ECTODERM-SPECIFIC GENE EXPRESSION IN DISSOCIATED CELLS OF SEA URCHIN EMBRYOS

by

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THESIS SUBMITTED IN PARTIAL FUFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCES

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

August 1995

APPROVAL

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Degree: **Master of Science**

Title of Thesis:

ECTODERM-SPECIFIC GENE EXPRESSION IN DISSOCIATED CELLS OF SEA URCHIN EMBRYOS

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ECTODERM-SPECIFIC GENE EXPRESSION IN DISSOCIATED CELLS OF SEA URCHIN EMBRYOS

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Abstract

The role of cell contact in the specification and differentiation of the aboral ectoderm cells was investigated by dissociating sea urchin embryos at various stages and culturing in calcium-free sea water as a single cell suspension. The expression of the aboral ectoderm-specific genes was monitored by RNA gel blot hybridization. In L. pictus embryos, the level of LpS1 mRNA declined considerably in the cells dissociated at mesenchyme blastula stage, somewhat at gastrula stage, but not at pluteus stage. The level of LpS1 mRNA began to decline almost immediately after dissociation at gastrula stage and continued for 13 hours. Readdition of Ca^{2+} ions, or addition of the peptide growth factors TGF-α and PDGF-BB, did not protect LpS1 mRNA from decline in dissociated gastrula stage embryo. Dissociation of cleavage stage embryos resulted in a much lower than normal increase in the level of LpS1 mRNA, which normally begins at hatching. The level of LpC2 actin mRNA showed a similar response to dissociation. Similar results were observed for the Spec1 gene, a homologue of the LpS1 gene, in S. purpuratus embryos, but the response was slower. These results indicate that while aboral ectoderm specification is autonomous, cell contact prior to hatching is required for full expression of LpS1 or Spec1 genes. These observations also suggest that cell contact is required for maintenance of the state of differentiation of the aboral ectoderm cells in L. pictus embryos at mesenchyme blastula and gastrula stages. Though efforts were made to establish whether the decline in LpS1 mRNA levels in dissociated L. pictus gastrulae was due to decreases in stability or rate of synthesis of the mRNA, no conclusive results were obtained.

To My Parents

Acknowledgments

I wish to thank my thesis supervisor, Dr. Bruce Brandhorst for providing me the opportunity of doing my thesis project in his laboratory. I have really been impressed by his ability of scientific judgment.

I thank members of my supervisory committee, Drs Michael Smith and Honda, for their interest, proper advice and guidance of the thesis. I really appropriate their ability to judge whether or not a project should be continued. I saved much time due to their right judgment.

I am indebted to all the members of our laboratory over the past three years. I thank Dr. Hung Fang for her enthusiastic assistance and friendship which made my starting time in the lab easy and enjoyable. I thank Diana Richer, Ruth Davenport, John Boom, Graham Corley-Smith, Bryan Crawford, and James Lim for their unselfish help and interesting discussions.

I also gratefully acknowledge many faculty members and graduate students from the Department of Biological Sciences and IMBB. In particular, I want to thank Dr. David Baillie, Dr. Kermode, Dr. Boone and Dr Gabe Kalmar.

I wish to thank all members of my family, my mother, my father, my brothers, my sisters and my wife. Especially I thank my wife for her love, support and sharing the happiness and unfortunates with me.

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Abbreviations

Ac	acetate
ATP	adenosine triphosphate
bp	base pair(s)
BSA	bovine serum albumin
°C	degree centigrade
Ci	curie
cpm	counts per minute
DEP	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol-bis (β-aminoethyl ether) N, N, N', N' - tetraacetic acid
g	gram(s) or gravity
GTP	guanosine triphosphate
hr(s)	hour(s)
kb	kilobase
l	liter
M	molar concentration
m	milli- (10^{-3}) or metre
MBL	Marine Biological Laboratory, Woods Hole, MA
min	minute(s)

mol	mole or molecule(s)
MOPS	3 - [N-morpholino]propanesulfonic acid
mRNA	messenger RNA
n	nano - (10^{-9})
p	pico - (10^{-12}) or page
PF	postfertilization
PMSF	phenylmethylsulfonyl fluoride
PP_i	sodium pyrophosphate
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
tRNA	transfer RNA
TTP	thymidine triphosphate
UTP	uridine triphosphate
w/v	weight/volume
μ	micro - (10^{-6})

Solutions

ACE	1M glycine, 2μM EGTA
ASW	artificial sea water
CFSW	calcium free sea water
CMFSW	calcium and magnesium free sea water
MFSW	Millipore filtered sea water
SET	0.15M NaCl, 0.03M Tris HCl, 2mM EDTA, pH8.0

SSC	0.15M NaCl, 0.015M triodium citrate
SSPE	20 × SSPE: 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA-Na_2, pH7.4

1 INTRODUCTION

1.1 Introduction to my project

During embryogenesis of the sea urchin, five spatially restricted territories are established: the small micromeres, the skeletogenic primary mesenchyme cells, the vegetal plate, the oral ectoderm, and the aboral ectoderm. Each territory is derived from several founder cells, and eventually show territory-specific gene expression and regulation (for review: Davidson, 1989; Brandhorst and Klein, 1992). Cell interactions play important roles in specification of the fates of many of the lineage founder cells. Both induction and repression appear to be involved in the specification of cell fate by interactions with neighboring cells (reviewed by Davidson, 1989; Brandhorst and Klein, 1992).

Many tissue-specific expressed genes have been identified and used as markers to study specification and cell differentiation in sea urchin embryos (for review: Davidson, 1986, 1989; Brandhorst and Klein, 1992). Spec1, Spec2 and CyIII actin genes of S. purpuratus are used as markers for aboral ectoderm; SM50 and msp130 for skeletogenic mesenchyme (Stephens *et al.*,1989; Grant *et al.*, 1985; Wessel and McClay, 1985; Anstrom *et al.*, 1987; Leaf *et al.*, 1987); Spec3 gene of S. purpuratus for oral and aboral ectoderm, and LpS1 genes and LpC2 actin gene of L. pictus for aboral ectoderm (Wikramanayake *et al.*, 1995).

In this thesis, I report on more detailed characterization of how the expression of the aboral ectoderm specific genes such as Spec1, LpS1 and LpC2 actin is affected by the dissociation of the embryos at several stages. I found that dissociation has little effect on the level of Spec1 mRNA in the cells cultured in CFSW after dissociation at mesenchyme blastula, gastrula and pluteus stages in S. purpuratus embryos except after prolonged culture. I found that sensitivity of Spec1 mRNA levels to dissociation begins to decline between hatching (20 hr after fertilization) and mesenchyme blastula stage(25 hr after fertilization) in S. purpuratus sea urchin. In contrast, for L. pictus embryos the level of LpS1 mRNA declined when the embryos were dissociated at mesenchyme blastula and gastrula stages. At pluteus stage the level of LpS1 mRNA was hardly affected by dissociation. It was found that dissociation has similar effect on the level of LpC2 actin mRNA and LpS1 mRNA. Efforts were made to establish whether the decline in LpS1 mRNA levels in dissociated L. pictus gastrulae was due to decreases in mRNA stability or transcription.

1.2 Embryogenesis of sea urchins

The embryogenesis of L. pictus is described in detail below, while the development of S. purpuratus is very similar.

The eggs of Lytechinus pictus are meiotically mature when shed with a diameter of 110 nm (for review: Giudice, 1973, 1986). Embryogenesis is initiated by the entry of a sperm, which triggers a series of events such as changes in membrane potentials, the release of cortical granules and the elevation of fertilization envelope. After fertilization, a period of rapid cleavage is initiated. If embryos are cultured in artificial sea water at 15°C, the first cleavage occurs about

90 minutes after insemination. The first two cleavages divide the embryo equally along the animal-vegetal axis and the third cleavage plane is equatorial, dividing the embryo into animal and vegetal halves. The fourth cleavage is equal in the animal half but unequal in the vegetal half, producing eight mesomeres of equal size in the animal half but four large macromeres and four small micromeres in the vegetal half. The rate of cell division slows at about 15 hours PF (postfertilization), with approximately 256 cells. At this time the embryo is at blastula stage. The ciliated epithelial cells of the blastula are arranged in a single layer around the blastocoel. Hatching usually takes place around 20 hours PF, with the fertilization membrane dissolved by hatching enzymes and a free swimming blastula produced. The hatched blastula has a tuft of long cilia restricted to the animal pole and shorter cilia around the rest of the embryo. At late blastula stage, the vegetal pole flattens to form the vegetal plate. The primary mesenchyme cells, derived from the 4 micromeres, ingress into the blastocoel and fuse with each other to form a branched syncytial ring. This is the mesenchyme blastula stage. At about 30 hours PF gastrulation begins and the vegetal plate invaginates into blastocoel to form the archenteron. At this stage the cells of the embryo become arranged into 3 germ layers: the ectoderm, endoderm (archnteron) and mesoderm (mesenchyme cells). The larval skeleton is generated by the primary mesenchyme cells. The tip of the archenteron contacts the ectoderm wall near the animal pole at the stomadaeum (mouth) around 44 hours PF, while the blastopore becomes the anus, as in all deuterostomes. Then the gut differentiates into esophagus, stomach, and intestine. The skeletal spicules form two oral arms, two anal arms, two transverse rods and two body rods of the pluteus larva. A ciliary band is formed at the boundary of the oral and aboral ectoderm. Until this stage of development, the mass of embryo does not change. The pluteus larva begins to feed and grows considerably during larval development. At

metamorphosis the specifically larval structures initially formed during embryogenesis are destroyed or discarded, including the larval skeloton, the anal, oral and esophageal structures, the ciliary band, and most of the collapsed larval ectoderm (Czihak, 1975; Horstadius, 1973).

The eggs of S. purpuratus sea urchin are also meiotically mature when shed. The diameter of the eggs is about 80 microns. The embryogenesis of S. purpuratus sea urchin is very similar to that of L. pictus sea urchin except for required culture temperature and time schedule of the events during the development. I cultured S. purpuratus embryos at 12°C. Under this temperature for S. purpuratus embryos, hatching usually occurred 20 hr after insemination, gastrulation began at 30 hr, prism formed by 68 hr, early plutei by 74 hr and feeding plutei by 96 hr.

1.3 Specification of cell fate during embryogenesis

In the undisturbed embryo there is a strict relationship between cell lineage and cell fate (Cameron and Davidson, 1987; Cameron and Davidson, 1991; reviewed by Davidson, 1989). In S. purpuratus, the founder cells of five spatially restricted territories are formed during the first six cell division: the small micromeres, the skeletogenic primary mesenchyme cells derived from the large micromeres, the vegetal plate, the oral ectoderm, and the aboral ectoderm. Each territory is derived from several founder cells, and eventually show territory-specific gene expression and regulation (for review: Davidson, 1989; Brandhorst and Klein, 1992). Several L. pictus genes show patterns of expression and territorial restrictions similar to their homologues in S. purpuratus embryos,

indicating that the L. pictus embryo may establish territories in a manner similar to the S. purpuratus embryo.

During the establishment of the five territories, cell interactions play important roles. The fates of many of the lineage founder cells can apparently be altered if the normal spatial interrelationships within the embryo are perturbed (reviewed by Davidson, 1989). Except for the micromere lineage, the fate of which appears to be irreversibly committed from the beginning, the fates of the progeny of all the other tiers of blastomeres in the 16-cell embryos can be altered by placing them in chimeric combinations with other blastomeres, indicating that the specification of these founder cells in the sea urchin embryos is considerably dependent upon intercellular interactions (reviewed by Davidson, 1986, 1989). The 64-cell sea urchin embryo has been regarded as a set of horizontal blastomere tiers (Horstadius, 1935). From the vegetal pole toward the animal pole these are the micromeres, veg_2, veg_1, an_2 and an_1, the latter two are often considered together as the "animal cap". In the embryos the animal cells and veg_1 tier normally produce ectoderm; the veg_2 layer gives rise to endoderm and some larval mesoderm; and the micromeres generate the skeletogenic mesenchyme (Horstadius, 1973). When isolated and cultured, veg_2 tier produces a ciliated embryoid with a large archenteron and minute skeletal elements; veg_1 produces a ciliated blastula which may or may not contain a rudimentary archenteron, and no skeleton; and the animal cap produces neither skeleton nor gut (Horstadius, 1935).

Transplantation experiments carried out by Horstadius also showed that cell interaction is critical (Horstadius, 1973). The isolated animal cap can with the addition of micromeres produce a complete larva including gut and skeleton; veg_1, with implanted micromeres, gives rise to a larva that while deformed, nonetheless

contains a prominent gut and forms a skeleton; and implantation of micromeres into veg_2 results in exogastrula consisting of archenteron, ectoderm and mesenchyme, but no skeleton. These experiments serve as superb indicators of inductive competence and plasticity of the blastomeres of the sea urchin embryo.

If implanted in ectopic positions on a whole early stage embryo (e.g. between veg_1 and an_1), micromeres induce the invagination and differentiation of a secondary gut (Horstadius, 1973; Ransick and Davidson, 1993). Therefore, micromeres have the capacity to induce gut formation in any tier of blastomeres with which they are placed in contact, and probably all blastomeres in the cleavage stage embryo (except other micromeres) are competent to respond to micromere induction. However, removal of micromeres just after they form, does not impair development (Horstadius, 1973).

The fate of all the lineage founder cells except the micromeres is plastic. Implanted veg_2 cells also induce adjacent an_2 cells to participate in gut formation (Horstadius, 1973). Another capacity of veg_2 progeny that is normally suppressed by the presence of micromeres and their derivatives is the formation of skeleton from the secondary mesenchyme that ingresses from the archenteron during gastrulation (Driesch, 1893; Ettensohn and McClay,1987). Implantation of the veg_1 tier on the vegetal surface of the animal cap causes the differentiation of oral structures (mouth and ciliated bands) which usually do not form from isolated animal caps (Horstadius, 1973). By implanting micromeres at successively later times into isolated animal caps, Horstadius also demonstrated that competence of animal cap cells to respond to induction is gradually lost (Horstadius 1973, pp.84-91). So the micromeres and the veg_2 and veg_1 tiers all possess inductive

capacities, and veg_2 tier, veg_1 tier and animal cap cells are all competent to respond to inductive signals during cleavage. In addition, there appears to be several suppressive interactions (Ettensohn and McClay, 1987; reviewed by Brandhorst and Klein, 1992; reviewed by Wilt, 1987).

Ransick and Davidson (1993) transplanted micromeres into the animal cap of an 8-cell sea urchin embryo. These skeletogenic mesenchyme precursor cells induced the formation of a secondary archenteron and the expression of the archenteron-specific genes. This suggests that induction plays a role in the specification of founder cell fate in the undisturbed embryo. This may be mediated by transcriptional regulatory proteins that are localized throughout the egg and that become activated by inductive interactions between adjacent cells (reviewed by Davidson, 1989).

1.4 Dissociation and reaggregation of sea urchin embryos

Cell dissociation and reaggregation experiments in sea urchins have been used to help understand the relevance of cell interactions in specification of cell fate and morphogenesis (reviewed by Giudice and Mutolo, 1970; Giudice, 1973 and 1986). Giudice (1961) developed a method to completely dissociate the sea urchin embryos into single cells which could reaggregate and form structures closely resembling normal larvae. This results applies to embryos from the blastula through the pluteus stages. When cells are dissociated from early blastula till late mesenchyme blastula stages, the pattern of reaggregation is always the same: formation of solid clumps that then become hollow spheres by means of a process of internal cavitation, appearance of cilia, with the formation of structures closely

resembling "swimming blastulae". Groups of cells inside the blastocoel form tubular structures which then attach to the blastula wall and, by opening outside, form one intestine-like structure. Triradiate spicules appear at this time, which elongate and bend in the characteristic way of the pluteus, while pigment cells differentiate. The shape and size of the reformed larvae vary with the aggregation conditions, i.e., concentration of the cells, rate of stirring or lack of stirring, and composition of the sea water. The efficiency of formation of new larvae also varies, but is close to 100% under optimal conditions (Giudice and Mutolo, 1970; and Giudice, 1973, for review). If the embryos are dissociated at the gastrula stage and the cells reaggregated, the reaggregation pattern is the same as that described for blastula, but the skeleton usually does not elongate to form rods. If cells are dissociated from the young plutei, they again reassociate according to the blastula cell pattern, but the skeleton is never formed. Embryos can also be dissociated into cells during stages earlier than blastula, i.e., before the cells have formed an epithelial shape and are still in the form of rounded blastomeres. In this case treatment with Ca^{2+}-free sea water followed by repeated pipeting is enough to achieve dissociation (Herbst, 1900). Reaggregates obtained by cells dissociated at the 16-blastomere stage have been reared through metamorphosis by Hinegardner (1975).

However, Freeman (1988) completely dissociated the embryos of Hemicentrotus pulcherrimus at 16-cell, 400-cell or mesenchyme blastula stage of development into single cells. The cells were allowed to reaggregate. Only aggregates from 16-cell embryos developed into pluteus-like larvae with radial or bilateral symmetry. If the embryos were incompletely dissociated at these three stages so that there were groups of undissociated cells, more aggregates from 16-

cell embryos developed in a pluteus-like manner than in aggregates from completely dissociated 16-cell embryos. And some aggregates from 400-cell embryos developed into pluteus-like larvae. Pieces of intact animal, lateral, or vegetal blastula wall were grafted to aggregates formed from completely dissociated blastula embryos. While each kind of graft improved the ability of the aggregate to develop in a pluteus-like manner, grafts of vegetal blastula wall were most effective. The pluteus-like development observed by other investigators (reviewed by Giudice, 1973) in aggregates from embryos dissociated from blastula stages and later stages of development may be because these aggregates contained a cell population which was not fully dissociated (Freeman, 1988).

Reaggregation of cells of sea urchin embryos shows some stage specificity. Though Giudice *et al.* (1969) found no strict stage specificity in the reaggregation process of the blastula cells with the prism cells, Spiegel *et al.* (1978) were able to prove that vitally stained micromeres dissociated from the 16-blastomere stage, if reaggregated with macro- and mesomeres, sort out and group separately from the latter blastomeres within the context of the same aggregate. Related to this cell-type specificity during reaggregation, new surface antigens appearing at gastrulation have been identified (McClay and Chambers 1978), and different surface antigens can be detected in different germ layers, at least at the pluteus stage (McClay and Marchase, 1979; McClay, 1979, 1982). During the course of development the cell surface properties of sea urchin embryos undergo changes (Krach *et al.*, 1973, 1974; Roberson and Oppenheimer, 1975; Neri *et al.*, 1975; Katow and Solursh, 1982; Sano, 1977; Timourian *et al.*, 1973; Turner *et al.*, 1977; Sasaki and Aketa, 1981). Sea urchin embryonic cells also reaggregate in a species-specific way (Giudice, 1962; Spiegel and Spiegel, 1975, 1978). Several kinds of molecules are involved in the intercellular adhesion (reviewed by Guidice, 1986).

Spiegel and Spiegel (1978) and Giudice (1963) showed that during reaggregation, the cells sort out and find their correct position in the aggregate, grouping among themselves according to cell types; cells do not dedifferentiate upon dissociation and redifferentiate again upon reaggregation, according to the position occupied in the new aggregate (reviewed by Giudice, 1986). Correct cell interactions are required for some developmental events to occur normally, such as changes in the activity of enzymes, synthesis of RNA, synthesis of DNA, synthesis of protein and ciliogenesis (reviewed by Giudice, 1973, 1986).

