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Expression of CyI Cytoplasmic Actin Genes in Sea Urchin Development

Jang-Hee Hahn† and Rudolf A. Raff*

Institute for Molecular and Cellular Biology, Department of Biology, Indiana University, Bloomington, IN 47405, USA

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Abstract: We present a study of evolutionary changes in expression of actin genes among closely related sea urchin species that exhibit different modes of early development. For this purpose, polyclonal antisera raised against peptides from the carboxyl terminus of the HeCyI cytoskeletal actin of *Heliocidaris erythrogramma* were used. *H. erythrogramma* is a direct developing sea urchin that proceeds from embryonic to adult stages without an intervening feeding larval stage. Expression patterns of the CyI actin isoform were compared with those of *Heliocidaris tuberculata* and to a related sea urchin *Strongylocentrotus purpuratus*, which both produce a feeding pluteus larval stage. The CyI actin of all three species is expressed in the same cell types. However, its expression patterns have been changed with reorganization of early cell lineage differentiation, which is apparent among the three species. Thus, evolutionary changes in CyI actin gene expression patterns are correlated with not only phylogenetic relationship, but developmental mode. The implication of this observation is that evolutionary changes in expression patterns of histospecific genes may underlie the emergence of novel developmental processes.

Key words: CyI actin gene, developmental mode, evolution of gene expression, *Heliocidaris*.

The goal of studies in evolutionary developmental biology is to define how programs of gene expression have evolved, and to utilize evolutionary data to understand underlying processes of development. An examination of the evolution of expression patterns of structural genes involved in the execution of histospecific developmental programs provides a test of how extensively developmental architecture can be modified. The use of closely related species that differ in major aspects of early development enables us to correlate gene expression changes with the remodeling of developmental processes of early development.

The two Australian sea urchin species, *Heliocidaris tuberculata*, and *H. erythrogramma* provide such a suitable experimental system for the study of evolution of developmental modes. *H. tuberculata* develops in the typical indirect manner and forms a feeding pluteus, whereas *H. erythrogramma* develops directly from the egg to the juvenile in about four days without producing a pluteus (William and Anderson, 1975; Raff, 1987). Comparative analysis of the development of the two *Heliocidaris* species has revealed a substantial reorganization of gametogenesis, reproductive ecology, and early development (Raff, 1992). Such complex developmental modifications strongly suggest that the *H. erythrogramma* larva is the product of an altered developmental program.

Gene structure, temporal and spatial expression patterns, and regulation of the actin gene family have been thoroughly characterized in the indirect developing sea urchin *Strongylocentrotus purpuratus* (Davidson, 1986, 1989). There are three functional cytoplasmic actin subgroups, and two pseudogenes as well as a single muscle actin gene. Although these different family members code for similar cytoskeletal actins, they are specifically and differentially expressed in particular embryonic cell types (Shott *et al.*, 1984; Cox *et al.*, 1986; Lee *et al.*, 1986). Information about the actin gene family of *S. purpuratus* provides a molecular basis sufficient to analyze changes in expression of actin genes of the *Heliocidaris* species. The actin gene family of the *Heliocidaris* species includes fewer representatives (3 genes) than the *S. purpuratus* family (8 genes), but includes expressed representatives of the three cytoplasmic actin groups identified in *S. purpuratus* and a muscle actin (Kissinger *et al.*, in preparation). The single *Heliocidaris* CyI homolog of the *S. purpuratus* family has been isolated and characterized (Hahn *et al.*, 1995).

Here we compare expression patterns of the CyI ac-

† Present address: Cancer Research Institute, School of Medicine, Seoul National University, Seoul, Korea
* To whom correspondence should be addressed.
Tel: 1-812-855-2791, Fax: 1-812-855-6082.
E-mail: rraff@sunflower.bio.indiana.edu

tins in embryos of the two *Heliocidaris* species and *S. purpuratus*, and show that evolutionary changes have occurred in some aspects of the expression patterns of homologous members of the actin gene family among relatively closely related sea urchin species.

