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Original Article

Transcriptional regulation of the gene for epidermal growth factor-like peptides in sea urchin embryos

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ABSTRACT Exogastrula-inducing peptides (EGIPs), which are epidermal growth factor-related peptides of the sea urchin *Anthocidaris crassispina*, are substances that elicit abnormal gastrulation (exogastrulation) during embryogenesis of the sea urchin. In the present study we have examined the regulation of the expression of the EGIP precursor gene (*EGIP*) in sea urchin embryos. Whole mount *in situ* hybridization showed that *EGIP* is zygotically expressed after the onset of gastrulation in subdomains of the embryonic and larval ectoderm. The expression is confined in early gastrulae to small ectodermal regions adjoining the vegetal plate, which progressively expand to almost the entire ectoderm except the oral hood and postoral tips of the arms in later stages. In adults the expression is restricted to the ovary. Zygotic *EGIP* expression is sensitive to dissociation of embryonic cells, as well as to disruption of the extracellular matrix (ECM) with 5-*cis*-hydroxyproline, suggesting requirements for interaction with neighboring cells and/or with the ECM. The expression of reporter genes (chloramphenicol acetyl transferase and green fluorescent protein) under the regulation of the 4.6 kb upstream region of *EGIP* is temporally and spatially similar to that of the endogenous gene, showing that EGIP expression is regulated at the transcription level during embryogenesis by the *cis*-elements within the 4.6 kb upstream region.

KEY WORDS: *exogastrula-inducing peptide, gene expression, transcription, cell-cell interaction, EGF motif*

Introduction

Epidermal growth factor (EGF) is a small peptide growth factor with a distinctive motif of six cysteines, and this motif is found in a variety of proteins with a broad spectrum of functions in animal development. EGF-type growth factors regulate various aspects of cell growth, differentiation and morphogenesis through receptors with protein tyrosine kinase activities (Wiley *et al.*, 1995; Schweitzer and Shilo, 1997; Riese and Stern, 1998). Membranous proteins with multiple EGF motifs (Notch family) are implicated in neurogenesis, somitogenesis, or establishment of the body plan (Davis, 1990; Jiang *et al.*, 1998). Recently, it has been found that a new type of EGF-related proteins (EGF-CFC proteins) are essential regulators in patterning of early embryos as well as in organogenesis (Shen and Schier, 2000). A number of extracellular matrix (ECM) proteins also contain EGF motifs (Davis, 1990).

Exogastrula-inducing peptides (EGIPs) are polypeptides identified in sea urchin embryos (*Anthocidaris crassispina*) with single copies of a novel type of EGF motif (Suyemitsu *et al.*, 1989, 1990; Yamasu *et al.*, 1995). They affect the gastrulation of sea urchin embryos; exposure to exogenous EGIP produces exogastrulae (Ishihara *et al.*, 1982). Structural analysis of the cDNA for EGIP showed the presence of a precursor of 36 kDa which is expected to generate three EGIPs (EGIP-A, C, D) by specific proteolysis (Yamasu *et al.*, 1995). Based on their structures and effects on gastrulation, it has been assumed that EGIPs are secreted into the ECM of embryonic cells where they associate with other ECM proteins (Hirate *et al.*, 1999).

We recently identified sea urchin ECM proteins that can physically associate with EGIP-D, which were termed EGIP-D-binding proteins (EBP; Fujita *et al.*, 1994; Hirate *et al.*, 1999). EBPs are similar in structure to the Bep proteins from *Paracentrotus lividus* (Di Carlo *et al.*, 1990), which are considered to be involved in cell-cell interactions (Romancino *et al.*, 1992). Each EBP protein contains two of the repeated structures that are also seen in fasciclin I from *Drosophila* (Zinn *et al.*, 1988) and several ECM proteins identified from mammals (OSF-2, Takeshita *et al.*, 1993; βig-h3, Skonier *et al.*, 1992). EBPs are observed on the surface of embryonic cells and in granular structures in the cytoplasm which are probably yolk granules (Hirate *et al.*, 1999), raising the possibility that EGIPs are secreted and interact with EBPs in the ECM. Such associations between proteins are considered to be a gen-

