted onto a Gene Screen membrane. The ethidium bromide pattern photographed resulted from ethidium bromide which blotted with the RNA; the filter was not restained.

A

B



APPENDIX III

ANALYSIS OF REASSOCIATION AND HYBRIDIZATION KINETIC MEASUREMENTS

A computer program was used for the rapid calculation of non-linear least square solutions for data fitted to functions normally used in reassociation and hybridization kinetic measurements (see Pearson, et al., 1977). The program is designed to converge on a solution yielding parameters which minimize the least squares deviation of the function from a set of data. Solutions generated by the program are independent of input parameters and insensitive to further iterations.

Data obtained from RNA-excess hybridizations were fit as pseudo first order reactions. DNA reassociation data were fit to a second order function modified for reactions assayed by S1-nuclease digestion (see Pearson, et al., 1977).

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