1.5 Tissue-specific genes expressed in sea urchin embryos

Spatially differential patterns of expression, in which specific genes are activated exclusively within specific cell lineages, were first discovered in the sea urchin embryo by Angerer and Davidson (1984). Batteries of genes and gene products that characterize the differentiated cells have been being identified (reviewed by Davidson, 1986, 1989; reviewed by Brandhorst and Klein, 1992). Nine or ten proteins constituting the skeletal (spicule) matrix and synthesized by skeletogenic mesenchyme cells were characterized by Benson *et al.* (1987). A number of other proteins that are synthesized specifically and exclusively by these cells have been detected by immunocytological methods and by cDNA cloning (reviewed by Davidson, 1986, pp. 224-228; Harkey *et al.*, 1988). Two cytoskeletal actin genes, CyIIIa and CyIIIb, the arylsufatase gene, the metallothionein A gene, the homeobox gene SpHB1, and a family of genes encoding about eight Spec1 and Spec2 proteins (related to the calmodulin/troposin C/myosin light chain group of calcium-binding proteins) are expressed specifically in aboral ectoderm of S. purpuratus (reviewed by Davidson, 1986, Hardin *et al.*, 1988; reviewed by Brandhorst and Klein, 1992). Two genes in L. pictus, LpS1α

and LpS1β, which are related to the Spec genes in sequence and the capacity of the encoded protein to bind calcium ions, are also exclusively expressed in the aboral ectoderm (Xiang *et al.*, 1988). The LpC2 actin gene of L. pictus is almost exclusively expressed in the aboral ectoderm (Fang, 1994).

Many tissue-specific expressed genes have been used as markers to study cell differentiation (reviewed by Davidson, 1986, 1989; reviewed by Brandhorst and Klein, 1992). Spec1, Spec2 and CyIII actin genes are used as markers for aboral ectoderm; SM50 and msp130 for skeletogenic mesenchyme (Stephens *et al.*, 1989; Grant *et al.*, 1985; Wessel and McClay, 1985; Anstrom *et al.*, 1987; Leaf *et al.*,1987); Spec3 for oral and aboral ectoderm; and LpS1 genes and LpC2 actin gene for aboral ectoderm (Wikramanayake *et al.*, 1995). Since the Spec1 gene, LpS1 gene, and LpC2 actin gene are used as aboral ectoderm markers in the research reported in the thesis, they are described further below.

Spec1 gene of S. purpuratus

In the S. purpuratus genome, there is a single Spec1 gene and six or seven related Spec2 genes (Hardin *et al.*, 1985; Hardin *et al.*, 1988; Klein *et al.*, 1987). Four of these genes (Spec1, Spec2a, Spec2c ,and Spec2d) have been cloned and studied in considerable detail. The Spec1 gene codes for a 16 kD troponin C-like protein that contains Ca^{2+}-binding domains. The 1.5Kb Spec1 mRNA is expressed in aboral ectoderm cells of the pluteus (Carpenter, 1984; Hardin *et al.*, 1988). Spec1 transcripts are initially present at low levels in all cells of the early embryo but accumulate to high levels in the 200 or so presumptive aboral ectoderm cells of the mesenchyme blastula embryos, which have about 500 cells (Cameron *et al.*, 1987; Carpenter *et al.*, 1984; Hardin *et al.*, 1988). Transcription

of the Spec1 gene is activated at the 4th-8th cleavage as the period of rapid division is ending (Tomlinson *et al.*, 1990).

cis-acting regulatory elements and *trans*-acting regulatory proteins involved in the aboral ectoderm-specific expression of Spec genes have been analyzed (reviewed by Brandhorst and Klein, 1992). A conserved repetitive sequence block of about 700 bp dominates the 5' flanking region of three Spec genes (Spec1, Spec2a, and Spec2c) (Gan *et al.*, 1990a; Gan *et al.*, 1990b). In Spec2a, this block abuts the start site of transcription, but in Spec2c and Spec1, several hundred base-pairs of DNA disrupt the block, drastically altering the immediate upstream region where *cis*-regulatory elements often reside. Positive *cis*-regulatory elements are present within the inserted DNA interrupting the block of Spec1 (Klein *et al.*, 1990). When a lacZ reporter gene fused to the 5' flanking regions of the Spec2a, Spec2c, or Spec1 genes is used to monitor spatial expression, only the Spec2a promoter shows a strict aboral ectoderm pattern of expression (Gan *et al.*, 1990a):a 1516 bp fragment of 5' flanking DNA from Spec2a is sufficient for proper spatial regulation, but fragments of up to 5600 bp of Spec2c and Spec1 are not sufficient, suggesting a lack of functional negative spatial regulatory elements (Gan *et al.*, 1990a).

Three subregions have been defined within the 1516 bp fragment of Spec2a: a distal region between base pairs -1516 and -697, necessary for preventing expression in mesenchyme cells; the RSR enhancer between base pairs -631 and -443, necessary and sufficient for aboral ectoderm and mesenchyme cell expression; and a proximal region from -1443 to +18, required for basal promoter activity (Gan *et al.*, 1990a, 1990b; Gan and Klein, 1993). Within the proximal region is a DNA-binding site for the transcription factor USF (between base pairs

-75 and -70) and a TATA box (between base pairs -30 and -26) (Hardin *et al.*, 1988; Kozlowski *et al.*, 1991). The su-USF transcription factor appears to be localized to ectodermal cells at the prism and pluteus stages, suggesting that it acts as a positive regulator of Spec gene activity (Kozlowski *et al.*, 1991). However, mutations that eliminate su-USF binding at these sites do not alter Spec2a (or LpS1) promoter activity (Gan *et al.*, 1990b). The RSR enhancer, when placed in front of a LacZ reporter gene, drives the reporter only in aboral ectoderm and mesenchyme cells, not in oral ectoderm or endoderm (Gan and Klein, 1993). These results suggest that positive aboral ectoderm/mesenchyme cell-specific elements or negative oral ectoderm/endoderm-specific elements are located within the enhancer. Within the RSR enhancer a postive *cis*-regulatory element with the core consensus sequence TAATCC has been defined, which is capable of binding the sea urchin orthodenticle-related homeobox protein SpOtx (Mao *et al.*, 1994; Gan and Klein, 1993; Gan *et al.*, 1995). The 188 bp enhancer region was further defined by Mao *et al.* (1994). Three Otx consensus binding sites were responsible for the activity of the enhancer, acting in a non-cooperative manner to yield full activity. Mutagenizing the three Otx sites and a fourth one just upstream abolished all activity in the context of the complete Spec2a control region. Otx sites were able to bind SpOtx, suggesting that this transcription factor mediates positive control at these sites. Non-SpOtx binding sites overlapping two of the Otx sites may also play a role in Spec2a expression. A 76-bp DNA fragment containing two of the Otx sites was sufficient for aboral ectoderm/mesenchyme cell expression. These results suggest that the RSR enhancer plus an upstream DNA element required for mesenchyme cell repression are necessary and sufficient for the proper temporal activation and aboral ectoderm expession of the Spec2a gene and that the Otx elements play a positive role in this process.

SpOtx, a cDNA clone encoding an orthodenticle-related protein from the sea urchin S. purpuratus has been characterized by Gan *et al.* (1995). Orthodenticle-related proteins bind with high affinity to DNA containing the sequence motif TAATCC/T. The Spec2a gene may be target gene for SpOtx. Two SpOtx transcripts accumulate during embryogenesis, an early transcript accumulating to highest concentration at gastrula stage. SpOtx transcripts were found initially in all cells of the cleaving embryo, but they gradually became restricted to oral ectoderm and endoderm cells. In contrast, SpOtx protein was found in nuclei of all cells at both blastula and pluteus stages. These results suggest that SpOtx plays a role in the activation of the Spec2a gene and most likely has additional functions in the developing sea urchin embryo.

There is also evidence that posttranscriptional regulation plays an important role in the aboral ectoderm specificity of expression of the Spec1 gene prior to gastrulation (Gagnon *et al.*, 1992).

Spec1 gene has been used as a marker for aboral ectoderm differentiation to study the role of cell-cell interactions in specification and differentiation of aboral ectoderm in S. purpuratus embryos (Wikramanayake *et al.*, 1995). When pairs of mesomeres or animal caps, which are fated to give rise to ectoderm, were isolated and cultured they developed into ciliated embryoids that were morphologically polarized (Wikramanayake *et al.*, 1995). In animal explants from S. purpuratus, the aboral ectoderm-specific Spec1 gene was activated at the same time as in control embryos and at relatively high levels (Wikramanayake *et al.*, 1995). The Spec1 protein was restricted to the squamous epithelial cells in the embryoids suggesting that an oral-aboral axis formed and aboral ectoderm differentiation occurred correctly (Wikramanayake *et al.*, 1995).

These results indicated that animal explants from S. purpuratus were autonomous in their ability to form an oral axis and to differentiate aboral ectoderm.

LpS1 genes of L. pictus

Two nearly identical genes in L. pictus, LpS1α and LpS1β, are distantly related to the Spec genes in sequence, immunological cross reaction, and the capacity of the encoded proteins to bind calcium ions (Xiang *et al.*, 1988). The LpS1 gene is transcriptionally activated during the late cleavage-early blastula stages, at a developmental stage similar to the activation of S. purpuratus Spec1 and Spec2 genes (Tomlinson *et al.*, 1990; Xiang *et al.*, 1988). The LpS1 gene and the Spec genes are likely to be derived from a common ancestor (Klein *et al.*, 1991). But the LpS1 gene has been tandemly duplicated (Xiang *et al.*, 1988), encoding a protein twice as large as Spec1. The LpS1 mRNA accumulates from extremely low levels in the unfertilized egg to high levels in postcleavage stage embryos and does so exclusively in aboral ectoderm cells. Transcription of the LpS1 genes is sensitive to the integrity of the collagenous extracellular matrix, while transcription of the Spec genes is not (Wessel *et al.*, 1989). PDGF-BB and TGF-*a* can rescue LpS1 expression in collagen-disrupted embryos of the sea urchin genus Lytechinus (Ramachandran *et al.*, 1993).

cis-regulatory elements and *trans*-regulatory factors involved in the temporal regulation and aboral ectoderm specific expression of the LpS1 gene have been characterized in detail (reviewed by Brandhorst and Klein, 1992). The

L. pictus LpS1 promoters lack an RSRA sequence block and their 5' flanking DNA shows little similarity to 5' flanking DNA of Spec genes. For the LpS1β gene, 762 bp of 5' flanking DNA is sufficient for proper temporal and aboral ectoderm specific expression (Xiang *et al.*, 1991b). Deletion of all but 109 bp of 5' flanking DNA results in a less than 3-fold decline in LpS1β promoter activity (Xiang *et al.*, 1991a). There is a sequence element involved in repressing expression in mesenchyme cells between -762 and -511 bp of the LpS1β promoter, since deletion of DNA of this region causes mesenchyme cells to express the gene (Xiang *et al.*, 1991b). A major positive *cis*-regulatory element having a core sequence of GGGGGGC is present in the LpS1β proximal promoter at -70 bp, as mutations in these six G's abolish promoter activity (Xiang *et al.*, 1991a). An identical G-rich sequence is present on the complementary DNA strand of LpS1β at -727 bp and may be responsible for the 3-fold drop in promoter activity observed by deletion analysis. These G-rich elements are referred to as proximal and distal G-strings. The proximal G-string element binds two distinct nuclear factors *in vitro*, one specific to aboral ectoderm cells (ecto-GSF) and the other to endoderm/mesoderm cells (endo/meso-GSF). The distal G-string element binds only ecto-GSF (Xiang *et al.*, 1991a). ecto-GSF is a positive regulatory protein that activates the LpS1β promoter at the proximal (and perhaps distal) G-string element. The role of endo-meso-GSF may be repression of the LpS1β expression in non-ectoderm cells. USF sites are also present on the LpS1 gene promoters and can bind sea urchin USF (Tomlinson *et al.*, 1990; Kozlowski *et al.*, 1991). But mutations at these sites do not alter LpS1 promoter activity (Gan *et al.*, 1990b). Thus the mechanisms regulating the aboral ectoderm specificity of transcription of the Spec1 and LpS1 genes are different.

LpS1 genes have also been used as markers of specification and differentiation of aboral ectoderm in investigating the role of cell-cell interactions in specification and differentiation of aboral ectoderm of L. pictus embryos (Wikramanayake *et al.*, 1995). Wikramanayake *et al.* (1995) found that no expression of LpS1 genes was detected in animal explants from L. pictus. Recombination of the explants with vegetal blastomeres or exposure to the vegetalizing agent LiCl restored activity of aboral ectoderm-specific genes, suggesting the requirement of a vegetal induction for differentiation of aboral ectoderm cells in L. pictus. This contrasts with the results of their similar experiments on S. purpuratus embryos.

LpC2 actin gene of L. pictus sea urchin

The LpC2 actin gene is a newly-identified actin gene of L. pictus sea urchin (Fang, 1994). Its mRNA accumulates almost exclusively in the aboral ectoderm territory, beginning at the gastrula stage. In cultured mesomere pairs its behavior is similar to that of the LpS1 mRNA. In the cultured animal cap no LpC2 actin mRNA was detected (Wikramanayake *et al.*, 1995).

1.6 Role of extracellular matrix in tissue-specific gene expression in the sea urchin embryos

The extracellular matrix (ECM) of the sea urchin embryo consists of an outer ECM and an inner ECM. The outer ECM surrounding the sea urchin embryo, also referred as hyaline layer, contains fibers and granules of various sizes (Spiegel *et al.*, 1989). The inner ECM includes the basal lamina and blastocoelar matrix (Spiegel *et al.*, 1989). The hyaline layer is a multi-laminate structure

composed of at least 10 polypeptides (Alliegro *et al.*, 1992) including collagen (Spiegel *et al.*, 1979), glycoprotein (echinonectin) (Alliegro *et al.*, 1990) and proteoglycan (Alliegro *et al.*,1992). Fibronectin and laminin have been found in the ECM and in the basement membrane of sea urchin embryos during early development (Spiegel *et al.*, 1983). They form a continuous matrix surrounding the cells which links the outer ECM to the inner ECM (Spiegel *et al.*, 1983).

The role of ECM in the differentiation of tissue types has been examined (Benson *et al.*, 1991). The lathrytic agent beta-aminopropionitrile fumarate (BAPN) inhibits collagen deposition in the ECM by specifically inhibiting lysyl oxidase, the enzyme involved in collagen crosslinking (Butler *et al.*, 1987). Treatment of sea urchin embryos with BAPN arrests gastrulation, suggesting that collagen is important for gastrulation and subsequent differentiation, but not for earlier developmental processes (Wessel *et al.*, 1987). The Spec1 mRNA, Spec2 mRNA, CyIIa actin, CyIIIa actin and collagen mRNAs in S. purpuratus, and metallothionine mRNA, ubiquitin mRNA and LpS3 mRNAs in L. pictus and L. variegatus accumulated normally during BAPN treatment, even though the embryos did not gastrulate (Wessel *et al.*, 1989). But LvS1 and LpS1 mRNA accumulation were inhibited by BAPN treatment. LpS1 transcription is selectively inhibited by BAPN treatment (Wessel *et al.*, 1989).

Benson *et al.* (1991) treated sea urchin embryos with BAPN and beta-D-xyloside, which disrupts proteoglycan metabolism. They found that the accumulation of SM50 transcripts was not affected by these agents. Spec1 mRNA, in contrast, accumulated to a level somewhat lower than that in control embryos when collagen and proteoglycan metabolism is disrupted. The postgastrula rise in gut-specific alkaline phosphatase is reversibly inhibited by

BAPN and xyloside treatment. These observations demonstrate a differential effect of the ECM on expression of tissue-specific molecular markers.

Ramachandran *et al.* (1993) found that human recombinant platelet derived growth factor-BB (PDGF-BB) and transforming growth factor-alpha (TGF-α) synergistrically rescue collagen disrupted/developmentally arrested L. pictus and L. variegatus embryos so that development and accumulation of LpS1 RNA proceed, and antagonists of PDGF block gastrulation and LpS1 RNA accumulation. The embryos recover and LpS1 RNA accumulation resumes when the antagonists are removed. Thus a growth factor mediated pathway, possibly associated with the ECM, appears to be required for sea urchin gastrulation, spiculogenesis, and LpS1 gene activation.

1.7 Expression of tissue-specific genes in dissociated sea urchin embryos

The Spec1 gene has been used as marker to examine the effects of embryo dissociation on the autonomy of aboral ectoderm specification and differentiation (Hurley *et al.*, 1989; Stephens *et al.*, 1989; Stephens *et al.*, 1990). Hurley *et al.* (1989) dissociated S. purpuratus sea urchin embryos into single cells at 16-cell stage and cultured them in calcium-free sea water until intact control developed to mesenchyme blastula stage. They found that Spec1 mRNA accumulation began at the appropriate time but reached much lower levels in dissociated cells than in control embryos. They also found that addition of Ca^{2+} or serum did not cause any increase in the level of Spec1 mRNA in dissociated cells. Stephens *et al.* (1989) dissociated S. purpuratus sea urchin embryos at 2-cell, 16-cell, 128-cell and mesenchyme blastula stages and then cultured the single cells in CFSW until intact control embryos reached the gastrula stage. They reported that average Spec1

expression is very low in dissociated embryos, only 10-20% of intact controls. The activation of Spec1 is temporally correct and remains restricted to the appropriate number of cells, even if the embryos is dissociated as early as the 2-cell stage and maintained as a suspension of single cells. This implies that Spec1 gene expression is properly activated in aboral ectoderm cells autonomously but not fully expressed.

Stephens *et al.* (1990) dissociated L. pictus sea urchin embryos at 4-cell stage and raised the single cells in Ca^{2+}-free sea water for 24 hours. They observed that accumulation of the aboral ectoderm-specific transcript (LpS1) is extremely low (about 2% of normal) in the dissociated cells. Readdition of Ca^{2+} to the sea water 8 hours after fertilization and allowing cells to reassociate into small, tight clusters restored Spec1 accumulation to 70% of control levels. When isolated mesomere pairs derived from dissociated 8-cell S. purpuratus embryos are cultured, Spec1 mRNA accumulated to a normal level (Wikramanayake *et al.*, 1995). These observations indicate that contact between the derivatives of two mesomeres is sufficient for full differentiation of aboral ectoderm, but this does not occur in the absence of this contact. In contrast, no LpS1 mRNA was detected by Wikramanayake *et al.* (1995) in the cultured animal cap or embryoids derived from mesomere pairs isolated at 8-cell stage of L. pictus, suggesting that contact between the animal cells and vegetal cells is required for differentiation of aboral ectoderm in L. pictus embryos.

Effect of dissociation on the expression of SM50, a primary mesenchyme specific transcript, was also examined by Stephens *et al.* (1989). Average SM50 expression in the cells dissociated at 2-cell, 4-cell, 16-cell, 128-cell is half that of intact embryos. The expression of SM50 is temporally correct and remains restricted to the appropriate number of cells after dissociation, suggesting that the expression of SM50 is autonomous.