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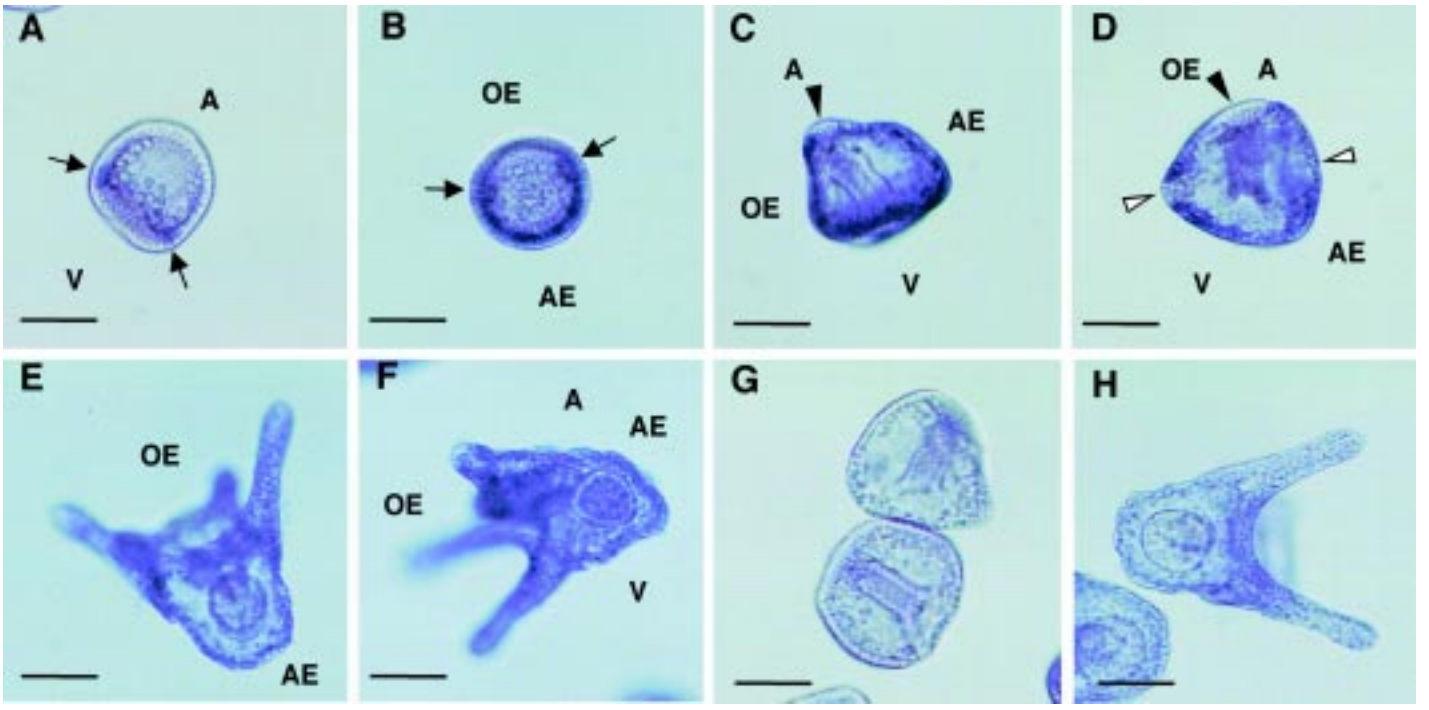

Fig. 1. Expression of *EGIP* in sea urchin embryos. (A,B) *Early gastrulae. Arrows show two subsets of ectodermal cells adjoining the vegetal plate. A frontal view (A) and an anal view (B) are shown. Faint reddish color in the vegetal plate tends to be seen without color reaction in the gut of this species and does not represent real staining (see also Fig. 1G). Since the anal ectoderm is flat and the boundary between the anal and oral ectoderm is close to straight in this stage, focus is on the entire anal ectoderm as well as on the lower part of the oral ectoderm in (B).* **(C,D)** *Prism embryos with high expression at the oral-aboral or anal-aboral boundaries. Black arrowheads represent oral lobes, while white arrowheads show the tips of prospective post oral arms, both of which lacked EGIP expression.* **(E,F)** *Anal (E) and lateral (F) views of plutei.* **(G,H)** *Control embryos (G, prism embryo; H, pluteus) treated with sense probes. A, animal pole; V, vegetal pole; OE, oral ectoderm; AE, aboral ectoderm. Bars, 50 μm.*

eral feature of many ECM proteins (Timpl *et al.*, 1984; Adams and Watt, 1993).