Materials and Methods

RNA gel blots and hybridization analysis

RNA was isolated from eggs and embryos with guanidine-HCl (Bruskin *et al.*, 1981). RNA samples of comparable developmental stages were loaded on gels. 5 μg samples of total RNA were electrophoresed on formaldehyde agarose gels and blotted to Nytran (S & S, Dassel, Germany). Blots were hybridized with a 0.65 kb *Xmn/Eco*RI fragment from the 3′ untranslated region UTR of a HeCyI cDNA clone (Hahn *et al.*, 1995). Hybridization conditions were moderate, 39℃ in a 50 % formamide hybridization buffer and final washes were carried out at 65℃ in 1X SSC, 0.1% SDS.

Embryo culture and fixation

H. erythrogramma and *H. tuberculata* were collected near Sydney, Austrailia. *S. purpuratus* was obtained from Pacific BioMarine (Venice, USA). Gametes were obtained by intra coelomic injection of 0.55 M KCl. *H. erythrogramma* embryos were cultured at low densities in 5 L beakers. *H. tuberculata* embryos do not remain suspended in stir-cultures, and were instead cultured as monolayers in covered glass dishes. Embryos were fixed at 4℃ for 1 h in 2% formalin in filtered sea water, washed three times in filtered sea water, and stored at 4℃ in 70% ethanol until prepared for examination.

Preparation of polysera against synthetic peptides

Peptides corresponding to amino acids 258-268 in the carboxyl terminal region of *Heliocidaris* actin CyI and CyII isoforms were synthesized by the Peptide Synthesis Facility, Department of Medical Chemistry at Purdue University or Bio-Synthesis, Inc. (Denton, USA). The peptides were dissolved in water at a concentration of 500 μg/μl. A 50 μl aliquot was added to 100 μg of keyhole limplet hemocyanin (KLH), and coupled to KLH activated by treating the hemocyanin with 1.5 μl of 25% glutaraldehyde for 1 h at room temperature. The reaction was terminated by adding an excess of glycine (Doolittle, 1986; E. M. Jakoi, personal communication). Young male New Zealand white rabbits were injected with a conjugate of approximately 140 μg of KLH coupled to 100 μg of peptide. Antigen was resuspended in about 1 ml of sterile phosphate-buffered saline (PBS) and emulsified with either 1 ml of Freunds complete adjuvant (for the first injection) or 1 ml of Freunds incomplete adjuvant (for all subsequent injections). Animals were injected three times at 2-week intervals prior to their first bleeding via the marginal ear vein 10 days after the third injection. Clarified sera were stored at −20℃. Such antibodies have been useful in determining expression patterns of conserved proteins such as actin (Bulinski, 1986; McLean *et al.*, 1990) and tubulin (Arevalo *et al.*, 1990; Kimble *et al.*, 1990). Actin isoform-specific polyclonal antibodies were prepared through affinity purification using nitrocellulose stripes (Harlow and Lane, 1988). Antibodies directed against KLH were removed in the same way by using KLH-bound nitrocellulose strips, and used in all investigations. Anti-actin monoclonal antibody that binds to an epitope in a highly conserved region of actin was purchased from Boehringer Manheim Co. (Manheim, Germany).

Protein gel blot analysis

Pelleted embryos or larvae were resuspended in 2~3 volumes of Laemmli sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.8). After vigorous mixing, the samples were boiled for 5 min, clarified by brief centrifugation and stored at −80℃. Proteins were separated on SDS-polyacrylamide gels according to Laemmli (1970) and electrophoretically transferred to nitrocellulose according Towbin *et al.* (1979). Nitrocellulose blots were incubated for 30 min to 1 h in TPBS, followed by incubation with primary polyclonal antibody for 2 h to overnight. The blots was washed in TPBS and the primary antibody located using a goat anti-rabbit secondary antibody conjugated to alkaline phosphatase (Hyclone; diluted 1:500 in PBS), and then in PBS and stained using the bromochloroindolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate for 30 min.