In addition to the Spec1 gene, Hurley *et al.* (1989) examined the expression of ten other genes in dissociated S. purpuratus embryos at the 16-cell stage. The genes were CyI actin, CyIIa actin, CyIIb actin, CyIIIa actin, Spec2a, Spec3, Collagen, SpHB1, SpMW5 and SpMW9. The expression of CyI actin gene was not affected by dissociation. The expression of CyIIa actin, CyIIb actin, SpMW5 and SpMW9 increased in dissociated embryos, while the expression CyIIIa actin, Spec2a, Spec3, Collagen and SpHB1 was reduced in dissociated embryos. Addition of Ca^{2+} or serum to the cultures of dissociated cells did not alter the expression of these genes in dissociated cells. Hurley *et al.* (1989) also tested the kinetics of accumulation of the mRNAs of these genes in dissociated cells up to 24 hr after dissociation. Two maternal RNAs, SpMW5 and SpMW9 decay in control embryos and in dissociated cells, although the latter maintain consistently higher levels. Three actin genes, CyI, CyIIa and CyIIb, show initial accumulation to higher levels in separated cells and then level off or decline while they continue to accumulate and decay with kinetics similar to those in control embryos, at all stages levels are reduced compared to controls. All other messages accumulate to lower (collagen), or much lower (Spec1, actin CyIIIa) levels in dissociated cells than in controls, but begin to accumulate at about the proper time.

The increase in the level of CyIIa actin mRNA in dissociated cells is due to an increase in the number of cells expressing CyIIa actin in dissociated embryos. The decline in the level of Spec1 mRNA in dissociated cells is not due to a decline in the number of cells expressing Spec1 mRNA, but due to decline in the level of Spec1 mRNA per cell. All these observations indicate that interactions among cells in the intact embryo are important for both negative and positive control of expression of different genes that are early indicators of the specification of cell fate.

1.8 Specific aims of this project

As discussed above, Spec1 and LpS1 genes have been used as markers to study the effect of dissociation on specification and differentiation of aboral ectoderm. But the previous investigations have been done only with embryos of early stages, and nothing is known about the mechanism by which dissociation affects the expression of these genes. For my project, there were two goals: (1) to establish whether or not dissociation affects the levels of LpS1 mRNA, LpC2 actin mRNA and Spec1 mRNA at later stages; (2) to establish whether the decline in the levels of LpS1 mRNA and LpC2 actin mRNA in L. pictus is due to decreases in mRNA stability or transcription.

2 MATERIALS AND METHODS

2.1 Materials

Lytechinus pictus sea urchins were purchased from Marinus, Long Beach, CA. S. purpuratus sea urchin were collected on Vancouver Island, BC. Trypan Blue was purchased from Sigma. Actinonymcin D was purchased from Sigma, and dissolved in sterile water. The plasmid containing Spec1 gene fragment was from Carpenter (1984). The plasmid containing LpS1 gene fragment was cloned by Xiang *et al.* (1991b). The plasmid bubT7 containing ubiquitin gene fragment was from Gong *et al.* (1991). The plasmid A7PCR containing LpC1 actin gene 3' non-coding sequence and the plasmid A5H(3') containing LpC2 actin gene 3' non-coding sequence were described by Fang (1994). The plasmid $P_{\beta 2}$ containing β-tubulin gene was cloned by Gong and Brandhorst (1988a). The two growth factors, TGF-α and PDGF-BB, were purchased from GIBCOBRL. ^{32}P-dCTP and ^{32}P-UTP were purchased from Amersham. Diethylpyrocarbonate (DEP) was purchased from Sigma. GeneSceen Nylon membrane was purchased from New England Nuclear. NEBlot Kit was purchased from New England Biolabs. Ficoll, DNaseI, dextran sulfate, Sephadex G-50 were purchased from Pharmacia. Proteinase K, HincII, EcoRI, PstI, Urea, glycerol, DTT, TGF-α, PDGF-BB and deionized formamide were purchased from BRL. Phenol, NaCl and KAc were purchased from Fisher. $CaCl_2$ was purchased from J.T.Baker. CTP and Tris were purchased from Boehringer. Glycine, agarose and SDS were purchased from BIO-RAD. Chloroform and LiCl were purchased from BDH Inc. $MgCl_2$, $MgSO_4$ and TCA (trichloroacetic acid) were purchased from Anachemia. All other chemicals used were purchased from Sigma except that some are specifically mentioned.

MBL artificial sea water contains 46.4g of NaCl, 7.3g of Na_2SO_4, 1.4g of KCl, 0.4g of $NaHCO_3$, 2g of $CaCl_2$ and 19.8g of $MgCl_2 \cdot 6H_2O$ in one liter of water. One liter of CMFSW (calcium/magnesium free sea water) contains 31g of NaCl, 0.8g of KCl, 0.2g of $NaHCO_3$, 1.6g of Na_2SO_4. One liter of CFSW (calcium free sea water) contains 26.5g of NaCl, 0.7g of KCl, 11.9g of $MgSO_4 \cdot 7H_2O$ and 0.5g of $NaHCO_3$. The reagent grade salts used for making the sea water were purchased from some specific companies, NaCl, KCl, $NaHCO_3$ and $CaCl_2$ from J.T.Baker; Na_2SO_4 from Fisher; $MgCl_2 \cdot 6H_2O$ from Anachemia.

2.2 Preparation and culturing of sea urchin embryos

Gametes were obtained by intracoelomic injection of 0.5M KCl or electrostimulation according to Fang (1994). After fertilization, the embryos were cultured in Millipore-filtered (0.45 μm filter) sea water MFSW with stirring at 12°C for S. purpuratus embryos, and at 15°C for L. pictus embryos. Under these conditions for L. pictus, hatching usually occurred 20 hr after insemination, gastrulation began at 30 hr, prisms were formed by 50 hr, early plutei by 70 hr, and feeding plutei by 90 hr. Under the conditions used for S. purpuratus, hatching usually occurred 20 hr after insemination, gastrulation began at 30 hr, prism formed by 68 hr, early plutei by 74 hr and feeding plutei by 96 hr.

2.3 Dissociation of S. purpuratus sea urchin embryos into single cells at early stages

Dissociation of S. purpuratus embryos at early stages was performed according to Stephens *et al.* (1989). After the eggs were washed at least twice with MFSW, they were fertilized in MFSW containing 5 mM sodium p-aminobenzonic acid, which prevents the hardening of the fertilization envelope. The fertilization membrane was removed by passage through a 55 μm Nitex screen at 30 min after fertilization. Embryos were then allowed to settle 2 times in MFSW, and cultured at 12°C to desired stage. The embryos were allowed to settle 2 times in CMFSW, or 3-4 times if necessary, and swirled vigorously in a 50 ml tube. Once separated, blastomeres were stirred at 60 revs/min and cultured in CFSW at 0.2 - 1 $\times 10^4$ initial cells per ml. Within the next 4 - 6 hr, and every 2 hr thereafter, cultures were poured through 35 μm Nitex to prevent reaggregation.

2.4 Dissociation of S. purpuratus embryos into single cells at mesenchyme blastula, gastrula and pluteus stages

The embryos of S. purpuratus at desired stages were collected by centrifugation at 500 rpm for 1 min in a SORVALL GLC-1 centrifuge. Then they were washed by resuspension in cold CMFSW once. After they were spun at 500 rpm for 1 min, and suspended in cold 40 volumes of AEC (1M glycine, 2μM EGTA). The embryos were incubated on ice for 3 - 6 min (gastrula:3 min; pluteus: 6 min), and collected at 500 rpm for 1 min in SORVALL (GLC-1). The

embryos were washed with cold CMFSW once or twice and resuspended in 20 volumes of cold CMFSW. They were dissociated into single cells by pipeting vigorously through a Pasteur pipette 10 times. The cell suspension was filtered through two layers of 35 μm Nitex to remove fragments of embryos. The cells were washed in CFSW twice, and counted by using a hemocytometer. Single cells were cultured in CFSW (7.5×10^5 cells /ml) with stirring 60 rpm at 12°C for the desired time. Cultures of cells showing a viability of over 95% were collected for preparation of cellular RNA.

2.5 Dissociation of L. pictus embryos at cleavage stages.

After the eggs were washed twice with MFSW, they were fertilized in 5 mM sodium p-aminobenzonic acid in sea water at 15°C. The fertilization envelope was removed by passage through a 55 μm Nitex screen at 30 min after fertilization. The embryos were washed once with cold CFSW. They were allowed to settle in CFSW at 4°C for 10 min. Then they were allowed to settle in CMFSW at 4°C for 10 min and swirled vigorously in a tube. Once separated, blastomeres were stirred at 60 revs/min and cultured in CFSW at 15°C at $0.2\text{-}1 \times 10^4$ initial cells ml^{-1} dilution. Within the next 4-6 hr, and every 2 hr thereafter, cultures were poured through 35 μm Nitex to prevent reaggregation. After the intact control embryos developed to gastrula stage, the single cells were collected for preparation of cellular RNA if their viability was over 95%.

2.6 Dissociation of L. pictus embryos at late stages

L. pictus embryos at mesenchyme blastula, gastrula, and pluteus stages were collected at 500 rpm for 1 min in SORVALL GLC-1 centrifuge. The embryos were washed with CFSW at 15°C once, resuspended in 25 volumes of cold AEC (glycine(1M) + 2 μM EGTA) and incubated on ice for 3-6 min. After centrifugation at 500 rpm for 1 min, the embryos were washed with cold CFSW at least once. The embryos were dissociated into single cells in cold CFSW by vigorous passage 6-10 times through a Pasteur pipe. The cells were filtered through two layers of 25 μm Nitex. They were cultured in CFSW at 15°C at 5-7.5 $\times$ 10^5 cells/ml with stirring at 60 rpm. After the cells were cultured for the desired time, the cells with viability of over 95% were collected to prepare cellular RNA.

2.7 Trypan blue exclusion

The viability of dissociated cells was tested by using a Trypan blue exclusion assay. 50μl of 0.4%(w/v) Trypan blue store solution in sea water was transferred to a test tube. Following addition of 30μl CFSW, 20μl of the cell suspension in CFSW was added and mixed thoroughly. The mixture was allowed to stand for 5 to 15 minutes. If cells are exposed to Trypan blue for extended periods of time, viable cells, as well as non-viable cells, may begin to take up dye. With the cover-slip in place, a small amount of Trypan blue-cell suspension mixture was transferred to both chambers of the hemocytometer. Non-viable cells stain blue. Viable cells and non-viable cells were counted separately. This

procedure was repeated for the second chamber. If less than 200 or greater than 500 cells (i.e.,20-50 cells/square) were observed in the 10 squares, the procedure was repeated after adjusting to an appropriate dilution factor. A second sample was then analyzed in the same way and cell viability calculated (percentage of cells excluding Trypan blue).

2.8 Treatment of the intact and the dissociated embryos with TGF-α and PDGF-BB

After the L. pictus sea urchin embryos developed to gastrula stage, some of them were dissociated into single cells as described above. Then the dissociated cells were divided into 4 samples. One was cultured in CFSW, one in CFSW containing 10ng/ml of TGF-α, one in CFSW containing 10ng/ml of PDGF-BB and one in CFSW containing 10ng/ml of TGF-α and 10ng/ml of PDGF-BB. Intact embryos were cultured in MFSW. After they were cultured for 20 hours, the cytoplasmic RNA was isolated and analyzed.

2.9 Preparation of cytoplasmic RNA

Cytoplasmic RNA was purified according to Fang (1994). About 10,000-20,000 embryos at desired stages or dissociated cells from about the same number of intact embryos were collected by light centrifugation. The intact embryos were collected at 500 rpm for 1 min in the SORVALL GLC-1. The single cells were collected at 2000 rpm for 3 min in the SORVALL GLC-1. The pellets were

washed once with cold CMFSW. After the CMFSW was removed, the pellet was resuspended in 1 ml of cold lysis buffer (50 mM Pipes, pH 6.5; 400mM NH_4Cl; 12 mM $MgCl_2$; 25 mM EGTA). Then the suspension was transferred to a 1.5 ml microfuge tube, and collected again by centrifugation in a microfuge (Fisher centrifuge, Model 59) at 1,000g for 20 seconds. The embryos were lysed in 400 µl lysis buffer containing 0.5% Triton X-100 by vigorous aspiration with a P-1000 Pipetman until the homogenized solution turned clear and no more intact embryos could be seen by microscopy. Nuclei and cell debris were removed by centrifugation in a microfuge at 14,000 rpm (Hermle 2302K, BHG) for 2 minutes at 0°C. RNA was precipitated from the supernatant by addition of an equal volume of prechilled LiCl-urea solution (4M LiCl; 8M Urea; 0.5mM EDTA; 20mM Tris, pH7.5) and incubation for at least 3 hours at -20°C, collected by centrifugation in a Hermle microfuge (14,000 rpm) for 5 minutes at 0°C, and dissolved in 200 µl RNA extraction solution (0.1M NaAc, pH5; 0.5% SDS; 25mM EGTA). It was extracted once with phenol/chloroform, and once with chloroform. RNA was precipitated by addition of 2.5 volume of prechilled ethanol for at least 1 hour at -20°C. The RNA pellet was collected by centrifugation in a Hermle microfuge for 10 min at 0°C. After it was washed twice with 70% ethanol, the RNA was dissolved in approximately 30-50 µl of ddH_2O. The RNA concentration was determined by a spectrophotometer (Pharmacia LKB, Ultrospec III). All aqueous solutions used were treated with 0.1% DEP (diethylpyrocarbonate, Sigma) overnight and autoclaved except the LiCl-Urea solution which was prepared in autoclaved DEP-treated H_2O.

2.10 Preparation of RNA blots

RNA blots were prepared as described by Fang (1994). RNA samples, approximately 5 μg based on A_{260} dissolved in dH_2O, were dried by vacuum desiccation (Speed Vac SVC 100), redissolved in 9 μl of 1 × MOPS (3 - [N-morpholino]propanesulfonic acid, sigma) buffer (20mM MOPS, pH7; 5mM NaAc, 1mM EDTA) containing 50% deionized formamide (BRL, ultrapure) and 6% deionized formaldehyde (Sigma), and denatured by heating at 65°C for 5 min and quickly chilled on ice. 1 μl of 10 × RNA loading buffer (Sambrook *et al.*,1989, pp 7.39-7.43) containing 1 mg/ml of ethidium bromide was added to each sample. RNA samples were separated by electrophoresis on a 1.2% agarose gel containing 1 × MOPS buffer and 3.3% formaldehyde; electrophoresis was carried out in 1 × MOPS with recirculation, using a voltage of 4-5 V/cm gel. After electrophoresis, the gel was soaked twice in distilled water for 10 minutes each, and blotted onto Gene Screen filter (New England Nuclear) in 10 × SSC overnight. RNA was UV cross-linked to the filter by using a Stratalinker (Stratagene) and baked for 2 hours under vacuum.

2.11 Labeling of DNA probes

DNA probes were labeled by the random priming reaction using the NEBlot Kit (New England Biolabs), according to the instruction manual. Briefly, 25-50ng of template DNA was dissolved in 33μl nuclease free H_2O, denatured in a boiling water bath for 5 minutes, then quickly placed on ice for 5 minutes. The

samples were centrifuged briefly, and combined with 5 μl 10 × labeling buffer (including random octodeoxyribonucleotides), 6 μl of dNTP mixture, 5 μl α-^{32}P-dCTP (3,000Ci/mmol, 50μci) and 1 μl DNA polymerase - Klenow fragment (5 units). The reaction was carried out at 37°C for 1 hour. Labeled DNA product was separated from unincorporated nucleotides by chromatography on a Sephadex G-50 column (Pharmacia) equilibrated with distilled H_2O. The labeling buffer, dNTP mixture and DNA polymerase were provided with the kit. 1 μl of labeled probe was taken to determine the specificity activity by using scintillation counter.

2.12 Northern blot hybridization

RNA blot hybridizations with labeled DNA probes were performed in a solution consisting of 6×SSPE (20×SSPE: 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA-Na_2,pH7.4), 5×Denhardt's solution (0.1% BSA, 0.1% Ficoll, 0.1% polyvinyl-pyrrolidone), 0.3% SDS and 100 μg/ml denatured salmon sperm DNA. Hybridizations were carried out overnight at 65°C. Posthybridization washes were usually carried out in 2 × SSPE, 0.5% SDS, twice for 30 minutes, at the hybridization temperature. Filters were rehybridized with desired probes after removal of the previous probe with two 15 minute washes in 0.1 × SET, 0.1% SDS at 95°C (1 × SET is 0.15M NaCl, 0.03M Tris HCl, 2mM-EDTA, pH8.0) according to Hurley *et al.* (1989). Blots were autoradiographed by exposure to Kodak X-ray XK-1 film at -80°C. The quantification of the density of the hybridization signals was performed by using a BIO-RAD GS250 - Molecular Imager. The hybridized probe was melted off (Hurley *et al.*, 1989) and the blot was rehybridized to ubiquitin DNA to normalize for variations in amount of RNA loaded onto each lane.

2.13 Slot blotting

Slot blotting was performed according to Sambrook *et al.* (1989, pp438-439). Samples of 5 μg DNA in 2.5 μl of 10mM sodium phosphate (pH6.5), were denatured for 5 minutes at 100°C. They were rapidly cooled on ice for 5 minutes before being applied to the slot template. A GeneScreen filter was first wet with sterile H_2O and then with 20 × SSC. The filter was dried by blotting on Whatman 3mmPaper. 5 μg DNA in 2.5 μl of 10 mM sodium phosphate (pH6.5) was applied to the membrane in each slot. The membrane was baked for 2 hr at 80°C (under vaccum) before prehybridization.

2.14 Preparation of nuclei for run-on and transcription assays

Nuclei were prepared by a modification of the method of Morris and Marzluff (1983) as described by Conlon *et al.* (1987). All steps are performed at 4°C. Embryos at the desired stage or dissociated cells were washed once with CMFSW, followed by another wash with 2-3 volumes of buffer I (0.32M sucrose; 5mM $MgCl_2$; 10mM Tris, pH8; 1mM EGTA; 1mM DTT; 1mM spermidine; 0.1 mM PMSF) at 4°C. The pellet was homogenized in Buffer I by 15 strokes in a Dounce homogenizer with a B pestle. An equal volume of Buffer II (the same as Buffer I but with 2M sucrose) was added and mixed at 4°C. The mixture was centrifuged at 6000g for 10 mins at 4°C. The pelleted nuclei were resuspended by homogenization in glycerol storage buffer (25% glycerol; 50mM Tris,pH8; 1mM

EGTA; 1mM spermidine; 1mM DTT; 0.1 mM PMSF) at 4°C. Samples of nuclei were counted by using a haemocytometer under a microscope. Aliquots of 100µl containing 3×10^7 nuclei were made. The aliquoted nuclei were immediately used for the RNA chain extension reaction or were quickly frozen in liquid nitrogen and used within 48 hours.

2.15 RNA run-on reaction and isolation of run-on RNA

All protocols were according to Gong and Brandhorst (1988a). The RNA chain extension reaction was performed in a total volume of 200µl which consists of 100µl of reaction buffer (40mM NaCl; 100mM KAc; 2.5 mM $MgCl_2$; 0.3M glycine; 10mM Tris, pH8.0) and 100µl of nuclei (2-3 $\times 10^7$ nuclei) in glycerol storage buffer. Unlabeled ATP, GTP and CTP were added to a final concentration of 50 µM and 100 µCi of α-^{32}P-UTP (410ci/mmol, Amersham) was added in each reaction. The reaction mixture was incubated at 20°C for 20 minutes, allowing for extension of nascent RNA. Chain-extended RNA was isolated essentially according to Groudine *et al.* (1981). The reaction mixture was treated with 20 µg DNaseI for 5 minutes at 20°C and then treated with 100µg/ml proteinase K at 42°C for 30 minutes following addition of SDS to 1% and EDTA to 5 µM. The mixture was extracted with phenol-chloroform once and 100 µg yeast RNA was added. RNA was precipitated with 5% TCA/1.5% PPi on ice for 30 minutes and collected on a 0.45 µm nitrocellulose filter by filtration under vacuum, followed by extensive washes with 3%TCA/1.5%PPi. RNA on the filter was incubated with 25 µg DNaseI in 1.8 ml DNase buffer (20mM Hepes, pH7.5; 5mM $MgCl_2$; 1mM

$CaCl_2$) at 37°C for 30 minutes. RNA was then eluted by adding EDTA to 15mM and SDS to 1% at 65°C for 10 minutes and eluted again with 1% SDS/10mM Tris, pH7.5/5mM EDTA for further 10 minutes. The combined eluate was incubated with 25μg/ml proteinase K for 30 minutes at 37°C. Phenol-chloroform extraction was performed 1-2 times and RNA was precipitated at -20°C with 0.1M NaCl and 2.5 volume of ethanol for greater than 2 hours. The final RNA pellet was dissolved in hybridization buffer containing 25% formamide for filter hybridization.