In order to define the role EGIPs play in the sea urchin development, it is essential to know when and where EGIPs are expressed. EGIPs are present in oocytes, eggs, and embryos through the pluteus stage, and they persist through the pluteus stage (Kinoshita *et al.*, 1992; Mizuno *et al.*, 1993). They exist mainly in vesicular structures in almost all the embryonic cells (Mizuno *et al.*, 1993). Transcripts of the EGIP precursor gene (*EGIP*) are present in unfertilized eggs, then disappear after fertilization, and again are expressed after the onset of gastrulation (Yamasu *et al.*, 1995). The constant presence of EGIP polypeptides, as well as the biphasic (maternal and zygotic) expression of the transcript, suggests that EGIPs might be involved in several aspects of the sea urchin embryogenesis. However, the spatial pattern of the zygotic expression has been unknown. In this regard, it should be noted that *SpEGFII,* which is a gene with some similarity to *EGIP* from another sea urchin species (*Strongylocentrotus purpuratus;* Yang *et al.*, 1989), is expressed only zygotically in subregions of the ectoderm (Grimwade *et al.*, 1991). Functional analysis of the product of *SpEGFII* has not been reported yet.

In the present study, we have examined the regulation of zygotic *EGIP* expression in sea urchin embryos. The result shows that *EGIP* is expressed specifically in subsets of ectodermal cells in embryos and that the expression of *EGIP* depends on cell-cell interaction in embryos. Gene transfer experiments have shown that the temporal and spatial regulation of the *EGIP* gene is governed transcriptionally by 4.6 kb DNA region including the upstream DNA and 5'-untranslated region.

Results

Expression of EGIP in subdomains of embryonic ectoderm

In order to know the spatial regulation of the zygotic *EGIP* expression during embryogenesis, we performed the whole mount *in situ* hybridization (WMISH) using DIG-labeled riboprobes. In keeping with our developmental northern analysis (Yamasu *et al.*, 1995), expression of *EGIP* transcript is first detected at the early gastrula stage in subsets of the ectoderm adjoining the vegetal

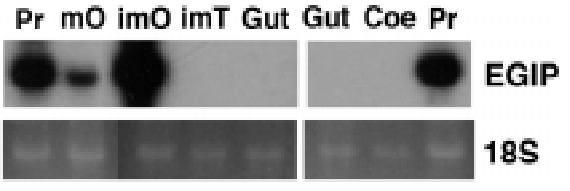

Figure 2. Expression of *EGIP* in adult organs. *The accumulation of EGIP mRNA with a size of 1.8 kb was examined by the northern analysis in different adult organs such as mature ovaries (mO), immature ovaries (imO), immature testes (imT), digestive tracts (Gut), and coelomocytes (Coe) in addition to prism embryos for comparison (Pr). One microgram per lane of total RNA was separated on gels. As a loading control, the ethidium-bromide pattern of 18S rRNA is shown at the bottom.*

plate (Fig. 1). Even though the oral-aboral axis is not morphologically apparent at this stage in the ectoderm, *EGIP* expression is relatively pronounced in two small spots (Fig. 1 A,B, arrows). By comparison with the expression pattern in later stage embryos, we assume that these two spots reside within the ectoderm at or near the boundaries between the prospective oral and aboral ectoderm.

From the late gastrula to the prism stage, the expression expands toward the animal pole (Fig. 1 C,D). The expression is relatively intense at the boundaries between the oral and aboral ectoderm, as well as between the anal and aboral ectoderm. It is striking that *EGIP* expression is not seen at the tip of future arms and the oral lobe. Later in development, the expression domain further enlarges, and the *EGIP* expression is seen throughout the ectoderm except in the oral hood and the tip of the arms (Fig.1 E,F). Consistent with the specific expression in the ectoderm, *EGIP* expression was significantly suppressed by lithium ions (data not shown) that are known to re-specify prospective ectoderm to the endoderm (Livingston and Wilt, 1989). Zinc ions, which severely inhibit cell differentiation in sea urchin embryos (Nemer, 1986), also abrogated the *EGIP* expression (data not shown). This is also in agreement with the restriction of *EGIP* expression to rather differentiated ectodermal cells.