Immunostaining of whole embryos

Embryos fixed with 4% formalin in artificial sea water (ASW) were washed and incubated in 10% normal goat serum in PBS containing 0.35% Tween-X100 for 3 h at room temperature. Following incubation in diluted serum (from 1:100 to 1:500) for 2~3 h or overnight at 4℃, embryos were washed two times with ASW and incubated in FITC-labelled goat-anti rabbit serum diluted 1:500 in ASW for 2~3 h. These were washed in ASW, mounted in ASW/glcerol (1:1), and viewed by confocal microscopy.

Results

Isoform-specific antibodies and specificity of staining

The putative protein sequences deduced from the

ISOFORM	AA258										AA268
SpCyI	CYS	PRO	GLU	ALA	LEU	PHE	GLN	PRO	ALA	PHE	LEU
*He/HtCyI	ALA	···	···	SER	···	···	···	···	SER	···	···
SpCyIIa	ALA	···	···	···	···	···	···	···	···	···	···
SpCyIIb	ALA	···	···	···	···	···	···	···	···	···	···
*HeCyII	ALA	···	···	···	···	···	···	···	SER	···	···
SpCyIIIa	ALA	SER	···	THR	···	···	···	···	SER	···	ILE
SpCyIIIb	···	SER	···	THR	···	LEU	···	···	SER	···	ILE
HeCyIII	SER	SER	···	THR	···	LEU	···	···	SER	···	ILE
SpM	···	···	···	THR	···	···	···	···	···	···	ILE
HeM	···	···	···	THR	···	···	···	···	···	···	ILE
HtM	···	···	···	THR	···	···	···	···	···	···	ILE

Fig. 1. Comparison of amino acid sequences of the most diversed region of actin isoforms. The amino acid sequences derived from residues 258 to 268 of SpCyI actin isoform are shown in the first line. Only residues that vary among the homologous sequences of the various sea urchin asctin isoforms are shown in the following lanes. Dotted lines represent conserved sequences. star (*) indicates amino acid sequences used for the production of synthetic peptides. Accession Nos. for the HeCyI and HtCyI coding region sequences in the GenBank/EMBL databases are U09635 and U09636. Abbreviation: Sp, *S. purpuratus*; He, *H. erythrogramma*; Ht, *H. tuberculata*; Cy, Cytoplasmic; M, muscle.

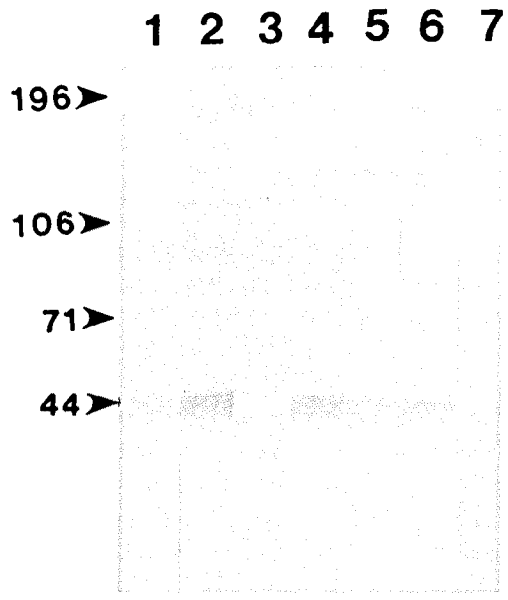

Fig. 2. Antibody specificity. The reactivities of antisera were assayed on protein gel blots containing 10 μg of late larval stage *H. erythrogramma* protein extract. Protein gel blots were probed with commercial anti-actin antibody (lane 1), anti-He/HtCyI antisera (lane 2-4), and anti-HeCyII antisera (lane 5-7). Lane 3 and 6 represent blots stained with antibodies preabsorbed at 1 mg/ml with He/Ht CyI:258-268 synthetic peptide. Lane 4 and 7 represent blots reacted with antisera competed with HeCyII:258-268 synthetic peptide (1 mg/ml). The mobilities of molecular weight markers are indicated on the left.

