CHLORATE DISRUPTS GASTRULATION AND AXIAL PATTERNING IN EMBRYOS OF THE SEA URCHIN *LYTECHINUS PICTUS*

by

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Chlorate disrupts gastrulation and axial patterning in embryos of the sea urchin *Lytechinus pictus*

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Abstract

Heparan sulfate proteoglycans (HSPGs) are abundant cell-surface molecules that have been shown to bind several growth factors, mediating their presentation to the appropriate receptors. These extracellular matrix molecules have been implicated in signaling and developmental patterning. To investigate the role of HSPGs in sea urchin development, an inhibitor of sulfation of proteoglycans, sodium chlorate, was used to treat embryos of the sea urchin *Lytechinus pictus*. Instead of two bilaterally symmetric skeletal spicules, treated embryos form multiple spicules in a ring, indicating radialization of the ectoderm. This was confirmed by the reduced and radialized expression of the *LpS1* gene, a marker for aboral (dorsal) ectoderm. Gastrulation was also inhibited: treated embryos form a short gut, lacking obvious differentiation. Embryos are sensitive to the effects of chlorate treatment from the time of hatching to late gastrula stage, implying that properly sulfated HSPGs are necessary during this period. These effects can be rescued by co-treating with platelet derived growth factor-BB (PDGF-BB), suggesting that growth factor signaling via HSPGs is involved in axial patterning and gastrulation.

Subtractive hybridizations of cDNAs were done to detect genes other than *LpS1* whose expression is altered in response to chlorate treatment. A gene that is considerably upregulated in response to

chlorate treatment was discovered, and is referred to here as *CS 1-1.13*. A sequence of approximately 1.4 kb of the *CS 1-1.13* cDNA was determined and appears to be part of the 3'-untranslated region. The *CS 1-1.13* gene is expressed throughout development, reaching a peak at the mesenchyme blastula stage.

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AMV-RT – Avian Myeloblastosis Virus reverse transcriptase

A/V – animal/vegetal

BAPN - β-aminoproprionitrile

BCIP – 5-bromo-4-chloro-3-indolyl phosphate

CSPG – chondroitin sulfate proteoglycan

DEPC – diethylpyrocarbonate

DIG – Digoxygenin

DNA – deoxyribonucleic acid

DNase – deoxyribonuclease

DMF – dimethylformamide

DTT – dithiothrietol

ECM – extracellular matrix

ECM RE – extracellular matrix response element

EDTA – ethylenediaminetetraacetic acid

EGF – epidermal growth factor

GSP – gene specific primer

HSPG – heparan sulfate proteoglycan

IPTG - isopropylthio-β-D-galactoside

kb – kilobases

LB – Luria-Bertani broth

MMLV-RT – Moloney Murine Leukemia Virus reverse transcriptase

NBT – nitro blue tetrazolium

NUP – nested universal primer

O/Ab – oral/aboral

ORF – open reading frame

PABA – para-aminobenzoic acid

PBS – phosphate buffered saline

PBST – PBS plus 0.1% Tween-20

PCR – polymerase chain reaction

PDGF – platelet derived growth factor

PMC – primary mesenchyme cell

RACE – rapid amplification of cDNA ends

RNA – ribonucleic acid

RNase – ribonuclease

RT-PCR – reverse transcriptase PCR

SDS – sodium dodecyl sulfate

SMC – secondary mesenchyme cell

TB – terrific broth

TGF – transforming growth factor

UPM – universal primer mix

UTR – untranslated region

UV – ultraviolet

X-gal – X-galactose

Chapter 1
Introduction

1.1 Embryogenesis of the sea urchin embryo

Sea urchin embryos have long provided a good model system for studying normal embryonic development. Embryos develop externally from spawned gametes and are nearly transparent, making observation extremely simple. The cleavage patterns and various stages of sea urchin embryo development are well known to developmental biologists such that any deviations are readily identified.

The spawned gametes from adult sea urchins may be combined in sea water to initiate fertilization. With fertilization, blocks to polyspermy are triggered, the visible one being the elevation of the fertilization envelope. The fertilized egg then begins its well-characterized cleavages. When *Lytechinus pictus* embryos are cultured at 16°C, first cleavage takes place after approximately 90 minutes.

In the case of sea urchin fertilization, sperm can fuse with the egg at any point on the surface of the egg. There is no correlation between the position of the first cleavage plane of the fertilized egg and the site of fertilization (reviewed by Wilt, 1987). However, the planes of division relate

to the location of the egg jelly canal, an area of discontinuity in the jelly layer surrounding sea urchin eggs formed by the extrusion of polar bodies during meiosis. The jelly canal marks the animal pole of the egg, and the first cleavage plane is along the animal-vegetal axis of radial symmetry (Schroeder, 1980).

Thus, the first cleavage is meridional, as is the cleavage occurring after it, and these first two cleavages are equal and perpendicular to each other. The third cleavage is equatorial, separating the animal and vegetal halves. Up to this point, cleavage has produced daughter cells of equal volume. At the fourth cleavage, however, the cells of the vegetal pole divide unequally and obliquely to produce four large cells, the macromeres, and four smaller cells, the micromeres; the cells of the animal pole divide equally to produce the mesomeres. After the sixth cleavage, the embryo is composed of 60 cells that are arranged in five cellular tiers: an1, an2, veg1, veg2, and the micromeres. The division of the micromeres is delayed, but they eventually cleave to form the small micromeres most vegetally, and the large micromeres. Subsequent cleavages produce a ciliated blastula.

At approximately 18 hours post fertilization, the blastula is able to dissolve its fertilization envelope due to the secretion of hatching enzyme, emerging as a free-swimming hatched blastula (Lepage *et al.*, 1992b). During the hatched blastula stage the vegetal pole flattens and forms the

vegetal plate. The primary mesenchyme cells (PMCs) begin to ingress from the vegetal plate during the mesenchyme blastula stage. The PMCs are descendents of the large micromeres, and they later fuse to form syncytial cables that lay down the calcium carbonate larval skeleton. After ingression, the PMCs begin to migrate along the epithelial wall of the blastocoel to the two ventrolateral regions where they will form the initial triradiate skeletal spicules.

Gastrulation normally begins about 30 hours after fertilization with the buckling of the thickened vegetal plate; this is the first stage of gastrulation. The second stage of gastrulation involves the elongation of the archenteron, which is thought to occur through the rearrangement and changes in shape of cells (Ettensohn, 1985). Secondary mesenchyme cells (SMCs) form at the tip of the archenteron and extend filopodia to make contact with the blastocoel wall. This process may be involved in the localization of the stomadeum, or future mouth (Wilt, 1987; Hardin and McClay, 1990). As the tip of the archenteron approaches the blastocoelar wall, SMCs ingress into the blastocoel. They later form coelomic pouches, pigment cells, blastocoelar cells, and muscle (reviewed by Ettensohn and Sweet, 2000). The tip of the archenteron fuses with the stomadeal ectoderm, forming the mouth. Gastrulation thus produces an embryo having three primary germ layers, including a digestive tube having a mouth and an anus, typical to deuterostomes.

During the prism stage the archenteron differentiates into a tri-partite gut consisting of the esophagus, stomach, and intestine. The skeletal rods also extend; these will provide structural support for the arms of the pluteus larva that are involved in feeding. The skeletal structures formed include two oral rods, two anal rods, two transverse rods, and two body rods. With the use of these arms and ciliary bands, the larva begins to feed and consequently grow. Eventually, the ectoderm on the left side of the larva invaginates to form a vestibule, which together with the hydrocoel develops into an adult rudiment. The rudiment grows inside the larva until metamorphosis occurs, when it is released as a juvenile sea urchin. Upon metamorphosis, many larval cells are destroyed through resorption.

1.2 The animal-vegetal axis

The sea urchin embryo has three axes of polarity: animal-vegetal, oral-aboral, and left-right. The primary axis, which is specified in the unfertilized egg, is known as the animal-vegetal (A/V) axis. The formation of this axis is due to the polarization of maternal factors within the sea urchin egg. This polarity is seen in the jelly canal, in the extrusion of the polar bodies at the animal pole, and in species of sea urchins whose unfertilized eggs contain polarized distributions of pigment granules

(Boveri, 1901; Schroeder, 1980). The polarity along this axis was demonstrated in early egg bisection experiments; the fertilized animal half of the egg gave rise to a ciliated Dauerblastula lacking endoderm and mesoderm, while the fertilized vegetal half of the egg had the ability to form a fairly normal pluteus larva (Hörstadius, 1939).

The classical model of patterning along the A/V axis was supported by the experiments of Hörstadius (1973). This model proposed two diffusible morphogens, each concentrated at either the animal or the vegetal pole. The animalizing morphogen would be concentrated at and diffuse from the animal pole while the vegetal morphogen would be concentrated in micromeres at the vegetal pole and diffuse toward the animal pole. The ratio of concentrations of these morphogens would determine the fate of the tiers of cells along the A/V axis. This model was later challenged by Ransick and Davidson (1993), who confirmed that micromeres from a 16-cell embryo that were transplanted to the animal pole of another embryo induced a second archenteron to form. They argued that there is no animalizing agent, and that micromeres can organize a vegetal plate in animal ectoderm by secreting inducing substances.

Vegetal morphology and expression of markers that are normally only expressed in vegetal blastomeres can be evoked in animal blastomeres by treatment with LiCl (Herbst, 1892; Livingston and Wilt,

1989). The pathway involved in vegetal signaling was later discovered to involve the nuclear localization of β-catenin in a vegetally graded fashion (Wikramanayake *et al.*, 1998; Logan *et al.*, 1999) that acts as a transcriptional co-activator with TCF (Vonica *et al.*, 2000). Lithium is thought to inhibit the activity of GSK-3, resulting in localization of β-catenin to nuclei and activation of expression of vegetal genes (Emily-Fenouil *et al.*, 1998). Entry of β-catenin into nuclei occurs by a cell autonomous mechanism that is yet uncharacterized, but is most likely maternally inherited (reviewed by Brandhorst and Klein, 2002). Recently, several proteins that are concentrated at the animal pole in early sea urchin embryos were identified, suggesting that the opposing gradient model may be correct. This discovery began with the identification of several genes that are activated as early as the 8-cell stage. These were found to be the genes encoding hatching enzyme (SpHE and HE) in *Strongylocentrotus purpuratus* (Reynolds *et al.*, 1992) and *Paracentrotus lividus* (Lepage *et al.*, 1992b) and a protease related to Tolloid and BMP1 in *S. purpuratus* (SpAN) (Reynolds *et al.*, 1992) and *P. lividus* (BP10) (Lepage *et al.*, 1992a). Having identified the regulatory regions of these two genes, it was possible to identify transcription factors involved in their expression. SpEts4 was found to bind the *SpHE* promoter region and positively regulate expression of *SpHE* in animal cells (Wei *et al.*, 1999), while SpSoxB1, an HMG-domain protein, is an essential positive regulator of

SpAN expression although it does not act as a transcription factor (Kenny *et al.*, 1999). The proteins encoded by these genes came to be referred to as animalizing transcription factors (ATFs). As would be expected of maternal factors, both *SpEts4* and *SpSoxB1* mRNAs are present in the unfertilized egg; however, what is strange is that they are evenly distributed in the egg and the embryo up until the fourth cleavage (reviewed by Angerer and Angerer, 2000).

With the discovery of the involvement of β-catenin and TCF in vegetal signaling, researchers began to look into possible downstream effectors. One such effector is the *S. purpuratus* Krox-like gene, *SpKroxl*. *SpKrox1* mRNA is first seen in macromeres of 16-cell stage embryos but is later restricted to cells of the developing vegetal plate. Its expression is lost with the invagination of vegetal plate cells, which suggests that its role may be in the initial establishment of the vegetal plate rather than in endoderm differentiation (Wang *et al.*, 1996). The *S. purpuratus* Krüppel-like gene, *SpKrl*, and the paired-class homeodomain protein, *pmar1*, are also activated by the β-catenin/TCF complex (Howard *et al.*, 2001; Oliveri *et al.*, 2002). However, both of these protein products appear to be acting as repressors at the vegetal pole. It has been suggested that SpKrl is involved in the clearing of ATFs such as SpSoxB1 from the mesendoderm region (reviewed by Angerer and Angerer, 2003), while Pmar1 is thought to

act in the micromeres to repress the action of a broadly distributed repressor of micromere fate (Oliveri *et al.*, 2002).

The mesendoderm region of the early sea urchin embryo must eventually differentiate into both secondary mesenchyme and endoderm. Thus, multiple studies have focused on the various signaling events involved in these specifications. At present, the best characterized pathway in this region involves the *L. variegatus* Notch pathway. At the early blastula stage, the Notch receptor is present on the surface of cells in the animal half of the embryo, but then shifts its localization in the mesenchyme blastula embryo to a ring surrounding the central vegetal plate. Notch is internalized into a ring of cells that will become SMCs, while it remains apical on a ring of cells that will become endoderm. Therefore, it is possible to define the boundary between presumptive SMC precursors and presumptive endoderm (Sherwood and McClay, 1997). *LvDelta*, the gene encoding the ligand for the Notch receptor, is expressed in the micromere descendents during the blastula stage, and in the macromere descendents during the mesenchyme blastula and early gastrula stages. It has been demonstrated that expression of *LvDelta* by micromere descendants is both necessary and sufficient for the development of mesodermal pigment cells and blastocoelar cells, while macromere-derived LvDelta is necessary for blastocoelar cell and muscle cell development. (Sweet *et al.*, 1999; 2002). Sherwood and McClay (1999)

demonstrated that overexpression of activated *LvNotch* increases SMC specification, whereas a loss of LvNotch signaling eliminates SMC specification. The expansion of SMCs seen with activation of LvNotch signaling was at the expense of presumptive endoderm cells, while loss of SMC specification resulted in the endoderm expanding into territory where SMCs usually arise. This suggests that signaling through LvNotch is necessary for the differentiation of SMCs in the mesendoderm region.

1.3 The oral-aboral axis

The secondary axis of the sea urchin embryo, the oral-aboral (O/Ab) (aka, the dorsal-ventral axis), does not seem to be established until after fertilization, and becomes evident during late cleavage with the localization of differential gene expression in the territories of the presumptive oral and aboral ectoderm (reviewed by Davidson *et al.*, 1998). The oral ectoderm eventually gives rise to the larval nervous system, mouth, and facial epithelium, and the aboral ectoderm forms squamous epithelium (Coffman and Davidson, 2001). The region that marks the border between the oral and aboral ectoderm forms the ciliary band of the pluteus larva (Wikramanayake and Klein, 1997).

The mechanism by which the O/Ab axis becomes specified remains somewhat of a mystery. There does appear to be a correlation between the

first cleavage plane and the future position of the O/Ab axis, at least in several sea urchin species. In *L. pictus*, the first cleavage plane normally corresponds to the plane of bilateral symmetry of the larva. Contrastingly, in *Strongylocentrotus purpuratus* embryos, the O/Ab axis usually forms 45° clockwise with respect to the A/V axis when viewed from the animal pole. However, there are some species that have no relationship between cleavage planes and the future O/Ab axis (Henry *et al.*, 1992).

The fact that the future O/Ab axis has a correlation with the first cleavage plane in some species of sea urchins suggests that in these particular species the O/Ab is specified before cell division begins. However, classical experiments demonstrate that this axis is certainly not determined, as it can be respecified under certain conditions. For example, when two or four cell embryos are dissociated, every blastomere has the ability to produce a pluteus larva with a normal O/Ab axis. Also, when meridional halves of eggs or embryos are fused, the pluteus larvae generated have a single O/Ab axis (Hörstadius, 1973).

There are differences between the oral and aboral side of the embryo quite early in development. Czihak (1963) demonstrated that at the 16-cell stage the blastomeres of the future oral pole exhibit a higher rate of respiration, as illustrated by a gradient of cytochrome oxidase. Later it was shown that this respiratory gradient can be used to respecify the O/Ab axis. Embryos can be immobilized in clusters of four such that a

redox gradient is then established. The side of the embryos facing the outside of the cluster tends to become the oral side, while the side facing inside tends to become aboral (Coffman and Davidson, 2001). However, the embryos used in this experiment were clustered at the 4-cell stage, which has already been shown to contain a plastic O/Ab axis. In fact, it has been suggested that the O/Ab axis does not become irreversibly fixed (determined) until gastrulation. Hardin *et al.* (1992) treated *L. variegatus* embryos with $NiCl_2$, an agent that appears to alter commitment of ectodermal cells along the O/Ab axis. When embryos were treated at various times, so as to determine the period during which $NiCl_2$ has an effect on patterning, it was found that embryos were sensitive from the hatched blastula stage to early gastrula stage. This result was quite surprising, as it implies that the O/Ab axis can be perturbed up until the early gastrula stage, which is relatively late in development.