2.16 Hybridization with run-on labeled RNA

Prehydridization, hybridization, and washing conditions are the same as described by Gagnon *et al.* (1992). Identical blots were prehybridized for 2 hr at 48°C in a solution containing 25% formamide, 0.3M NaCl, 2mM EDTA, 20mM Tris HCl, pH8.0, 1% SDS, 1 × Denhardt's solution, 10% dextran sulfate, and 1mg yeast RNA/ml. Fresh hybridization solution (0.5 ml) containing equal input of run-on labeled RNA (6×10^6 cpm) was added to each blot. Hybridization was carried out in a sealed plastic bag for 3.5 days at 48°C. Blots were washed in 0.3 × SSC (1 × SSC is 0.15M NaCl, 0.015M trisodium citrate), 0.1% SDS three times at room temperature and once at 58°C, each for 15 minutes. Blots were finally washed in 2 × SSC, 0.1% SDS for 15 minutes at 58°C, and exposed to Kodak X-ray XK-1 film or Biorad GS250 Molecular Imager.

2.17 Description of hybridization probes

The Spec1 gene sequence, LpS1 gene sequence, LpC2(3') gene sequence and ubiquitin gene sequence were used as probes for Northern hybridizations. A 390 bp EcoRI fragment containing 130 codons of the Spec1 gene (Carpenter *et al.*, 1984) inserted in the EcoRI site of Gem1 (Promega Biotec. Inc) was excised with EcoRI and purified by using a spin column containing Sephedex G-50. The isolated fragment was used as probe. The 0.44 Kb EcoRI fragment from the LpS1 (λ)gt10 clone was subcloned into Bluescript by Xiang *et al.* (1991). The fragment inserted in the EcoRI site of Bluescript was digested with EcoRI and isolated as probe. LpC2(3') sequence is from the plasmid A5H(3') which was constructed by Fang (1994). A HincII/EcoRI fragment from the 3' untranslated region of LpC2 gene inserted in HincII site and EcoRI site of Bluescript(KS+) was digested out with HincII and EcoRI and isolated as probe. The ubiquitin gene probe is a 1.95 Kb fragment inserted into the PstI site of Bluescript KS+/-. The plasmids used for run-on assay are pβ_2, A$_7$PCR, A5H(3'), LpS1, bubT7 and Bluescript plasmid. pβ_2 contains β-tubulin gene (Gong and Brandhorst,1988a). A$_7$PCR was constructed by inserting the PCR fragment of the 3' untranslated region of LpC1 actin gene into EcoRV site in Bluescript (Fang, 1994). A5H(3') contains a 3' untranslated region of LpC2 in the HincII site and EcoRI site of Bluescript(KS+) (Fang, 1994). The LpS1 plasmid contains a 0.44 Kb fragment of LpS1 gene inserted in the EcoRI site of Bluescript. The bubT7 plasmid contains a 1.95 Kb fragment of ubiquitin gene inserted in the PstI site of Bluescript KS+/-.

2.18 Isolation of probes from the gel

After the plasmid DNA was digested with appropriate restriction enzymes, the fragments of DNA were separated by agarose gel electrophoresis. The band containing the desired gene sequence was cut out under UV. The slice of the gel was cut into $1mm^3$ blocks and put into a spin column. The spin column was made of one 1.5ml centrifuge tube and one 0.5ml centrifuge tube. The small tube was punctured its bottom by using a 26G3/8 needle which was heated in a flame. The hole was covered from inside with some siliconized glass fibers. The small tube was put into the big tube. The blocks of gel were put over the glass fibers and were centrifuged at 6,000 rpm at room temperature for 45 minutes in a Hermle microfuge. The solution spun down into the big tube was collected. The concentration of the isolated gene sequence was estimated by agarose gel electrophoresis. It was used directly for labeling.

2.19 Quantification of relative levels of mRNAs

The level of mRNA relative to total RNA was quantitatively measured by using RNA gel blot (Northern). The intensity of each band was taken by using the Biorad Molecular Imager. There may be some variation in the amount of RNA loaded for agarose gel electrophoresis and in the amount of RNA really blotted onto the membrane during Northern transfer. To normalize for such variations, I hybridized the same blot with ubiquitin DNA. Whether or not it is reliable to use ubiquitin gene hybridization to adjust the amount of RNA blotted depends on the

constancy of the amount of ubiquitin mRNA to that of total cellular RNA during development both for the intact embryos and the dissociated single cells. Since the mass of RNA per embryo is constant during embryogenesis, and since the polyubiquitin gene is ubiquitously expressed in embryonic cells and ubiquitin mRNA prevalence is constant during embryonic development (Gong *et al.*, 1991), the cloned ubiquitin DNA probe provides a useful control in investigations of the temporal and spatial patterns of ontogenetic gene activity, serving to normalize RNA inputs in blot and solution hybridization assays. Moreover, the prevalence of ubiquitin mRNA is unaltered by perturbations such as inhibition of protein synthesis, deciliation of embryos, or heat shock, providing a useful control for investigation of the effects of such treatments on transcript prevalences (Gong *et al.*, 1991). If the prevalence of ubiquitin mRNA is unaltered by dissociation, ubiquitin hybridization provides a useful way to normalize RNA inputs in blots. Since I observed that the ubiquitin RNA appears to be directly related to the amount of rRNA loaded onto the gel, the prevalence of ubiquitin mRNA does not appear to be altered by dissociation. Thus it is reasonable to use ubiquitin DNA hybridization to adjust the RNA inputs on Northern blots. The amount of hybridization signal in each lane is divided by the ubiquitin mRNA signal, as quantified by the Phoshpor-Imager.

3 RESULTS

3.1 Dissociated cells remained viable in culture.

Sea urchin embryos were dissociated into single cells and cultured in CFSW as a single cell suspension according to the methods described in the Materials and Methods. The viability of the cells was tested immediately after dissociation and after they were cultured for the desired time by using a Trypan blue exclusion assay. As shown in Table 1 and 2, the viability of cells immediately after dissociation was 97.5%, 98.2% and 94.3% for S. purpuratus dissociated at mesenchyme blastula, gastrula and pluteus stages, respectively. After they were cultured in CFSW for 5 to 6 hours the cell viability remained about 95%. Table 2 shows the viability of the single cells of S. purpuratus sea urchin embryos dissociated at mesenchyme blastula stage and cultured for various periods. The cell viability decreased slightly from 96% immediately after dissociation to 91.5% for the cells cultured for 24 hours. Thus single cells of S. purpuratus embryos dissociated at these stages remain alive for at least 24 hours in CFSW. Cells of S. purpuratus embryos dissociated at 4-cell, 8-cell, 16-cell and hatching (20 hr PF) stages were also assessed. They could be kept alive at least until the intact control embryos developed to gastrula stage. The viability of the dissociated cells of L. pictus embryos is shown in Table 3. The viability of the single cells cultured in CFSW was over 90% for the cells immediately after dissociation at mesenchyme blastula stage, gastrula stage and pluteus stages. Viability was over 95% for these cells after 23-27 hr culture. The viability of single cells of L. pictus embryos

Viability of Dissociated Cells of S. purpuratus Sea Urchin Embryos

Stages	Primary Mesenchyme Blastula				Gastrula				Pluteus			
Time of Culture (hrs)	0		5		0		5		0		6	
No of cells / Samples	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells
1	5.675×10^7	1.25×10^6	1.35×10^7	1.0×10^6	4.25×10^7	1.0×10^6	4.45×10^7	0.75×10^6	3.15×10^7	2.0×10^6	3.175×10^7	0.50×10^6
2	6.0×10^7	1.75×10^6	1.30×10^7	1.25×10^6	4.15×10^7	0.5×10^6	3.625×10^7	2.75×10^6	3.0×10^7	1.75×10^6	3.625×10^7	0.75×10^6
Mean	5.838×10^7	1.5×10^6	1.325×10^7	1.125×10^6	4.2×10^7	0.75×10^6	4.038×10^7	1.75×10^6	3.075×10^7	1.875×10^6	3.4×10^7	0.625×10^6
Total No. of Cells	5.988×10^7		1.438×10^7		4.275×10^7		4.213×10^7		3.263×10^7		3.463×10^7	
Cell Viability	97.5%		92.1%		98.2%		95.8%		94.3%		98.2%	

Table 1

Viability of Single Cells of S. purpuratus Sea Urchin Embryos Dissociated at Primary Mesenchyme Blastula Stage

Time of Culture (hrs)	0		2		4		6		24	
No. of cells / Samples	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells
1	4.825×10^7	2.50×10^6	1.025×10^7	7.5×10^5	1.50×10^7	1.00×10^6	1.55×10^7	5.0×10^5	1.55×10^7	1.25×10^6
2	3.40×10^7	1.00×10^6	0.375×10^7	2.5×10^5	1.55×10^7	1.00×10^6	0.775×10^7	7.5×10^5	1.525×10^7	1.50×10^6
Mean	4.113×10^7	1.75×10^6	0.70×10^7	5.0×10^5	1.525×10^7	1.00×10^6	1.163×10^7	6.25×10^5	1.533×10^7	1.38×10^6
Total No. of Cells	4.288×10^7		0.750×10^7		1.625×10^7		1.225×10^7		1.675×10^7	
Cell Viability	95.9%		93.3%		93.8%		94.9%		91.5%	

Table 2

Viability of Dissociated Cells of L. pictus Sea Urchin Embryos

Stages	Primary Mesenchyme Blastula				Gastrula				Pluteus			
time of Culture (hrs)	0		26.5		0		27		0		23	
No of cells / Sample	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells	Living Cells	Dead Cells
1	5.0×10^5	6.0×10^4	2.8×10^5	0.0×10^4	3.2×10^5	2.0×10^4	3.2×10^5	0.0×10^4	4.4×10^5	6.0×10^4	4.4×10^5	2.0×10^4
2	4.6×10^5	0.0×10^4	5.4×10^5	2.0×10^4	3.0×10^5	4.0×10^4	2.8×10^5	2.0×10^4	4.8×10^5	4.0×10^4	4.6×10^5	2.0×10^4
Mean	4.8×10^5	3.1×10^4	4.1×10^5	1.0×10^4	3.1×10^5	3.0×10^4	3.0×10^5	1.0×10^4	4.6×10^5	5.0×10^4	4.5×10^5	2.0×10^4
total No of cells	5.1×10^5		4.2×10^5		3.4×10^5		3.1×10^5		5.1×10^5		4.7×10^5	
Cell Viability	94.1%		97.6%		91.2%		96.8%		90.2%		95.7%	

Table 3

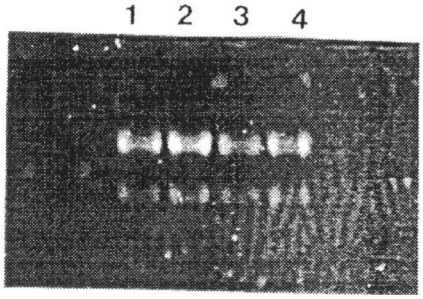
dissociated at 4-cell, 8-cell, 16-cell and hatching (20 hr PF) stages was generally over 95%. So under the conditions we used to dissociate the embryos and culture the dissociated cells, the viability of the dissociated cells from the two species of sea urchin used was generally about 95%. All cultures used were tested and had at least 95% live cells.

3.2 Dissociation of embryos into single cells has little effect on Spec1 mRNA levels in hatched embryo of S. purpuratus

S. purpuratus sea urchin embryos were dissociated into single cells at mesenchyme blastula (24 hr PF) or gastrula (48 hr PF) stages and cultured at 12°C in CFSW for 6 hours. Cellular RNA was isolated from the dissociated cells and the corresponding intact embryos for the same period of time. Northern blot hybridization assays with the cloned Spec1 DNA showed that there was no obvious difference in the level of Spec1 mRNA between the intact embryos and the single cells dissociated at these two stages after 6 hours of culture (Fig.1). Dissociated cells cultured in CFSW for over 20 hours (with the viability of more than 95%) showed a somewhat reduced level of Spec1 mRNA (Fig.2). The level of Spec1 mRNA in the intact embryos increases from 44 hours after fertilization to 74 hours and decreases from 74 hours to 120 hours after fertilization (Fig.2). The pattern of accumulation of Spec1 mRNA in the intact embryos of S. purpuratus sea urchin during this period of development was similar to that reported by others (Bruskin *et al.*, 1981, 1982; Hardin *et al.*, 1988).

Fig.1. Levels of Spec1 mRNA in single cells of dissociated S. purpuratus embryos. The single cells were cultured in CFSW for 6 hours after dissociation. **A**, Agarose gel electrophoresis of cytoplasmic RNA, ethidium stained; **B**, Northern blot hybridization with Spec1 DNA probe; **1**, intact control embryos at gastrula stage; **2**, embryos dissociated at gastrula stage; **3**, intact control embryos at pluteus stage; **4**, embryos dissociated at pluteus stage.

A



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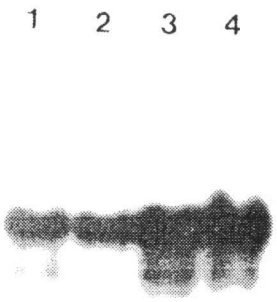
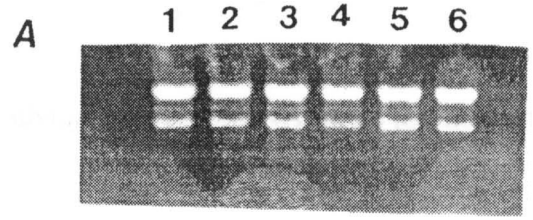


Fig.2. Levels of Spec1 mRNA in the single cells of dissociated S. purpuratus embryos dissociated at mesenchyme blastula, gastrula and pluteus stages and the intact embryos at the corresponding stages. Dissociated cells were cultured in CFSW for 20 hours before preparing RNA. **A**, Agarose gel electrophoresis of cytoplasmic RNA, ethidium stained; **B**, Northern blot hybridization with Spec1 DNA probe. **C**, Levels of Spec1 mRNA in dissociated embryos and intact control embryos. Compared to intact embryos Spec1 mRNA levels in dissociated embryos were 46% for mesenchyme blastula stage, 23% for gastrula stage and 70% for pluteus stage. The values were not normalized by ubiquitin hybridization. **1**, intact embryos as control for mesenchyme blastula stage; **2**, single cells dissociated at mesenchyme blastula stage; **3**, intact embryos as control for gastrula stage; **4**, single cells dissociated at gastrula stage; **5**, intact embryos as control for pluteus stage; **6**, single cells dissociated at pluteus stage.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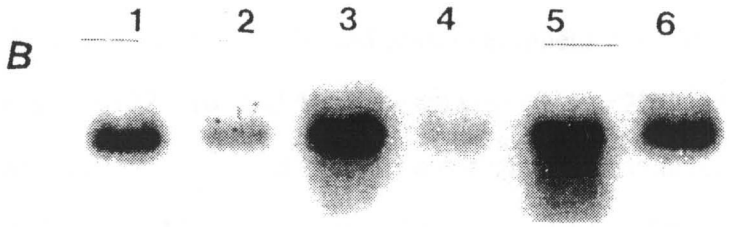
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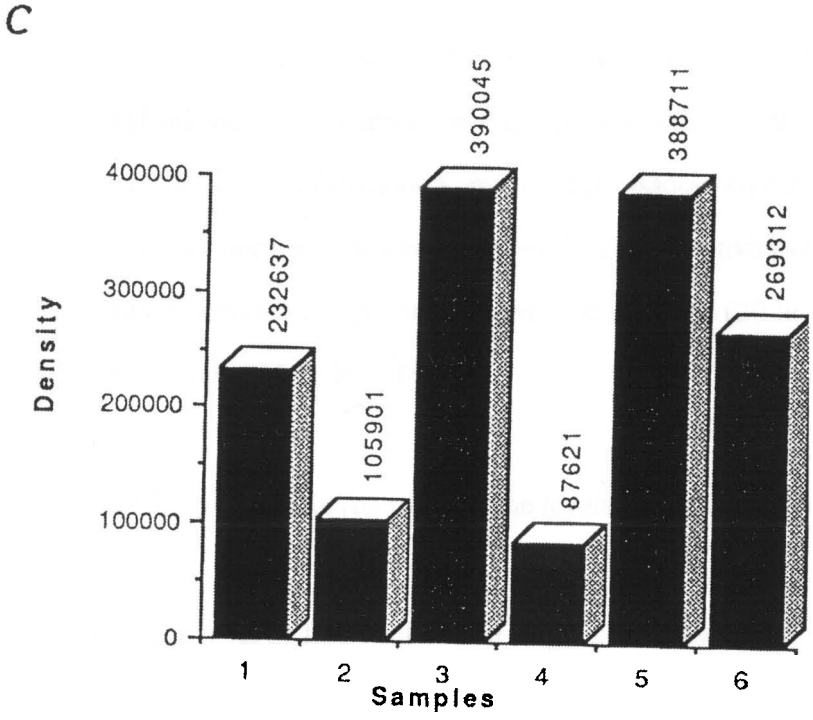
C
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105901
390045
87621
388711
269312
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Samples

3.3 Loss of sensitivity of Spec1 mRNA levels to dissociation

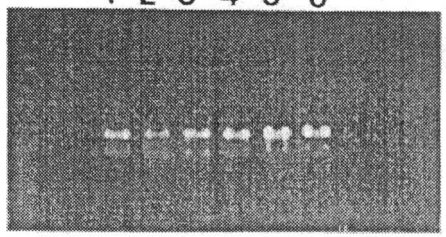
Embryos of S. purpuratus dissociated during early cleavage stages express tissue-specific genes, such as SM50 and Spec1 (Stephens *et al.*,1989). Dissociation at the 128-, 16- and 4-cell stages yielded only 22%, 13% and 9% levels of Spec1 mRNA compared to intact mesenchyme blastula embryos. I inferred that there should be a stage at which the sensitivity of Spec1 mRNA level to dissociation declines. To define the stage, S. purpuratus embryos were dissociated at 4-cell, 8-cell, 16-cell, hatching (20 hr PF) and mesenchyme blastula stages and cultured in CFSW until the intact control embryos reached to gastrula stage. Northern hybridization blots shown in Fig.3 confirms the results observed by Stephens *et al.* (1989) that embryos of S. purpuratus dissociated during early cleavage stages express Spec1, but at a reduced level. The sensitivity of Spec1 mRNA to dissociation declined at a point between blastula stage (20 hr PF) and mesenchyme blastula stage (25 hr PF) (Fig.3).