open reading frame of the HeCyI and HeCyII mRNA were used for production of polyclonal antisera against the CyI and CyII actin proteins (Hahn *et al.*, 1995; Kissinger *et al.*, in preparation). The synthetic peptides used as immunogens (He/HtCyI:258-268 and HeCyII: 258-268) are presented in Fig. 1. This region is the most divergent region among the various actin isoforms (Hightower and Meagher, 1986; Lee *et al.*, 1986). As shown in Fig. 1, three out of eleven amino acid residues in the region are different between SpCyI and He/HtCyI actins. The He/HtCyI and HeCyII actin isoforms differ by one amino acid. The He/HtCyIII isoform is similar to the SpCyIII isoforms (Kissinger *et al.*, in preparaton).

Fig. 2 shows the reactivity of anti-He/HtCyI antibody (lane 2) and anti-HeCyII antibody (lane 5) with *H. erythrogramma* larval stage proteins of 44~46 kD, the approximate molecular weight expected for actin on protein gel blots of extracts. A 44~46 kD band was labelled in the same extract by the commercial monoclonal anti-actin antibody that recognizes an epitope in a highly conserved region of actin (lane 1). Specificity of the antibodies was demonstrated by competition experiments. When the anti-He/HtCyI antibody was preabsorbed with an excess of peptide He/HtCyI:258-268, the 46 kD band disappeared (lane 3), but an excess of the HeCyII:258-168 peptide did not block labelling of the actin band by the anti-He/HtCyI antibody (lane 4). These results indicate that the affinity-purified anti-He/HtCyI antibody reacts specifically with the He/HtCyI peptide. Similar results were obtained with the anti-HeCyII actin antibody (see lanes 5-7), and support the specificity of antibodies to these similar actin peptides. The specificity of the antibodies was also determined in staining embryos by using the same competition assay (data not shown).

Comparison of expression patterns of CyI actin genes

Fig. 3 shows confocal microscopic views of a series of fixed *H. tuberculata* embryos reacted with the anti-He/HtCyI antibody. Very little staining was observed in eggs (data not shown). A surface view of an *H. tuberculata* blastula reacted with the anti-HeCyI antibody shows a staining pattern which looks like dot surrounded by a ring (Fig. 3A). Staining with preimmune serum does not reveal the ring and dot (data not shown). The ring pattern apparently corresponds to a contractile bundle of actin filaments which lies at the apical ends of ectodermal cells adjacent to the adhesion belt, running parallel to the plasma membrane. The brightly stained dotted structure apparently represents the ciliary root in an ectodermal cell. Both of these structures are associated with actin. The staining pattern of the actin ring and dot closely resembles visualization of this structure in the ectoderm cell layer of *S. purpura-*