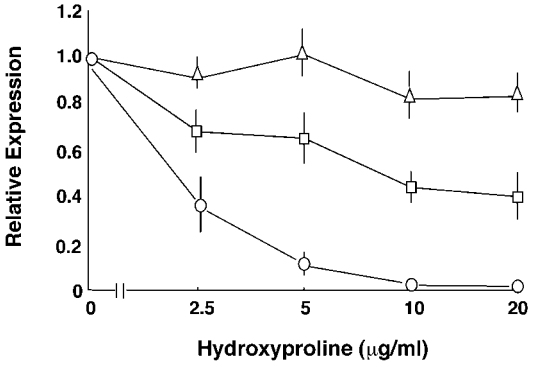

Fig. 3. Expression of *EGIP* is suppressed as a result of the disruption of the ECM. *Embryos were treated during the entire culture (24 h) with 5-cis-hydroxyproline at the indicated concentrations and examined for the accumulation of EGIP mRNA (1.8 kb, circles), AcSrc1 mRNA (8.2 kb, squares), and AcEFP (2.2 kb, triangles). Ordinates represent expression of the three genes at different drug concentrations relative to those in untreated embryos. Single blots were probed for the three genes sequentially after removing the probes used before. Experiments were repeated three times, and vertical bars represent the corresponding standard deviations.*

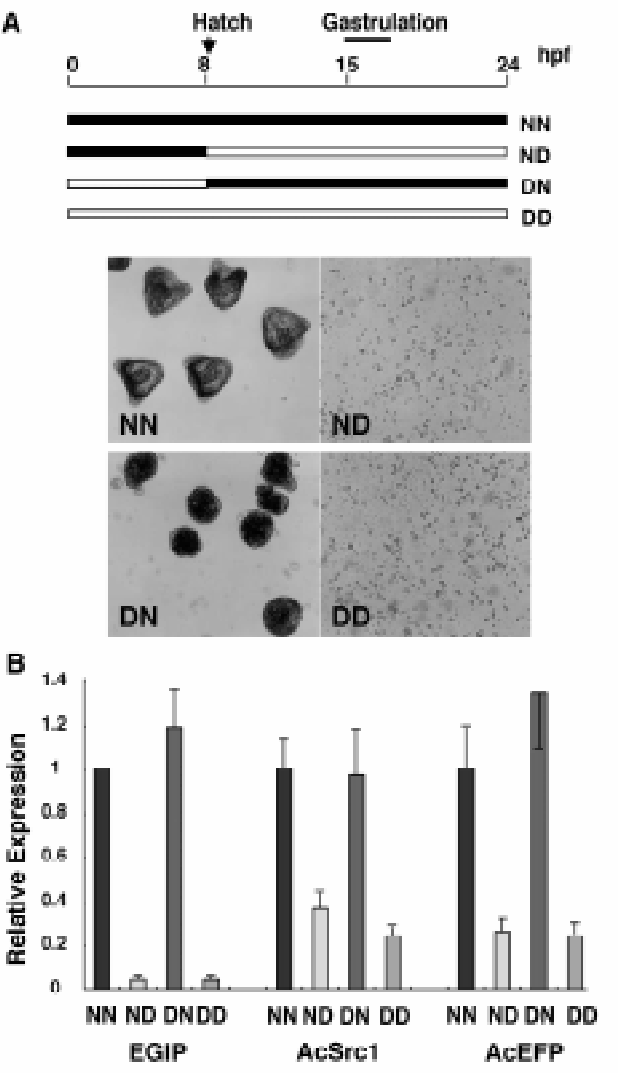

Fig. 4. Expression of *EGIP* is strongly suppressed in dissociated embryonic cells. (A) *Upper panel: Time schedule of the treatment of embryos. Embryos were cultured until 24 hpf in normal seawater (NN), dissociated with CMFSW at 8 hpf and cultured as dissociated cells in CFSW until 24 hpf (ND), cultured as dissociated cells until 8 hpf and then allowed to reaggregate by being transferred to normal seawater (DN), or cultured as dissociated cells from fertilization through 24 hpf in CFSW (DD). Embryos or dissociated cells for respective schedules were fixed at 24 hpf with 10% formaldehyde in seawater and shown in the lower panels. After the treatment DD, embryonic cells kept dissociated until 24 hpf when control sibling embryos (NN) were in the prism stage. Similarly, embryos after the treatments ND were in a completely dissociated state. By contrast, after the treatment DN, dissociated cells had reaggregated to form embryoids. When they were allowed to develop further until 48 hpf, deformed guts and spicules were observed in the embryoids (data not shown).* **(B)** *Accumulation of mRNA for EGIP, AcSrc1, and AcEFP in dissociated embryonic cells. mRNA levels were examined by Northern analysis of a single blotted membrane, which was probed for the three genes sequentially after removing old probes. Experiments were repeated three times, and vertical bars represent the corresponding standard deviations.*

Expression of EGIP in adult tissues

The distribution of the *EGIP* transcript in adult tissues was then examined by the northern analysis (Fig. 2). The transcript can be detected exclusively in both immature and mature ovaries, though immature ovaries contain much more transcript than mature ones. No transcript is seen in gut, muscle, testis, and coelomocytes. Thus, *EGIP* seems to be expressed only during oogenesis and embryogenesis.