Until recently, genes specifically involved in driving the differentiation of the ectoderm were unknown. With the discovery of a positive regulatory region in the enhancer region of the aboral ectoderm-specific gene *Spec2a*, the *S. purpuratus* orthodenticle-like gene, *SpOtx*, came to light. *Spec2a* was shown to contain three SpOtx consensus binding sites, and *Spec2a* expression was lost when these sites were mutated (Mao *et al.*, 1994). Two SpOtx proteins have been identified, α and β, and these are generated by alternative RNA splicing from the single

SpOtx gene. The spatial and temporal patterns of *SpOtx* mRNA were studied, and showed a surprising pattern. *SpOtxα* transcripts are initially present in all cells of the embryo during cleavage, but they gradually become restricted to oral ectoderm; whereas, the three *SpOtxβ* transcripts accumulate primarily in ectoderm at the mesenchyme blastula stage, with restriction to oral ectoderm and vegetal plate territories later (Gan *et al.*, 1995; Li *et al.*, 1997). The SpOtx proteins are initially present in the cytoplasm of early cleavage stage embryos but are then translocated into nuclei during the early blastula stage, which corresponds to the time of *Spec* gene activation (Mao *et al.*, 1996). The role of SpOtx as an activator of *Spec2a* transcription was also shown by the injection of *SpOtx* mRNA into eggs. Once fertilized, these embryos developed into epithelial balls of aboral ectoderm suggesting that SpOtx redirected all cells to an aboral ectoderm fate (Mao *et al.*, 1996).

SpGsc, a homologue of *goosecoid*, has been identified by Angerer *et al.* (2001), and has been shown to also play a role in the differentiation of ectoderm in the sea urchin embryo. Morpholino-mediated inhibition of SpGsc translation produced embryos that failed to gastrulate or establish O/Ab polarity. These embryos were shown to be expressing *Spec1* in all ectodermal cells, which suggests that the role of SpGsc is as a repressor of aboral ectoderm differentiation in the oral region. As SpGsc transcripts are first detectible at the hatched blastula stage, quite a bit later than the

transcriptional activation of SpOtx, the authors suggest that SpOtx may first specify all ectoderm as aboral, but that SpGsc may then act to respecify the oral region.

As the establishment of the O/Ab axis appears to be related to the position of the A/V axis, there have been several investigations into how disrupting the A/V axis affects the O/Ab axis. Wikramanayake *et al.*(1995) cultured animal caps from both *S. purpuratus* and *L. pictus*, and compared the expression of markers for the differentiation of oral and aboral ectoderm in the *Dauerblastulae* that formed Intriguingly, even though a marker for oral ectoderm was detected throughout the *S. purpuratus* animal cap embryoids, and no stomodeum or ciliary band was formed, they did express an aboral specific marker at about the same time as control embryos. However, *L. pictus Dauerblastulae* did not express the aboral ectoderm specific marker, suggesting that the animal cap in this species is not autonomous in its ability to form the O/Ab axis and to form aboral ectoderm. When *L. pictus* embryos were treated with lithium, vegetal structures were formed, and the ectoderm began to differentiate. To test whether endoderm formation is necessary for the differentiation of aboral ectoderm when animal caps are treated with lithium, Wikramanayake and Klein (1997) optimized the lithium treatment to ensure that no endoderm formed, and demonstrated that the treatment resulted in the polarized expression of the aboral ectoderm specific gene.

In contrast, Yoshikawa (1997) showed that when normal sea urchin embryos are vegetalized by treatment with lithium, the differentiation of ectoderm into oral and aboral is also disrupted.

Because SpOtx and SpGsc are thought to act upstream of the oral and aboral tissue specific markers, the effects of disrupting nuclear localization of β-catenin on the expression of *SpGsc* was also studied. It was discovered that the accumulation of *SpGsc* in oral ectoderm depends on proper A/V patterning involving the nuclear localization of β-catenin. This was determined through the dissociation of embryos and the injection into embryos of cadherin, an inhibitor of nuclear localization of β-catenin; in both of these cases, *SpGsc* transcripts were undetectable (Angerer *et al.*, 2001). The transcript *BMP2/4*, which is normally enriched in oral ectoderm, also links the patterning of the two axes. *BMP2/4* mRNA injection into both *L. pictus* and *S. purpuratus* eggs not only suppresses the commitment of cells to a vegetal fate, but it also causes differentation of ectoderm into the aboral type. On the other hand, injection of *NOGGIN* mRNA, which encodes an inhibitor of BMP4, causes the opposite effects. That is, the ectoderm/endoderm border is shifted toward the animal pole and causes ectodermal differentiation into ciliated band and oral ectoderm. Thus, all of these reports imply that the proper establishment of the A/V axis is necessary for the appropriate differentiation of ectoderm into oral and aboral types.

Recently, three T-box genes involved in O/Ab patterning were characterized in two species of sea urchins: *LvBrac* (*bracyury*) and *LvTbx2/3* in *L. variegatus* (Gross and McClay, 2001; Gross *et al.*, 2003), and *coquillette*, a member of the Tbx2 subfamily, in *P. lividus* (Croce *et al.*, 2003). These genes encode transcription factors that are part of the T-domain family. LvTbx2/3 was localized using a polyclonal antiserum and nuclear localization was seen in the aboral territories of all three germ layers starting at the mesenchyme blastula stage (Gross *et al.*, 2003). *Coquillette* had a dynamic localization pattern: at the blastula stage it was expressed in prospective aboral ectoderm and endoderm, wheras starting from the gastrula stage, it was only expressed in aboral primary mesencyme cells (Croce *et al.*, 2003). Both of these genes appear to be downstream of the O/Ab axis-specifying proteins. *LvTbx2/3* expression is lost when BMP2/4 is overexpressed (Gross *et al.*, 2003), and overexpression of goosecoid blocks *coquillette* expression (Croce *et al.*, 2003). *Brachyury*, on the other hand, is expressed in endoderm vegetally, and oral ectoderm animally. Vegetal expression of *Lvbrac* requires proper β-catenin signaling in the micromeres (Gross and McClay 2001); thus *LvBrac* appears to be another gene involved in the correct positioning of the O/Ab axis with respect to the A/V axis.

1.4 Aboral ectoderm-specific genes

With the discovery of tissue specific genes in sea urchin embryos, researchers were able to rely on these markers of differentiation rather than on only observations of morphological differences in perturbed embryos. Since the first discoveries of tissue specific genes by Lynn *et al.* (1983), Carpenter *et al.* (1984) and Angerer and Davidson (1984), many tissue specific genes have been characterized. One of the first tissue specific genes to be classified is *Spec1*, an aboral ectoderm specific marker (Carpenter *et al.*, 1984). Therefore, when focusing on the specification of the O/Ab axis, the most common genes studied are the *Spec* family and their homolgue in *L. pictus*, *LpS1*.

The *S. purpuratus Spec* gene family is made up of *Spec1* and six or seven related *Spec2* genes (Hardin *et al.*, 1985; Hardin *et al.*, 1988). It is suspected that the multiple copies of genes in the family arose from fairly recent gene duplications within the species. The products encoded by the Spec family belong to the troponin C/calmodulin/myosin light chain group of calcium binding proteins and vary in size from 14 kD to 17 kD. The Spec proteins each contain four EF-hand (or helix-loop-helix) domains, which are involved in binding calcium ions. Although the function of the *Spec* genes remains unknown, it has been suggested that they may be

involved in the transport of calcium ions across the ectoderm to be used in the construction of the calcium carbonate spicules (Hardin *et al.*, 1987).

Spec1 mRNA is present at low levels in the early embryo, but begins to accumulate in the prospective aboral ectoderm at the early blastula stage (Bruskin *et al.*, 1981; Bruskin *et al.*, 1982; Hardin *et al.*, 1985; Hardin *et al.*, 1988). This is before the differentiation of aboral ectoderm, as detected by its squamous nature, is even visible. This makes *Spec1* a good molecular marker in the study of aboral ectoderm territory specification during the course of development. Hardin *et al.* (1988) made measurements of the *Spec* transcripts during *S. purpuratus* embryogenesis and showed that *Spec1* is the most abundant mRNA of the family. *Spec1* expression reaches a peak at the midgastrula stage, but then declines two- to three-fold by the pluteus larva stage. The transcripts of most of the various other members of the family, the *Spec2* mRNAs, begin to accumulate detectably several hours later at the late blastula-early gastrula stage; however, collectively they only reach about 40 to 60% the levels of *Spec1*. The *Spec2d* gene has a different expression timeline – the transcripts accumulate mostly during the gastrula and pluteus stages with levels reaching only 2% those of *Spec1* (Hardin *et al.*, 1988).

The *L. pictus* Spec homologue, *LpS1*, was identified and cloned by Xiang *et al.* (1988). It was later discovered that there are two *LpS1* genes of almost identical sequence, *LpS1α* and *LpS1β*, that generate transcripts

of about 2.1 kb (Xiang *et al.*, 1991b). Two similar LpS1 proteins were detected by Brandhorst *et al.* (1991) using two-dimensional electrophoresis. The LpS1 protein is able to cross-react with Spec1 antibodies, and *LpS1* and *Spec1* mRNAs display a similar spatial and temporal pattern of expression. However, the LpS1 protein was found to be 34 kDa rather than 17 kDa in size. LpS1 was also shown to contain eight EF-hand domains which share structural homology with the Spec1 or Spec2 EF-hand domains; the rest of the sequence is highly divergent, suggesting that the overall structural features of the Spec family of proteins are more conserved than the amino acid sequences (Xiang *et al.*, 1991b). Therefore, it is believed that the *LpS1* genes arose from a duplication of an ancestral Spec gene (Xiang *et al.*, 1988; 1991b).

Proper temporal and spatial expression of the *LpS1β* gene requires only 762 bp of the 5' flanking DNA sequence and 17 bp of 5'-untranslated leader sequence, based on expression promoter-reporter transgenes (as both *LpS1* genes have indistinguishable upstream sequences, it is assumed that LpS1α is regulated in an identical manner). Deletions to either -511 or -368 bp resulted in a 3-4 fold decrease in chloramphenicol acetyltransferase (CAT) reporter gene activity and perturbed the exclusive expression of a lacZ reporter gene in aboral ectoderm (Xiang *et al.*, 1991b). Two guanine-rich DNA elements (G-strings) appear to be involved in transcriptional regulation of the *LpS1* genes: a proximal positive cis-

regulatory element at 70 bp to -75 bp and a similar, more distal element at -721 bp to -726 bp (Xiang *et al.*, 1991a). The proximal G-string element is able to bind the mammalian transcription factor IF1, and a similar transcription factor may activate *LpS1* expression in aboral ectoderm (Xiang *et al.*, 1991a). Seid *et al.* (1996) reported that mutations in the distal G-string cause over a two-fold increase in CAT gene activity and the expression of a green fluorescent protein reporter in non-aboral ectoderm cells in *L. pictus* embryos. These results suggest that a repressor normally binds to the distal G-string in non-aboral ectoderm cells to inhibit *LpS1* expression. Wang and Klein (1996) later reported that there is a G/C-rich positive regulatory sequence beside the proximal G-string, and that both G-strings and the G/C-rich region are required for the positive control of *LpS1β* transcription. In accordance with Seid *et al.* (1996), they stated that the distal G-string also appears to act as a negative regulator as it contains a mesenchyme cell repressor element. This indicates that the LpS1 genes are positively regulated in aboral ectoderm cells and negatively regulated in mesenchyme cells.

George *et al.* (1996) determined that *Lytechinus* embryos synthesize two distinct upstream stimulatory factors (USFs) that bind to other DNA regulatory sites - USF sites - within the *LpS1* promoter region. Reporter gene constructs containing a mutated USF binding site were expressed in all cell types in gastrula and pluteus stage embryos, while wild-type

constructs were expressed primarily in the aboral ectoderm (Seid *et al.*, 1996). The USF proteins are expressed in all cells of the early embryo and in all tissues except the aboral ectoderm in later embryos. Therefore, the USF proteins appear to be acting as a repressor of *LpS1* expression in *Lytechinus* embryos.

1.5 The extracellular matrix

The extracellular matrix (ECM) is composed of an interlocking network of macromolecules. The composition and organization of the ECM has been implicated in influencing cell migration, adhesion, and differentiation in many biological processes including development (reviewed by Hay, 1989). The sea urchin blastula is made up of a spherical epithelium with two faces – the apical and basal epithelia. Both of these surfaces are coated with complex multilayered ECMs made up of macromolecules such as collagens, heparan sulfate proteoglycans, laminins, and fibronectin-like proteins (reviewed by Har-el and Tanzer, 1993; Spiegel *et al.*, 1983; Spiegel *et al.*, 1989).

The lathrytic agent β-aminopropionitrile (BAPN) has been shown to reduce the cross-linking of collagen molecules by inhibition of lysyl oxidase activity, leading to the proteolytic digestion and removal of collagen from the matrix (Kleinman *et al.*, 1981). When sea urchin embryos were treated

with BAPN, no invagination of the archenteron occurred, but when BAPN was removed gastrulation was initiated with the acculmulation of collagen. The failure of treated embryos to gastrulate was interpreted as a lack of endoderm differentiation, and was confirmed with staining for expression of a cell surface glycoprotein associated with differentiating endoderm, Endo1 (Wessel and McClay, 1987). When the expression of *LpS1* was examined in BAPN treated *L. pictus* embryos, it was found that disruption of the ECM inhibits transcription of these genes. When the agent was removed, however, transcripts began to accumulate once more. Interestingly, transcription of the *Spec* genes in *S. purpuratus* was unaffected by BAPN treatment (Wessel *et al.*, 1989). These results suggested that the *LpS1* genes may be regulated by a growth factor signaling pathway mediated by the ECM in *L. pictus* embryos; however, this does not appear to be the case in *S. purpuratus*. Seid *et al.* (1997) reported that the *LpS1β* gene contains an ECM response element (ECM RE) in the proximal G-string regulatory sequence that appears to bind an ECM-regulated repressor. They suggest that the intact ECM normally transmits signals to inhibit repressor activity at the proximal G-string regulatory sequence in aboral ectoderm cells. This repressor was found to normally be active in oral ectoderm, as embryos injected with mutated proximal G-string-LpS1-CAT constructs showed staining for CAT in both the aboral as well as the oral ectoderm. Inconsistencies with respect to

whether the proximal G-string is a positive or negative regulator have yet to be worked out.

1.6 Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPGs) have been shown to have profound roles in both vertebrates and invertebrates (Esko and Lindahl, 2001). They have been implicated in developmental processes such as cell proliferation, differentiation, motility, and morphogenesis (reviewed by Bernfield *et al.*, 1999). Although HSPGs were previously thought to lack specificity for binding particular ligands at the cell surface, that idea is now changing as their functions become better characterized (Perrimon and Bernfield, 2000). Due to the potential for numerous variations in the structure of these molecules, HSPGs have been shown to have extremely specific functions.

An HSPG is a complex structure, composed of a core protein coupled to several glycosaminoglycan (GAG) chains. There are five main classes of HSPGs, based on their core proteins: syndecans, glypicans, perlecans, agrins, and type XVIII collagen (Iozzo, 2001). These proteins have heparan sulfate (HS) chains of various lengths attached to them at particular serine-glycine residues found within defined amino-acid sequences (Lindahl *et al.*, 1998). HS is composed of alternating

glucuronic/iduronic acid and glucosamine disaccharide units connected to the core protein by a linkage region (galactose-galactose-xylose). The individual saccharide units of the HS chain can undergo numerous modifications such as N-sulfation, O-sulfation, epimerization, and deacetylation (Perrimon and Bernfield, 2000). It is apparent that due to the diversity of core protein type and HS chain composition, length, and modification, individual HSPGs are extremely distinct. The expression of the core proteins is tissue-specific and the molecules bound by the HS side chains are extremely exact; therefore, a precise signaling pathway can be initiated. HS chains, and therefore HSPGs, have been shown to be implicated in several signaling pathways due to their ability to bind growth factors such as of the fibroblast growth factors (FGFs), platlet derived growth factor (PDGF), heparin-binding epidermal growth factor (HB-EGF), transforming growth factor β (TGF-β), bone morphogenetic protein 2 (BMP 2), as well as members of the Wnt and Hedgehog families (Guimon *et al.*., 1993; Kelly *et al.*, Joseph *et al.*, 1996; Paria *et al.*, 1999; Jackson *et al.*, 1997; Ruppert *et al.*, 1996; Dhoot *et al.*, 2001; Lin and Perrimon, 1999).

HSPGs are generally found associated with cell surfaces, basement membranes or extracellular matrices (ECMs), where they present their extracellular ligands to the appropriate receptors. Syndecans have transmembrane domains, glypicans contain a carboxy-terminal glycosyl phosphatidylinositol (GPI) linkage to plasma membrane lipids, and

perlecans and agrins are secreted into basement membranes (Iozzo, 1998). HSPG protein cores range from about 32 to almost 500 kDa, while HS chains vary from approximately 5 to 70 kDa. The HS chains also have the ability to move laterally fairly freely, allowing bound growth factors to reach receptors that may be located several hundred nanometers away (Iozzo, 2001). It is interesting to note that syndecan core proteins also have the capability of being proteolytically cleaved close to their transmembrane domain, releasing the ectodomain of the protein, which contains the HS chains. This process could be involved in mediating movement of HSPG-bound growth factors within extracellular spaces (Subramanian *et al.*, 1997).

The roles of HSPGs in developmental processes have recently gained more attention. Studies have been conducted on the role of HSPGs in the normal development of *Drosophila*, *Xenopus*, quail, and mice. It has been shown that defects in the genes or disruptions in the accumulation of mRNA from the genes encoding either the core proteins to which HS chains are attached, or the enzymes involved in HS chain modification have profound effects on development (Nakato *et al.*, 1995; Kramer and Yost, 2002; Haerry *et al.*, 1997; Binari *et al.*, 1997; Bullock *et al.*, 1998; Dhoot *et al.*, 2001; Sen *et al.*, 1998).