3.4 Dissociation of L. pictus embryos reduces the levels of LpS1 mRNA

Fig.4 shows the level of LpS1 mRNA in single cells dissociated at mesenchyme blastula, gastrula and pluteus stages and cultured in CFSW for 20 hours compared to corresponding intact control embryos of L. pictus. The level of LpS1 mRNA in the dissociated cells is only 5% for cells dissociated at mesenchyme blastula stage, and 19% for gastrula stage compared to intact embryo. Thus the levels of LpS1 mRNA in the single cells dissociated at

Fig.3. Levels of Spec1 mRNA in the cells of S. purpuratus embryos dissociated at early stages. After dissociation cells were cultured in CFSW until the intact control embryos developed to gastrula stage, when cytoplasmic RNA was purified. **A**, Agarose gel electrophoresis of the RNA, ethidium stained; **B**, Northern blot hybridization with Spec1 gene DNA probe; **C**, Densitometeric levels of Spec1 mRNA in the dissociated embryos; **D**, Relative levels of Spec1 mRNA in dissociated and intact embryos. The level of Spec1 mRNA in the intact gastrula embryos is taken as 1 unit. The values have not been adjusted according to ubiquitin gene DNA hybridization. **1**, intact gastrula; **2**, dissociated at 4-cell stage; **3**, dissociated at 8-cell stage; **4**, dissociated at 16-cell stage; **5**, dissociated at hatching stage; **6**, dissociated at mesenchyme blastula stage.

A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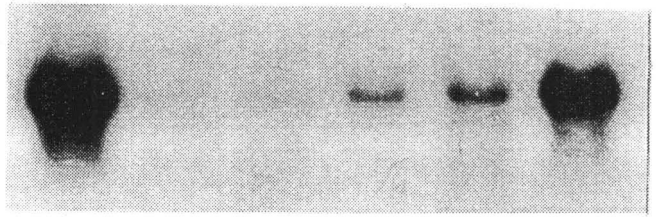
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2
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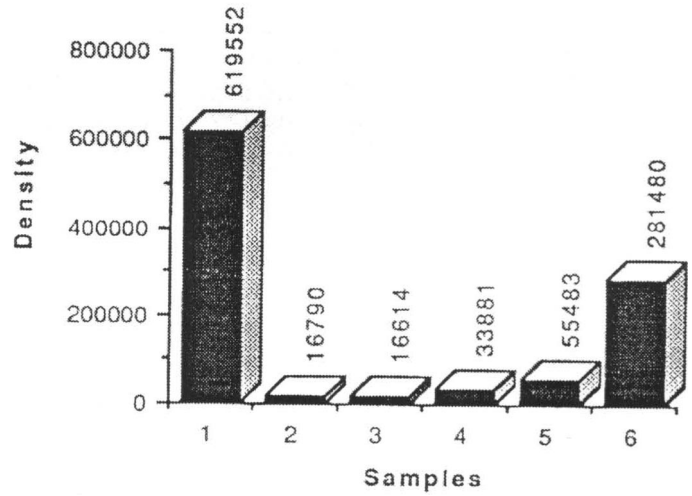
C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2
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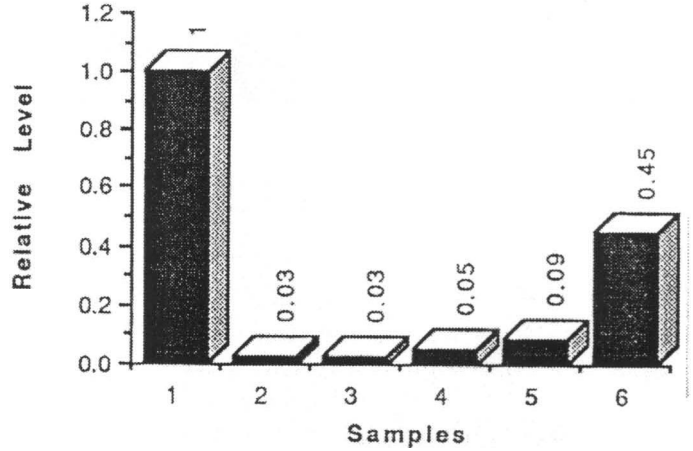
4
5
6
Samples

D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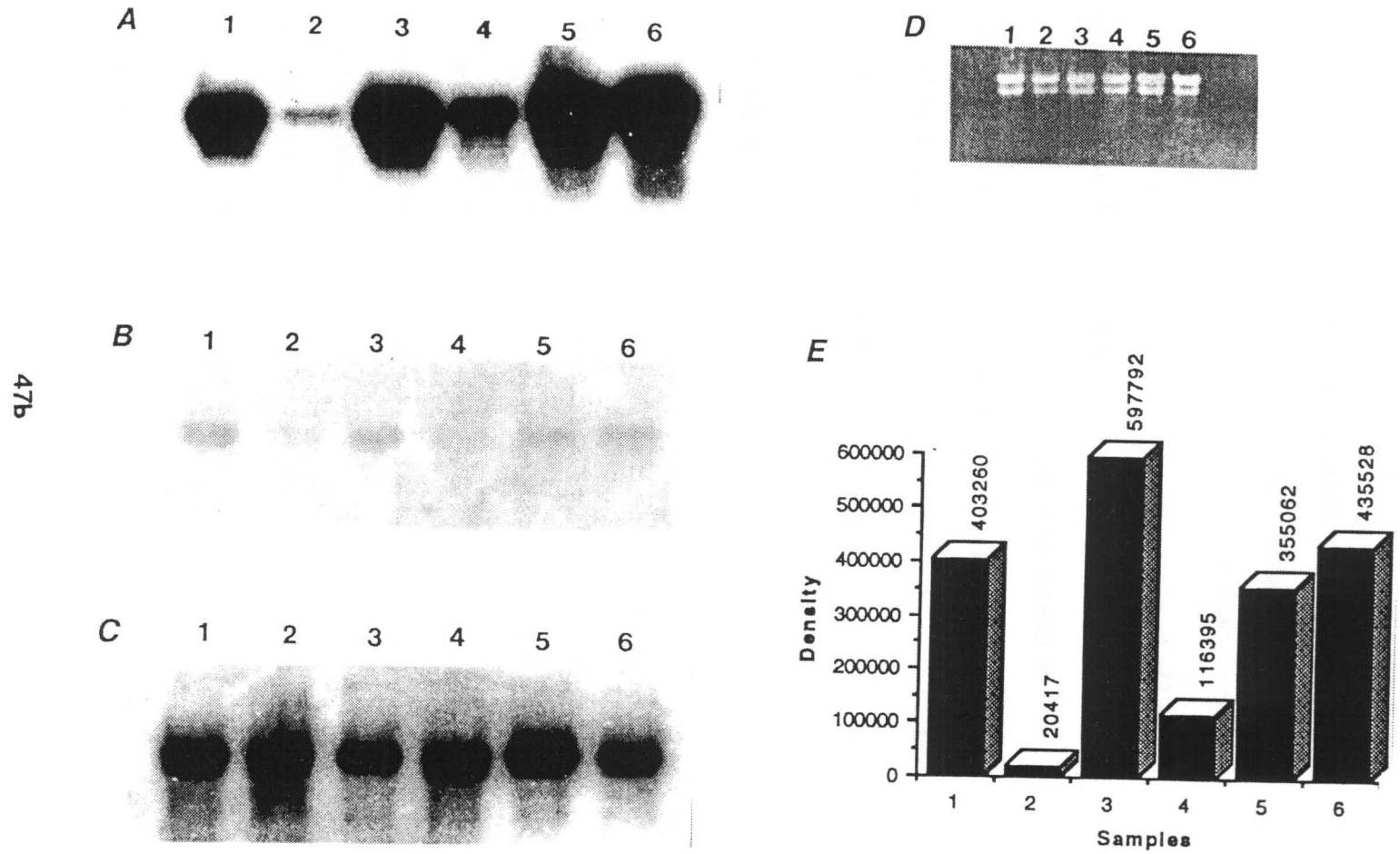
0.09
0.45
1
2
3
4
5
6
Samples

Fig.4. Levels of LpS1 mRNA and LpC2 actin mRNA in dissociated L. pictus embryos. Embryos were dissociated at mesenchyme blastula, gastrula, or pluteus stages and cultured for 20 hours before preparing RNA. Intact controls were also cultured for 20 hours beyond the stage indicated. **A**, Northern blot hybridization with LpS1 DNA probe; **B**, Northern blot hybridization with LpC2 actin DNA probe to the same blot; **C**, Northern blot hybridization with L. pictus ubiquitin DNA probe to the same blot; **D**, Agarose gel electrophoresis of the RNA, ethidium stained; **E**, Densitometric levels of LpS1 mRNA in dissociated cells and intact control embryos at these stages. Compared to intact embryos the levels of LpS1 mRNA in dissociated embryos were 5% for mesenchyme blastula stage, 19% for gastrula stage and 123% for pluteus stage. The density has been normalized according to ubiquitin DNA hybridization. **1**, intact mesenchyme blastulae; **2**, dissociated mesenchyme blastulae; **3**, intact gastrulae; **4**, dissociated gastrulae; **5**, intact plutei; **6**, dissociated plutei.


A
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E
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400000
300000
200000
100000
0
403260
20417
597792
116395
355062
435528
1 2 3 4 5 6
Samples

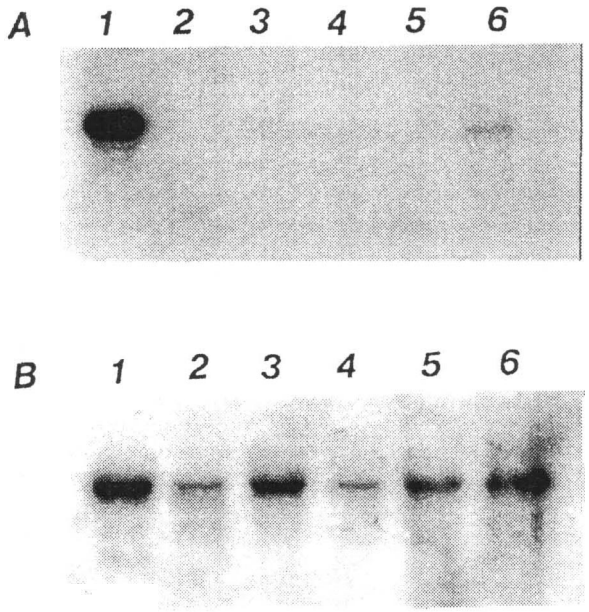
47b

mesenchyme blastula stage and gastrula stages are greatly reduced as a result of dissociation. A slight increase in dissociated cells at pluteus stage is probably not significant. As shown in Fig.5, when L. pictus embryos were dissociated at 4-cell, 8-cell, 16-cell, hatching and mesenchyme blastula stages and cultured in CFSW until the intact control developed to gastrula stage (48 hr PF), the level of LpS1 mRNA was considerably reduced by dissociation. Thus cleavage stage embryos of both L. pictus and S. purpuratus show a considerable reduction in level of accumulation of LpS1 or Spec1 mRNA after dissociation, but S. purpuratus embryos are less sensitive to dissociation at mesenchyme blastula stage, while L. pictus remain quite sensitive until after gastrula stage.

3.5 LpC2 actin mRNA is reduced in dissociated embryos

The blots used to detect the LpS1 mRNA were stripped as described in Materials and Methods and hybridized with cloned LpC2 actin DNA, using a 3'- untranslated gene specific probe. Fig.4 shows that the levels of LpC2 mRNA in the dissociated cells are quite reduced at mesenchyme blastula stage and gastrula stages. At pluteus stage dissociation had no effect on the level of LpC2 mRNA. The effect of dissociation on the levels of LpC2 mRNA in the dissociated cells at early stages was also investigated. LpC2 mRNA was detected at very low levels in dissociated cells cultured to a time corresponding to gastrula stage.

Fig.5. Relative levels of LpS1 mRNA in L. pictus embryos dissociated at early stages. Embryos were dissociated at various stages and cultured in CFSW until control embryos had reached gastrula stage(40 hours after fertilization). **A**, Northern blot hybridization with LpS1 DNA probe; **B**, Northern blot hybridization with ubiquitin DNA probe to the same blot; **C**, Relative levels of LpS1 mRNA in dissociated cells at these stages based on densitometry with Phosphor Imager. The level of LpS1 mRNA in the intact gastrula embryos is taken as 1 unit. The values have been normalized to ubiquitin DNA hybridization. **1**, intact gastrulae; **2**, single cells dissociated at 4-cell stage; **3**, single cells dissociated at 8-cell stage; **4**, single cells dissociated at 16-cell stage; **5**, single cells dissociated at early blastula stage(20 hr after fertilization); **6**, single cells dissociated at mesenchyme blastula stage(25 hr after fertilization).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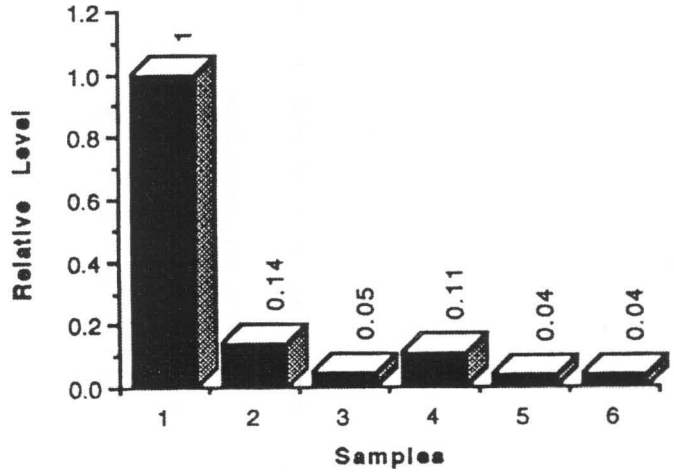
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0.11
0.04
0.04
1
2
3
4
5
6
Samples

3.6 Time course of decline in LpS1 mRNA after dissociation.

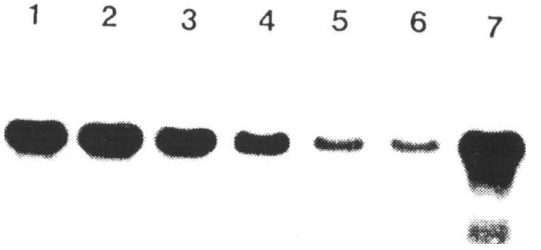
Fig.6 shows the time course of decline of LpS1 mRNA in single cells of L. pictus dissociated at gastrula stage. Northern blot hybridization showed that the level of accumulation of LpS1 mRNA gradually declined after dissociation. In contrast, the level of LpS1 mRNA in the intact embryos during this period increased 50%. I observed a similar result when I dissociated L. pictus embryos at mesenchyme blastula stage, gastrula stage and pluteus stage (Fig.4). From about 44 hrs after fertilization to about 60 hrs the level of LpS1 mRNA also increased about 50% in the intact embryos. It took about 9 hr for LpS1 mRNA in the single cells dissociated at gastrula stage to decrease by half.

3.7 Readdition of Ca^{2+} ions failed to rescue LpS1 mRNA in dissociated cells.

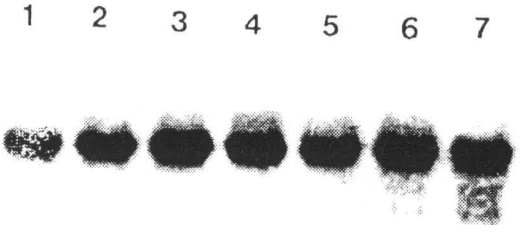
Stephens *et al.* (1990) cultured embryos in CFSW but allowed the cells to remain closely associated within the fertilization envelope. Under these conditions, cell division behavior is the same as in stirred, dissociated cultures but Spec1 is expressed at near normal levels. Hence, neither failure to complete all the cell divisions, nor the lack of Ca^{2+}, seems to be responsible for the low level of Spec1 accumulation in dissociated cultures. Hurley *et al.* (1989) reached a similar conclusion on the basis of experiments in which readdition of Ca^{2+} to rapidly stirred cultures of dissociated cells (in which no reaggregation occurred) did not enhance Spec1 expression. I dissociated L. pictus embryos at gastrula stage. The

Fig.6. Time course of decline in the level of LpS1 mRNA in L. pictus embryos dissociated at gastrula stage. L. pictus embryos at gastrula stage were dissociated into single cells and cultured in CFSW. Samples of dissociated cells and intact embryos were collected at desired time intervals. Cellular RNA was purified and separated by agarose gel electrophoresis for Northern blot hybridization. **A**, Northern blot hybridization with LpS1 DNA probe; **B**, Northern blot hybridization with ubiquitin DNA probe. **C**, Time course showing decline in the level of LpS1 mRNA after dissociation. Values were normalized according to ubiquitin gene hybridization. **1**, intact control embryos at 0 hour; **2**, dissociated cells at 0 hour after dissociation; **3**, dissociated cell at 3 hours after dissociation; **4**, dissociated cells at 6 hours after dissociation; **5**, dissociated cells at 13 hours after dissociation; **6**, dissociated cells at 23 hours after dissociation; **7**, intact control embryos at 23 hours after dissociation.

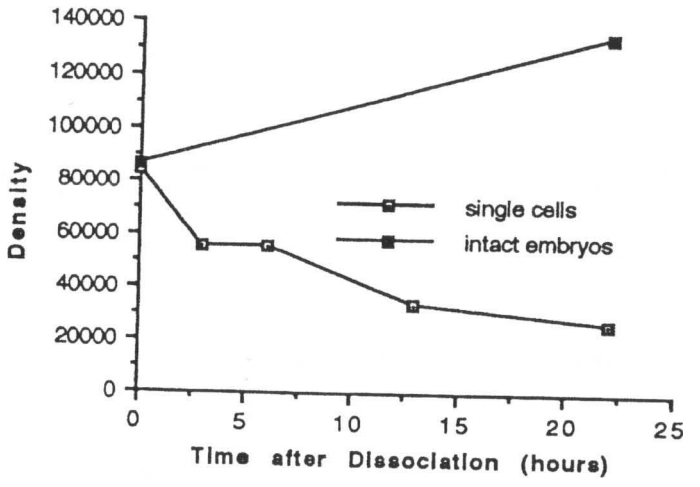
A


1
2
3
4
5
6
7

B


1
2
3
4
5
6
7

C


140000
120000
100000
80000
60000
40000
20000
0
Density
0
5
10
15
20
25
Time after Dissociation (hours)
single cells
intact embryos

dissociated cells were cultured in CFSW or ASW at dilute concentrations with stirring at 60 rpm which prevented most cells from reaggregating. Fig.7 shows that the levels of LpS1 mRNA in dissociated cells cultured in CFSW and ASW after 20 hrs are almost the same. Thus the decline in level of LpS1 mRNA is due to dissociation rather than absence of Ca^{2+} ions.

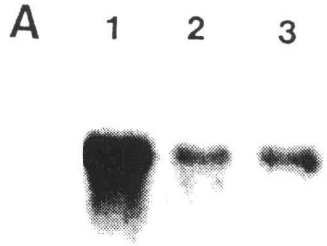
3.8 Effect of TGF-α and PDGF-*BB* on the levels of LpS1 mRNA in dissociated embryos.

