The gene encoding the sea urchin complement protein, SpC3, is expressed in embryos and can be upregulated by bacteria

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Abstract

Sea urchins have an innate immune response that functions in the absence of adaptive capabilities. It is mediated, in part, by components of the complement system, an important subsystem of the innate response in mammals. A homologue of complement C3, SpC3, has been identified in adult *Strongylocentrotus purpuratus* and is expressed in coelomocytes. In this study, transcript levels from the gene, *Sp064*, which encodes SpC3, were examined in developing embryos and found to be present in unfertilized eggs and throughout embryogenesis with a peak in transcript levels just prior to and during gastrulation. In addition, continuous exposure of embryos, beginning with the hatched blastula stage, to heat killed *Vibrio diazatrophicus*, a marine pathogen of sea urchins, significantly increased *Sp064* message content in plutei compared to unexposed controls. These results suggest that sea urchin embryos may use a complement-based immune system for defense against pathogens in their aquatic environment.
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1. Introduction

The innate immune response of higher vertebrates consists of many well developed defense systems, and among these one of the best characterized effector systems is complement. The complement system, composed of thioester proteins, enzymes, cofactors, receptors and regulatory proteins, acts in concert through a set of enzyme cascades to generate a local inflammatory response, opsonize foreign particles to enhance phagocytosis, and initiate the membrane attack complex which lyses microbial pathogens (reviewed in Refs. [1–3]). Each of the three activation pathways, the classical, alternative and lectin pathways produce a C3-convertase enzyme which cleaves additional C3, producing the complement fragment C3b that is deposited on the surface of foreign cells, targeting them for removal by phagocytes [2,4–6]. The C3-convertases are readily

modified to become C-5 convertases that activate the terminal pathway [2].

Although the deuterostome invertebrates and the cyclostome fish do not have demonstrable adaptive immunity, these animals posses simpler complement systems in the form of the alternative pathway that has been identified in sea urchins [7–9], tunicates [10], cephalochordates [11], and agnathans [12–16], and the lectin pathway that has been identified in tunicates [17,18]. Homologues of C3 have not been identified in the *C. elegans* genome [19], however, thioester containing proteins (TEPs) have been characterized in *Drosophila* [20] and mosquito [21]. Although the mechanism of forming covalent bonds with foreign molecules through the thioester site in complement proteins, alpha 2 macroglobulin, or TEPs, is found throughout the entire animal kingdom [2,22–24], a complement cascade with an amplification feedback loop may be limited to the deuterostome lineage of animals.

The sea urchin immune response is mediated by the phagocyte class of coelomocytes [25–28]. Investigations of the molecular immune response of *Strongylocentrotus purpuratus* showed that coelomocytes express transcripts of the gene encoding SpC3 (*Sp064*) [7] and that two subfractions of the phagocyte fraction of these cells contain the SpC3 protein [26]. A significant amount of SpC3 within phagocytes was found to be in a pre-processed form [29] and the protein appeared to be present within vesicles, which is typical of secreted proteins [26]. The amount of SpC3 protein in the coelomic fluid is quite variable, can be induced to increase in concentration as quickly as 15 min after LPS challenge [29], and has a functional thioester site that is typical of the vertebrate complement proteins C3 and C4 [30–32].

While the defence capabilities of the adult sea urchin have received some attention, very little is known about the defense system of sea urchin embryos. In a landmark experiment by Metchnikoff [33] that has been viewed as establishing the field of invertebrate immunology, he provided the first evidence of a cell-based immune system that was also the first evidence of an embryonic immune system in echinoderms. Metchnikoff inserted a rose prickle into the larva of a sea star and the next day found phagocytic cells in the blastocoel attempting to engulf the foreign object. In a modern replication of

this experiment, Silva [34] showed that when yeast cells were microinjected into the blastocoel of the embryonic sea urchin, *Lytechinus variegatus*, mesenchyme cells in the gastrula phagocytosed the yeast cells. Mesenchyme cells share amoeboid, phagocytic and chemotactic properties with phagocytic coelomocytes of the adult animal [35]. Amoeboid activity has also been inferred from increases in profilin transcripts in these cell types under circumstances when cytoskeletal modulation is required, including ingression of mesenchyme cells prior to and during gastrulation [36] and coelomocyte activation by immune challenge or injury [37,38]. Currently, little is known about the defense systems in the embryo and larval stages of sea urchins and other echinoderms. Therefore, to learn more about the immune capabilities of the purple sea urchin during embryonic development, the timing of *Sp064* expression was investigated under normal culture conditions and under conditions in which a heat killed marine pathogen of sea urchins, *Vibrio diazatrophicus*, was added to the culture. Unchallenged cultures showed that transcripts from *Sp064* were present in the uncleaved egg and persisted throughout embryogenesis to the pluteus stage with peak levels at mesenchyme blastula and gastrula stages. Immune challenge resulted in increased expression of *Sp064* in the pluteus after continuous exposure to bacteria starting at the hatched blastula stage. These results suggest that sea urchin embryos have a defence system that appears to respond to immune challenge with increased levels of *Sp064* transcripts.