Several genetic studies on *Drosophila* have implicated HSPGs in various signaling pathways due to the effects of any alterations in enzymes

involved in biosynthesis of HS chains. Embryos having mutations in a gene encoding a UDP-glucose dehydrogenase were discovered in several different labs. This is a protein that produces UDP-glucuronic acid from UDP-glucose, which is essential for heparan side chain elongation. When the structures of GAG chains were examined in *Drosophila* embryos carrying mutations in the gene that encodes this protein – *sugarlesss* – there were shown to be great reductions in heparan sulfate levels (Toyoda *et al.*, 2000). Previously, two papers had reported the effects of mutation of *sugarless*. Cuticles prepared from mutant *Drosophila* embryos showed a loss of naked cuticle and a mirror-image duplication of denticle belts. The expression of both *wg* and *engrailed* (*en*) was examined because they mutually support each other's expression through a feedback loop that involves *hedgehog* (*hh*) (review in Martinez Arias, 1993). Haerry *et al.* (1997) found that the phenotype caused by a mutation in a gene they referred to as *suppenkasper* (*ska*) showed a marked similarity to the phenotype due to a mutant *wg* gene. Binari *et al.*(1997) also analyzed the role of this gene, which they called *kiwi*, and showed that its activity is critical for Wg signaling. They also found that the phenotypes observed due to the different mutant alleles of *kiwi* showed similarity to *wg* mutant phenotypes. *Sugarless*, *ska*, and *kiwi* are the same gene.

Furthermore, the biochemical effects of two other *Drosophila* mutations that are know to have an impact on signaling involving Wnt,

TGF-β, Hh, and FGF families have been examined. Mutations in the gene *sulfateless*, which encodes a protein with similarity to the vertebrate N-deacetylase/N-sulfotransferase, have a strong impact on the normal production of HSPGs: embryos were found to have an absence of N-, 6-O- and 2-O-sulfated disaccharides (Toyoda *et al.*, 2000a). *Sulfateless* is essential for Wg signaling, and mutations in this gene show a similar segment-polarity cuticle phenotype (Lin and Perrimon, 1999). The Drosophila homologue of the vertebrate gene *EXT1*, *tout-velu*, has been shown to be selectively involved in Hh signaling (The *et al.*, 1999). EXT1 encodes a heparan sulfate co-polymerase that has been shown to be a transmembrane glycoprotein that functionally alters the synthesis and display of HS chains (McCormick *et al.*, 1998). Analysis of HSPGs from *ttv* mutant larvae has revealed a dramatic reduction in the levels of HS chains (Toyoda *et al.*, 2000b).

While the enzymes required for the synthesis of HS chains have been shown to be necessary for the proper function of HSPGs during development, mutations in the core proteins can also cause developmental problems. The *Drosophila* gene *division abnormally delayed* (*dally*) has homology to a family of glypican-related integral proteins, and it has been shown to be required for proper cell division patterning in the development of the nervous system. *Dally* mutants have a delayed first division in lamina precursor cells and failure of the second divion to enter S phase

(Nakato *et al.*, 1995). In a later study, Lin and Perrimon (1999) demonstrated that Dally is the protein core of an HSPG that is involved in Wg signaling, and that Dally cooperates with the Wg receptor, Frizzled, to mediate this pathway.

HSPGs have also been shown to play a role in axis patterning in *Xenopus* embryos (Kramer and Yost, 2002; Kramer *et al.*, 2002). Injection of truncated, dominant-negative syndecan-2 mRNA results in randomized left-right patterning, as does injection of syndecan mRNA containing mutated serine HS attachment sites. This suggests that the syndecan-2 core protein and the attached HS chains are necessary for correct left-right patterning. Through co-immunoprecipitation, sydencan-2 was also shown to bind Vg1, a TGFβ family member known to be involved in left-right patterning (Kramer and Yost, 2002). Kramer *et al.* (2002) then demonstrated that PKCγ phosphorylation of syndecan-2 on the right side of the *Xenopus* embryo is necessary for correct left-right patterning. Through immunostaining using antibodies against phospospecific syndecan-2 and a pan-syndecan antibody, they found that the phosphorylation state of the cytoplasmic domain of syndecan-2 differs on the left and right sides of the ectoderm in the embryo. The differential phosphorylation is essential for establishing left-right asymmetry.

The first putative connection between the ECM and HSPGs in sea urchin embryos was revealed by Ramachandran *et al.* (1993). They found

that human recombinant platelet derived growth factor-BB (PDGF-BB) and transforming growth factor-α (TGF-α) synergistically rescue BAPN treated *L. pictus* and *L. variegatus* embryos so that development and accumulation of *LpS1* proceed. Later, Govindarajan *et al.* (1995) used anti-human PDGF-BB and TGF-α antibodies to treat *L. pictus* and *L. variegatus* embryos, and discovered that addition of these antibodies from the four-cell to the hatching blastula stage inhibited gastrulation and spiculogenesis in these embryos. When embryos were treated with a synthetic peptide containing the HSPG binding sequence on human PDGF-BB, gastrulation was inhibited and multiple radialized spicules were formed, suggesting that *Lytechinus* embryos normally use PDGF-BB bound to HSPGs in the ECM for signaling involved in gastrulation and patterning of the O/Ab axis. Ramachandran *et al.* (1995) used immunostaining to localize PDGF-like receptors in *Lytechinus* embryos during the gastrula stage, and found that they are located on the primary mesenchyme cells, the gut, and most prominently on the secondary mesenchyme cells and the stomadeum. EGF-like receptors were also discovered in the ECM of embryos; however, these receptors stained less intensely and were present on the gut and the primary and secondary mesenchyme cells.

A gene encoding a sea urchin syndecan core protein was cloned from the Japanese species *Anthocidaris crassispina* (Tomita *et al.*, 2000).

Immunostaining indicated that this proteoglycan is present at a constant level from the unfertilized egg stage to the pluteus larva stage, and it is localized on the apical and basal surfaces of the epithelia in embryos at the blastula and gastrula stages. The effects of culturing *A. crassispina* embryos in the antibody against the syndecan core protein were later, and it was concluded that syndecan is necessary for proper elongation of the postoral arms during the late pluteus stage (Tomita *et al.*, 2002).

1.7 Chlorate as an inhibitor of sulfation

The sulfation of various molecules *in vivo* involves a complex series of steps which are driven by various enzymes. Chlorate has been shown to be an inhibitor of ATP-sulfurylase, the first enzyme in the biosynthesis of the high energy sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Farley *et al.*, 1978). The *in vivo* sulfation pathway involves the transport of sulfate to the cytosol, where it reacts with adenosine triphosphate (ATP) to form adenosine 5'phosphatesulfate, and then phosphorylation of this molecule to form PAPS (Hirchsberg *et al.*, 1998). Sulfate is transferred from PAPS onto various substrates by the action of the enzyme tyrosylprotein sulfotransferase (Lee and Huttner, 1983). In this pathway, chlorate acts as a competitive inhibitor due to its structural

similarity to sulfate, thereby interfering with formation of the sulfate donor (Farley *et al.*, 1978; Hortin *et al.*, 1988).

Due to the importance of sulfation in biochemical events, chlorate has been used to investigate the effects of the inhibition of this process in cultured cells (Baeuerle and Huttner, 1986; Hortin *et al.*, 1988; Greve *et al.*, 1988; Delehedde *et al.*, 1996; Fjeldstad *et al.*, 2002). Baeuerle and Huttner (1986) showed that chlorate is an extremely potent inhibitor in the sulfation of proteins by studying the incorporation of [^{35}S] when cultured cells were treated with an assortment of sulfation inhibitors. Of significance is the fact that chlorate was shown to have no toxic effects, unlike other commonly used sulfation inhibitors. Chlorate had no effect on protein synthesis, and cell morphology and cell number did not vary from controls. A dose dependent response in inhibition of sulfation was apparent, as well as a synergistic inhibitory effect when cells were cultured in the presence of chlorate and a medium low in sulfate. Hortin *et al.* (1988) also looked at the effects of various sulfate inhibitors on cultured cells. They investigated the incorporation of [^{35}S] into proteins, but studied various other sulfated macromolecules including glycoproteins, and proteoglycans. Again they found that chlorate is an effective inhibitor of the sulfation of proteins, as well as of glycoproteins and proteoglycans. Therefore, several studies to date have used chlorate to investigate the various roles of sulfated glycoproteins and proteoglycans.

Dhoot et al. (2001) used chlorate to determine the relationship between Qsulf1, the avian orthologue of a family related to heparan-spcific N-acetyl glucosamine sulfatases, and Wnt signaling using muscle progenitor cells and a quantitative TCF luciferase reporter gene. They found that Wnt signaling was activated 17-fold when C2C12 cells were cocultured with Wnt1-expressing cells, as compared to controls. However, Wnt1 induction was inhibited by 1mM chlorate in all circumstances.

The role of HSPGs in various aspects of mouse development has also been studied using chlorate to inhibit N- and O-linked sulfation of HS chains. Gritli-Linde *et al.* (2001) used chlorate to study the diffusion of SHH in mouse tooth cultures due to the known association between Hh proteins and HSPGs. Molars cultured in chlorate showed a dramatic decrease or even a total ablation of SHH immunostaining in dental papilla and in the inner enamel epithelium/preameloblasts as compared to control molars; however, the expression of *shh* was unaffected. An unexpected finding was an upregulation in *patched 1* (*Ptc 1*) expression in chlorate treated molars. Yip *et al.* (2002) looked at the effects of chlorate on spinal neurulation in the mouse embryo. It was found that treated embryos experience accelerated neuropore closure, which can be rescued with the addition of HS to the medium in which the embryos were cultured. Interestingly, they also found that *Ptc 1* expression is strongly

upregulated in treated embryos. There was strong *Ptc 1* staining in the notochord, with less intense staining in the midline neuroepithelium.

Although chlorate has been shown to inhibit the formation of PAPS, the universal sulfate donor in cellular sulfation reactions, there have been several experiments on cultured cells with results that suggest that chlorate may be having a selective effect on sulfation. Safaiyan *et al.* (1999) analyzed the effect of various concentrations of chlorate on the structure of HS. They isolated HS chains from cultured cells, which were then subjected to nitrous acid cleavage. It was discovered that when cells were treated with 50 mM chlorate, overall O-sulfation was inhibited by about 70% while N-sulfation was not significantly affected. At lower concentrations of chlorate (5 and 20 mM), 6-O-sulfation was selectively inhibited while 2-O-sulfation was unaffected. On the other hand, Fjeldstad *et al.* (2002) found that varying concentrations of chlorate have an effect on what molecules are not being sulfated. At low concentrations of chlorate (2-5 mM), they showed that HSPG sulfation was not affected whereas sulfation of proteins and chondroitin sulfate proteoglycans (CSPGs) was almost completely eliminated. At 15-30 mM chlorate, HSPG sulfation was greatly decreased. It was suggested that HSPG sulfation operates at a lower PAPS concentration than protein and CSPG sulfation. Chondroitin sulfate is composed of alternating glucuronic acid and

glucosamine disaccharide units connected to the core protein by glucosamine (Pedersen *et al.*, 2000)

1.8 The specific objectives of this thesis

In this project chlorate was used as an inhibitor of sulfation of HSPGs during the normal developmental time course of *L. pictus* embryos. In particular, I set out to answer several questions:

1) If chlorate has an effect on *L. pictus* embryos, what is this effect with regard to morphology?
2) When is the period of sensitivity of *L. pictus* embryos to chlorate?
3) Can the effects of chlorate on *L. pictus* embryos be rescued by treatment with HS or growth factors?
4) Are there any genes that experience either a downregulation or upregulation due to chlorate treatment?

I report on the results of my experiments and investigations in the next chapters of this thesis.

Chapter 2
Perturbation of gastrulation, spiculogenesis, and axial patterning in chlorate-treated *L. pictus* embryos and rescue with PDGF-BB

2.1 Introduction

The use of chlorate has increased our understanding of the effects of a lack of sulfation of HSPGs during embryo development. The ECM of animals contains various macromolecules, including HSPGs, which are made up of a protein core and heparan sulfate chains. The HSPGs have been implicated in the binding and presentation of several growth factors to their receptors (reviewed by Bernfield *et al.*, 1999 and Perrimon and Bernfield, 2000). The benefits of chlorate, in particular, lie in the fact that it is a powerful inhibitor of sulfation, yet it does not have the toxic effects of previously used sulfation inhibitors (Baeuerle and Huttner, 1986; Hortin *et al.*, 1988).

While chlorate has recently been used to treat both mouse and quail embryos (Yip *et al.*, 2002; Dhoot *et al.*, 2001), its effects on sea urchin embryos had never been investigated. The experiments done by Ramachandran *et al.* (1993, 1995), and Govindarajan *et al.* (1995) revealed a possible connection between the ECM and growth factors such

as PDGF-BB, TGF-α, and EGF. Because the presence of intact HSPGs has been shown to be necessary for the proper binding of various growth factors to their receptors, and therefore in the function of signaling pathways (reviewed by Bernfield *et al.*, 1999 and Perrimon and Bernfield, 2000), a putative relationship between unaffected HSPGs and normal development in the sea urchin embryo was proposed. Especially of interest were the results that Govindarajan *et al.* (1995) obtained when they treated *L. pictus* and *L. variegatus* embryos with a synthetic peptide containing the HSPG binding sequence for human PDGF-BB. These results implicated HSPG-mediated PDGF-BB signaling in multiple developmental processes.

The previous data suggest an important role for HSPGs in sea urchin development. We hypothesized that treating *L. pictus* embryos with chlorate would alter normal development if it inhibits sulfation of HSPGs. Thus, the first goal of this part of my research project was to establish whether chlorate has an effect on the development of *L. pictus* embryos, and if it does, to determine the time frame during which it exerts this effect. Embryos were first cultured with several concentrations of chlorate from fertilization to larval stage so that the consequences of these treatments on the morphology of the embryos could be examined. Then, embryos were treated with a set concentration

of chlorate for selected periods during their development. Chlorate treatment disrupted gastrulation and radialized embryos.

It had been previously reported that the disruption of collagen cross-linking in the ECM by the treatment of *L. pictus* embryos with BAPN inhibits gastrulation and the expression of *LpS1* (Wessel *et al.*, 1989), and that the *LpS1β* gene actually contains an ECM RE (Seid *et al.*, 1997). With this in mind, the second goal of this part of my project was to assess the effects of disrupting the ECM with chlorate on *LpS1* gene expression. This was determined by RNA electrophoretic gel blot hybridization (Northern blot hybridization) and by reverse transcription-polymerase chain reaction (RT-PCR) (a more sensitive assay). *LpS1* expression was also assessed in chlorate treated and control embryos through *in situ* hybridization to see if the positioning and differentiation of aboral ectoderm, and therefore the patterning of the O/Ab axis are altered by chlorate treatment. *LpS1* expression was reduced and radialized in chlorate-treated embryos.

The final goal of this part of my project was to determine whether the morphology of chlorate treated embryos could be rescued with PDGF-BB and/or TGF-α. Govindarajan *et al.* (1995) found that treatment of *L. pictus* and *L. variegatus* embryos with antibodies to these two growth factors had a strong effect on the morphology of treated embryos. Therefore, these growth factors were used individually and concurrently

to attempt to rescue the morphology of chlorate treated *L. pictus* embryos. PDGF-BB almost completely rescued chlorate-treated embryos.

2.2 Materials and Methods

2.2.1 Embryo Culture

Adult *Lytechinus pictus* were obtained from Marinus in Long Beach, California. These animals were kept in aquarium tanks with recirculating sea water at 12-17°C. Gametes from adult animals were collected through intracoelomic injection of approximately 200 µl of 0.5 M KCl. Eggs were collected in sea water and were then filtered into 4 l of sea water through a 153 µm mesh Nitex filter to remove debris such as fallen spines. They were washed by settling and pouring off excess water 3 times in approximately 4 l of sea water. Sperm was collected "dry" and was stored on ice in a 1.5 ml microcentrifuge tube. Several drops of sperm were activated by dilution in sea water; the activity of the sperm was verified using microscopy. Eggs were fertilized by adding several drops of diluted sperm to the suspension of eggs. A sample of the suspension was taken to view fertilization, which was indicated by the elevation of the fertilization envelope. Eggs with low fertilization rates were discarded. Once fertilized, the suspension was washed once more by filtering sea water through a 44 µm Nitex filter to remove any excess sperm that may be present. The culture was diluted to a density of approximately $1x10^2$ embryos/ml, and divided up into beakers of

appropriate volumes. Embryos were cultured in an incubator at 15-16°C with stirring at a speed of 60 rpm. Generally, under these conditions embryos hatch 20 hours post fertilization. The mesenchyme blastula stage is reached approximately 24 hours after fertilization, and gastrulation begins at around 32 hours. The pluteus larvae stage is reached after approximately 72 hours; however, embryos were generally cultured for at least 96 hours to verify that development was not retarded by any treatments that were used.

2.2.2 Removal of the fertilization envelope

Before fertilization, eggs were suspended in sea water containing 10 mM para-aminobenzoic acid (PABA), an inhibitor of the peroxidase involved in hardening of the fertilization envelope. The PABA sea water was aspirated through a 44 μm Nitex filter so as to prevent aspiration of the eggs. The eggs were resuspended in PABA-sea water and fertilized. To remove the soft fertilization envelope, fertilized eggs were passed through a 54 μm Nitex filter.