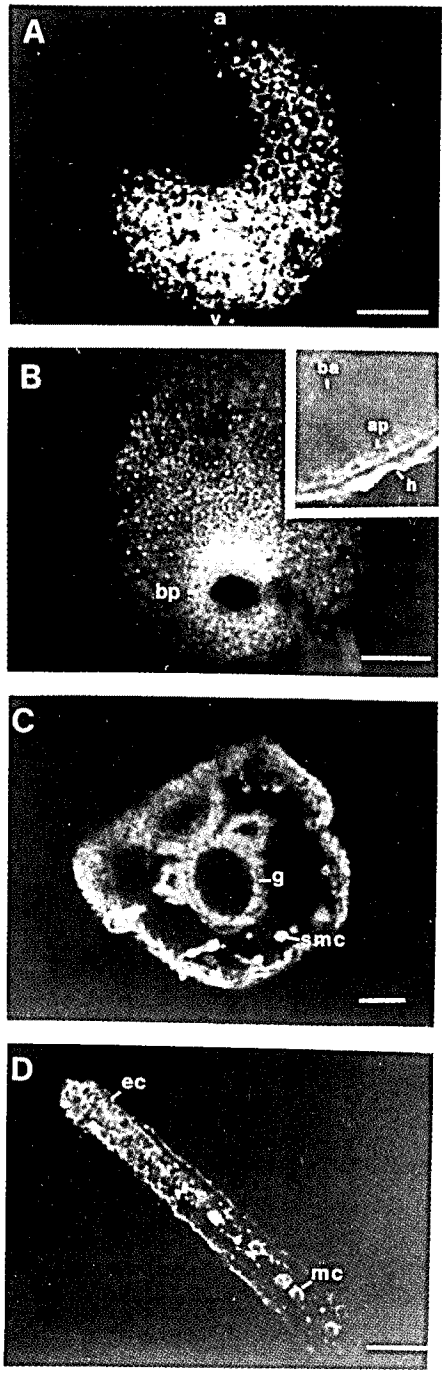

Fig. 3. Immunofluorescence confocal micrographs of *H. tuberculata* embryos stained with anti-He/HtCyI antibody. Sequential developmental stages of *H. tuberculata* were stained with anti-He /HtCyI antibody; the stages illustrated are (A) grazing section of sub-space of mesenchyme blastula ectoderm, (B) grazing section of sub-surface of gastrula (B inset, cross section through gastrula ectoderm showing staining of apical ends of ectodermal cells beneath the hyaline layer), (C) section through prism and (D) section through arm of pluteus. (A) and (B) embryos are observed with the vegetal pole down. The section in (C) passes approximately through the oral-aboral axis, Abbreviation: a, animal pole; ap, apical region; ba, basal region; mc, mesenchyme cells; bp, blastopore; ec, ectoderm cells; g, gut; h, hyaline layer; smc, secondary mesenchyme cells; v, vegetal pole. Scale bar represents 50 μm.

tus blastula and gastrula embryos revealed with the F-actin stain phalloidin.

In gastrulae, anti CyI antibody stains the ectoderm (Fig. 3B), and very brightly the secondary mesenchyme cells at the tip of the archenteron (data not shown). Their filopodia are distinctively stained. Staining of archenteron and primary mesenchyme cells is faint, but evident. Distinct staining of the ring and dot structure is shown over the ectoderm cells (Fig. 3B). The inset in Fig. 3B shows that the apical region of each ectoderm cell is stained, whereas the basal region is not. Apical staining probably represents a contractile bundle of actin filaments in the ectoderm cells. In the prism stage (Fig. 3C), some secondary mesenchyme cells which have departed from the tip of the archenteron are strongly stained. Staining intensity of ectoderm cells has lessened, but at the prism stages the ring pattern of brightly stained structures was still visible in ectodermal cells (data not shown). Gut and primary mesenchyme cells are faintly stained. Fig. 3D shows antibody staining patterns of the inside and surface of the pluteus embryo. The antiserum reacts specifically with ectoderm and some secondary mesenchyme cells. The strongest staining is detected in the ectoderm cells in the tip of arms and in mesenchyme cells. As in blastula and gastrula ectoderm cells, a regular ring and dotted pattern is observed lying beneath and parallel to the surface of the pluteus. Less staining is observed in oral ectoderm cells as compared with arm tips. Relative to oral ectoderm, very little staining is observed in aboral ectoderm cells.