2. Materials and methods

2.1. Embryo culture

Adult sea urchins were purchased from Marinus (Long Beach, CA) or The Southern California Sea Urchin Company (Corona del Mar, CA) and maintained at 14 °C in a 60-gallon Instant Ocean aquarium (Aquarium Systems, Eastlake, OH). Animals were artificially spawned by injection of 0.53 M KCl into the perivisceral coelom. Eggs from an individual female were collected in Herbst [39] artificial seawater as modified by Motomura [40] (HMSW: 450 mM NaCl, 48.3 mM $MgSO_4 \cdot 7H_20$, 9.4 mM KCl,

10.2 mM CaCl$_2$·2H$_2$0, 5.4 mM NaHCO$_3$, pH 8.1), washed once in HMSW by settling and fertilized with a dilute sperm suspension from a single male. Batches of fertilized eggs were discarded if less than 98% showed raised fertilization membranes. Egg counts were performed on eggs fixed in 10% formalin in HMSW using a Leitz Diavert inverted microscope (Bunton Instr., Rockville, MD).

Zygotes were suspended at 1000 zygotes/ml in each of 3 one gallon jars and cultured in a Freas 815 low temperature incubator (Precision Scientific Co., Baltimore, MD) at 14 °C with continuous agitation by a Teflon paddle rotated by an electric motor at 30 rpm. Embryos were cultured to the stages given in Table 1 and were examined and photographed using a Leitz Diavert inverted microscope equipped with a Weiss Polaroid camera (Bunton Instr.) containing Type 665 P/N film (Eastman Kodak Co., Rochester, NY). Approximately 7.3×10^5 eggs or embryos were collected in 20 ml by settling at 2 °C and then pelleted from 1 ml aliquots by centrifugation (Eppendorf Centrifuge 5810R, Westbury, NY) at 1500 rpm for 3 min at 4 °C and stored at −70 °C prior to RNA isolation (see below).

Table 1
Developmental stages of *S. purpuratus* embryos at 14 °C

Developmental stage	Abbreviation	Hours post-fertilization
Unfertilized egg	unf	–
Fertilized egg	fert	0
Four cell	4 cell	3.75
16 cell	16 cell	6.00
Morula	morula	8.75
Mid blastula	mid blas	13.75
Late blastula	late blas	16.25
Hatched mesenchyme blastula	hmb	21.75
Hatched mesenchyme blastula with thickened vegetal plate	hmb/vp	25.25
Early gastrula	early gast	31.50
Mid gastrula	mid gast	33.50
Late gastrula	late gast	36.00
Early prism	early prism	43.00
Mid prism	mid prism	51.00
Late prism	late prism	56.00
Early pluteus	early plut	65.75
Pluteus	plut	72.75

Eggs were fertilized as described above and suspended in HMSW to 230 zygotes/ml and cultured in 2 one-gallon jars at 14 °C with constant stirring until exposure to immune challenge from a heat-killed marine bacterium, *V. diazotrophicus* (#33466 American Type Culture Collection, Manassas, VA). Bacteria were grown in marine broth 2216 (Difco, Detroit, MI) augmented with 3 g/l yeast extract (Difco) and 5 g/l proteose peptone (Difco) at 25 °C with shaking. After 24 h, bacteria were pelleted, resuspended in HMSW and killed by incubation at 60 °C for 30 min. Aliquots of embryo culture (400 ml) in 800 ml beakers were treated by adding 8.7×10^5 *Vibrio* per ml of culture beginning at hatching or early gastrula and then collected at late gastrula, pluteus and feeding pluteus stages. The concentrations of heat killed bacteria used in these studies were estimated from investigations of LPS challenges in vivo in adult sea urchins as assayed by coelomocyte responses [29, 38,41]. With the culture conditions employed in these studies, synchronous development of embryos within and among beakers was observed. Control embryo cultures received HMSW without bacteria.