2.2.3 Chlorate treatment of embryos

The appropriate amount of $NaClO_3$ was weighed out using a fine scale. The chemical was dissolved in 1 ml of sea water before adding the

appropriate volume to each beaker of embryos. To define the sensitivity period for chlorate, the chemical was added to and removed from cultures at various stages during development. The removal of chlorate from an embryo culture required the aspiration of the sea water through a 44 μm Nitex filter to concentrate the embryos and the addition of fresh sea water to the culture. This was done at least 3 times so as to decrease the concentration of chlorate to a negligible amount. For chlorate treatments, volumes of 150 ml were generally used.

2.2.4 Cytoplasmic RNA isolation from embryos

Total cytoplasmic RNA was extracted using Trizol (Invitrogen, catalogue #15596-026) and chloroform. Suspensions of pluteus stage embryos were transferred to 50 ml conical tubes and centrifuged at around 300 g to loosely pellet embryos. Much of the sea water was aspirated off; about 2 ml of water were left in each tube to resuspend embryos. About 500 μl of embryos were transferred to each 1.5 ml microcentrifuge tube and centrifuged again to pellet embryos; all excess water was removed. The embryos were resuspended in 1 ml of Trizol, and this mixture was repeatedly pipetted using a P-1000 Pipetman until it appeared homogenous. The mixture was incubated at room temperature for 5 minutes. Then 200 μl of chloroform was added and the tubes were vigorously shaken for approximately 15 seconds to mix

the Trizol and chloroform. The tubes were again left to incubate at room temperature for 3 minutes. To separate the two phases, the tubes were centrifuged at 12,000 g for 15 minutes at 2-8°C. The colourless upper phase was transferred to a clean tube and 0.5 ml of isopropanol was added. The solution was left to incubate at room temperature for 10-20 minutes so that the RNA would precipitate. The tubes were centrifuged at 12,000 g for 10 minutes at 2-4°C such that the precipitated RNA could be pelleted. The pellets were washed twice with 500µl of 75% ethanol, centrifuging at 7,500 g for 5 minutes at 2-4°C each time. Pellets were then air-dried for about 10 minutes, redissolved in DEPC (diethylpyrocarbonate)-treated water, and aliquotted. Tubes of RNA were frozen at –80°C to inhibit RNase activity.

2.2.5 Agarose gel electrophoresis of RNA

RNA samples were quantified using an Ultrospec III (Pharmacia) spectrophotometer. Samples containing 10 µg of RNA were dried in a vacuum spin drier until most of the DEPC-water had evaporated. The RNA was resuspended in 50% (v/v) deionized formamide (BRL, ultrapure), 6% (v/v) deionized formaldehyde (Sigma), 1 x MOPS (3-[N-morpholino]propanesulfonic acid, Sigma) buffer (20 mM MOPS at pH 7, 5 mM sodium acetate, 1mM EDTA), and heat-denatured at 65°C for 15 minutes. RNA samples were separated by electrophoresis on 1.2%

agarose (Bio-Rad, molecular analytical grade) gel containing 1 x MOPS buffer and 3.3% formaldehyde and 1 μg/ml ethidium bromide in 1 x MOPS buffer at 3-4 V/cm. After electrophoresis, the gel was washed twice, for ten minutes each, in distilled water to remove formaldehyde. Gels were visualized using a 300 nm UV transilluminator and photographed before blotting. Photographs were taken using the Scion Image system.

2.2.6 Overnight bacterial cultures

To produce *LpS1* or *ubiquitin* plasmid, 5 ml of either Luria-Bertani broth (LB) or terrific broth (TB) containing 100 μg/ml ampicillin was inoculated with frozen transformed DH5α cells. The cells were cultured overnight in 15 ml Falcon round-bottom tubes at 37°C in a 225 rpm shaking incubator.

2.2.7 Plasmid DNA isolation and restriction enzyme digestion

Plasmid DNA was extracted from bacterial cultures using The Magic Minipreps DNA Purification Kit (Promega), as described in the manufacturer's instructions. DNA was eluted with 50 μl of distilled water. To check the extracted plasmid DNA, restriction digests were carried out with several enzymes (Invitrogen) known to cut the plasmid.

Typical digestions were carried out at 37°C for approximately 90 minutes at an enzyme concentration of 1 unit per μg recombinant plasmid DNA. The sizes of the DNA fragments produced from the digests were analyzed by electrophoresis on agarose gels.

2.2.8 DNA agarose gel electrophoresis

A 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1mM EDTA) containing 1 μg/ml ethidium bromide was used to separate DNA fragments according to size. Loading buffer was added to the samples at a ratio of 1:6 before gel loading. Gels were run at 1-5 V/cm and were visualized using a 300 nm transilluminator. Photographs were taken by using the Scion Image system.

2.2.9 Description of hybridization riboprobes

In this project, both Digoxigenin- and ^{32}P-labeled riboprobes were used for Northern blot hybridization and *in situ* hybridization assays. For this chapter, only ^{32}P-labeled riboprobes were used for Northern blots; therefore, a description of Northern blotting using DIG-labeled probes can be found in the next chapter. A plasmid containing an *LpS1* DNA insert was used to produce both types of riboprobes. This plasmid includes a 0.44 kb *LpS1* DNA fragment corresponding to part of the

coding sequence of the *LpS1* gene from the (λ)gt10 clone. The sequence was subcloned into the EcoRI site of Bluescript (BRL) by Xiang *et al.* (1988).

2.2.10 Preparation of ^{32}P-labeled LpS1 *hybridization probe*

To prepare ^{32}P-labeled probes, the Rediprime II DNA Labeling System (Amersham, catalogue #27-9250-01) was used according to the manufacturer's instructions with some modifications. To begin, approximately 1 μg of *LpS1* plasmid was cut using the restriction enzyme EcoRI to excise the insert from the vector. The DNA was separated by electrophoresis on an agarose gel, and the fragments were visualized using a 360 nm gel box, to minimize UV damage to the DNA. The band corresponding to the 0.44 kb coding sequence was excised from the gel using a clean razor blade, and the DNA was purified using the QIAquick Gel Extraction Kit (Qiagen, catalogue #28704) according to the manufacturer's instructions. The DNA was eluted into a microcentrifuge tube using only 5 μl of warmed distilled water so that it would be more concentrated.

The purified coding sequence DNA was denatured by heating to 95°C for 5 minutes, and placed on ice for 2 minutes to prevent reannealing. The tube was briefly centrifuged and 10 μl of reagent mix, 5 μl (50 μCi) of [α-32P]-dCTP (Perkin-Elmer NEN), 29 μl of distilled water

and 1 µl of 5-10 units/µl *FPLC*pure Klenow fragment were added. The tube was placed in a 37°C water bath and the reaction was left to proceed for 60-90 minutes. Unincorporated nucleotides from the labeling reaction were removed using Micro Bio-Spin 30 Chromatography Columns (Bio-Rad, catalogue #732-6202). The probe was heated to 95°C for 5 minutes, placed on ice for 2 minutes, and added directly to hybridization buffer.

2.2.11 Preparation of DIG-labeled LpS1 *hybridization riboprobe*

DIG-labeled riboprobes were used for the whole mount *in situ* hybridization assays in this section of the project. Riboprobes were synthesized by using labeling components from Roche Molecular Biochemicals and T7 or T3 RNA polymerase (Ambion, catalogue #2082 and #2063, respectively).

To begin, the *LpS1* plasmid was linearized with either the restriction enzyme BamHI or HindIII to produce antisense or sense riboprobes, respectively. 10-20 µg of plasmid DNA were digested, producing 5' overhangs at the appropriate restriction site. Each restriction site is in the multiple cloning site on either side of the *LpS1* coding sequence. The antisense riboprobe was transcribed from the T7 phage promoter, while the sense riboprobe was transcribed from the T3 phage promoter.

After restriction enzyme digestion, the linearized template was purified using chloroform. An equal volume of chloroform was added to each tube and vortexed. The tubes were briefly centrifuged to separate the aqueous and organic phases. The top phase, containing the purified DNA was removed to a clean tube. The linearized plasmid could be used as a template for the *in vitro* transcription reaction to make DIG-labeled riboprobe.

Briefly, DEPC-treated water was added to 1-2 μg of linearized template DNA to bring the volume up to 13 μl. Then 2 μl of DIG-labeling mix, 2 μl of 10 x transcription buffer, 1 μl of RNaseOUT RNase inhibitor (Invitrogen, catalogue #10777019), and 2 μl RNA polymerase were added so that the final volume was 20 μl. The solution was quickly centrifuged, and the reaction was carried out at 37°C for 2 hours. After this time, the DNA template was digested by adding 1 μl of DNase I (Invitrogen, catalogue #18047019) and incubating at 37°C for 15 minutes. Unincorporated nucleotides were removed from labeled RNA by passing the reaction mixture through Micro Bio-Spin 30 Chromatography Columns (Bio-Rad). The riboprobe was aliquotted and stored at –80°C for future use.

The DIG-labeled riboprobe was characterized before use to ensure that the probe is full length and to verify the yield of the *in vitro* transcription. To confirm the size of the riboprobe, electrophoresis was

carried out. To quantify the yield of the *in vitro* transcription, serial dilutions (1/1, 1/10, 1/100, 1/1,000 and 1/10,000) of both the probe and the DIG-labeled control RNA were spotted onto a positively charged nylon membrane and fixed onto the membrane with UV light using a Stratalinker 2400 (Stratagene) on the Auto Cross Link setting (120 mJoules). After cross-linking, the procedure used to treat the membrane was identical to that used in DIG Northern blotting after hybridization (see section 3.2.6). After the film was developed, the dilutions of riboprobe were compared to the dilutions of DIG-labeled control RNA to estimate the yield of riboprobe.

DIG-labeled probes may be used several times once they are diluted in hybridization buffer. After 2 or 3 uses, more probe may be added to the hybridization buffer to replenish the concentration. The riboprobe in hybridization buffer may be stored at –80°C until the next time it is used.

2.2.12 ^{32}P Northern blotting

The RNA gel was blotted overnight onto a positively charged nylon membrane (Hybond) in 10X SSC (1.5 M NaCl and 0.15 M sodium citrate, pH 7.0) using the unidirectional capillary method. After it had been transferred, RNA was UV cross-linked to fix it onto the membrane.

The membrane was prehybridized at 65°C in hybridization buffer consisting of 50 mM PIPES (piperazine-N-N'-bis [2-ethane sulfonic acid]), 100 mM NaCl, 50 mM sodium phosphate, and 1 mM EDTA. The prehybridization buffer was discarded, and the membrane was hybridized overnight at 65°C in fresh hybridization buffer containing 10^6 cpm/ml of probe. All hybridizations were carried out in glass tubes using a rotating hybridization incubator.

Following hybridization, the membrane was washed for 10 minutes in 50 ml of wash buffer (5% SDS in 1 X SSC) at room temperature. After this, the membrane was washed twice at 65°C for 30 minutes each time in wash buffer. The membrane was wrapped in Saran Wrap and exposed to Kodak BioMax MS film (Amersham) overnight at –80°C.

2.2.13 cDNA synthesis

cDNA was prepared from extracted total RNA to be used for RT-PCR. Briefly, 2 μg of RNA was combined with 1 μl of oligo dT primer (Invitrogen), 1 μl of RNaseOUT RNase inhibitor (Invitrogen) diluted 1:4 in DEPC-treated water, and enough DEPC-treated water to bring the reaction volume up to 10 μl. This solution was heated to 70°C for 10 minutes to denature the RNA and then chilled on ice. To the same tube was added 2 μl of 10 x first-strand buffer (500 mM Tris-Cl, pH 8.3, 750 mM KCl, and 30 mM $MgCl_2$), 1 μl of 10mM dNTPs (purchased from

Amersham as 100 mM each, and diluted to the final concentration), 1 µl diluted RNase inhibitor, 2µl of 100 mM dithiothreitol (DTT), 2 µl DEPC-treated water, and 2 µl AMV-RT (Invitrogen, catalogue #18020-016). The tube was briefly centrifuged and incubated at 37°C for approximately 2 hours to allow the reverse transcription to proceed. After this time, the RNA templates were digested by adding RNase A and incubating at 37°C for 15 minutes. The tube was centrifuged, and the solution was aliquoted and frozen at –20°C to be used for PCR.

2.2.14 RT-PCR analysis of cDNA expression

RT-PCR was carried out to quantify *LpS1* gene expression, with *polyubiquitin* expression acting as a loading control; the prevalence of *polyubiquitin* mRNA is constant during embryonic development and is not altered by many experimental treatments. The *LpS1* primers used were taken from Xiang *et al.* (1988), and are shown below:

Forward 5'-GACAACTATGACACAAACAAAGACG-3'

Reverse 5'-ACCATCGCCATCCTTGTCTGCTTCT-3'

It should be noted that these primers are outside of the coding sequence contained in the *LpS1* plasmid; therefore, a positive control was not possible for *LpS1* PCR.

The *polyubiquitin* primers were designed using the program Oligo 4.1 to verify that primers are not capable of self-annealing. The primers

made were within the repeat sequences of *polyubiquitin*. The sequences are shown below:

Forward 5'-CTCACAGGCAAGACCATCACTC-3'

Reverse 5'-CACGGAGCCTGAGAACAAGATG-3'

Both sets of primers were ordered from Invitrogen Custom Primers.

PCR reaction solutions were made up as a master mixture, and 23 µl of this mixture was added to 2 µl of each template cDNA. For each PCR reaction, the following was added to the master mixture: 2.5 µl of 10X Idaho PCR buffer, 2.5 µl of 2 mM dNTPs, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 15.8 µl of PCR-grade water, and 0.2 µl of Taq DNA Polymerase (Invitrogen, catalogue #18038-018).

All PCR reactions were carried out using capillary tubes in a Idaho Technology thermal cycler. The conditions used for the *LpS1* PCR were 94°C for 1 minute for the denaturation, 60°C for 1 minute for the annealing, and 72°C for 45 seconds for the elongation; these conditions were taken from Wikramanayake and Klein (1997). The PCR conditions used for *polyubiquitin* were determined empirically. They were 94°C for 30 seconds for the denaturation, 55°C for 30 seconds for the annealing, and 72°C for 90 seconds for the elongation. The reactions were carried out separately due to the differences in PCR conditions.

2.2.15 Whole mount in situ *hybridization*

L. pictus embryos were fixed at desired stages in 10 volumes of ice-cold fix containing 2.5% gluteraldehyde in phosphate buffered saline (PBS), pH 7.4 for 2 hours. Embryos were washed 3 times with 10 volumes of PBS at room temperature. They were then dehydrated with washes of 20%, 35%, 50%, and 70%. At this point, fixed embryos were stored in 70% ethanol at –20°C.

Aliquots of approximately 300 µl of fixed embryos were further dehydrated by 1 ml washes of 80%, 90%, and 2X 100% ethanol. To delipidate embryos and therefore increase probe accessibility, they were resuspended in 100% toluene for 10 minutes. After the delipidation, embryos were washed in 100% ethanol, and then rehydrated with 1 ml washes of 90%, 80%, 70%, 50%, 35%, and 10% ethanol. Embryos were washed twice in PBST (PBS, 0.1% Tween-20) to remove all traces of ethanol, and were resuspended in 500 µl of proteinase K solution (40 µg/ml proteinase K in PBST) for 10 minutes at room temperature. The digestion was stopped by removing the proteinase K solution and adding 1 ml of stop solution (2 mg/ml glycine in PBST). Embryos were washed twice in PBST and were post-fixed for 30 minutes on ice in freshly prepared 4% paraformaldehyde in PBST. To remove paraformaldehyde, embryos were thoroughly washed in PBST 4-5 times.

After washing, hybridization buffer consisting of 50% deionized formamide (Sigma), 10% polyethylene glycol (Sigma), 0.6 M NaCl, 5 mM EDTA, 20 mM Tris-Cl, 500 μg/ml yeast tRNA (Boehringer Mannheim), 2X Denhardt's solution (Sigma), and 0.1% Tween-20 was added sequentially to the embryos at 0.25X, 0.5X, 0.75X, and 1X concentrations. Embryos were prehybridized for 1-4 hours at 50°C. To hybridize, an equal volume of hybridization buffer containing 100 ng/ml of riboprobe was added to the embryos. Thus, the final concentration of riboprobe was 50 ng/ml. Embryos were hybridized for 4-5 days at 50°C with gentle rotation. After hybridization, the buffer was diluted by adding PBST drop wise. Embryos were washed twice in PBST for 20 minutes at 50°C, three times in 0.5X SSC for 30 minutes at the same temperature, and once more in PBST at room temperature.

To block non-specific antibody binding, the embryos were resuspended in blocking solution (PBST containing 2% sheep serum) for 30 minutes at room temperature. They were then incubated in a 1:500 dilution of anti-Digoxigenin Fab fragments (Roche) in blocking solution overnight. To remove unbound antibody, embryos were washed twice in PBST and twice in alkaline phosphatase buffer (100 mM NaCl, 100 mM Tris pH 9.5, 50 mM $MgCl_2$, 1mM levamisole, 0.1% Tween-20). To stain, embryos were incubated in the dark in 1 ml of alkaline phosphatase buffer containing 3.375 μl NBT (100 mg/ml nitro blue tetrazolium in 70%

dimethylformamide, DMF) and 3.5 μl BCIP (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100% DMF). Samples were checked often for colour development, which usual took several hours. If staining was proceeding slowly, embryos would sometimes be left overnight. The reaction was stopped with 1 mM EDTA in PBST.