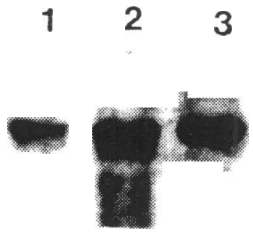
Ramachandran *et al.* (1993) found that application of peptide growth factors PDGF-BB and TGF-α could rescue accumulation of LpS1 mRNA in the L. pictus embryos in which the extracellular matrix had been disrupted. I surmised that these growth factors might protect dissociated embryos from the loss of LpS1 mRNA. However the results shown in Fig.8 indicated that the level of LpS1 mRNA in dissociated cells cultured with 10ng/ml of PDGF-BB, 10ng/ml of TGF-α, or a combination of 10ng/ml of TGF-a and PDGF-BB, was nearly the same as that in the untreated dissociated cells. Thus these growth factors do not appear to protect embryos from the effects of dissociation on LpS1 gene expression.

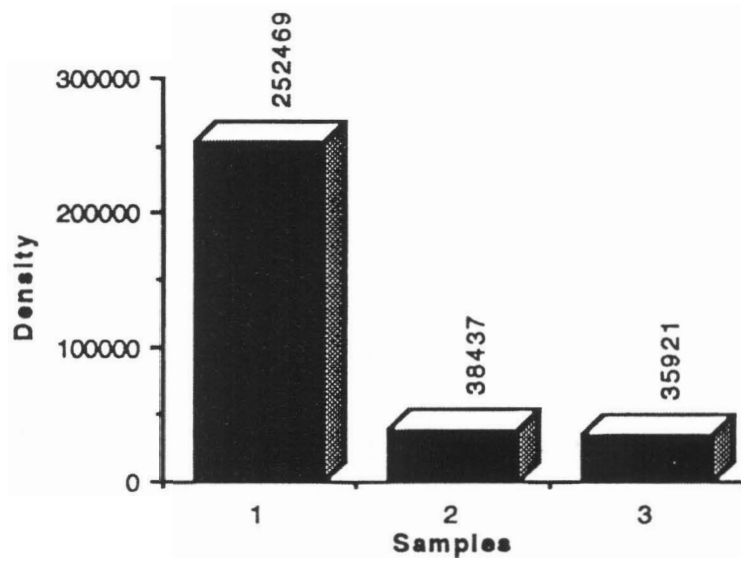
3.9 LpS1 mRNA levels in embryos treated with Actinomycin D

As dissociation of L. pictus hatched embryos results in a decline in the level of LpS1 mRNA, I wondered if this decline was due to a decrease in the stability of the mRNA or a decline in the production of the mRNA in the dissociated cells. I

Fig.7. Effect of Ca^{2+} on the level of LpS1 mRNA in the single cells of L. pictus embryos dissociated at gastrula stage. The dissociated cells were cultured in CFSW and MBLSW that contains Ca^{2+}. **A**, Northern blot hybridization with LpS1 DNA probe; **B**, Northern blot hybridization with ubiquitin DNA probe to the same blot; **C**, Densitometric levels of LpS1 mRNA in dissociated cells. The values have been adjusted according to ubiquitin DNA hybridization; **D**, Relative levels of LpS1 mRNA in dissociated cells. The level of LpS1 mRNA in the intact control embryos is taken as 1. **1**, intact control; **2**, dissociated cells in CFSW; **3**, dissociated cells in MBLSW.


A
1
2
3


B
1
2
3


C
252469
38437
35921
300000
200000
100000
0
Density
1
2
3
Samples

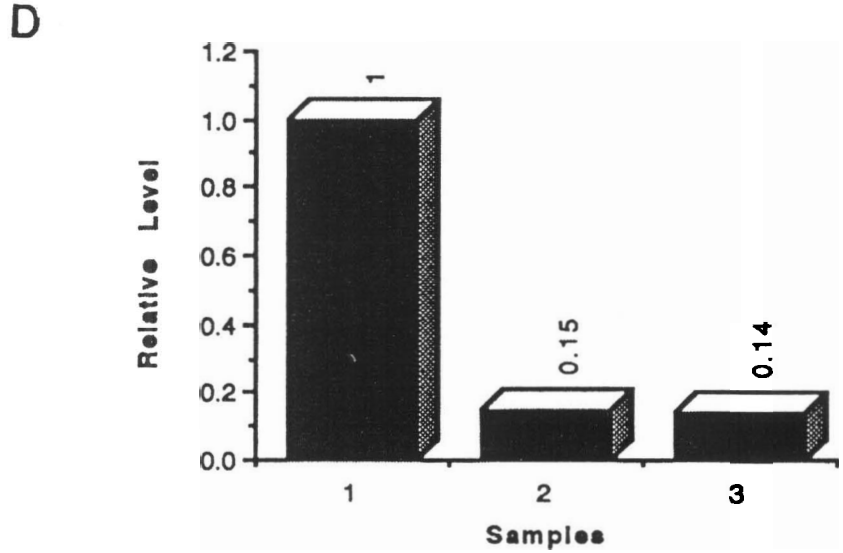
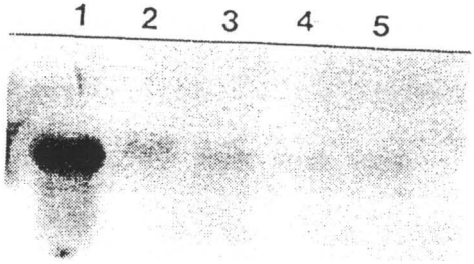
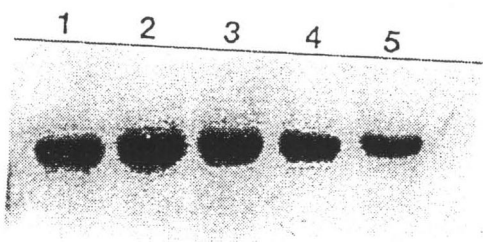

D
1
0.15
0.14
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Relative Level
1
2
3
Samples

Fig.8. Levels of LpS1 mRNA in the dissociated cells treated with TGF-α and PDGF-BB. After L. pictus sea urchin embryos were dissociated at gastrula stage the growth factors were immediately added and cultured for 20 hours. **A**, Northern blot hybridization with LpS1 DNA probe; **B**, Northern blot hybridization with ubiquitin DNA probe; **C**, Relative levels of LpS1 mRNA in dissociated cells. The level of LpS1 mRNA in the intact control embryos is taken as 100%. Values were adjusted according to ubiquitin DNA hybridization. **1**, intact control; **2**, dissociated cells cultured in CFSW; **3**, dissociated cells treated with TGF-α(10ng/ml); **4**, dissociated cells treated with PDGF-BB(10ng/ml); **5**, dissociated cells treated with TGF-α(10ng/ml) and PDGF-BB(10ng/ml).

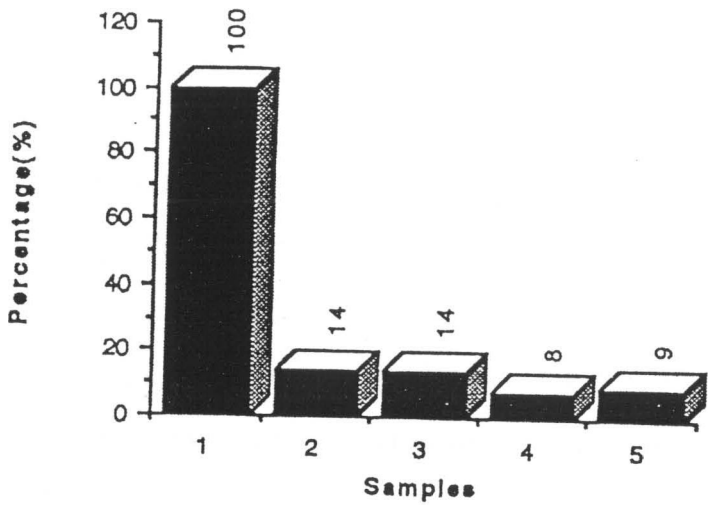
A


1 2 3 4 5

B

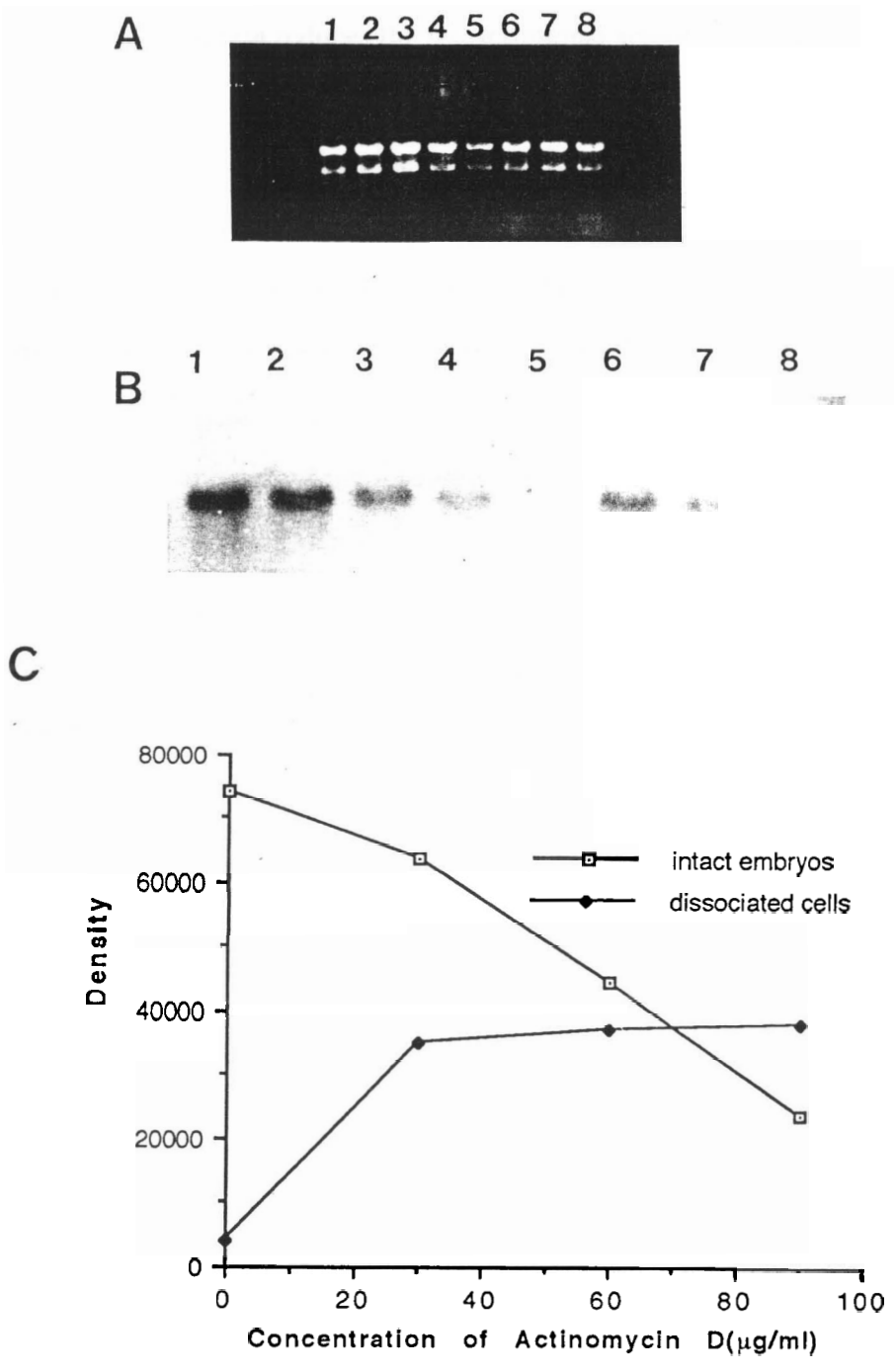

1 2 3 4 5

C


Percentage(%)
120
100
80
60
40
20
0
100
14
14
8
9
1
2
3
4
5
Samples

decided to use Actinomycin D to inhibit the synthesis of RNA and observe the kinetics of decay of LpS1 mRNA in dissociated cells and intact embryos. I first treated the intact embryos and the dissociated embryos of L. pictus sea urchin with different concentrations of Actinomycin D. The embryos of L. pictus were dissociated at mesenchyme blastula stage and treated with Actinomycin D immediately. After culture in the presence of Actinomycin D for 6 hours, cytoplasmic RNA was isolated and blots analyzed on Northern blots for LpS1 mRNA. Since the level of ubiquitin mRNA decreases in embryos treated with Actinomycin D in sea urchin embryos (Gong *et al.*,1991), I could not use ubiqutin DNA hybridization to normalize for the amount of RNA loaded. Ethidium bromide staining was fairly consistent among lanes, as shown in Fig.9. In intact embryos the level of LpS1 mRNA was reduced as the concentration of Actinomycin D increased, indicating that LpS1 RNA synthesis continued in the presence of Actinomycin D. In dissociated cells treated with Actinomycin D the level of LpS1 mRNA was increased as compared to that in the untreated cells. Actinomycin D treatment seems to stabilize LpS1 mRNA at least in dissociated cells. I treated the intact embryos and the dissociated embryos with 30μg/ml of Actinomycin D and measured the level of accumulation of LpS1 mRNA over several hours. LpS1 mRNA did not change in the intact embryos or dissociated cells treated with Actinomycin D as compared to the corresponding untreated control embryos or cells (data not shown). It appears from the two experiments that synthesis of LpS1 mRNA continues in the presence of Actinomycin D and probably Actinomycin D can stabilize LpS1 mRNA in both intact embryos and dissociated cells of L. pictus embryos at mesenchyme blastula stage. The stability of LpS1 mRNA cannot be assessed by use of Actinomycin D to inhibit RNA synthesis.

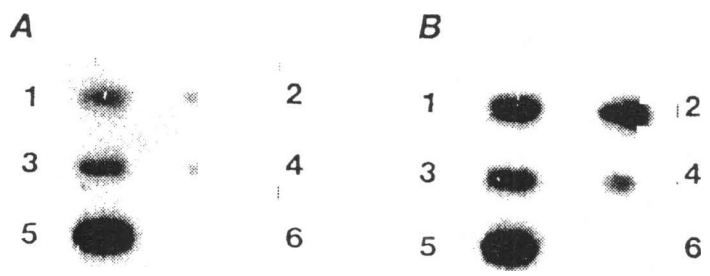
Fig.9. Levels of LpS1 mRNA in intact and dissociated embryos of L. pictus treated with different concentrations of Actinomycin D. Once L. pictus sea urchin embryos were dissociated at mesenchyme blastula stage, both intact control embryos and dissociated cells were divided into several aliquots and cultured with different concentrations of Actinomycin D for 6 hours. Cellular RNA was purified and Northern blot hybridization was performed. **A**, Agarose gel electrophoresis of the cellular RNA, ethidium stained; **B**, Northern blot hybridization with LpS1 DNA probe; **C**, Curve showing the levels of LpS1 mRNA in the intact and dissociated embryos treated with Actinomycin D. The values were not normalized to ubiquitin DNA hybridization. **1**, intact embryos untreated; **2**, intact embryos treated with 30μg/ml of Actinomycin D; **3**, intact embryos treated with 60μg/ml of Actinomycin D; **4**, intact embryos treated with 90μg/ml of Actinomycin D; **5**, dissociated cells untreated; **6**,dissociated cells treated with 30μg/ml of Actinomycin D; **7**, dissociated cells treated with 60 μg/ml of Actinomycin D; **8**, dissociated cells treated with 90 μg/ml of Actinomycin D.

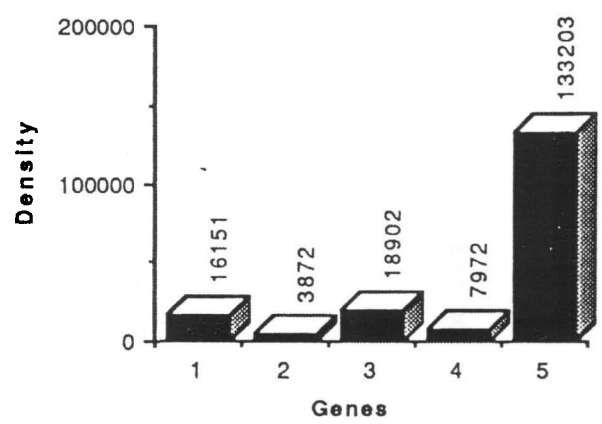

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8
C
80000
60000
40000
20000
0
Density
intact embryos
dissociated cells
0 20 40 60 80 100
Concentration of Actinomycin D(μg/ml)

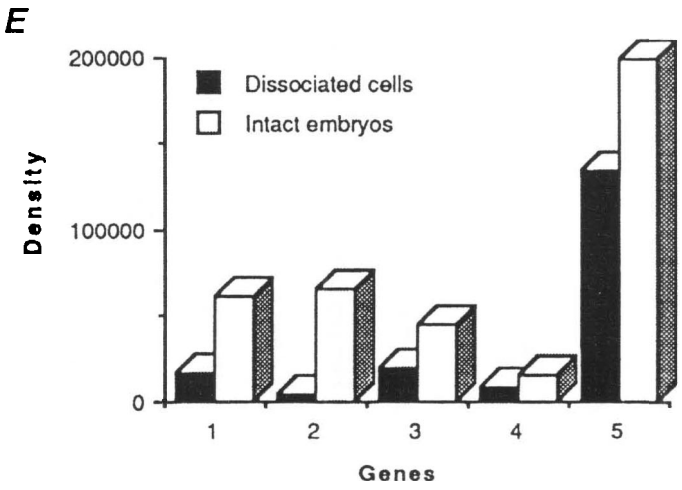
3.10 Transcriptional activity of LpS1 gene in dissociated cells

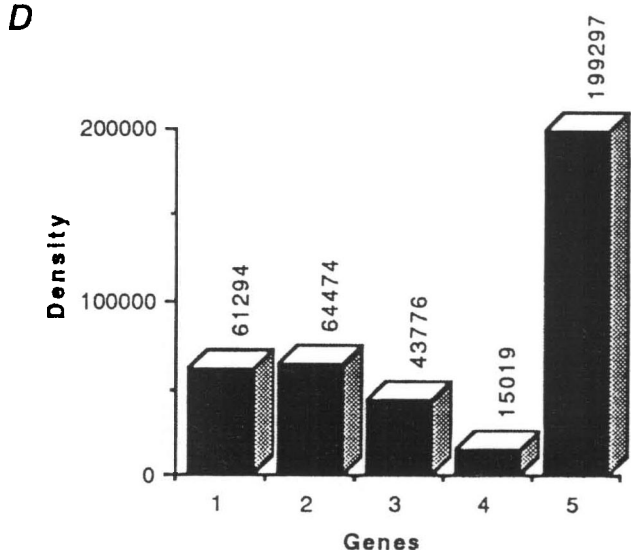
To see if dissociation reduces the transcriptional activity of the LpS1 gene, I performed run-on assays in isolated nuclei prepared from dissociated and intact embryos at gastrula stage. Based on the number of nuclei tested, the nuclei from the dissociated cells and intact control incorporated almost the same amount of radioactive nucleotide per nucleus. Fig.10 shows the results of a run-on assay. Panel A shows the relative transcriptional activity of the β-tubulin gene, LpC1 actin gene, LpC2 actin gene, LpS1 gene and ubiquitin gene in the nuclei of dissociated cells. Panel B shows the relative transcriptional activity of these genes in the nuclei of intact embryos. The transcriptional activity of all these genes tested were somewhat reduced after dissociation (Fig.10 C). This suggests that dissociation may result in a decline in transcription of the LpS1 gene, but a repeat of the experiment gave inconsistent results. Thus no firm conclusion was reached.