2.2. RNA isolation and cDNA synthesis

Total RNA from embryos was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, embryos were lysed in guanidine isothiocyanate buffer, the lysate was bound to a silica-gel-based column, washed, and the RNA was eluted in RNase-free water containing 1.33 units (U) of RNasin RNase Inhibitor (Promega, Madison, WI) per μl of eluate. First strand cDNA synthesis reactions were performed with 1–3 μg total RNA, 300 ng random hexamer primers, 1.25 mM each of dATP, dGTP and dTTP, 0.5 mM dCTP and 10 μCi of ^{32}P-dCTP (Amersham Pharmacia Biotech, Arlington Heights, IL) in a volume of 12 μl. Reactions were heated to 65 °C for 5 min, chilled on ice, and to which was added company supplied buffer, 10 mM DTT (Gibco/BRL, Bethesda, MD) and 40 U RNasin (Promega). Reactions (20 μl) were incubated at 42 °C for 2 min followed by the addition of 200 U Superscript II reverse transcriptase (Gibco/BRL), incubated at 25 °C for 10 min, 42 °C for 50 min, and 70 °C for 15 min to

deactivate the enzyme. Reactions were stored at $-70\,°C$ until used for PCR.

The ^{32}P-labelled first strand cDNA from the RT reactions was used for quantitating the amount of cDNA produced, which was inferred from the incorporation of ^{32}P-dCTP. Radio-labeled cDNA–RNA heteroduplexes (1 μl) were electrophoresed on a 1.1% agarose/0.5% NuSieve (BioWhittaker Molecular Applications, Rockland, ME) gel containing 0.5 μg/ml ethidium bromide in 1X Tris acetate EDTA buffer (TAE; 40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.3) at 70 V for 3 h. The cDNA was transferred by capillary action to a GeneScreen nylon filter (NEN Research Products, Boston, MA) with 10X SSC (1.5 M NaCl, 0.15 M Na citrate). After transfer, filters were exposed to X-OMAT AR film (Eastman Kodak Co.) and the cDNA, which appeared as a smear in each lane, was analyzed by densitometry using an Astra 4000U scanner (Eastman Kodak Co.) and Kodak 1D Scientific Analysis Software (Scientific Imaging Systems, New Haven, CT). Variations in smears were quatitated and used to calculate equal amounts of cDNA template for initial PCR reactions.

2.3. Polymerase chain reaction

To compare mRNA levels between developmental stages and between treatments, equal amounts of cDNA template were used in PCR reactions as calculated from the smears of cDNAs (see above). PCR reactions were performed using a 9600 thermocycler (Perkin Elmer Inc, Boston, MA) with cDNA templates (0.8–3.2 μl of the RT reaction product) to which was added 0.5 or 1.0 U Taq polymerase (Gibco/BRL), 0.15 or 0.2 mM each of dATP dCTP dGTP and dTTP, 1.0 μM primer pairs (see below), 1X company-supplied buffer and 2.0 or 2.5 mM $MgCl_2$. The lower concentrations of dNTPs and $MgCl_2$ were used in PCR reactions with primers for actin, and the higher concentrations with primers for *Sp064*. The reaction volume was adjusted to 20 μl and heated to $94\,°C$ for 5 min. Amplifications were performed as follows: $94\,°C$ for 30 s, $64\,°C$ for 2 min, 2 min ramp to $72\,°C$ for 1 min for 25 cycles (actin) or 30 cycles (*Sp064*) to ensure that reactions were in the exponential amplification phase and that no reagents were

limiting (data not shown). To ensure that the RNA samples were not contaminated with genomic DNA that might support amplification with the primers, the equivalent amount of RNA (and contaminating DNA, if present) that would be present in 0.8–3.2 μl of the RT reaction (see above) was calculated (40–480 ng) and used directly in PCR reactions prior to the reverse transcriptase step. Results did not show detectible products indicating that no genomic DNA contamination was present in the RNA samples (data not shown).