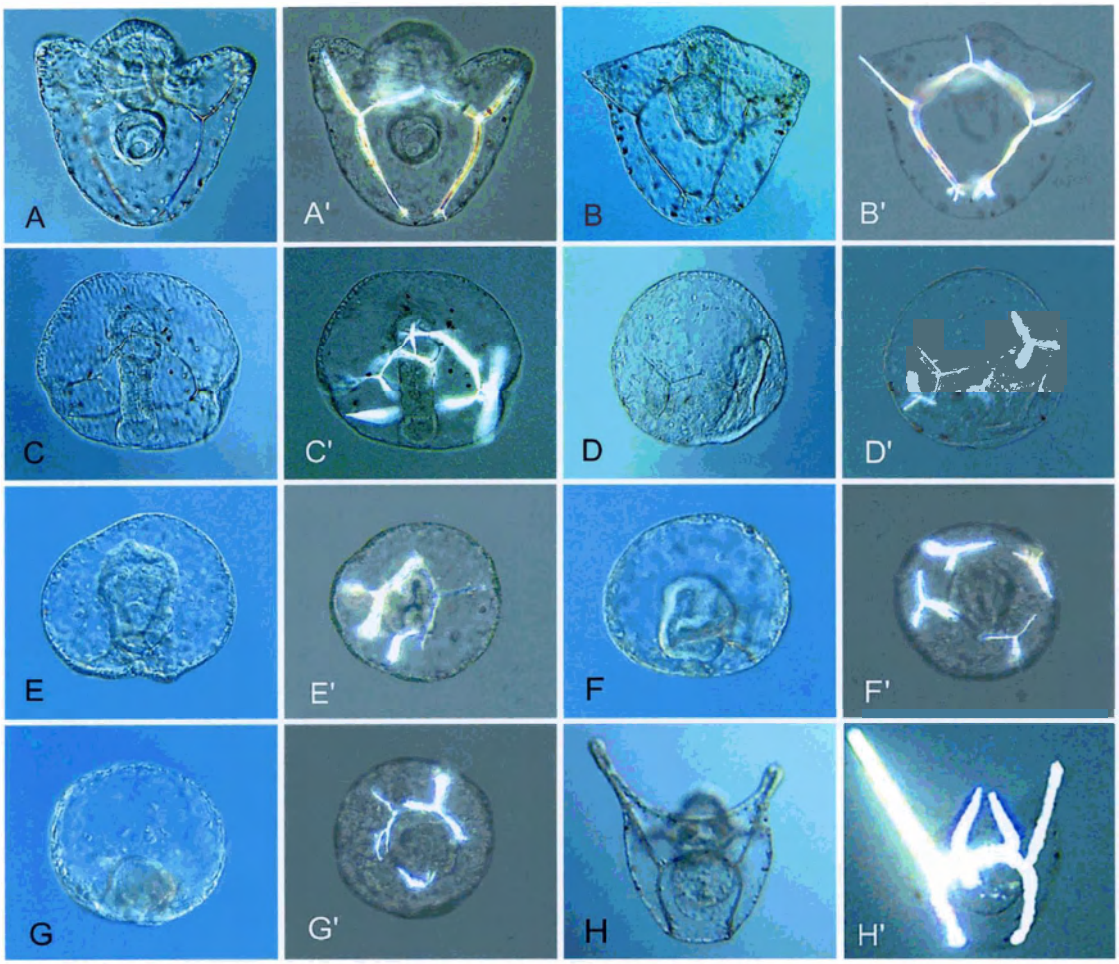
Results

2.3.1 Chlorate interferes with development: concentration dependence

L. pictus embryos were cultured in various concentrations of sodium chlorate to assess its effects on the morphology of embryos. The concentrations of chlorate used were 1 mM, 2.5 mM, 5 mM, 10 mM, 20 mM, 30 mM, 40 mM, and 50 mM. Embryos were cultured for at least 1 day after the control embryos had reached pluteus larva stage to verify that chlorate is not simply slowing development. Morphology was recorded using differential interference contrast and polarized light imaging on an Olympus Vanox microscope.

As can be seen in Fig. 1, as the concentration of chlorate was increased, the ventrolateral pair of birefingent spicules in treated embryos began to form extra branches and then increased in number. This implied a radialization of the embryos with a loss of bilateral symmetry and the O/Ab axis at higher concentrations of chlorate. With respect to gastrulation, the gut became shorter, broader, and apparently failed to differentiate into pharynx, stomach, and intestine as the chlorate concentration was increased. The concentration of 50 mM chlorate appeared to be lethal to *L. pictus* embryos.

Figure 1: The effects of various concentrations of sodium chlorate on *L. pictus* embryos. Embryos were treated from fertilization until 24 hours after controls had reached the pluteus larval stage. Each embryo was photographed using differential interference contrast (X) and polarized light imaging (X'). (A, A') 1 mM chlorate, (B, B') 2.5 mM chlorate, (C, C') 5 mM chlorate, (D, D') 10 mM chlorate, (E, E') 20 mM chlorate, (F, F') 30 mM chlorate, (G, G') 40 mM chlorate. (H, H') are control pluteus larvae. While E, F, and G are shown from a lateral view, E', F', and G' which are shown from a vegetal view to better illustrate spicule arrangement.


A
A'
B
B'
C
C'
D
D'
E
E'
F
F'
G
G'
H
H'

2.3.2 Chlorate sensitivity period

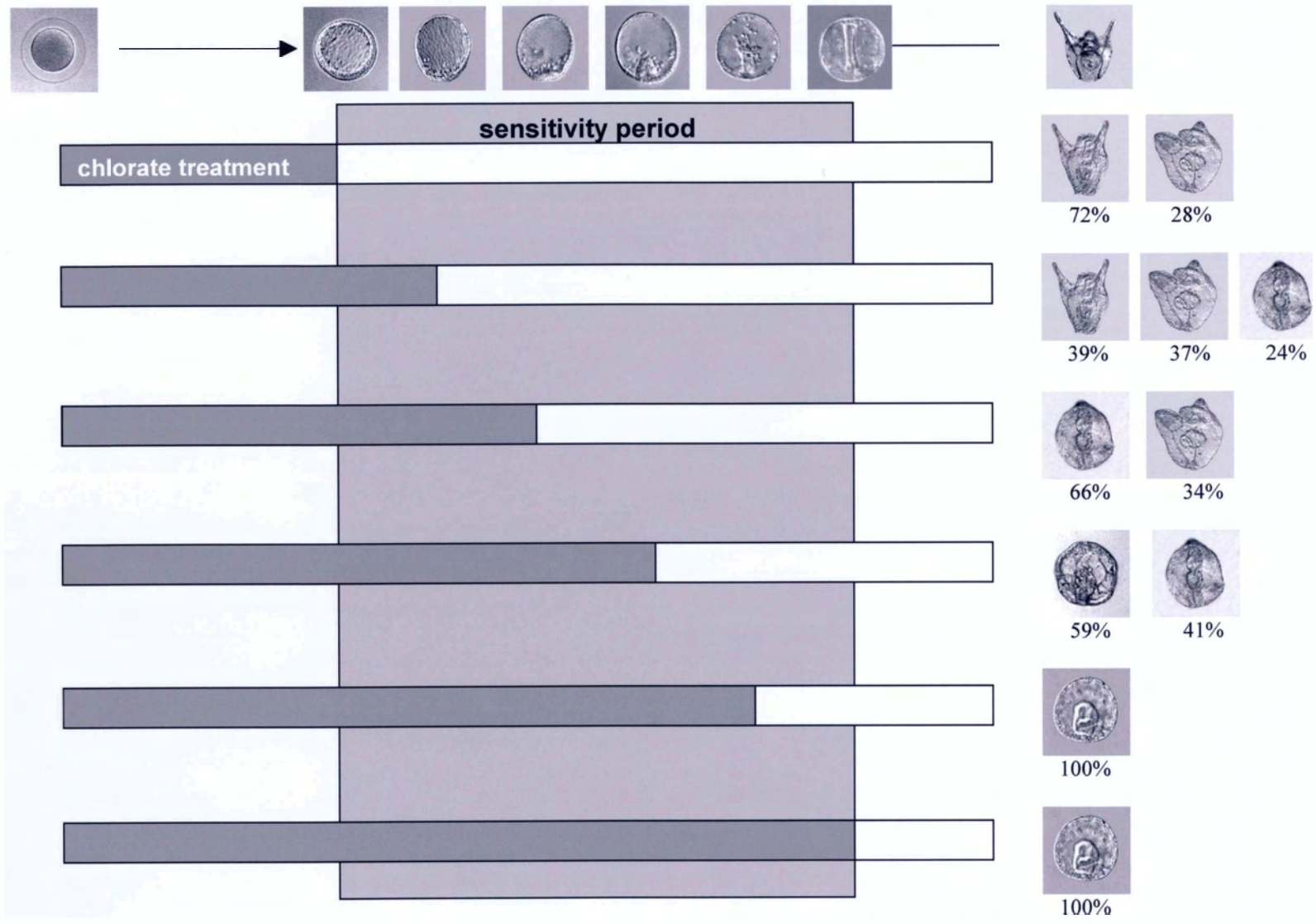
Chlorate was either added to (Fig. 2), or removed from (Fig. 3), cultures of *L. pictus* embryos at set times during embryonic development. The stages used were hatched blastula, early mesenchyme blastula (when primary mesenchyme cells have just begun to ingress), late mesenchyme blastula (when primary mesenchyme cells have begun to migrate), early gastrula, mid gastrula, and late gastrula.

It was discovered that chlorate could be removed and have no effect up until the hatched blastula stage. To verify that this was not due to an inability of chlorate to pass through the fertilization membrane prior to hatching, the membrane was removed prior to treatment; this made no difference. Thus, the hatched blastula stage signified the beginning of the chlorate sensitive period. Chlorate could be added to cultures and produce an effect up until the late gastrula stage; therefore, this signified the end of the sensitive period. This implies that there is a window of sensitivity to chlorate in *L. pictus* embryos that extends from hatched blastula to late gastrula.

Figure 2: The effects of the addition of 30 mM chlorate at different stages (hatched blastula, early mesenchyme blastula, late mesenchyme blastula, early gastrula, mid-gastrula, and late gastrula) during development. Percentages of the various morphologies observed from each treatment are shown to the right.

sensitivity period
chlorate treatment
100%
100%
59%
41%
82%
18%
97%
3%
79%
21%

Figure 3: The effects of the removal of 30 mM chlorate at different stages during development. Percentages of the various morphologies observed from each treatment are shown to the right.


sensitivity period
chlorate treatment
72%
28%
39%
37%
24%
66%
34%
59%
41%
100%
100%

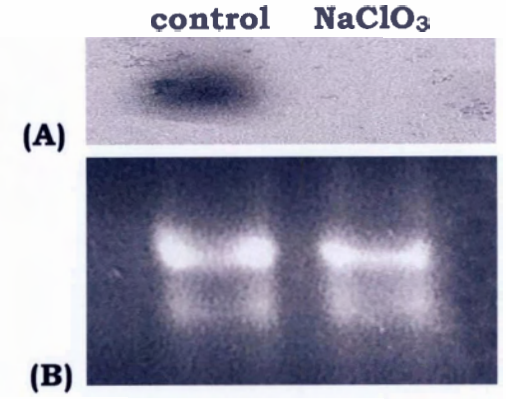
2.3.3 LpS1 expression in 30 mM chlorate-treated embryos

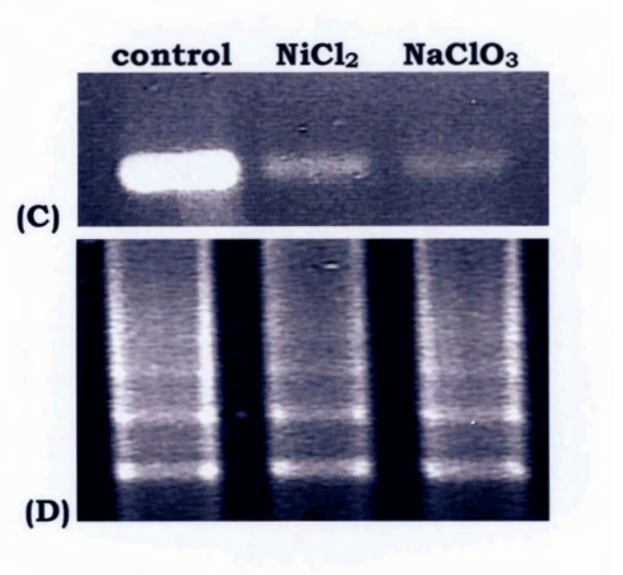
The concentration of 30 mM chlorate was used to treat embryos and examine changes in the expression of *LpS1* as compared to control embryos. Chlorate-treated embryos appeared radialized, and Hardin *et al.* (1992) had previously reported a decrease in *LpS1* expression after treatment of *L. variegatus* embryos with the radializing agent, $NiCl_2$. Northern blotting and RT-PCR detected a decrease in *LpS1* RNA per embryo, implying a reduction in aboral ectoderm, as is illustrated by Fig. 4.

By Northern blots there appeared to be a complete loss of *LpS1* in chlorate-treated embryos, but the more sensitive RT-PCR method detected *LpS1* mRNA in chlorate-treated embryos, although at a much lower level. Using RT-PCR, *LpS1* expression in chlorate-treated embryos was also compared to its expression in nickel-treated embryos, as nickel is known to cause a severe reduction in LpS1 transcripts. Chlorate appeared to reduce *LpS1* expression even more strongly than nickel treatment.

The loading control for the Northern blot was the ethidium bromide stained rRNA that was visualized prior to blotting, while for RT-PCR *polyubiquitin* primers were used to confirm that equivalent amounts of RNA were compared.

Figure 4: Expression of the *LpS1* gene in embryos treated with $NaClO_3$ or $NiCl_2$. (A) Northern blot using an *LpS1* ^{32}P-labeled probe. (B) rRNA visualized with ethidium bromide to serve as a loading control for the Northern blot. (C) RT-PCR using *LpS1* primers. (D) RT-PCR using *polyubiquitin* primers to serve as a control for the *LpS1* RT-PCR. Multiple bands are seen due to the amplification of different numbers of *ubiquitin* repeat units.


control
NaClO3
(A)
(B)


control
NiCl2
NaClO3
(C)
(D)

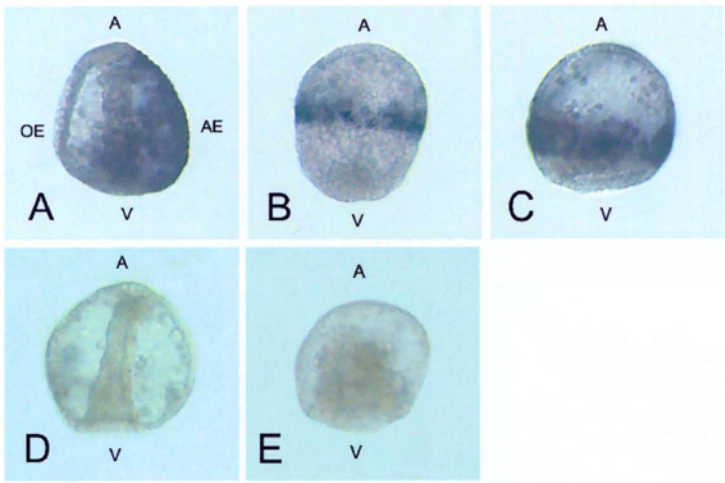
2.3.4 In situ *hybridizations of 30 mM chlorate-treated embryos*

In situ hybridizations using the *LpS1* antisense riboprobe were preformed on 30 mM chlorate-treated embryos and control embryos to determine the localization of *LpS1* expression. Embryos hybridized with sense riboprobe served as controls. Normally, spicules form at the boundary between oral and aboral ectoderm, which is why only two spicules form in the control embryos. Because 30 mM chlorate-treated embryos form a ring of spicules, it was anticipated that aboral ectoderm, which expresses LpS1, would also be found in a ring around these embryos. As was expected, LpS1 expression did encircle the embryos, but its position along the A/V axis varied (see Fig. 5). This indicates that the chlorate-treated embryos are radialized: that is, they lack bilateral symmetry and an O/A axis, and a reduced amount of aboral ectoderm appears to form.

2.3.5 PDGF rescues of 30 mM chlorate-treated embryos

Based on the literature regarding HSPGs and their involvement in signaling, we hypothesized that growth factors might rescue the effects seen from chlorate treatment. Embryos were cultured for at least 96 hours in 30 mM of chlorate and 25 ng of PDGF-BB or TGF-α, or a combination of the two growth factors. Although TGF-α did not have a

Figure 5: *In situ* hybridizations using an *LpS1* probe. (A) Control embryo showing the normal, polarized pattern of *LpS1* expression in aboral ectoderm. (B) and (C) *LpS1* expression patterns in chlorate-treated embryos illustrating how the position of the ring of its expression varies along the A/V axis. (D) Control embryo hybridized with *LpS1* sense riboprobe. (E) Chlorate-treated embryo hybridized with *LpS1* sense riboprobe.