Fig.10. Run-on assay of transcriptional activity in nuclei from dissociated cells and intact L. pictus embryos. Embryos were dissociated at gastrula stage. The single cells were cultured in CFSW for 3 hours. Nuclei were then prepared from dissociated embryos and intact embryos. The same amount of radioactively labeled RNA run-on product in disintegrations per minute was used to hybridize to cloned DNA samples blotted onto filter. **A**, Hybridization with the run-on product from the nuclei of dissociated cells; **B**, Hybridization with the run-on product from the nuclei of the intact control embryos; **C**, Density of hybridization signal of run-on product from the nuclei of dissociated cells to the DNA blotted on the filter; **D**, Density of hybridization signal of run-on product from the nuclei of intact control embryos to the DNA blotted on the filter; **E**, comparison of the transcriptional activity of the genes between dissociated cells and intact embryos. **1**, ß-tubulin gene; **2**, LpC1 actin gene; **3**, LpC2 actin gene; **4**, LpS1 gene; **5**, Ubiquitin gene; **6**, plasmid of Bluescript(KS+).


A
1
2
3
4
5
6
B
1
2
3
4
5
6


C
200000
100000
0
Density
16151
3872
18902
7972
133203
1
2
3
4
5
Genes


E
200000
100000
0
Density
Dissociated cells
Intact embryos
1
2
3
4
5
Genes


D
200000
100000
0
Density
61294
64474
43776
15019
199297
1
2
3
4
5
Genes

58b

4 DISCUSSION

4.1 Dissociation does not cause substantial death or injury.

Dissociation of embryos has been used for many years to investigate some aspects of cellular interactions and morphogenesis of sea urchin embryos (reviewed by Giudice, 1986). Recently, this technique has been used to study the role of cell-cell interaction in cellular specification and differentiation (Hurley *et al.*,1989; Stephens *et al.*,1989; Stephens *et al.*,1990). In S. purpuratus embryos, DNA synthesis and incorporation of radioactive amino acids into protein were severely reduced by dissociation treatment (Stephens *et al.*, 1990). In L. pictus embryos, both DNA synthesis and incorporation of radioactive amino acid into protein were less severely reduced by dissociation treatment (Stephens *et al.*, 1990). The reduced levels of DNA and protein synthesis do not appear to be due to significant cell death or damage since 95% of the cells exclude Trypan blue (Stephens *et al.*,1990). Hurley *et al.* (1989) also found that cells of completely dissociated sea urchin embryos could be kept alive for at least 12 hours in CFSW as a single cell suspension. The results of my Trypan blue exclusion tests are consistent with their observations. The dissociated cells of both S. purpuratus sea urchin and L. pictus sea urchin embryos can be cultured in CFSW as a single cell suspension with cell viability of over 95% for at least 20 hours. These results indicates that dissociation does not cause substantial death or injury.

4.2 Pattern of response to dissociation by Spec1 gene in S. purpuratus.

Cleavage stage embryos of S. purpuratus showed a considerable reduction in Spec1 mRNA after dissociation (Fig.3), consistent with the results observed by Stephens *et al.* (1989). But S. purpuratus embryos show reduced sensitivity before mesenchyme blastula stage (Fig.1, Fig.2, Fig.3). The level of Spec1 mRNA begins to decline latter than 6 hour after dissociation at gastrula and pluteus stages(Fig.1, Fig.2). The pattern of response to dissociation by Spec1 mRNA indicates that cell contact is more important at early stages than at late stages in maintaining the level of Spec1 mRNA in intact S. purpuratus embryos. The decline in the level of Spec1 mRNA may be due to a decline in the stability of Spec1 mRNAs or transcriptional activity of the Spec1 gene after dissociation or both of the stability of Spec1 mRNA and transcriptional activity of Spec1 gene. Since at late stages of development the stability of Spec1 mRNA increases and the rate of transcription of Spec1 gene decreases considerably in S. purpuratus (Tomlinson *et al.*, 1990), if dissociation reduced the transcriptional activity of Spec1 gene, the temporal pattern of response to dissociation by Spec1 mRNA in S. purpuratus might be expected. But as there is no direct measurement of the stability of Spec1 mRNA and transcriptional activity of Spec1 gene in dissociated embryos, it can not be excluded that dissociation may reduce the stability of Spec1 mRNA or both the stability and rate of production of Spec1 mRNA.

There is a positive temporal enhancer present in the 5' half of the block region of Spec1 gene (Gan *et al.*, 1990b). Deletion of this element causes a substantial drop in promoter activity of Spec2a (Gan *et al.*, 1990b). Dissociation

may affect some factors such as SpOtx (Gan *et al.*, 1995) which mediate the interaction between the enhancer and other *cis*-regulatory elements of Spec1 gene promoter. Gagnon *et al.* (1992) reported that there is a transition from posttranscriptional to transcriptional regulation of tissue-specific mRNA accumulation during the gastrula stage. Prior to gastrula stage the Spec1 gene is also transcribed in the endoderm and mesenchyme (E/M) cells, which do not accumulate Spec1 mRNA. After gastrula stage, transcription of the Spec1 gene is restricted to ectoderm cells. Dissociation might reduce the level of Spec1 mRNA in dissociated cells of S. purpuratus embryos prior to gastrula stage by reducing the number of the cells which accumulate the mature Spec1 mRNA, the rate of transcription of Spec1 gene in the cells which accumulate the mature Spec1 mRNA, the rate of processing of Spec1 premature transcripts within the nuclei of the cells which accumulate the mature Spec1 mRNA, the rate of transportation of the mature Spec1 mRNA from the nuclei to the cytoplasm of the cells which accumulate the mature Spec1 mRNA or the stability of Spec1 mRNA in the cells which accumulate the mature Spec1 mRNA. Stephens *et al.* (1989) found that Spec1 was expressed in the correct number of cells, and transcripts accumulated at the correct time during mid-cleavage stage. Thus dissociation does not reduce the number of cells which accumulate Spec1 mRNA. But no experiment to date allows exclusion of any other possibilities.

4.3 Pattern of response to dissociation by LpS1 mRNA in L. pictus.

L. pictus embryos showed considerable reduction in the level of LpS1 mRNA after dissociation at cleavage stages(Fig.5). Stephens *et al.* (1990) also found that accumulation of LpS1 mRNA in the cells of L. pictus embryos dissociated at 4-cell stage was very low (about 2% of normal). The level of LpS1 mRNA declines almost immediately after dissociation at gastrula stage(Fig.6). The sensitivity to dissociation by LpS1 mRNA remained very high until after gastrula stage. At pluteus stage the level of LpS1 mRNA was hardly affected by dissociation(Fig.4). The pattern of response to dissociation by LpS1 mRNA in L. pictus suggests that cell contact is important prior to pluteus stage in maintaining the level of LpS1 mRNA in intact L. pictus embryos. But at pluteus stage cell contact appears to be not required for maintaining the level of LpS1 mRNA in intact L. pictus embryos. As the rate of transcription of LpS1 gene remains high in L. pictus at late stages (Tomlinson *et al.*, 1990), dissociation may reduce the level of LpS1 mRNA by reducing the transcriptional activity of LpS1 gene, or stability of LpS1 mRNA or both of the stability of LpS1 mRNA and transcription of LpS1 gene. The run-on assay shown in Fig.10 suggests that the transcriptional activity of LpS1 gene was reduced about 53% in the cells cultured for three hours after dissociation at gastrula stage. This suggests that dissociation may reduce the transcriptional activity of LpS1 gene. But no experiment to date allows to draw a firm conclusion about the effect of dissociation on the transcriptional activity of LpS1 gene or the stability of LpS1 mRNA.

4.4 Difference in response to dissociation of Spec1 mRNA in S. purpuratus and LpS1 mRNA in L. pictus embryos

Generally speaking, the levels of both Spec1 mRNA and LpS1 mRNA were negatively affected by dissociation. But quantitatively the patterns of response to dissociation by Spec1 mRNA in S. purpuratus and by LpS1 mRNA in L. pictus are different. The sensitivity of the level of LpS1 mRNA to dissociation in L. pictus declines at gastrula stage (Fig.4, Fig.5), while the sensitivity of Spec1 mRNA to dissociation in S. purpuratus begins to decline by hatching (Fig.3). At gastrula and pluteus stages dissociation has almost no effect on the levels of Spec1 mRNA in dissociated cells of S. purpuratus cultured for 6 hours (Fig.1) and shows some effect in dissociated cells cultured for 20 hours(Fig.2), while in L. pictus the level of LpS1 mRNA at gastrula stage declines almost immediately after dissociation and is reduced about 50% at 6 hours after dissociation (Fig.6), LpS1 mRNA reaches much lower level after 20 hours(Fig.6, Fig.4), and is hardly affected at pluteus stage even 20 hours after dissociation(Fig.4). These results indicate that at gastrula stage the level of LpS1 mRNA in L. pictus is more sensitive to dissociation than the level of Spec1 mRNA in S. purpuratus, while both are not or almost not sensitive to dissociation at pluteus stage. At mesenchyme blastula stage the level of LpS1 mRNA is more sensitive to dissociation in L. pictus than that of Spec1 mRNA in S. purpuratus(Fig.2, Fig.4). The difference between Spec1 and LpS1 in these two species of sea urchins in response to dissociation may result from differences in the regulation of expression of the genes (reviewed by Brandhorst and Klein, 1992; Gan *et al.*, 1995)). At late stages of development the stability of Spec1 mRNA increases and the rate of transcription of Spec1 gene decreases considerably in S. purpuratus (Tomlinson *et*

al., 1990), while the rate of transcription of LpS1 gene remains high in L. pictus at late stages (Tomlinson *et al.*, 1990).

Since Spec1 gene and LpS1 gene products are markers of aboral ectoderm differentiation, cell contact may be required for full differentiation of aboral ectoderm cells in both S. purpuratus and L. pictus embryos prior to hatching while the specification of aboral ectoderm cells in these two species of sea urchin may be autonomous. At mesenchyme blastula and gastrula stages cell contact appears to be required for maintenance of the state of differentiation of the aboral ectoderm cells of L. pictus embryos. At pluteus stage cell contact is no longer required for maintenance of the state of differentiation of aboral ectoderm cells of S. purpuratus embryos or L. pictus embryos.

4.5 No inhibition of the activation of transcription of Spec1 gene in S. purpuratus and LpS1 gene in L. pictus by dissociation.

In intact S. purpuratus embryos, during early cleavage stages, transcription from the Spec1 gene is very low or absent. The earliest detection of Spec1 transcription, at 12 hr, implies that the Spec1 gene is activated sometime between 6 and 12 hr, which would represent a time somewhere between the fourth and eighth cleavage divisions, that is between 16-cell and 256-cell stages (Tomlinson *et al.*, 1990). Does dissociation of the embryos inhibit the activation of Spec1 gene? It has been reported that there are low levels of Spec1 mRNA in unfertilized eggs and early cleaving embryos and they presumably represent maternal mRNA made during oogenesis (Bruskin *et al.*, 1981, 1982; Hardin *et al.*, 1988; Tomlinson *et al.*, 1990). I dissociated S. purpuratus embryos into single cells and maintained

them as a suspension of single cells at 4-cell, 8-cell, 16-cell, hatching (20 hr PF) and mesenchyme blastula (25 hr PF) stages. These showed 3%, 3%, 5%, 9% and 45%, respectively, of the intact gastrula Spec1 transcript level. There is some difference in the values between our results and those of Stephens *et al*'s (1989). That is probably because I used a different stage of intact control embryos. They used intact primary mesenchyme blastula embryos as control, while I used intact gastrula embryos as control. They used acridine orange staining of gels prior to transfer or hybridization of blots with a mitochondrial 16S rRNA probe to confirm equal loading of RNA, while I used ubiquitin gene hybridization to the same blot to adjust for variations in the RNA applied to the blot. There is an agreement that there was a very low level of Spec1 mRNA in the single cells of embryos dissociated during cleavage stages. The Spec1 mRNA detected in the single cells dissociated at 16-cell, 128-cell, hatching and mesenchyme blastula stages is not maternal Spec1 mRNA, since none was detected in intact 16-cell stage embryos (Bruskin *et al.*, 1981). The Spec1 mRNA present in the single cells dissociated at 4-cell and 8-cell stages might all be maternal Spec1 mRNA or might be of zygotic origin. In my experiment (Fig.3), there was detectable Spec1 mRNA present in the single cells from the 16-cell embryos in which no Spec1 mRNA was detected by Bruskin *et al.* (1981). Thus the Spec1 mRNA in the cells dissociated at 16-cell stage and cultured until the intact control embryos developed to gastrula stage must result from the transcription of Spec1 gene in dissociated cells. Thus dissociation does not inhibit the activation of transcription of the Spec1 gene. Most of the Spec1 mRNA detected in the cells dissociated at 4-cell and 8-cell stages should be also of zygotic origin. This is consistent with the observations of Stephens *et al.* (1989) that there was zygotic accumulation of Spec1 mRNA in the cells dissociated at the 4-cell stage. Thus dissociation does not inhibit the

activation of transcription of the Spec1 gene in S. purpuratus. Nuclear run-on assays of transcription showed that synthesis of the LpS1 transcripts was not detectable at the early cleavage stage (4 hr) but could be detected by the late cleavage-early blastula stage (7 hr) (Tomlinson *et al.*, 1990). No LpS1 mRNA was detected in L. pictus embryos at early cleavage stage(4 hr) and late cleavage-early blastula stage(7 hr) by Tomlinson *et al.* (1990). As there is detectable LpS1 mRNA in the cells dissociated at early stages (Fig.5), dissociation does not inhibit the activation of transcription of LpS1 genes in L. pictus, either.

4.6 The level of LpC2 actin mRNA is reduced by dissociation.

Like the LpS1 gene, the LpC2 actin gene of L. pictus is also aboral ectoderm specific (Fang, 1994). As shown in Fig.4 ,the level of LpC2 actin mRNA declined after dissociation at mesenchyme blastula and gastrula stages. At pluteus stage the level of LpC2 actin mRNA was not affected by dissociation. Thus LpS1 and LpC2 actin genes show a similar temporal pattern of sensitivity to dissociation. Since these genes are markers of aboral ectoderm differentiation, dissociation appears to inhibit or reverse this differentiation.

4.7 Intercellular factors involved in regulation of expression of Spec1 gene and LpS1 gene.

Transcription of the LpS1 genes is sensitive to integrity of the collagenous extracellular matrix, while transcription of the Spec genes is not (Wessel *et al.*, 1989). Disruption of the extracellular matrix by BAPN treatment inhibits the transcription of LpS1 gene in L. pictus, but does not inhibit the transcription of Spec1 gene in S. purpuratus (Wessel *et al.*, 1989). I found that dissociation during

which extracellular matrix was removed had little effect on the accumulation of Spec1 mRNA in S. purpuratus embryos at late stages (Fig.1, Fig.2). For LpS1 gene, the effect of dissociation on the level of LpS1 mRNA in hatched embryos was considerable (Fig.4) and might be related to the disruption of extracellular matrix during dissociation in addition to, or instead of, disruption of cell contacts. Hurley *et al.* (1989) found that the presence of Ca^{2+} in the sea water is not sufficient to cause any increase in Spec1 accumulation as long as the cells are maintained as a completely dispersed cell culture. This is consistent with my observation that Ca^{2+} alone did not restore LpS1 mRNA (Fig.7). Stephens *et al.* (1990) forced the cells to maintain close physical proximity , in the absence of Ca^{2+}, by retention of the fertilization membranes. With L. pictus, retention of the fertilization membranes and culture in CFSW resulted in moderate increases in LpS1 accumulation. It has been shown that belt desmosomes and septate junctions disappear from blastula stage embryos within 10 minutes in the absence of Ca^{2+} and reappear within several hours when Ca^{2+} is added back and contact restored (Turner, 1987). The partial restoration of LpS1 mRNA levels observed by Stephens *et al.* (1990) is not due to formation of Ca^{2+}-dependent cell-cell contact. It may be due to reconstruction of extracellular matrix, contacts and connections by the cells. Stephens *et al.* (1990) also found that adding Ca^{2+} to the dissociated cells of L. pictus embryos and allowing them to form small ciliated clusters of 4-8 tightly packed cells resulted in a higher level of LpS1 mRNA than in the cells kept contact within the fertilization envelope in the absence of Ca^{2+}. This suggests that Ca^{2+}-dependent cell junctions are more important than the contact in the absence

of Ca^{2+} in maintaining the level of LpS1 mRNA in the L. pictus sea urchin embryos. Thus the effect of dissociation on the level of accumulation of LpS1 mRNA in L. pictus sea urchin embryos may be the result of disruption of Ca^{2+}-dependent cell junctions, disruption of extracellular matrix , loss of physical contact, or a combination of these effects.

Ramachandran *et al.* (1993) found that PDGF-BB and TGF-α rescued gastrulation, spiculogenesis, and LpS1 expression in collagen-disrupted embryos of the sea urchin genus Lytechinus. They speculated that the ECM may bind these growth factors which are required for morphogenesis. As shown in Fig.8, I found that use growth factors alone or in combination did not protect LpS1 mRNA from decline after dissociation. Ramachandran *et al.* (1993) found that collagen-disrupted embryos did not always respond to treatment with these growth factors. On the basis of a single experiment I cannot draw a firm conclusion about the role of these growth factors in mediating cellular interactions.

4.8 Effect of Actinomycin D on the synthesis and stability of LpS1 mRNA in L. pictus.

Synthesis of LpS1 mRNA continues even at high concentrations of Actinomycin D and Actinomycin D probably stabilizes LpS1 mRNA both in the intact embryos and the dissociated embryos of L. pictus at mesenchyme blastula stage (Fig.9). Gong and Brandhorst (1988b, 1988c) treated L. pictus embryos with Actinomycin D and found that 30μg/ml of Actinomycin D inhibited RNA synthesis

by 70%. Tubulin mRNA and ubiquitin mRNA decayed in Actinomycin D and synthesis of tubulin mRNA was inhibited by Actinomycin D. Thus Actinomycin D has a range of effects on, and does not cause a generalized stabilization of, mRNA. "Superinduction" is a phenomenon frequently observed in eukaryotic cells, whereby the inhibition of RNA synthesis (usually with Actinomycin D) results in an increase in the concentration (activity) of a specific protein (Palmiter *et al.* 1973; Steinberg *et al.*, 1975; Thompson *et al.*, 1970; Killewich *et al.*, 1975). Steinberg *et al.* (1975) found that Actinomycin D could slow overall protein synthesis and inhibit the degradation of both tyrosine aminotransferase and its mRNA. Palmiter *et al.* (1973) demonstrated that Actinomycin D could stabilize some mRNAs. These mRNAs increased in proportion to the total mRNA population remaining. Thus stabilization of LpS1 mRNA by Actinomycin D might result in the superinduction of LpS1 protein. Actinomycin D may stabilize mRNA by inhibiting synthesis of the factors which are responsible for degradation of the mRNA. Actinomycin D inhibition of RNA synthesis is not a useful approach to investigating the stability of LpS1 mRNA.

4.9 Effects of dissociation on transcription of the LpS1 gene

It is quite possible that the effects of dissociation are mostly or entirely due to a reduction in the transcriptional activity of LpS1 genes in L. pictus and Spec1 gene in S. purpuratus. For the ubiquitin gene, LpC1 actin gene, LpC2 actin gene and β-tubulin gene, the run-on results shown in Fig. 10 indicate that their transcriptional activity may be reduced by dissociation. The decline in the level of

LpC2 actin mRNA after dissociation (Fig.4) at gastrula may result from the effect of dissociation on the transcriptional activity of LpC2 actin gene. As the level of ubiquitin mRNA does not decline in dissociated embryos (Fig.4, Fig.5), a decline in transcriptional activity of ubiquitin gene after dissociation suggests that the level of ubiquitin mRNA in dissociated embryos is regulated at posttranscriptional level. For the β-tubulin gene, the level of β-tubulin mRNA was reduced by dissociation (data not shown). This may be caused by the decline in the transcriptional activity of β-tubulin gene after dissociation. No firm conclusions can be reached until more run-on assays are performed. These should be done on earlier stages as well.