EGIP expression is dependent on external signals

Ectoderm-specific expression shows that regulation of *EGIP* expression is under the influence of the embryonic cell lineage. Dynamic changes of the *EGIP* expression domain, on the other hand, could point to an additional regulatory mechanism(s) by cell-cell interaction and/or cell-ECM interaction. Formation of *EGIP*-expressing subdomains in the ectoderm may depend on signals from adjacent tissues or surrounding ECM. To test this possibility, we first examined the *EGIP* expression in embryos treated with 5-

cis-hydroxyproline, which is known to inhibit the deposition of collagen molecules, resulting in disruption of the ECM (Wessel and McClay, 1987). This treatment strongly affected the *EGIP* expression, while *AcSrc1* gene, which is specifically expressed in the gut (Onodera *et al.*, 1999), was only moderately suppressed (Fig. 3). The ubiquitously expressed gene, *AcEFP,* was least susceptible to this substance, as had been shown before (Saito and Yamasu, 1999).

In order to further address the roles of extracellular signaling, we also performed cell dissociation experiments. When embryonic cells were dissociated immediately after fertilization and allowed to develop until control sibling embryos reached the prism stage (24 hpf, Fig. 4, DD), the expression of *EGIP* was strikingly lowered to less than 5% of controls. On the other hand, both *AcEFP* and *AcSrc1* still showed 25 to 40% expression compared to the control embryos. Cell viability was monitored by dye exclusion test, and it was greater than 95% throughout the experiments; no sign of RNA degradation was seen on a formaldehyde gel (data not shown). A similar extent of suppression was observed even when embryos were dissociated at the hatching blastula stage (8 hpf, Fig. 4, ND), while restoration of the expression was obtained when dissociated cells were allowed to reaggregate by transfer to normal seawater at 8 hpf (Fig. 4, DN). Basically the same result was obtained when the level of *EGIP* mRNA was examined one more day later (48 hpf, data not shown).

It seems, therefore, that cell-cell interaction after hatching, not that in the cleavage stage, is essential for the expression of *EGIP* at later stages. Alternatively, it is possible that the absence of divalent cations on its own might affect the gene expression. Though this latter view cannot be excluded at present, treatment of the embryos with CMFSW/CFSW does not seem to be toxic to cells. In fact, restoration of the *EGIP* expression after transfer to normal seawater shows that the CMFSW/CFSW effect is reversible with respect to the expression of *EGIP*. It should be noted that restoration of the expression requires only aggregation, not normal morphology of the embryos (Fig. 4, compare NN to DN). At any rate, taken together these data from ECM disruption and cell dissociation experiments, we assume that expression of *EGIP* is non-autonomously regulated, being strongly dependent on the external signaling.

EGIP expression is regulated at the transcriptional level

To know if the regulation of the *EGIP* expression is regulated at the transcriptional level, we constructed two types of expression plasmids. The upstream DNA region of 4.6 kb excised from the genomic clone (λGEG8) of the *EGIP* gene was ligated either to the gene for chloramphenicol acetyl transferase (CAT) or green fluorescent protein (GFP) (*EGIP-CAT*, *EGIP-GFP*, Fig. 5). The 4.6 kb DNA also contains, at its downstream end, the 5'-terminal 95 bp of the *EGIP* cDNA (Yamasu *et al.*, 1995). Sea urchin eggs were injected with *EGIP-CAT* and allowed to develop for 12-35 h, and were then subjected to the CAT assay. The *CAT* expression was standardized by the amount of incorporated reporter construct. The standardized *CAT* expression is significantly activated at the onset of gastrulation (15 hpf; Fig. 6B), when zygotic expression of *EGIP* mRNA is first observed faintly in embryos (Fig, 6B; Yamasu *et al.*, 1995). The activation of the *CAT* expression thus foreshadowed the appearance of the *EGIP* mRNA, showing that activation of the endogenous *EGIP* gene is regulated temporally mainly at the transcriptional level by the upstream 4.6 kb region.