Fig. 4 shows the staining of fixed *H. erythrogramma* embryos with the anti-He/HtCyI antibody. No staining is observed in eggs (data not shown). In the blastula, staining is still weak, however, actin filament ring and dot labelling appears in the ectoderm cells (Fig. 4A). As in *H. tuberculata* gastrula embryos, strong staining is detected in some secondary mesenchyme with filopodia (Fig. 4B). Throughout larval development, secondary mesenchyme cells in the animal region of larva continue to show strong staining (Fig. 4F). The characteristic staining pattern of an actin ring lying just below the apical surface is present throughout the ectoderm cell layer of gastrula and larval stage embryos (Fig. 4C and E). As in the *H. tuberculata* embryos, the apical region is stained in each ectodermal cell (Fig. 4D). There is little or no expression in the archenteron until 32 h of development (data not shown). CyI expression continues as the archenteron closes and begins to form the stomach.

CyI transcripts

RNA gel blot analysis of CyI expression was performed

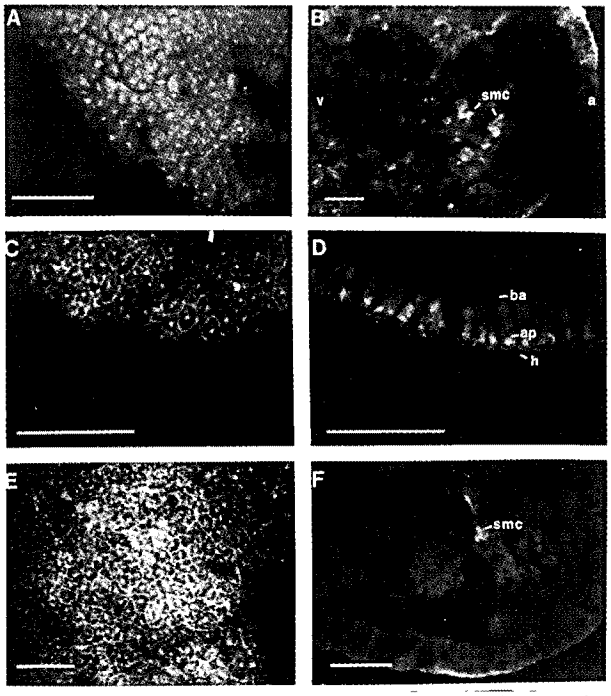

Fig. 4. Immunofluorescence confocal micrographs of *H. erythrogramma* embryos stained with anti-He/HtCyI antibody. Sequential developmental stages of *H. erythrogramma* were stained with anti-He/Htcyl antibody; the stages illustrated are (A) mesenchyme blastula ectoderm, (B), (C) and (D) late gastrula, and (E) and (F) 32 h larva. Frames B, D, and F are internal views. The others are grazing views of the subsurface of the ectoderm. Sections in frame B, D, and F are observed through the animal-vegetal axis Abbreviation: a, animal pole; ap, apical region; ba, basal region; h, hyaline layer; smc, secondary mesenchyme cells; v, vegetal pole. Scale bar represents 50 μm.