Based on known sequences of sea urchin cDNAs, the following primer pairs were designed: SpC3 (EST064 from [8]; accession # R61937) forward, 5′-ACTTACAGGGCTCAAACAGGTGGTGAACG-3′; reverse, 5′-TCCTTCCCGGTCAAATCTTGTA-TATGGCC-3′ which amplified a 352 base pair fragment, and actin (EST278 from Ref. [8]; accession # R62049, matches to CyI, see Ref. [42]) forward, 5′-ACGACGATGTTGCCGCTCTTGTCAT-3′; reverse, 5′-GCTGTCCTTCTGTCCCATACCGACCA-3′ which amplified a 152 base pair fragment. Amplified products were separated on a 1.1% agarose/0.5% NuSieve (BioWhittaker Molecular Applications) gel containing ethidium bromide in 1X TAE buffer at 70 V for 3 h. For developmental profile studies, PCR bands were visualized by ultraviolet light and photographed with a Kodak 120 digital camera (Eastman Kodak Co.). Gel images were formatted using Adobe Photoshop v.5.5 (Adobe Systems, San Jose, CA) and analyzed by densitometry using 1D Scientific Analysis Imaging Systems (Eastman Kodak Co.).

For studies in which embryos were exposed to bacteria, PCR bands were transferred to an Immobilon-Ny$^+$ nylon filter (Millipore Corp., Bedford, MA) by capillary action and exposed to X-OMAT AR film (Eastman Kodak Co.) with an intensifying screen at $-70\,°C$. Transcripts were detected based on the incorporation of radiolabelled dCTP that was introduced into the PCR reaction with the cDNA template. Densitometric analyses of autoradiograms were carried out as described above. Relative levels of *Sp064* message between treatments were calculated by comparing *Sp064* band intensities to those for actin.

3. Results

3.1. Profile of gene expression during embryonic development

The *Sp064* message content was determined in unfertilized and fertilized eggs and in embryos cultured to 15 different developmental stages (Table 1). Results showed that *Sp064* messages were low in unfertilized eggs through late blastula, however, beginning with hatched mesenchyme blastula, a pronounced increase in transcript levels was observed through gastrulation (Fig. 1). Levels of *Sp064* message at hatched mesenchyme blastula with thickened vegetal plate (hmb/vp), early gastrula and late gastrula were significantly higher than at earlier stages ($p < 0.05$). The data showed a peak in *Sp064* message at hmb/vp, which immediately precedes gastrulation and is the point in development when the primary mesenchyme cells ingress from the vegetal plate into the blastocoel of the embryo. This was also

the point at which the embryos hatch from the fertilization membrane. The elevation in *Sp064* message was not maintained at the same level throughout development and tended to decrease from early prism to late pluteus.

In our initial searches to identify a gene expressed in the embryo that did not vary in transcript content during development and that we could use to quantify the amount of cDNA in PCR reactions, our first choice was the sea urchin homologue of the ribosomal protein L8, called *SpL8* (EST219 from Ref. [8]; accession # R62029). However, we found that it also had a variable expression pattern during development that paralleled the changes observed for *Sp064*. *SpL8* transcripts were present at low levels throughout early embryogenesis with a distinct increase at the time of gastrulation (data not shown). In subsequent searches for a control, the expression pattern of cytoplasmic actin was examined. In accordance with Lee et al. [41], who first demonstrated the low abundance of actin transcripts during early sea urchin development,
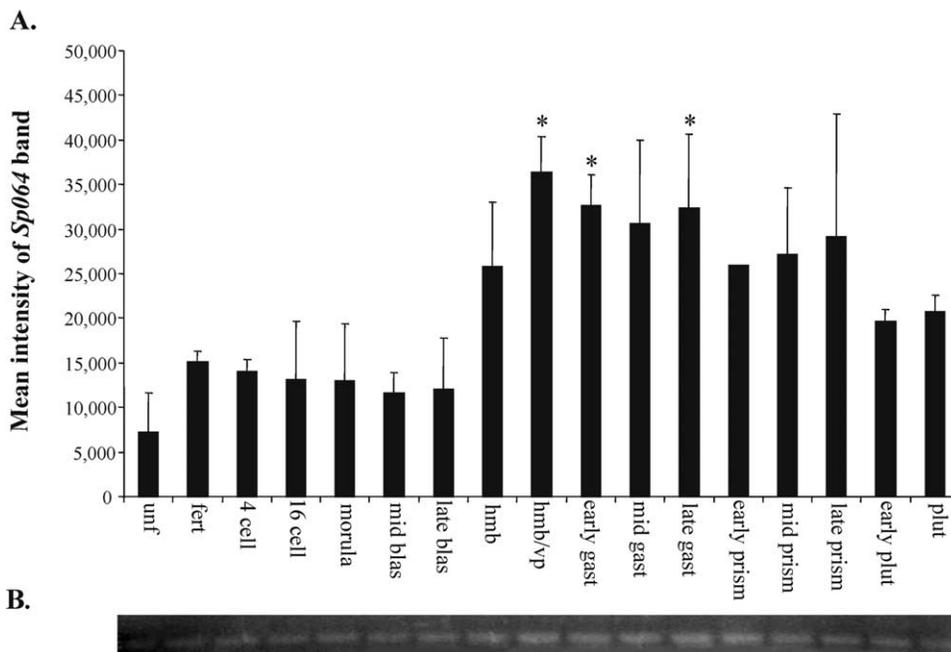