5 CONCLUSION

In summary, in L. pictus embryos dissociation reduced the level of LpS1 mRNA considerably at mesenchyme blastula stage, somewhat at gastrula stage and not at pluteus stage. The level of LpS1 mRNA began to decline almost immediately after dissociation at gastrula stage. Readdition of Ca^{2+} ions, or addition of the peptide growth factors TGF-α and PDGF-BB, did not protect LpS1 mRNA from decline in dissociated gastrula embryos. Dissociation of cleavage stage embryos resulted in a much lower than normal increase in the level of LpS1 mRNA, which begins at the time of hatching. LpC2 actin mRNA showed the same response to dissociation as LpS1 mRNA to response to dissociation. Similar results were observed for Spec1 mRNA in dissociated S. purpuratus embryos. However, the Spec1 mRNA declined more slowly and embryos became less sensitive to dissociation sooner than observed for LpS1. These results indicate that cell contact prior to hatching is required for full expression of Spec1 or LpS1 genes in S. purpuratus or L. pictus embryos. Since LpS1 mRNA, LpC2 actin mRNA, and Spec1 mRNA are markers of differentiation of aboral ectoderm, cell

contact appears to be required for maintenance of the state of differentiation of the aboral ectoderm cells in echinoid embryos at mesenchyme blastula and gastrula stages. It is possible that the decline in the levels of these mRNAs after dissociation is due to a decline in the transcriptional activity of these genes. The difference in the patterns of response to dissociation by Spec1 mRNA in S. purpuratus and LpS1 mRNA in L. pictus may be due to the differences in the regulation of Spec1 and LpS1 gene expression.

Though I made efforts to investigate the mechanism by which dissociation affects the levels of LpS1 mRNA, there are still many problems to be solved. As LpS1 mRNA did not decay in the presence of Actinomycin D, I could not measure the stability of LpS1 mRNA in dissociated embryos of L. pictus. The stability of LpS1 mRNA in dissociated cells may be tested by analyses of RNA labeling kinetics *in vivo* (Galau *et al.*, 1977; Cabrera *et al.*, 1984; Lee *et al.*, 1992). Several factors which determine mRNA prevalence, such as rate of synthesis of the RNA, processing and nuclear export efficiencies and mRNA stability, can be measured in the same labeling experiment (Lee *et al.*, 1992). Since the level of LpS1 mRNA is more sensitive to dissociation at earlier stages than at gastrula stage and I did not assess the transcriptional activity of the LpS1 gene in the cells dissociated at cleavage stages, it would be better to assess the mechanisms which determine the prevalence of LpS1 mRNA in the cells dissociated at earlier stages. It would be informative to use run-on assays of transcription to establish whether or not dissociation affects the stability or synthesis of Spec1 mRNA. Ultimately, it may become possible to identify the molecules involved in the cellular interactions which are disrupted by dissociation of embryos.

6 REFERENCES

Alliegro MC, Black SD and McClay DR (1992) Deployment of extracellular matrix protein during sea urchin embryogenesis. Microscopy Research and Technique 22:2-10

Alliegro MC, Burdsal CA and McClay DR (1990) *In vitro* biological activities of echininectin. Biochemistry 29:2135-2141

Angerer RC and Davidson EH (1984) Molecular indices of cell lineage specification in the sea urchin embryo. Science 226:1153-1160

Benson S, Rawson R, Killian C and Wilt F (1991) Role of the extracellular matrix in tissue-specific gene expression in the sea urchin embryo. Molecular Reproduction and Development 29:220-226

Benson S, Sucov H, Stephens L, Davidson E and Wilt F (1987) A lineage-specific gene encoding a major matrix protein of the sea urchin embryo spicule. I Authentication of the cloned gene and its developmental expression. Devel. Biol. 120:499-506

Brandhorst BP and Klein WH (1992) Territorial specification and control of gene expression in the sea urchin embryo. *Seminars in* Developmental Biology, Vol. 3, pp175-186

Bruskin AM, Bedard PA, Tyner AL, Showman RL, Brandhorst BP and Klein WH (1982) A family of proteins accumulating in ectoderm of sea urchin embryos specified by two related cDNA clones. Dev. Biol. 91:317-324

Bruskin AM, Tyner AL, Wells DE, Showman RM and Klein WH (1981) Accumulation in embryogenesis of five mRNAs enriched in the ectoderm of the sea urchin pluteus. Dev. Biol. 87:308-318

Butler E, Hardin J and Benson S (1987) The role of lysyl oxidase and collagen crosslinking during sea urchin development. Experimental Cell Research 173:174-182

Cabrera CV, Lee JJ, Ellison JW, Britten RJ and Davidson EH (1984) Regulation of cytoplasmic mRNA prevalence in sea urchin embryos: Rates of appearance and turnover for specific sequences. J. Mol. Biol. 174:85-111

Cameron RA, Hough-Evans BR, Britten RJ and Davidson EH (1987) Lineage and fate of each blastomere of the eight-cell sea urchin embryos. Genes and Dev. 1:75-84

Carpenter CD, Bruskin AM, Hardin PE, Keast MJ, Anstrom J, Tyner AL, Brandhorst BP and Klein WH (1984) Novel proteins belonging to the troponin C superfamily are encoded by a set of mRNAs in sea urchin embryos. Cell 36:663-671

Citkowitz E (1971) The hyaline layer:its isolation and role in echinoderm development. Developmental Biology 24:348

Conlon RA., Tufaro F and Brandhorst BP (1987) Post-transcriptional restriction of gene expression in sea urchin interspecies hybrid embryos. Genes and Development 1:337-346

Davidson EH (1986) Gene activity in early development,Third edition, Academic Press,Inc.,New York.

Davidson EH (1989) Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: a proposed mechanism. Development 105:421-445

Driesch H (1893) Zur verlagerung der blastomeren des echinideneies. Anat. Anz. 8:348-357

Ettensohn CA and McClay DR (1987) A new method for isolating primary mesenchyme cells of the sea urchin embryo. Expl Cell Res 168:431-438

Fang H (1994) Evolution and expression of the actin gene family of the sea urchin Lytechinus pictus. Ph.D. thesis, Department of Biological Sciences, Simon Fraser University

Freeman G (1988) The factors that promote the development of symmetry properties in aggregates from dissociated echinoid embryos. Roux's Arch Dev. Biol. 197:394-405

Galau GA, Lipson ED, Britten RJ and Davidson EH (1977) Synthesis and turnover of polysomal mRNAs in sea urchin embryos. Cell 10:415-432

Gagnon ML, Angerer LM and Angerer RC (1992) Post-transcriptional regulation of ectoderm-specific gene expression in early sea urchin embryos. Development 114:457-467

Gan L and Klein WH (1993) A positive *cis*-regulatory element with a bicoid target site lies within the sea urchin Spec2a enhancer. Developmental Biology 157:119-132

Gan L, Mao CA, Wikramanayake A, Angerer LM, Angerer RC and Klein WH (1995) An orthodenticle-related protein from S. purpuratus. Developmental Biology 167:517-528

Gan L, Wessel GM and Klein WH (1990a) Regulatory elements from the related Spec genes of S. purpuratus yield different spatial patterns with a *LacZ* reporter gene. Dev. Biol. 142:346-359

Gan L, Zhang W and Klein WH (1990b) Repetitive DNA sequences linked to the sea urchin Spec gene contain transcriptional enhancer-like elements. Dev. Biol. 139:186-196

Giudice G (1962) Restitution of whole larva from disaggregated cells of sea urchin embryos. Developmental Biology 5:402

Giudice G (1963) Aggregation of cells isolated from vegetalized and animalized sea urchin embryos. Experientia 19:83-86

Giudice G (1973) Developmental Biology of the sea urchin embryo. Academic Press, Inc. New York and London, pp 131-155

Giudice G (1986) The sea urchin embryo: A developmental biological system. Springer-Verlag , Berlin, Heidelberg,New York, Tokyo

Giudice G and Mutolo V (1970) Reaggregation of dissociated cells of sea urchin embryos. Advan. Morphop. 8:115

Giudice G, Mutolo V, Donatuti G and Bosco M (1969) Reaggregation of mixture of cells from different developmental stages of sea urchin embryos. Exp. Cell. Res. 54:279-281

Gong ZY and Brandhorst BP (1988a) Autogenous regulation of tubulin synthesis via RNA stability during sea urchin embryogenesis. Development 102:31-43

Gong ZY and Brandhorst BP (1988b) Stabilization of tubulin mRNA by inhibition of protein synthesis in sea urchin embryos. Molecular and Cellular Biology 8:3518-25

Gong ZY and Brandhorst BP (1988c) Multiple levels of regulation of tubulin gene expression during sea urchin embryogenesis. Developmental Biology 130:144-53

Gong ZY, Cserjesi P, Wessel GM and Brandhorst BP (1991) Structure and expression of the polyubiquitin gene in sea urchin embryos. Molecular Reproduction and Development 28:111-118

Groudine M, Peretz M and Weintraub H (1981) Transcriptional regulation of hemoglobin switching in chicken embryos. Mol. Cell Biol. 1:281-288

Gurdon JB, Brennan S, Fairman S and Mohun TJ (1984) Transcription of muscle-specific actin genes in early Xenopus development : nuclear transplantation and cell dissociation. Cell 38:691-700

Gurdon JB (1987) Embryonic induction-molecular prospects. Development 99:285-306

Hardin PE, Angerer LM, Hardin SH, Angerer RC and Klein WH (1988) Spec2 genes in Strongylocentrotus purpuratus: Structure and differential expression in embryonic aboral ectoderm cells. J Mol.Biol. 202:417-431

Hardin SH, Carpenter CD, Hardin PE, Bruskin AM and Klein WH (1985) Structure of the Spec1 gene encoding a major calcium-binding protein in the embryonic ectoderm of the sea urchin, Stronglyocentrotus purpuratus. J. Mol. Biol. 186:243-255

Herbst C (1900) Uber das Auseinandergehen Wilhelmin in Furchungs-und Gewebezellen in Kalkfreiem Medium. Arch EntwicK Wngsmech org 9:424-463

Hinegardner RT (1975) Morphology and genetics of sea urchin development. Am. Zool. 15:679-689

Horstadius S (1935) Uber die Determin im Verlaufe der Eiachse bei Seeigeln. Publ. Staz. Zool. Napoli. 14:251-479

Horstadius S (1973) "Experimental embryology of Echinderms" Oxford Univ. Press(Clarendon) London and New York

Hurley DL, Angerer LM and Angerer RC (1989) Altered expression of spatially regulated embryonic genes in the progeny of separated sea urchin blastomeres. Development 106:567-579

Katow H and Solursh M (1982) In situ distribution of con A-binding sites in mesenchyme blastulae and early gastrula of the sea urchin L. pictus. Exp. Cell. Res. 139:171-180

Killewich L, Schutz G and Feigelson P (1975) Functional level of rat liver tryptophan 2,3-dixoygenase messenger RNA during superinduction of enzyme with Actinomycin D. Proc. Nat. Acad. Sci, USA 72:4285-4287

Klein WH, Hardin P, Hardin S and Flytzanis C (1987) Structure and expression of the sea urchin Spec genes. in "Proceedings of the 5th International Symposium on Calcium-Binding Proteins in Health and Disease" (Norman AW, Vanaman TC, Means AR,eds), pp243-249, Academic Press, San Diego

Klein WH, Tomlinson CR, Zhang W, Xiang W,Kozlowski M, Gan L., Nguyen J and Wessel GM (1990) Ectoderm differentiation and the Spec gene family of sea urchins. in"Developmental Biology", UCLA Symposium on Molecular and Cellular Biology, New series, Vol125 (Davidson EH, Ruderman J, Posakon J, eds), pp 123-134, Alan R Liss, New York

Klein WH, Xiang M and Wessel GM (1991) Spec proteins: calcium binding proteins in the embryonic ectoderm of sea urchins. in "Novel Calcium Binding Proteins: Structures, Principles and Chemical Relevance" (Heizmann CW, eds). pp465-479, Springer, Heidelberg

Krach SW, Green A, Nicolson GL and Oppenheimer SB (1973) Cell surface changes occuring during development. J Cell Biol 59, no2, part2:176(Abst)

Krach SW, Green A, Nicolson GL and Oppenheimer SB (1974) Cell surface changes occuring during sea urchin embryonic development monitored by quantitative agglutination with plant lectins. Exp. Cell. Res. 84:191-198

Lee JJ, Calzone FJ and Davidson EH (1992) Modulation of sea urchin actin mRNA prevalence during embryogenesis: nuclear synthesis and decay rate measurements of transcripts from five different genes. Developmental Biology 149:415-431

Lynn DA, Angerer LM, Bruskin AM, Klein WH and Angerer RC (1983) Localization of a family of mRNAs in a single cell type and its precursors in sea urchin embryos. Proc.Natl.Acad.Sci. USA 80:2656-2660

Mao CA, Gan L and Klein WH (1994) Multiple Otx binding sites required for expression of the S. purpuratus Spec2a gene. Developmental Biology 165:229-242

McClay DR (1979) Surface antigens involved in interactions of embryonic sea urchin cells. Curr Top Dev Biol 13:199-214

McClay DR (1982) Cell recognition during gastrulation in the sea urchin. Cell Differ. 11:341-344

McClay DR and Chambers AF (1978) Identification of four classes of cell surface antigens appearing at gastrulation in sea urchin embryos. Dev. Biol. 63:179-186

McClay DR and Marchase RB (1979) Separation of ectoderm and endoderm from sea urchin pluteus larvae and demonstration of germ layer specific antigens. Dev. Biol. 71:289-296

Morris GF and Marzluff WF (1983) A factor in sea urchin eggs inhibits transcription in isolated nuclei by sea urchin RNA polymeraseIII. Biochemistry 22:645-653

Neri A, Roberson M, Connolly DT and Oppenheimer JB (1975) Quantitative evaluation of concannavalin A receptor site distributions on the surfaces of specific populations of embryonic cells. Nature(London) 258:343-344

Palmiter RD and Schimke RT (1973) Regulation of protein synthesis in chick oviduct, III Mechanism of ovalbumin "superinduction" by Actinomycin D. Journal of Biological Chemistry 248:1502-1512

Ramachandran RK, Seid CA, Lee H and Tomlinson CR (1993) PDGF-BB and TGF-*a* rescue gastrulation, spiculogenesis, and LpS1 expression in collagen-disrupted embryos of sea urchin genus Lytechinus. Mechanisms of Development 44:33-40

Ransick A and Davidson EH (1993) A complete second gut induced by transplanted micromeres in the sea urchin embryo. Science 259:1134-1138

Roberson M and Oppenheimer SB (1975) Quantitative agglutination of specific populations of sea urchin embryo cells with concanavalin A. Exp. Cell Res. 91:263-268

Sano K (1977) Changes in cell surface charges during differentiation of isolated micromeres and mesomeres from sea urchin embryos. Dev. Biol. 60:404-415

Sambrook J, Fristsch EF and Maniatis T (1989) Molecular cloning ,A laboratory Manual. Cold Spring Harbor Laboratory Press, Second Edition, pp 7.37-7.43

Sasak H and Aketak K (1981) Purifications and distribution of a lectin in sea urchin(Anthocidaris crassispina) egg before and after fertilization. Exp.Cell Res. 135:15-19

Spiegel E, Burger MM and Spiegel M (1983) Fibronectin and laminin in the extracellular matrix and basement membrane of sea urchin embryos. Experimental Cell Research 144:47-55

Spiegel E, Howard L and Spiegel M (1989) Extracellular matrix of sea urchin and other marine invertabrate embryos. Journal of Morphology 199:7 1-92

Spiegel E and Spiegel M (1979) The hyaline layer is a collagen-containing extracellular matrix in sea urchin embryos and reaggregating cells. Experimental Cell Research 123:434-441

Spiegel M and Spiegel E (1975) The reaggregation of dissociated embryonic sea urchin cells. Am Zool 15:583-606

Spiegel M and Spiegel E (1978a) The morphology and specificity of cell adhesion of echinoderms embryonic cells. Exp Cell Res 117:261-268

Spiegel M and Spiegel E (1978b) Sorting out of sea urchin embryonic cells according to cell type. Exp. Cell Res 117:269-271

Steinberg RA, Levinson BB and Tomklins GM (1975) "Superinduction" of tyrosine aminotransferase by Actinomycin D: a reevaluation. Cell 5:29-35

Stephens LE, Kitajima T and Wilt FH (1989) Autonmous expression of tissue-specific genes in dissociated sea urchin embryos. Development 107:299-307

Stephens LE, Shiflet GW and Wilt FH (1990) Gene expression, DNA synthesis and protein synthesis in cells from dissociated sea urchin embryos. Develop. Growth. and Differ. 32:103-110

Thompson EB, Granner DK and Tomkins GM (1970) Superinduction of tyrosine aminotransferase by Actinomycin D in rat hepatoma(HTC) cells. J. Mol. Biol. 54:159-175

Timourian H and Watchmaker G (1975) The sea urchin blastula: extent of cellular determination. Am Zool 15:607-627

Tomlinson CR and Klein WH (1990) Temporal and spatial transcriptional regulation of the aboral ectoderm-specific Spec genes during sea urchin embryogenesis. Molecular Reproduction and Development 25:328-338

Tomlinson CR, Kozlowski M and Klein WH (1990) Ectoderm nuclei from sea urchin embryo contain a Spec DNA binding protein similar to the vertebrate transcription factor USF. Development 110:259-272

Turner RS (1987) Cell-cell association in echinoderm embryos. In invertebrate Models: Cell receptors and cell communication. (Ed. A.H.Greenberg), pp143-171, Karger

Turner R S Jr, Watanabe M and Schnitman JR (1977) Cell adhesive changes during sea urchin development. J Cell Biol 75:67(Abstr)

Wessel GM and McClay DR (1987) Gastrulation in the sea urchin embryo requires the deposition of crosslinked collagen within the extracellular matrix. Developmental Biology 121:149-165

Wessel GM, Zhang W, Tomlinson CR, Lennarz WJ and Klein WH (1989) Transcription of the Spec-like gene of Lytechinus is selectively inhibited in response to disruption of the extracellular matrix. Development 106:355-365

Wilt FH (1987) Determination and morphogenesis in the sea urchin embryo. Development 100:559-576

Wikramanayake AH, Brandhorst BP and Klein WH (1995) Autonomous and non-autonomous differentiation of ectoderm in different sea urchin species. Development 121:1447-1505

Xiang M, Bedard A-P, Wessel G, Filion M, Brandhorst BP and Klein WH (1988) Tandem duplication and divergence of a sea urchin protein belonging to the troponin C superfamily. J. Biol. Chem. 263:17173-17180

Xiang M, Lu S-Y, Musso M, Karsenty G and Klein WH (1991a) A G-string *cis*-regulatory element in the LpS1 promoter binds two nuclear factors distributed non-uniformly in Lytechinus pictus embryos. Development 113, 1345-1355

Xiang M, Tong G, Tomlinson CR and Klein WH (1991b) Structure and promoter activity of the LpS1 genes of Lytechinus pictus duplicated exons account for LpS1 proteins with eight calcium-binding domains. J. Biol. Chem. 266:10524-10533