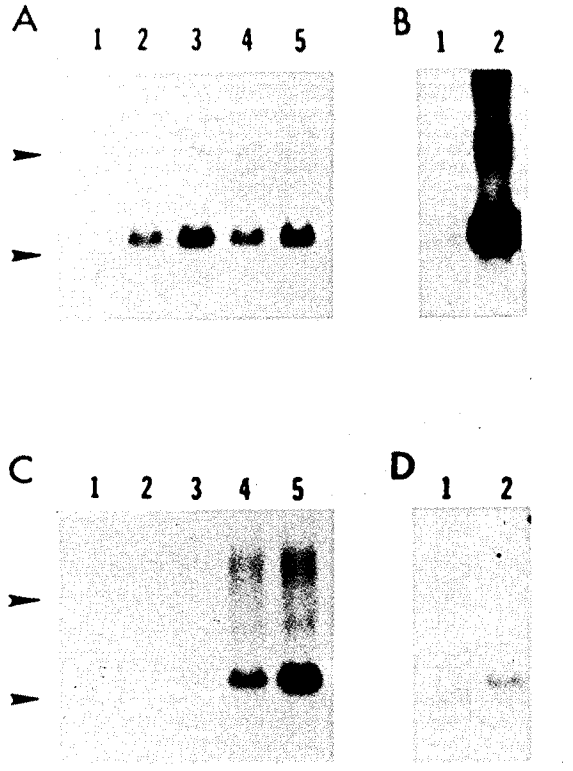

Fig. 5. Comparative analysis of CyI actin mRNA accumulation between *H. tuberculata* and *H. erythrogramma*. Panel A: *H. tuberculata*, lane 1, unfertilized egg; lane 2, mesenchyme blastula; lane 3, midgastrula; lane 4, prism; lane 5, pluteus. Panel B: a longer exposure of lanes 1 and 2 of panel A. Panel C: *H. erythrogramma*, lane 1, unfertilized egg; lane 2, blastula; lane 3, midgastrula; lane 4, early larva; lane 5, metamorphosing larva. Panel D: a longer exposure of lanes 1 and 2 of panel C. Arrows indicates the position of large and small subunit rRNAs.

using an HeCyI isoform-specific 3′ untranslated region probe on blots containing total RNA. The 3′ UTR sequences of CyI gene were fairly conserved between both *Heliocidaris* species (Hahn *et al.*, 1995). Fig. 5 shows the expression of CyI in stages ranging from unfertilized eggs to pluteus or metamorphosed larva in both *Heliocidaris* species. The CyI transcript is approximately 2.1 kb and can be detected in unfertilized eggs and early stages with longer exposures. *H. tuberculata* CyI mRNA begins to accumulate in the blastula stage with slightly higher levels than in the gastrula and pluteus stages. This pattern of expression is similar to that of the SpCyI gene of *S. purpuratus* (Shott *et al.*, 1984). *H. erythrogramma* differs in that significant levels of CyI mRNA do not begin to accumulate until the gastrula stage, and the levels steadily increase through larval stages to metamorphosis. High transcript levels are present in the stages in which we observed antibody staining of the CyI proteins.

Discussion

The amino acid sequences of HtCyI and HeCyI actins are identical (Hahn *et al.*, 1995). Thus, the two closely related, but developmentally different *Heliocidaris* species utilize the same CyI actin isoform during embryogenesis. Fig. 6 represents a schematic comparison of the spatial expression patterns of the CyI actin gene of the *Heliocidaris* species with the homologous CyI gene of *S. purpuratus*. In the blastula stage of *S. purpuratus*, the CyI messages accumulate at a relatively high level in the cells of the vegetal pole including the presumptive archenteron and some primary mesenchyme (Cox *et al.*, 1986). In contrast, CyI proteins seem to be uniformly distributed at a low level in the ectoderm cell layer of blastula stage embryos of two *Heliocidaris* species. In the gastrula, the SpCyI gene is expressed most abundantly in secondary mesenchyme cells at the tip of the archenteron and oral ectoderm progenitor cells. Some secondary mesenchyme cells with filopodia are also strongly stained in the gastrula of two *Heliocidaris* species, indicating that the CyI actin isoforms are a component of cytoskeleton in the filopodia of secondary mesenchyme cells in all three