Fig. 1. Developmental profile of *Sp064* messages. (A) Densitometric analysis of *Sp064* PCR-amplified bands from ethidium bromide stained gel images. Means and standard deviations of band intensities from three experiments were calculated for each developmental stage. Stages showing a statistically significant increase in *Sp064* transcript levels (*, $p < 0.05$) compared to early stages (unfertilized egg through late blastula; see Table 1 for stage abbreviations) were determined by one-way ANOVA and the Student–Newman–Keul's test. (B) Image of a representative gel showing PCR-amplified bands of *Sp064* transcripts during development.

we also found that actin transcripts were present in low amounts in unfertilized eggs through morula (Fig. 2). However, a significant ($p < 0.05$) increase in actin message was observed at the mid blastula stage, a point earlier in development than that observed for *Sp064*. Beginning with mid blastula, actin mRNA content remained elevated, but with no significant variations throughout the rest of embryogenesis, and therefore, actin transcripts provided an appropriate control for comparisons to *Sp064* expressed in embryos cultured under normal conditions and those exposed to immune challenge.

3.2. Sp064 message levels in response to immune challenge

Sea urchin embryos were exposed to *V. diazatrophicus* at two developmental time points: either hatched blastula or early gastrula. Embryos treated at the hatched blastula stage were collected for analysis at late gastrula and at pluteus, while embryos treated during early gastrulation were collected at late gastrula and at feeding pluteus. *Sp064* transcript levels were calculated by comparing *Sp064* band intensities from both challenged and untreated control embryos to intensities of actin bands. Actin was used as the control because when the intensities of actin bands from the RT-PCR analyses were compared to the total ^{32}P-labeled cDNA for each developmental stage, the ratios indicated that actin messages remained unchanged after the morula stage (Fig. 2). Therefore, actin was used as an internal control for both PCR reactions and as a gel loading control. Results of comparisons between challenged and control cultures showed that plutei, when exposed beginning at hatching to *V. diazatrophicus* showed a significant ($p < 0.05$) increase in *Sp064* message content (Fig. 3). However, when embryos were treated beginning at early gastrula, a similar increase was not observed. Embryos collected at late gastrulation irrespective of treatment timing also did not show increases in *Sp064* transcripts. This suggests that given enough contact
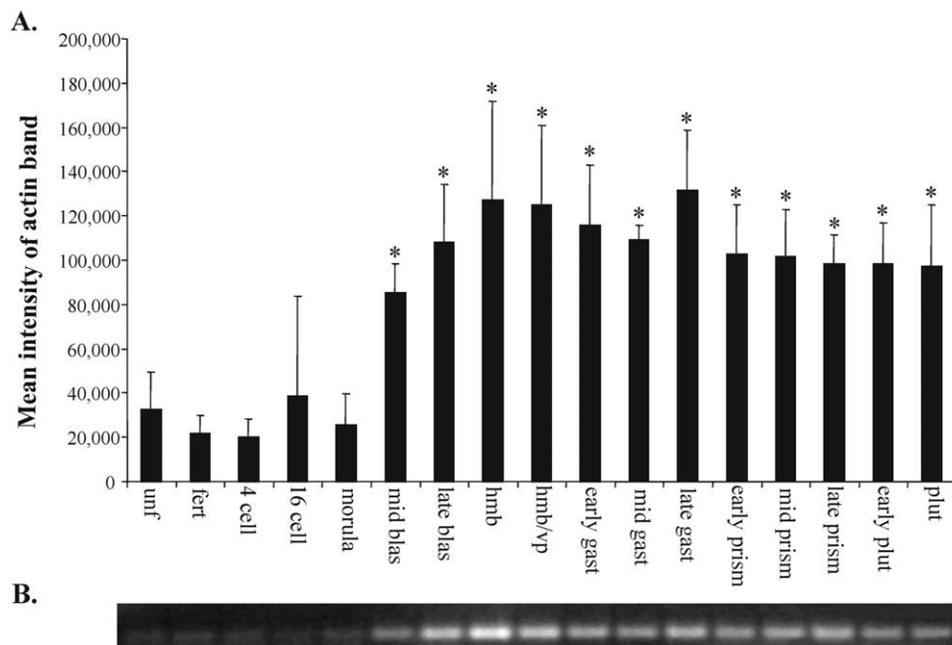