A
OE
AE
V
A
B
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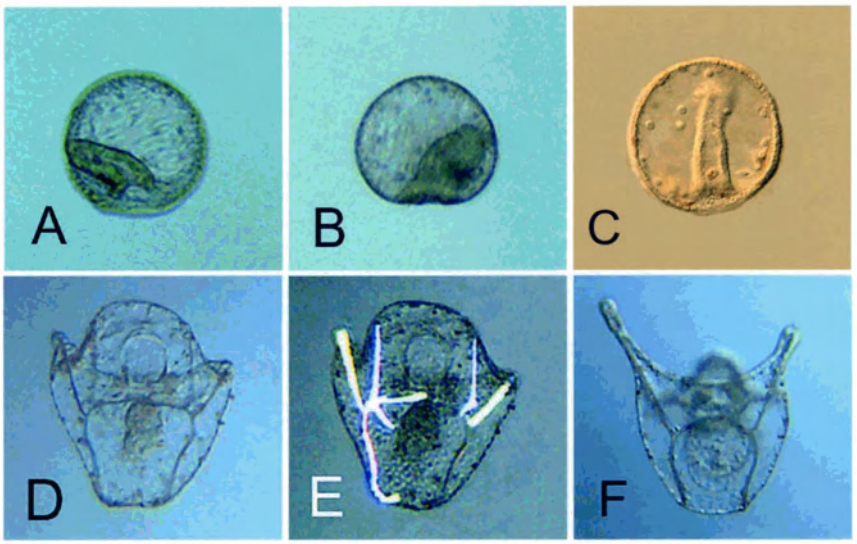
A
C
V
A
D
V
A
E
V

significant effect, PDGF-BB (or PDGF-BB and TGF-α) had an effect on chlorate-treated embryos. In these cultures, gastrulation was partially rescued as was seen by a more elongated gut (see Fig. 6), when compared to embryos treated only with 30 mM chlorate. The gut appeared to attach randomly in these cultures, and apparently did not go on to differentiate. Skeletal and ectodermal patterning did not appear to be rescued, as multiple spicules were still seen.

Because PDGF-BB seemed to have a more dramatic rescuing effect on chlorate-treated embryos than TGF-α, it was used in later experiments. Chlorate and PDGF-BB were added to a culture after fertilization and were removed at the end of the chlorate sensitivity period (late gastrula). When this was done, the morphology of the embryos formed was similar to control embryos. Fig. 6 illustrates that gut differentiation and proper attachment were rescued and bilateral spiculogenesis was rescued as well. Some abnormal branching of the spicules was still visible when larvae were viewed under polarized light, as can be seen in Fig. 6.

Figure 6: Rescues done with growth factors on chlorate-treated embryos. (A) *L. pictus* embryo cultured in PDGF-BB and chlorate, which were added after fertilization and left for at least 96 hours. (B) Embryo cultured in PDGF-BB, TGF-α, and chlorate, which were added after fertilization and left for at least 96 hours. (C) Control late gastrula embryo included to illustrate normal gastrulation. (D) Embryo cultured in PDGF-BB and chlorate, which were added after fertilization but removed at late gastrula stage. (E) Same embryo as in (D), but visualized with polarized light to show spicule morphology. (E) Control pluteus larva.


A
B
C
D
E
F

2.4 Discussion

2.4.1 The effects of chlorate on L. pictus *embryos*

Sea urchin embryos have been used extensively to observe the effects of various drugs on development. With the discovery of chlorate's effectiveness as an in vivo inhibitor of sulfation (Baeuerle and Huttner, 1986; Hortin *et al.*, 1998), researchers began to use this drug to investigate the role of sulfation. In this project, chlorate was used to observe the consequences of putative inhibition of sulfation of HSPGs.

Chlorate appears to disrupt two major processes during *L. pictus* development – gastrulation and patterning of the O/Ab axis, and as a result, spiculogenesis. There are few known treatments that perturb the O/Ab axis in sea urchin embryos; $NiCl_2$ has been shown to do this (Hardin *et al.*, 1992). The treatment of *L. pictus* embryos with 30 mM chlorate produces a morphology which resembles that of *L. variegatus* embryos treated with 0.5 mM $NiCl_2$. The most striking similarity is the excessive number of spicules formed, their radial distribution, and their reduced size. In both cases there are always more than two triradiate spicules present, and these form in a ring inside the blastocoel. As it is clear that the O/Ab axis is disrupted, it is not surprising that *LpS1/LvS1* expression is reduced and radialized. This finding suggests that with

both chlorate and nickel treatment, excess oral ectoderm is formed at the expense of aboral ectoderm.

While some of the similarities between the morphology of chlorate-treated embryos and the morphology of nickel-treated embryos are quite evident, it is unknown whether the underlying biochemical effects of the two treatments are connected, as the underlying action of nickel is unknown. As chlorate has been shown to act as an inhibitor of sulfation, treated embryos might be expected to resemble those cultured in sulfate-free sea water. An obvious similarity is that embryos in sulfate-free sea water do not gastrulate properly, but often undergo a limited form of invagination (reviewed by Wilt, 1987), as is the case in chlorate-treated embryos. However, gastrulation occurs normally in nickel-treated embryos, forming a fully extended archenteron, although targetting of the tip of the stomadeum may be altered (Hardin *et al.*, 1992).

When embryos are cultured in sulfate-free sea water, primary mesenchyme cells ingress, but their capacity to migrate is greatly reduced (Katow and Soursh, 1981). Solursh *et al.* (1986) found a similar effect when embryos were treated with D-β-xyloside, which inhibits the processing of CSPGs: PMC migration was again disrupted. As chlorate is a more potent inhibitor of HS than CS sulfation, it is possible that CSPGs are necessary for PMC migration while HSPGs are involved in the specification of the location to which PMCs should migrate.

As HSPGs are modified through both sulfation and glycosylation, it is interesting to consider the morphology of sea urchin embryos in which glycosylation has been disrupted. Schneider *et al.* (1977) treated *Arbacia punctulata* sea urchin embryos with tunicamycin, an inhibitor of N-linked glycosylation. Tunicamycin has been shown to affect both glycoprotein and proteoglycan synthesis (Yanagishita, 1986). Treatment of *A. punctulata* embryos with tunicamycin early in development (unhatched blastula to early gastrula) seemed to have no detrimental effects on morphology until embryos reached the early gastrula stage. At this point, it became evident that the PMCs in the blastocoel were unable to migrate, and that extension of the archenteron (which had invaginated slightly) was arrested. When embryos were treated after the completion of gastrulation, there was an arrest of spicule formation and arm outgrowth. As with chlorate, disruption of HSPGs by tunicamycin may have a significant inhibitory effect on gastrulation and spiculogenesis in sea urchin development. Although chlorate-treated embryos are capable of forming multiple spicules, they are reduced in size. This is similar to the effects of post-gastrula tunicamycin treatment on spicule formation.

2.4.2 The sensitive period to chlorate

Hardin *et al.* (1992) discovered that *L. variegatus* embryos are sensitive to 0.5 mM nickel treatment from the hatched blastula stage

until early gastrula. While chlorate treatment also appears to exert its earliest effects at hatched blastula, the end of the sensitive period of *L. pictus* to chlorate extends to late gastrula. However, different aspects of development are affected by the presence of chlorate at various stages. As previously mentioned, gastrulation is always inhibited by the presence of chlorate until late gastrula stage. If chlorate is acting to disrupt the sulfation of HSPGs, then this result suggests that HSPGs are crucial for the elongation of the archenteron.

In the case of spiculogenesis, multiple spicules were generally formed if chlorate was present prior to primary cell migration. Therefore, when chlorate is added at the late mesenchyme blastula stage (when primary mesenchyme cells have ingressed and migrated but gastrulation has not yet begun) the majority of treated embryos form only two spicules. HSPGs of the extracellular matrix are probably involved in patterning migrating PMCs, so adding chlorate after PMCs have already migrated to the sites where the first two triradiate spicules form produces a majority of embryos with only two spicules. Transplantation experiments using embryos treated with $NiCl_2$ performed by Armstrong *et al.* (1993) reinforce this idea. They recombined normal or $NiCl_2$-treated PMCs with either normal or $NiCl_2$-treated PMC-less host embryos to establish whether spicule patterning is regulated by the PMCs themselves or the ectoderm of the blastula wall. They observed that it is

the ectoderm that is responsible for specifying the number and arrangement of spicules made by the PMCs. They also determined that spicule size is controlled by the ectoderm, as cultured PMCs produce larger spicules than *in vivo*. Spicule size also responds to experimental perturbation of embryo size.

Interestingly, even when chlorate is present later in development (after primary mesenchyme cells have migrated), spicules still become branched and twisted. This effect is seen up until the late gastrula stage. Ettensohn and McClay (1986) injected primary mesenchyme cells into hosts of the same sea urchin species that were at different stages in development. They were able to demonstrate that the migration of PMCs and the correct deposition of the carbonate skeleton were dependent upon the stage of the host, and that the interactions of PMCs with the blastocoel environment (especially the inner wall of the ectoderm) are important until the late gastrula stage. Thus, chlorate may be affecting the patterning of HSPGs in the ECM later in development such that spicule elongation and patterning is abnormal.

2.4.3 The role of growth factors in development

There are several growth factors that have been shown to be involved in the proper development of sea urchin embryos. Through previous work, TGF-α, EGF, and PDGF-BB have all been implicated in

gastrulation and the formation of spicules (Ramachandran *et al.*, 1993; 1995; 1997; Govindarajan *et al.*, 1995). The addition of anti-human PDGF-BB and TGF-α antibodies to *Lytechinus* embryos inhibits gastrulation, spiculogenesis, and differentiation along the O/Ab axis (Govindarajan *et al.*, 1995), as does the expression of a dominant-negative human PDGF receptor-β lacking the cytoplasmic domain (Ramachandran *et al.*, 1997). The most striking connection was seen between chlorate and PDGF-BB. The morphology of embryos treated with a synthetic peptide representing the HSPG binding sequence on human PDGF-BB (Govindarajan *et al.*, 1995) showed a remarkable similarity to the morphology of embryos treated with chlorate. This peptide presumably competed for binding sites with the endogenous PDGF-like growth factor, thereby inhibiting PDGF signaling. Treatment of *Lytechinus* embryos with this peptide inhibited gastrulation and caused radially arranged multiple spicules to form. The rescue of chlorate-treated embryos by PDGF-BB treatment further illustrates the importance of PDGF signaling, which probably occurs through HSPGs, during sea urchin development.

Katow and Aizu (2002) demonstrated a connection between growth factors and their effects on downstream proteins. They determined that suramin, an inhibitor of growth factor receptors, inhibits the migration of PMCs in the blastocoel and the second phase of gastrulation in *H.*

pulcherrimus sea urchin embryos. Cotreatment with PDGF-AB significantly restored the length of the archenteron in these suramin-treated embryos. These rescued embryos had a remarkable similarity to embryos that were cultured in both chlorate and PDGF-BB: the archenteron was partially extended, but it attached randomly along the blastocoel wall rather than extending straight toward the animal pole. Suramin treatment was shown to decrease the incorporation of BrdU (indicating decreased cell division), but PDGF-AB did not rescue this effect. Thus, the partial restoration of archenteron length was not associated with restoration of cell proliferation, but probably rather had to do with the rearrangement of cells that occurs during convergent extension of the archenteron. It was also suggested that PDGF signaling might have a role in the activation of extacellular signal-regulated kinase 1 (ERK-1), as PDGF-AB treatment brought about a re-activation of the kinase.

It is interesting that adding chlorate and PDGF to embryo cultures produces only a partial rescue, while their removal at the end of the chlorate sensitive period results in a nearly complete rescue. One of the problems with introducing growth factors into the sea water culture medium is that this is not a site-specific process; the exogenous growth factor is presented throughout the embryo which is not representative of the precise binding of growth factors by HSPGs that presumably

normally occurs. It is possible that an overall distribution of a low concentration of PDGF is sufficient to partially rescue gastrulation (archenteron extension), but that a higher concentration of PDGF at certain sites is necessary for full rescue, including O/Ab polarization, to occur. Another possible explanation lies in the role of HSPGs as co-receptors in signaling (reviewed by Perrimon and Bernfield, 2000). For proper signaling to occur, PDGF may require binding to both its appropriate receptor and HSPGs. Once chlorate is removed, HSPGs may be assembled and then locally bind the exogenous PDGF that is present in the blastocoel leading to a more complete rescue.

Ramachandran *et al.* (1995) used antibodies against mammalian receptors for immunostaining to visualize *Lytechinus* PDGF-like receptors. They revealed that these receptors are concentrated on the PMCs, the gut, and most evidently on the SMCs and at the stomadeum (where the mouth forms after fusion with the tip of the gut). The putative localization of PDGF-like receptors on the gut and the stomadeum suggest a possible role of growth factor signaling in locating the correct position for archenteron attachment. If PDGF signaling is, in fact, involved in this process, this might explain why the archenteron attached at random sites on the blastocoel wall when *L. pictus* cultures were treated with chlorate and PDGF for the duration of their development.

Chapter 3

The use of subtractive hybridization and 5'-RACE to characterize a gene that is upregulated in chlorate-treated *L. pictus* embryos

Introduction

Subtractive hybridization is a powerful technique for discovering genes that are differentially expressed in two populations. Originally, Sargent and Dawid (1983) used a primitive version of subtractive hybridization to determine transcripts that are present in a developmental stage or tissue, but are not present or are much less abundant in another stage or tissue in *Xenopus laevis* embryos. The subtractive hybridization techniques currently available are extremely sensitive to even slight changes in gene expression, making it possible to characterize even rare messages.

Although several marker genes have been characterized in *Lytechinus* embryos, in the strictest sense there are presently no RNA markers for oral ectoderm. Because I discovered that chlorate treatment appears to cause a decrease in the expression of *LpS1*, a marker gene for aboral ectoderm (Xiang *et al.*, 1988) and potentially causes the formation of oral ectoderm at the expense of aboral ectoderm, it is possible that

expression of oral ectoderm-specific genes might increase in chlorate-treated embryos. A subtractive hybridization screen might uncover markers of oral ectoderm. Moreover, it might detect genes involved in extension of the archenteron and specification of the O/Ab axis, processes that are poorly understood.

3.2.1 Poly-A$^+$ RNA isolation

Poly-A$^+$ RNA was extracted from *L. pictus* embryos using a QuickPrep Micro mRNA Purification Kit (Amersham, catalogue #27-9255-01) following the manufacturer's instructions. Approximately 500 µl of embryos were homogenized in 400 µl of Extraction Buffer, and then diluted with 800 µl of Elution Buffer. This solution was centrifuged for 1 minute at 13,000 rpm to separate the two phases. The oligo(dT)-cellulose pellet that resulted from centrifuging a tube provided by the manufacturer and removing its supernatant was overlain with the clear supernatant from the homogenate. The tube was inverted to resuspend the cellulose. The mixture was incubated for 5-10 minutes and then centrifuged at 13,000 rpm briefly. At this point, the polyadenylated RNA (mRNA) should be bound to the cellulose, so the supernatant was removed and the pellet was washed three times with 1 ml of High-Salt Buffer and three times with 1 ml of Low-Salt Buffer. The tube was centrifuged for several seconds and the supernatant removed between each washing. Finally, the pellet was resuspended in 300 µl of Low-Salt Buffer and transferred to a Microspin column. The column was briefly centrifuged at 13,000 rpm, and it was placed into a sterile 1.5 ml

microfuge tube. To elute the poly-A^+ RNA, 200 µl of pre-warmed Elution Buffer was added to the resin bed and the column was centrifuged for 5 seconds. In order to increase the yield of mRNA, the elution was repeated. A volume of 16 µl was removed to be used for quantifying the mRNA using a Ultrospec III (Pharmacia) spectrophotometer, and then 10 µl of glycogen, 38.4 µl of potassium acetate and 1 ml of 95% ethanol was added to the tube. This solution was placed at -20°C for 30 minutes to precipitate the mRNA. The poly-A+ RNA was pelleted at 4°C at 14,000 rpm for 5 minutes. The supernatant was removed, and the pellets were allowed to air dry before being redissolved in DEPC-treated water.