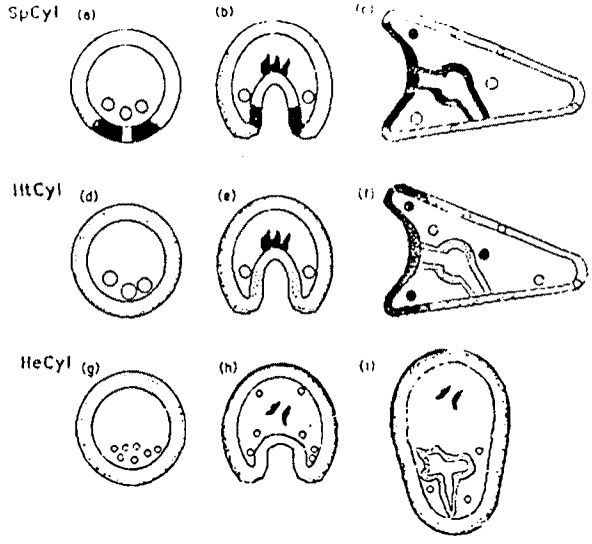

Fig. 6. Diagrammatic representation of expression of CyI actin isoform genes in three sea urchin species. The cell expressing CyI actin gene is indicated by a black dot. Sequential developmental stages are mesenchyme blastula (a, d, and g), gastrula (b, e, and h), and in (c) and (f), pluteus. For *H. erythrogramma*, the final stage (i) is an early larva. The vegetal pole is down.

species. In contrast to the regional expression pattern of SpCyI in the presumptive oral ectoderm, uniform staining is observed all over the ectoderm cells of *Heliocidaris* gastrula, representing phylogenetic relationship. The strongest antibody staining is observed over the oral ectoderm and the ectodermal cell layer of the four arms of the *H. tuberculata* pluteus. Thus, regional confinement of CyI gene expression in the ectoderm cells does not occur until the pluteus stage in *H. tuberculata*. Because the relationship between the first cleavage and the oral-aboral axis of *H. tuberculata* differs from that of *S. purpuratus* (Henry *et al.*, 1992), the boundary between oral and aboral ectoderm in the pluteus is different. Unlike ectodermal cells of two indirect developers, those of early larva of *H. erythrogramma* are uniformly stained. This suggests that the *H. erythrogramma* larva has no pattern of CyI actin gene expression corresponding to the oral ectoderm of the pluteus of *S. purpuratus* and *H. tuberculata* (Cox *et al.*, 1986). Furthermore, this difference in gene expression patterns may represent a change in developmental mode. In summary, the cell types in which the CyI gene is expressed in the embryos of three sea urchin species are similar to one another. However, evolutionary changes in expression have occurred not only between *Strongylocentrotus* and *Heliocidaris*, but also between the two *Heliocidaris*. Some changes are correlated with phylogenetic relationship, others with developmental mode. These types of changes in the closely related species are unexpected for such a highly conserved gene.

The indirect mode of sea urchin development has specific larval and adult programs. This is the primitive mode of development from which the direct development of *H. erythrogramma* arose (Strathmann, 1978; Raff, 1987; Wray and Raff, 1991). In the evolution of direct development in *H. erythrogramma*, there was an extensive reorganization of early cell lineage differentiation, including changes in localization of maternal factors, in cell lineage and in the timing of cell fate decisions (Wray and Raff, 1989, 1990; Henry *et al.*, 1990). Alteration in origination of cell lineage founder cells may produce novel inductive interactions between blastomeres, which create new regulatory spatial domains, possibly containing novel sets of *trans*-acting regulatory factors. Nevertheless, the same cell types are produced in the *H. erythrogramma* embryos as in *H. tuberculata* and *S. purpuratus*. Each cell type is defined by a pattern of expression of structural genes, with the expression of histospecific structural genes controlled by the interaction between their *cis*-acting regualtory regions and cell type-specific set of *trans*-acting factors (Davidson, 1989; Dynan, 1989; Thiebaud *et al.*, 1990). To recognize novel sets of *trans*-acting factors that result from a change of developmental mode, *cis*-acting regulatory sequences of cell type-specific structural genes must have changed during evolution in developmetal modes of *Heliocidaris* species. In fact, a comparison of 5′ regulatory regions of the CyI actin gene in *S. purpuratus*, and both *Heliocidaris* species suggested evolutionary changes in organization of *cis*-regulatory elements (Hahn *et al.*, 1995). Thus, evolutionary modifications in regulation of expression of histospecific genes may underlie the remodeling of early developmental process from which morphological evolution can derive.

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