Fig. 2. Developmental profile of actin messages. (A) Densitometric analysis of PCR-amplified actin bands from ethidium bromide stained gel images. Means and standard deviations of band intensities from three experiments were calculated for each developmental stage. Embryos older than the morula stage showed significantly increased levels of actin transcripts (*, $p < 0.05$) compared to that in earlier stages, as calculated by one-way ANOVA and the Student–Newman–Keul's test. After morula, levels of actin message were not statistically different from one stage to another. (B) Image of a representative gel showing PCR-amplified bands of actin transcripts during development.
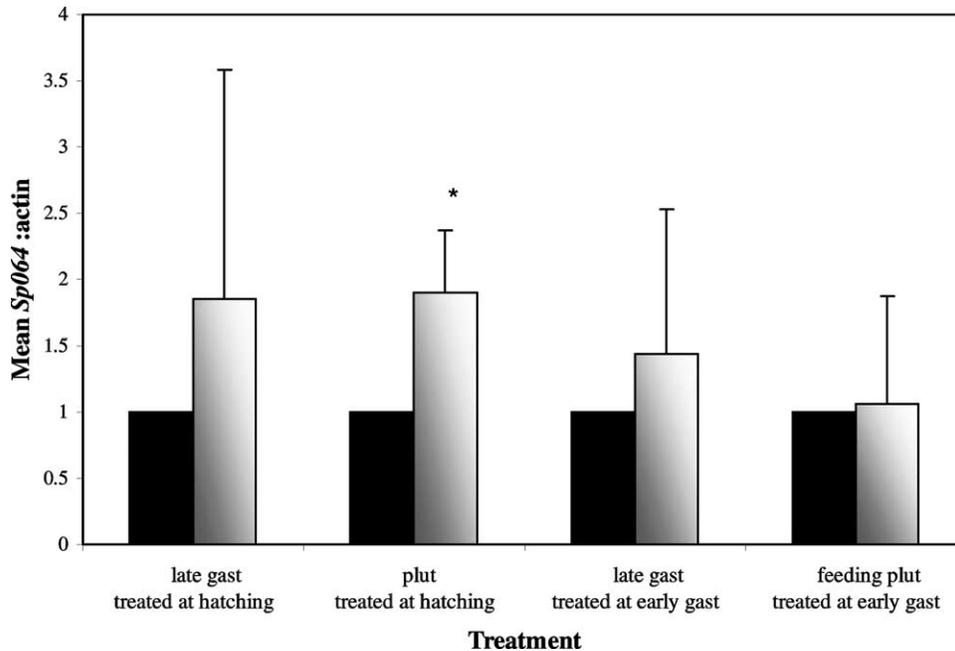
Fig. 3. Effects of immune challenge with heat killed *V. diazatrophicus* on *Sp064* transcript levels in sea urchin embryos. Ratios of *Sp064*: actin band intensities from autoradiograms of PCR fragments on two gels are shown compared to the control culture for each treatment. Control cultures were incubated in HMSW without bacteria and *Sp064* message content in these cultures was set to 1 (black bars). Embryos incubated with bacteria (gray bars) showed a statistically significant increase in *Sp064* levels (*, $p < 0.05$, Student's *t*-test) in cultures challenged from hatched blastula to pluteus compared to control cultures.

time, early sea urchin embryos can detect the presence of bacteria and respond with accumulations of *Sp064* message that are detectible at later stages.