3.2.2 Subtractive hybridization

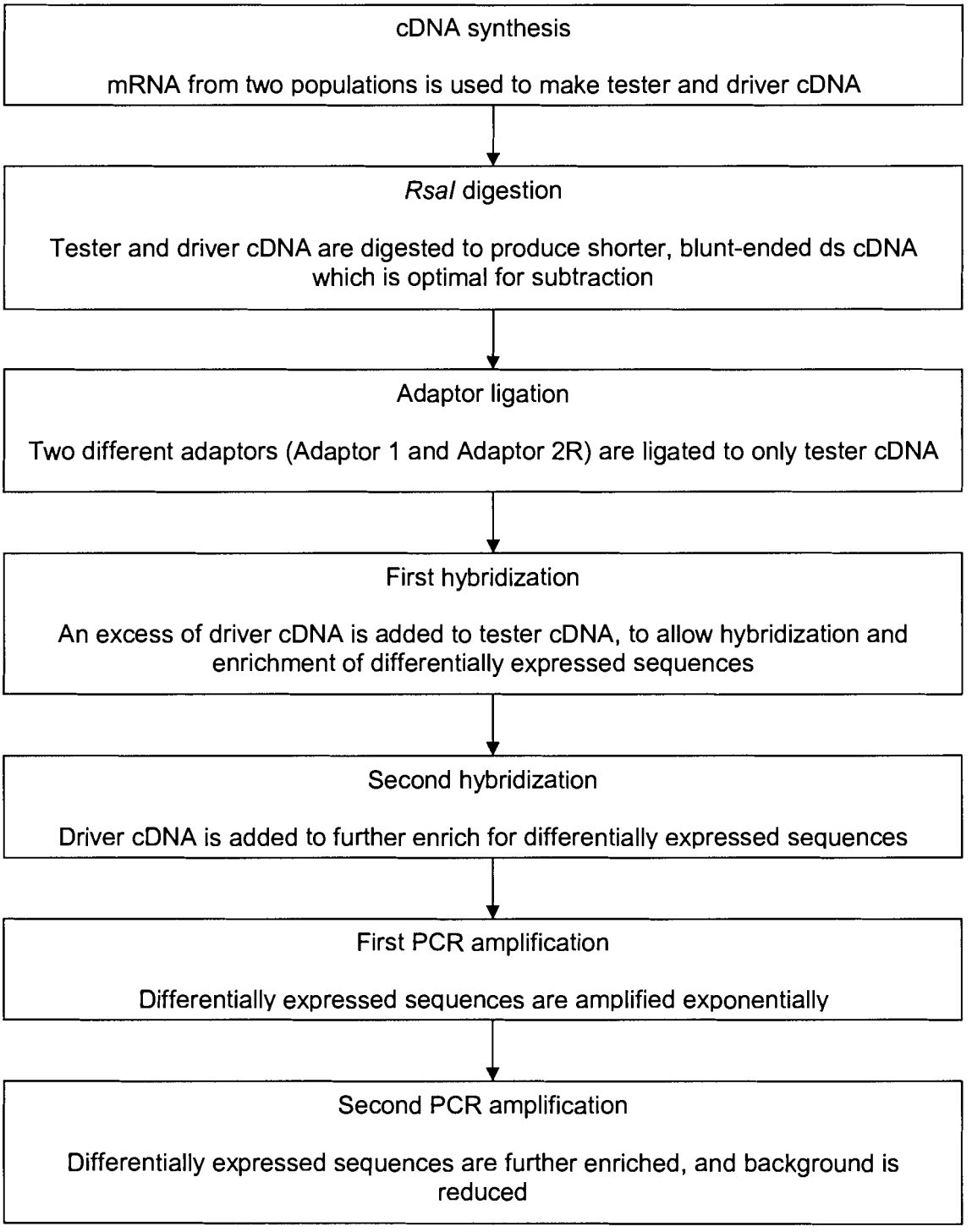
Clontech's PCR-Select cDNA Subtraction Kit (catalogue #K1804-1) was used as recommended by the manufacturer. This protocol allows the comparison of two populations of mRNA and the cloning of portions of genes that are expressed at different levels in the populations. As the protocol is complex, a flowchart is shown to illustrate the steps taken during this procedure. Note that the reference cDNA is referred to as the "driver" cDNA, and the sample with upregulated transcripts is known as the "tester" cDNA.

The significance of the Clontech system is that only target molecules are exponentially amplified. After digestion of both tester and

driver cDNA with the blunt cutter *RsaI*, the tester is subdivided into two populations which are each ligated with a different cDNA adaptor (Adaptor 1 and Adaptor 2R). Two hybridizations are performed following ligation. In the first, an excess of driver is added to each sample tester; the samples are then heat denatured and allowed to anneal. Already, the pool is enriched for differentially expressed sequences as some tester cDNA cannot reanneal with driver cDNA and remains single stranded. In the second hybridization, the two primary hybridization samples are mixed together but are not denatured. At this point, the single stranded tester cDNA from both primary subtractions is able to reassociate, and double stranded hybrids with different ends (due to the two adaptors) are formed. After filling in the ends of the double stranded cDNAs, the differentially expressed tester sequences will be the only ones that contain annealing sites for the two nested primers. cDNAs with the same adaptor on both ends undergo suppression PCR. In this case, during each primer annealing, the formation of a secondary structure resembling a pan handle (due to annealing of the adaptors on the same strand) is kinetically favored to primer annealing.

In this project, cDNA made from chlorate treated embryos was used as both tester and driver cDNA. In this way, it was possible isolate genes that are both upregulated and downregulated in chlorate treated embryos.

Figure 7: Flowchart illustrating the steps taken to carry out a subtractive hybridization.


cDNA synthesis
mRNA from two populations is used to make tester and driver cDNA
RsaI digestion
Tester and driver cDNA are digested to produce shorter, blunt-ended ds cDNA which is optimal for subtraction
Adaptor ligation
Two different adaptors (Adaptor 1 and Adaptor 2R) are ligated to only tester cDNA
First hybridization
An excess of driver cDNA is added to tester cDNA, to allow hybridization and enrichment of differentially expressed sequences
Second hybridization
Driver cDNA is added to further enrich for differentially expressed sequences
First PCR amplification
Differentially expressed sequences are amplified exponentially
Second PCR amplification
Differentially expressed sequences are further enriched, and background is reduced

3.2.3 Preparing cDNA probes

DIG-labeled cDNA probes were prepared through the reverse transcription of cytoplasmic RNA using DIG-labeled dUTP (Boehringer Mannheim). Before beginning the reaction, 1.5 μg of RNA were denatured at 65°C for 15 minutes and placed on ice for 5 minutes. For the reverse transcription, the following were added to a sterile microfuge tube: 3 μl of 10 x first strand buffer, 1.5 μl of RNase inhibitor, 3 μl of 5 mM dNTP mixture (dATP, dCTP, and dGTP only), 6 μl of 0.65 mM dTTP, 6 μl of 0.35 mM DIG-dUTP, 3 μl of oligo-dT, and the heat-denatured RNA. The volume was made up to 28 μl with DEPC water, and then 2 μl of AMV-RT was added for a final volume of 30 μl. The tube was briefly centrifuged, and the reaction was left to proceed for 2 hours at 37°C. After this time, RNase A was added to the tube to degrade the template RNA at 37°C for 15 minutes.

3.2.4 Plasmid DNA blotting

The plasmid samples were first heated to 95°C for 10 minutes and snap-cooled on ice to denature the DNA. Each sample was spotted onto a positively-charged membrane and cross-linked with UV light. The membrane was pre-hybridized at 65°C for 30-60 minutes, and hybridridized using cDNA probes made from control embryos. The procedure followed for detection is the same as for RNA blots. The same membrane was stripped and reprobed using cDNA probes from chlorate treated embryos to enable a comparison of expression levels.

3.2.5 Preparation of polyubiquitin *and* CS 1-1.13 *DIG-labeled riboprobes*

DIG-labeled riboprobes were made using the *ubiquitin* and *pCR1-1.13* plasmids. The ubiquitin plasmid was digested with EcoR1 to linearize the DNA, while either HindIII or BamHI was used with the *pCR1.1-13* plasmid. Both riboprobes were transcribed using T7 RNA polymerase. The transcription was carried out as described in section 2.2.11 of the previous chapter.

3.2.6 DIG Northern blotting

RNA was resolved by electrophoresis as was described in section 2.2.5. The RNA gel was blotted overnight onto a positively charged nylon membrane (Boehringer Mannheim) in 20X SSC (3.0 M NaCl and 0.3 M Sodium citrate, pH 7.0) using the unidirectional capillary method. After it had been transferred, RNA was UV cross-linked to fix it onto the membrane.

The membrane was then pre-hybridized in pre-warmed hybridization buffer [which consists of 50% deionized formamide, 5 x SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (v/v) SDS, 2% blocking reagent (Boehringer Mannheim)], for 30-60 minutes at 55°C. After the pre-hybridization, the membrane was hybridized with denatured DIG-labeled riboprobe at a concentration of 100 ng/ml in pre-warmed

hybridization buffer overnight at 55°C. The next day, the membrane was washed twice with low stringency wash buffer (2 x SSC plus 0.1% SDS) at room temperature for 10 minutes each time, twice with pre-warmed high stringency wash buffer (0.1 x SSC plus 0.1% SDS) at 55°C for 30 minutes each time.

To detect the labeled riboprobe, the membrane was washed briefly in maleic acid buffer [0.1 M maleic acid (Sigma) and 0.15 M NaCl, pH 7.5] plus 0.3% (v/v) Tween-20 (Sigma)] for several minutes, then incubated in 1X blocking solution [1% (w/v) blocking reagent in maleic acid buffer] for 30-60 minutes. After blocking, the membrane was incubated in anti-DIG-alkaline phosphatase conjugate diluted 1:10,000 in blocking solution for 30-60 minutes. To remove unbound antibody, the membrane was washed twice in maleic acid buffer plus 0.3% (v/v) Tween-20 for 30 minutes each time. The membrane was equilibrated in detection buffer (0.1 M Tris-Cl and 0.1 M NaCl, pH 9.5), and it was incubated for 15 minutes in CSPD, a chemiluminescent alkaline substrate (Boehringer Mannheim) diluted 1:100 in detection buffer. After the membrane was removed from the CSPD solution, it was wrapped up in Saran Wrap to keep it from drying out. The luminescent signal was enhanced by incubating the membrane at 37°C for 15 minutes. The membrane was placed onto Hyperfilm ECL (Amersham) for 5 seconds to 30 minutes, and the film was developed.

3.2.7 Removing the riboprobe from Northern blots for rehybridization

To rehybridize a membrane with a different probe, it was necessary to remove the previously used DIG-labeled riboprobe. Therefore, 100 ml of 0.1% SDS was heated until it was almost boiling, and was poured onto the membrane. The membrane was incubated in this solution for 10 minutes on a rocker without further heating. After the membrane had been stripped, it was washed for 5 minutes in washing buffer at room temperature. At this point, the membrane was ready to be prehybridized for the next hybridization.

3.2.8 Depurination and denaturation of DNA gel before blotting

DNA was resolved by electrophoresis as was described in section 2.2.8. Before blotting, DNA was depurinated and denatured to make transfer and hybridization easier. Depurination is achieved with a controlled acid treatment, and is optional for DNA fragments smaller than 10 kb in size. I chose to use this treatment to increase transfer efficiency.

The gel was submerged in 250 mM HCl with shaking for 10 minutes, and was rinsed with distilled water. To denature the DNA, the gel was submerged in denaturation solution (0.5 N NaOH and 1.5 M NaCl) twice, for 15 minutes each time. The gel was again rinsed with water before its pH was neutralized by being submerged in neutralization

buffer (0.5 M Tris-HCl, pH 7.5 and 3 M NaCl) twice, for 15 minutes each time.

DNA was blotted in the same was as RNA, and DNA blots using DIG-labeled probe were treated in the same way as RNA blots using DIG-labeled probe (see section 3.2.6) except that any water used with DNA was not DEPC-treated.

3.2.9 5'-RACE

Clontech's SMART RACE cDNA Amplification Kit (catalogue #K1811-1) was used as recommended by the manufacturer, with some deviations. Briefly, 1 μg of poly A^+ RNA prepared from *L. pictus* at pluteus larva stage was combined with 1 μl of 10 μM 5'-cDNA Synthesis Primer, 1 μl of 10 μM SMART II oligo (5'-AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG-3'), and sterile water to bring the volume up to 5 μl. The reaction mixture was heated to 70°C and cooled on ice. The first strand synthesis was carried out by adding 2 μl of 5 x first strand buffer, 1 μl of 20 mM DTT, 1 μl of 10 mM dNTP mix, and 1 μl of Superscript II MMLV reverse transcriptase (Invitrogen, catalogue #18064-022) to the reaction mixture and incubating the tube for 1.5 hours at 42°C in an air incubator to reduce evaporation. The Superscript II MMLV-RT possesses a terminal transferase activity that adds 3-5 dC residues to the 3' end of the first strand cDNA. This allows the 3' dGs of the SMART II oligo to anneal at the end of the template mRNA and provide template for further extension. As a positive control for the cDNA synthesis, the kit contained control human placental total RNA to be used to make RACE-Ready cDNA. After the first strand synthesis, the first strand reaction product

was diluted with Tricine-EDTA buffer (10 mM Tricine-KOH, pH 8.5 and 1 mM EDTA) to 250 µl and heated to 72°C for 7 minutes to destroy MMLV-RT activity.

5' RACE was carried out using several gene specific primers (GSP) to extend the 5' end of the cloned cDNA. The various GSPs also served as nested primers as the RACE PCRs appeared to generate clearer bands when a secondary PCR was done. For the primary PCR, a master mix was made containing 17.25 µl PCR-grade water, 2.5 µl 10 x Advantage 2 PCR Buffer, 0.5 µl of 10 mM dNTP mix, and 0.5 µl 50 x Advantage 2 Polymerase Mix per PCR being done. The 5' RACE sample comprised of 20.75 µl of this master mix combined with 1.25 µl of 5'-RACE-Ready cDNA, 2.5 µl of 10 x universal primer mix (UPM), and 0.5 µl of GSP. A positive control was done on cDNA made from the control human plactental RNA using a 10 µM 5'-RACE TFR Primer. The negative controls used only the GSP or only the UPM.

After the primary PCR, the product was purified using the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. During the purification, DNA was eluted using 50 µl of water which was diluted into 200 µl of Tricine-EDTA buffer. 2.5 µl of diluted primary PCR product was used for a nested PCR using 1 µl of the nested universal primer (NUP) and a nested GSP.

The PCR reactions were carried out following a hotstart PCR protocol consisting of 30 cycles of 5 seconds at 94°C for the denaturation, 10 seconds at 66°C for the annealing, and 3 minutes at 70°C for the elongation. All PCR reactions were done using a Perkin-Elmer GeneAmp Systems 2400 thermal cycler. The various GSPs used

for the 5'-RACE reactions were designed using Oligo 4.1 and were purchased from Sigma Genosys. They are shown below:

GSP 1 5'-CCT ACM CCA CTC TTG CAA ATT CAT TC-3' (M = A or C)

GSP 2 5'-CTG GCA GTA AAA CCT ATC GCT ATG T-3'

GSP 3 5'-TAA AGA ATG CTG TTG ACC TAC CA-3'

GSP 4 5'-CCT TTA CCA GCT TCT TCG TTC GC-3'

3.2.10 Cloning of subtractive hybridization and 5'-RACE products

The various products from the subtractive hybridization and 5'-RACE reactions were cloned into the pCR 2.1 vector using the Original TA Cloning Kit (Invitrogen, catalogue #45-0046) according to the manufacturer's directions. The TA cloning system takes advantage of the activity of Taq polymerase that adds a single dA to the 3' ends of PCR products; therefore the vector contains 3'-overhangs of dT residues.

PCR products were first ligated into the pCR 2.1 vector using components from the kit. The reaction mixture contained 1 μl of 10 X Ligation Buffer, 2 μl of pCR vector (25 ng/μl), 1 μl of fresh PCR product (approximately 10 ng), 5 μl of sterile water, and 1 μl of T4 DNA Ligase. The reaction was incubated at 14°C overnight.

The next day, a vial of One Shot cells was thawed on ice, and 4 μl of the ligation mixture was pipetted into the vial and stirred gently. The vial was incubated on ice for 30 minutes and then heat shocked for 30 seconds in a 42°C water bath. 250 μl of SOC medium was added to the vial before putting it into a 37°C shaking incubator at 225 rpm for exactly 1 hour. The vial was placed on ice to discourage more bacterial growth. TB plates containing 100 μg/ml ampicillin were prepared by

spreading 40 µl of 40 mg/ml X-Gal and 40 µl of 100 mM IPTG onto their surfaces. Varying volumes of cells (generally 50 µl-100 µl) were then plated onto them. Plates were incubated at 37°C for at least 18 hours; they were then incubated at 4°C to aid with blue colour development in negative colonies. Plasmid DNA extractions were performed, and the DNA was digested with EcoRI to verify that an insert of the expected size was present. All sequencing was done at the University of Calgary.

3.3 Results

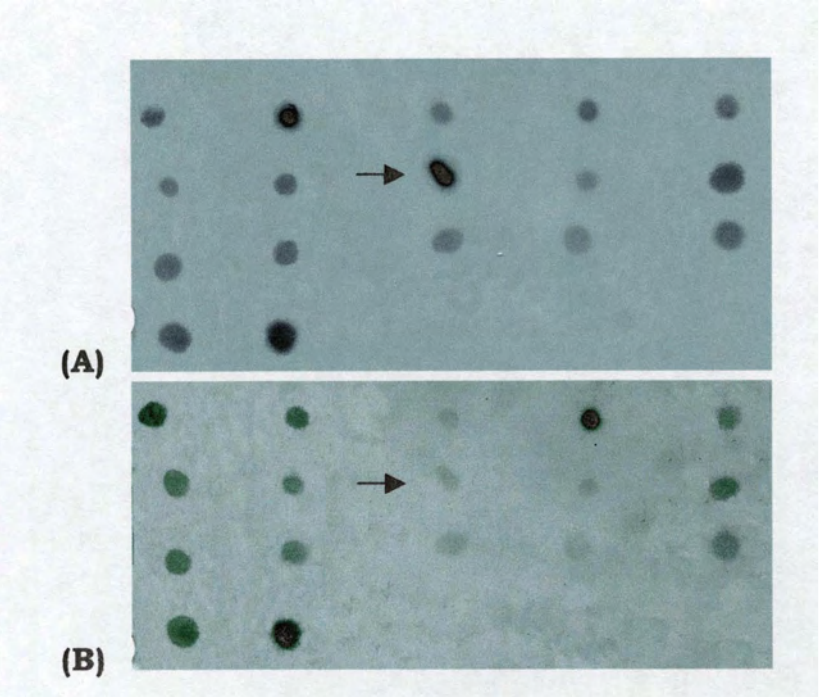
3.3.1 Subtractive hybridizations of control and chlorate-treated embryo cDNA

Subtractive hybridizations were performed using cDNA made from control and chlorate-treated *L. pictus* embryos to determine whether any other genes besides *LpS1* experience a change in expression. Both forward and reverse subtractive hybridizations were done such that decreases and increases in gene expression of chlorate-treated embryos would be detected.

Once the cDNAs were subtracted, the products were amplified by PCR. These subtraction products were then cloned, and approximately 20 colonies were chosen at random from both the forward and reverse reaction for dot blotting using cDNA probes made from chlorate-treated and control embryos.

Fig. 8 shows the results of the dot blots done to compare the various expression levels. In the dot blots for the forward subtraction (not shown), which represents genes that are potentially downregulated in chlorate-treated embryos, there were no substantial differences in gene expression observed. However, in the dot blots for the reverse subtraction, which represents genes that are upregulated in chlorate-treated embryos, there were two cDNAs that had noticeable differences in expression. I chose to focus on the cDNA with the largest apparent

Figure 8: Dot blot analysis of the reverse subtracted cDNA library. The blot was spotted with plasmids from randomly chosen colonies containing inserts from the reverse subtraction. (A) Dot blot hybridized with cDNA probes prepared from RNA of chlorate-treated embryos. (B) The same dot blot hybridized with cDNA probes prepared from RNA of control embryos.


(A)
(B)

increase in expression, which is denoted by an arrow. From this point on, this cDNA will be referred to as *pCR1-1.13*. Most cDNAs in the subtracted library have not been characterized.

3.3.2 Verification of subtractive hybridization

As a test of the effectiveness of the subtractive hybridization, the pool of amplified cDNA products from the forward and reverse subtractions were run on a gel, blotted and probed with *LpS1* riboprobe. *LpS1* riboprobe was used because its expression was known to decrease as a result of chlorate treatment. It was predicated that the *LpS1* probe would recognize a band in the forward subtraction pool of approximately 1 kb in size due to the RsaI digestion used in the subtractive hybridization protocol. Fig. 9 confirms that the subtractive hybridization effectively detected the downregulation of *LpS1*.

3.3.3 Northern blot with CS 1-1.13 *riboprobe*

To verify the difference in gene expression of *pCR1-1.13* seen in the cDNA dot blots, a Northern blot hybridization was performed using mRNA from control and chlorate-treated embryos and *pCR1-1.13* riboprobe. The blot was stripped and reprobed with *polyubiquitin* riboprobe, which served as a loading control. Again, a significant upregulation of *pCR1-1.13* was evident in chlorate-treated embryos as compared to control embryos (see Fig. 10).

Figure 9: Southern blot using pooled forward and reverse subtracted cDNA digested with RsaI, and hybridized with an *LpS1* riboprobe.

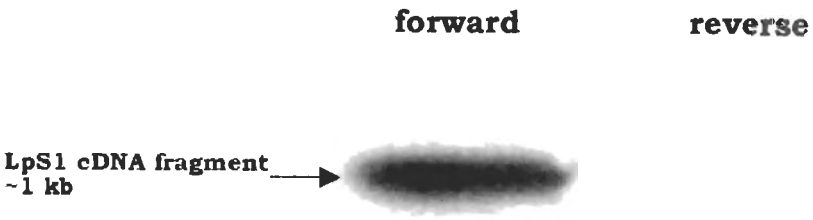
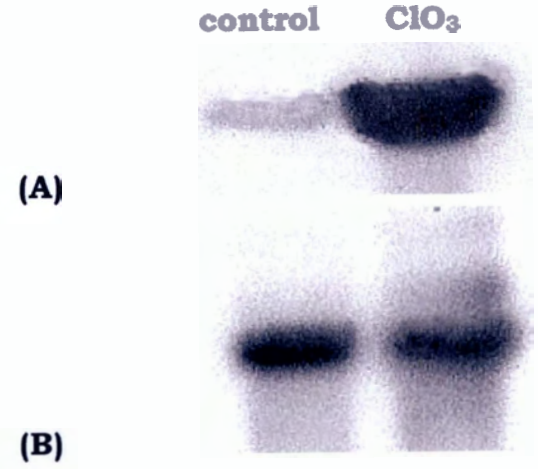

forward
reverse
LpS1 cDNA fragment
~1 kb

Figure 10: Expression of *CS 1-1.13* in chlorate-treated embryos. (A) Northern blot hybridized with *CS 1-1.13* riboprobe. (B) Northern blot hybridized with *polyubiquitin*, used as a loading control.


control
ClO3
(A)
(B)

3.3.4 Sequence obtained from 5'-RACE

Approximately 1.4 kb of pCR1-1.13 sequence was obtained by using 5'-RACE, as is shown in Fig. 11. Because the cloned sequences all overlapped somewhat, this 1.4 kb sequence was verified several times. The original clone from the subtractive hybridization contained a poly-A^+ tail, indicating that this clone contained the 3' end of the mRNA. There is no long open reading frame (ORF); thus, the sequence obtained so far appears to be part of the 3' untranslated region (UTR) of the mRNA.

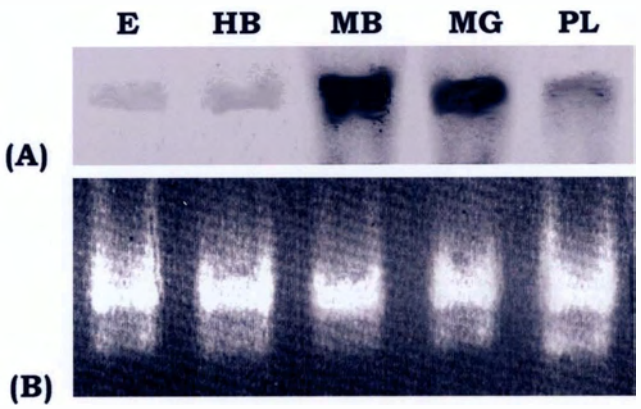
3.3.5 Northern blot of various developmental stages using CS 1-1.13 riboprobe

In order to see the temporal expression levels of *CS 1-1.13* during various stages of development, an ontongeny Northern blot was done, with rRNA being used as a loading control (see Fig. 12). It appears that *CS 1-1.13* is expressed throughout embryonic development, reaching a peak in expression at the mesenchyme blastula stage, and declining during the later stages of development.

Figure 11: The 1.4 kb sequence of *CS 1-1.13* compiled from fragments that were obtained through 5'-RACE, with highlighted GSPs. The sequence reads 5' to 3' starting in the top left corner.


5′-ATCCAACAAAAAGGGGAATGTCGAAGGTATTTTTTTCTTCAGTGCTTAGGTCTCAGGGTGTACTAATTGAGGAAAAGTTAT
CCTCTTTTAATAGTTGTTCATATTATGTTTTTAATCCGTAATGATAAAAAGATACACTTCATACGGTTTCTTTTTATCTTTCAA
GTTTTGAATCATCTAATGAAAAGAAAATTATAATGGGATCTTGTTGTTGGGTTACAAACAAAATGCGAACGAAGAAGCTGGTAA
AGGATTATTTAAGTTGAATGGTATATTTTGTACAGAAATTTTGCACTTTTATGATGTATCTTTGCACAACGTTTTAGCCTTTTG
ATTTTTTTTTTCTTGATTGTACTTTAGTTTTATTTTTACCTTGAAAAGAGAACATTGTGTACTTAAGACTGAATTAAATCTTCC
ATGTAATCTTCATTTTGAAGAACACTGAATTGGATTATACATGTAGCTCCAATCACCCGGTAGGTCAACAGCATTCTTTCATTA
ATTCATTCCACACTTCATATGTTTATTCTCCAGTAAGAATACATTTGACATGATACATTGTAGATTCTTTTATATGAATTAGAA
AATCGCTGTTTTCATTCAGAATATCGAAATCAAAATAGGAAATAAAGCAAGATCTTTCCCAAAATAATCTATGATACCGTATAA
GATATTGAAGCCTGTTTTCATCCGGAACTCTTTCCTTTCTTCACTTTATTCTCTTAATTATTACTATGAAATGTGCCTCCATCA
AAAACAAAACACCAACAAGAGAAATGTGTATATGTATAAATGAAGTATGATAGATGAAATGGCTGTAGACTTCGTCTAGTTATA
TTTGTATTAGTGAACTCGGTTGTTTGATGTTTCCTCTAATAGTTTCAGACCTCCCTGTGATCAATCATGTATCGATTGCATACC
AGTATGTTGGATGTTTTTATAAGAAAAAATATCAAGATTATTATCAAGCATGAAATATGGAGTTGTACTTATTAATGGCTACTG
TGTGTTGATGATAAACACATGGCACTAAATGTAAGTGTTAACAGTAAGTTATTGCACTCTCATTTTTATACAACATAGCGATAG
GTTTTACTGCCAGTGAAGAAATTTAATTTGTTAGCTTGATTGTGTGGATGAACTTATTTTTATGGGATGAAATGAATTTGCAAG
AGTGGTGTAGGTATTTGTTTATTTTGTCTGGAAAAATAAACCCAGATATACTGAAAAGAATGGCAAAATTGCATCTTGACATTT
GATCAACCCAAAAGTTTACAGTATTTTTATTGTTTGATGGATTTAAGTAGATAAAAGATATATAAAAGCTTTTTGTTTCACGAT
ACTACATGGATACTAAAAAAAAAAAAAAAAAAAAAAAAAA-3′
Gene Specific Primer 1
Gene Specific Primer 2
Gene Specific Primer 3
Gene Specific Primer 4

Figure 12: Expression of *CS 1-1.13* at various developmental stages. (A) Northern blot showing *CS 1-1.13* expression. E : egg, HB : hatched blastula, MB : mesenchyme blastula, MG : mid gastrula, PL: pluteus larva. (B) Ethidium bromide stained rRNA, used as a loading control.


E
HB
MB
MG
PL
(A)
(B)

3.4 Discussion

3.4.1 Subtractive hybridization using control and chlorate-treated cDNA

The forward and reverse subtractive hybridizations gave some significant results. The 1 kb *LpS1* cDNA fragment that would be recognized by the 0.44 kb sequence used to make *LpS1* riboprobe was shown to be present in the forward subtraction pool, but absent in the reverse subtraction pool, which was a confirmation of the validity of the procedure. The forward subtraction clones that were chosen did not show any significant differences in gene expression when they were probed with control and chlorate-treated cDNA probes; however, there were two cloned plamids in the reverse subtraction that illustrated an obvious upregulation of gene expression when they were hybridized with chlorate-treated cDNA probes as compared to control cDNA probes.

I chose to focus my work on the plasmid that demonstrated the largest difference in expression, which was named *CS 1-1.13*. The clone obtained from the subtractive hybridization contained the poly-A^+ tail and no open reading frame, suggesting that this was the 3'-UTR of the mRNA.

3.4.2 5'-RACE sequence

With 5'-RACE, it was possible to obtain a total of approximately 1.4 kb of sequence. This sequence was used in BLAST searches on both the NCBI database and the sea urchin genome database (*S. purpuratus*), but

no significant matches were found. It appears that the sequence I have gathered up until this point does not contain an open reading frame (ORF). This makes it extremely difficult to compare the *CS 1-1.13* sequence with anything in the databases as UTRs vary significantly between species.

This project will be continued in order to acquire sequence from the ORF that can be used to determine which gene is upregulated as a result of chlorate treatment.

3.4.3 The temporal expression of CS 1-1.13

The expression level of *CS 1-1.13* appears to change during various stages of *L. pictus* embryo development, making the gene potentially interesting for studying development. It seems that the *CS 1-1.13* mRNA is present at all stages, although its levels are extremely low in eggs and early embryos. Expression appears to peak at the mesenchyme blastula stage and then decline until the pluteus stage. It is interesting that the period of peak expression corresponds to the period of sensitivity of the embryos to chlorate. The gene may have a role in oral ectoderm specification or differentiation, or gastrulation.

Chapter 4
Conclusions and Future Work

4.1 Conclusions

Through this project I set out to address the following questions:

1) If chlorate has an effect on *L. pictus* embryos, what is this effect with regard to morphology?
2) When is the sensitive period of *L. pictus* embryos to chlorate?
3) Can the effects of chlorate on *L. pictus* embryos be rescued by treatment with HS or growth factors?
4) Are there any genes that experience either a downregulation or upregulation due to chlorate treatment?

Using the results that have been presented in this thesis, several conclusions can be drawn.

Chlorate has several clear effects on the development of *L. pictus* embryos, and the severity of these effects increase with higher concentrations of chlorate. At 30 mM chlorate, embryos are unable to complete gastrulation and normal O/Ab axis patterning is disrupted, resulting in multiple radially distributed spicules.

L. pictus embryos appear to be sensitive to chlorate for an extensive period of time. Embryos first become sensitive at the hatched blastula stage, which was confirmed by treating embryos that had had their fertilization envelopes removed. They remain sensitive until the late gastrula stage, after which the addition of chlorate has no effect on development. The extent to which gastrulation and spiculogenesis are affected depends on when chlorate is present during development.

It was possible to rescue the effects of chlorate treatment by also adding PDGF-BB to embryo cultures. The presence of chlorate and PDGF throughout development produced only a partial rescue, as the archenteron was more elongated in this case. However, the removal of chlorate and PDGF-BB at the end of the chlorate sensitive period brought about a nearly full rescue. Addition of HS to sea waterdid not seem to rescue the effects of chlorate, although it is unclear whether this was due to the inability of HS to penetrate into the blastocoel or the inability of HS to actually rescue chlorate-treated embryos.

Through the use of subtractive hybridization, gene expression variations in chlorate-treated and control embryos were studied, and a gene whose expression is upregulated in chlorate-treated embryos was discovered. Although it is unknown what protein encoded by this particular gene and how this protein is involved in development, there

appears to be a peak in expression of *CS 1-1.13* during the mesenchyme blastula stage.

4.2 Future Work

There are several further investigations that can be carried out to further our understanding of the effects of chlorate.

Although we assume that the effects of chlorate are seen due to an inhibition of HSPG sulfation, this has not been verified. It is probable that chlorate is acting in this manner based on previous research reviewed in Chapter 1. This could be verified by doing experiments using ^{35}S to label HSPGs in control and chlorate-treated embryos. Similarly, it would be beneficial to know whether HS has the capacity to rescue the effects of chlorate. Although HS added to chlorate-treated sea urchin embryo cultures did not provide a rescue, it is possible that this is due to the inability of HS to cross the blastocoel wall. Thus, injection of HS into the blastocoel might bring about a rescue.

It is quite interesting that chlorate-treated embryos form multiple spicules, and that embryos that are exposed to chlorate after PMC migration form branched and twisted spicules. There appears to be some patterning signal present on the blastocoel wall, and HSPGs may have a role in providing this signal. It would be beneficial to be able to visualize the pattern of HSPGs binding PDGF, perhaps by immunostaining for PDGF or core proteins in the blastocoel. This may provide some insight into how proper growth factor binding to HSPGs mediates normal development.

With respect to the O/Ab axis, due to the expression pattern of *LpS1* and spicule formation in chlorate-treated embryos, it is clear that the normal patterning of the O/Ab axis is perturbed. However, it would be useful to verify that *LpC2 actin*, another aboral ectoderm specific gene, is also affected in a similar manner by chlorate treatment.

The DNA sequence of the cDNA or gene corresponding to *CS 1-1.13*, is not finished. It would be interesting to determine the identity of this gene, and how it is involved in normal development. This could be accomplished through a continuation of the 5'-RACE that was originally carried out to determine its sequence or through hybridization screening of a cDNA library for full length inserts. Once *CS 1-1.13* is characterized, the potential for further experiments would increase.

Presumably, several genes that experience a difference in expression due to chlorate treatment were discovered through subtractive hybridization. While I chose to focus on a gene that experienced a large upregulation in chlorate-treated embryos, there is at least one other gene that is certainly upregulated and several others that appear to be upregulated. In addition, there are probably genes that become downregulated with chlorate treatment, as is the case with *LpS1*, although they were not among the few subtracted cDNA clones randomly chosen for characterization. A hybridization screen using a cDNA library for *L. pictus* would be a high-throughput method for identifying other genes that are up- or down-regulated in response to chlorate treatment. Such genes have potentially interesting roles in axis specification, ectoderm differentiation, gastrulation, and ECM function.

Joseph Yost (University of Utah) has offered our lab polyclonal antibodies against the HSPG syndecan-2 core protein and two of its

phosphorylated forms. His lab has found a connection between left-right patterning in *Xenopus* embryos and the phosphorylation state of syndecan-2 (Kramer *et al.*, 2002). Although chlorate has only been shown to inhibit sulfation of HSPGs (and not phosphorylation), it would be interesting to immunostain normal and chlorate-treated embryos to assess the distribution of syndecan-2 and its state of phosphorylation. The putative inhibition of sulfation that may be occurring in *L. pictus* embryos due to the addition of chlorate may also affect localization and modification of HSPGs. It will be interesting to know the distribution of other HSPG and CSPG core proteins in urchin embryos. Creation of appropriate reagents will be facilitated by the completion of the sea urchin genome project.

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