4. Discussion

The presence of *Sp064* transcripts in unfertilized eggs, their persistence throughout embryogenesis, and their accumulation in response to contact with *V. diazatrophicus*, provides the first evidence of a putative complement based defense capability in sea urchin embryos. *Sp064* transcripts in unfertilized eggs and early embryos have been identified previously by EST analysis of mid cleavage embryos (accession # AF122324; see Ref. [43]). Although many different transcripts are loaded into the egg from the ovary during oogenesis, and some are translated during early embryogenesis (reviewed in Refs. [44,45]), it is not known if maternally derived *Sp064* messages are translated. It is possible that embryos prior to hatching

may not require a functional defense system because they are protected from foreign contact by the fertilization envelope. It is noteworthy that the increase in *Sp064* transcripts corresponds not only with gastrulation, but also with hatching. Because *Sp064* transcripts are supplied to eggs this may imply (1) that complement mediated defense may function in unfertilized eggs and early embryos, and/or (2) that *Sp064* messages may be delivered to the oocytes in preparation for defence capabilities that might not be needed until after hatching or when the pluteus begins to feed.

The increase in *Sp064* transcript content in the unchallenged embryo, perhaps due to the onset of zygotic *Sp064* expression, corresponds with the initiation of gastrulation. At this time, primary mesenchyme cells ingress into the blastocoel [46], and in an EST study of these cells, *Sp064* was identified 12 times (accession #: BG781881, BG781758, BG785784, BG784497, BG781085, BG783152, BG785352, BG780359, BG785194,

BG786755 BG786433, BG780257; [47]). However, it is the secondary mesenchyme cells that are of interest with regard to the defence system in the embryo. Some of these cells emerge from the vegetal plate just prior to gastrulation, while most migrate from the tip of the archenteron during late gastrulation [35,48]. Descendents include pigment cells that become established in the ectoderm [49–51] and blastocoelar cells that are found in the blastocoel [52]. Secondary mesenchyme cells can phagocytose yeast [34], pigment cells contain echinochrome that has anti-bacterial properties [53], and blastocoelar cells were perhaps the cells attempting to encapsulate Metchnikoff's rose prickle. Based on these studies, secondary mesenchyme cells and their descendants, in addition to the primary mesenchyme cells, are perhaps the best candidates for mediating the immune responses in the developing echinoderm embryo. Therefore, we speculate that some or all of these cells may express *Sp064*.

A number of genes have been identified that are expressed in the coelomocytes of the adult sea urchin and that show message accumulation in response to injury or immune challenge. These include profilin [37,38], several transcription factors, scavenger receptors with cysteine rich domains, several uncharacterized messages [27,54,55], and *Sp064* [28,29]. An increase in *Sp064* content in coelomocytes has been interpreted as an indication of an immune response induced by lipopolysaccharide [29]. Similarly, our results from embryos cultured with bacteria suggest an immune response that is upregulated in response to microbial contact. The mechanism(s), however, for increasing the *Sp064* message in either embryos or adult coelomocytes is not clear and could involve either an increase in gene transcription, increased message stabilization, or both. The available sequence from the 3′ untranslated region of *Sp064* has six AUUUA repeats, or AU-rich elements (AUre) [7], which may be involved in message stabilization/destabilization. Messages with multiple AUre's are normally turned over quickly, but in response to stress or immune challenge, AUre-binding proteins are induced and stabilize messages, decreasing their turnover rates that results in message accumulation [56,57]. This suggests that the increase in *Sp064* transcripts in embryos after exposure to *V. diazatrophicus* may be attributed to zygotic gene transcription observed under control culture conditions plus induced message stabilization observed in response to contact with bacteria.

Based on the life history of the sea urchin, it is not surprising that the embryo and larva appear to have defense capabilities. In an indirect developing echinoderm, the result of embryogenesis, which takes about three days for *S. purpuratus*, is to produce a bilateral pluteus larva that becomes a member of the omnivorous zooplankton population feeding on single celled phyto- and zooplankton in addition to prokaryotes [58]. The larval life stage of the sea urchin, which lasts approximately 6 weeks for *S. purpuratus* under normal conditions, facilitates the dispersal of this sessile species and enables the larva to feed so that the adult rudiment can develop prior to settling and metamorphosis into a small, pentamerous juvenile. Because the larval sea urchin is a free-living, feeding organism, defense responses to contact between microbes in the water and the ectoderm of the hatched embryo might be mediated by the pigment cells. Contact with microbes through feeding by the larva, which could include both opportunistic and virulent pathogens, suggests defences might be mediated by the gut or cells associated with the gut such as the blastocoelar cells. The presence of and the increase in *Sp064* messages in response to contact with bacteria suggests the existence of an embryonic immune system in the sea urchin which may be similar, although not necessarily identical to that in the adult.

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