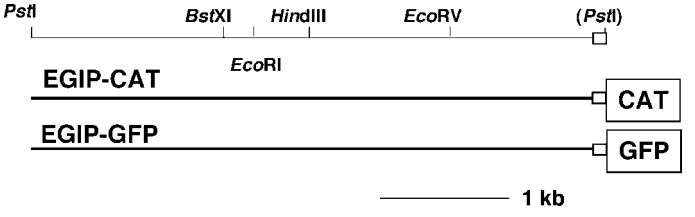

Fig. 5. Reporter constructs injected into sea urchin embryos. *The 4.6 kb upstream sequence of EGIP was ligated to CAT or GFP as reporter genes (EGIP-CAT and EGIP-GFP, respectively). Small white rectangles at the 3'-end show the 5'-terminal 95 bp of the EGIP cDNA clone (pEG32; Yamasu et al., 1995).*

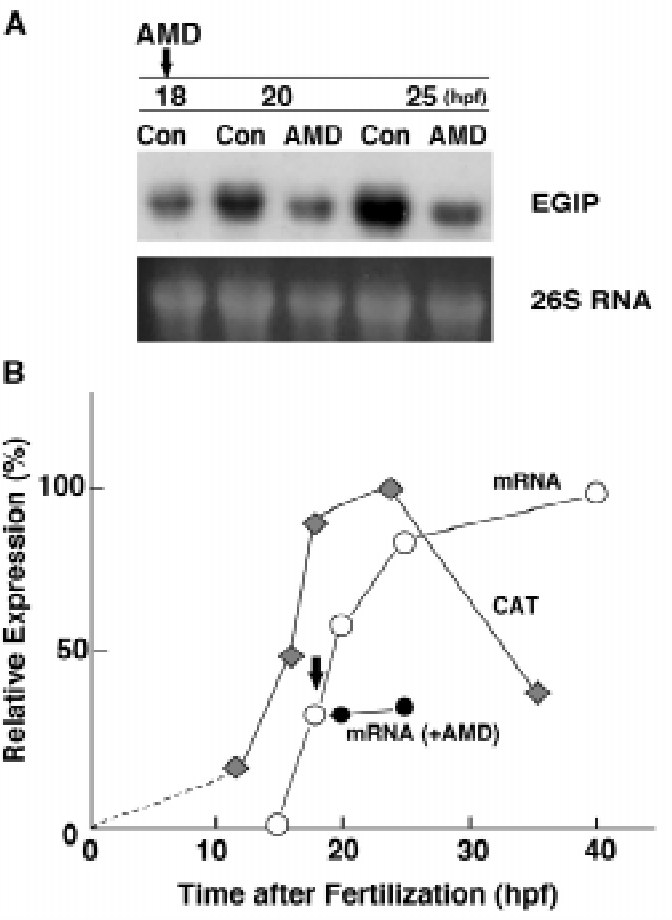

Fig. 6. Temporal regulation of *EGIP* expression. (A) *Stability of EGIP mRNA in sea urchin embryos. Actinomycin D was added at 10 μg/ml to the embryonic culture at 18 hpf and then further cultured for 2 h or 7 h, and examined for the expression of EGIP mRNA (AMD). Expression in normal sibling embryos (Con) was also examined in parallel.* **(B)** *Expression of the EGIP-CAT construct during development. The expression of the CAT gene in embryos was standardized with the amount of the incorporated construct and shown with gray diamonds. Superimposed are the quantified results of Northern analysis shown in B for normal embryos (open circles) and AMD-treated embryos (solid circles).*

The level of the *EGIP* transcript continues to increase further even after the prism stage (Fig. 6B; Yamasu *et al.*, 1995), while the expression of the *CAT* reporter gene standardized with respect to the template declined to a much lower level after the prism stage (Fig. 6B). Since the product of *EGIP-CAT* is known to be rather

labile in sea urchin embryos with a half time of 40 min (Flytzanis *et al.*, 1987), CAT activities can be regarded as the transcription rates of the reporter gene at given times, not representing accumulated enzymes. Therefore, the discrepancy after later stages between the *EGIP* mRNA level and the *CAT* expression might be explained by the lack of the regulatory element in the expression construct that would maintain the transcriptional level in later stages of development or might reflect real downregulation of the endogenous *EGIP* transcription. In the latter case, the sustained increase of the transcript in embryos would occur because of the stability of the mRNA.

In order to test this possibility, we examined the level of the transcript after treatment of embryos with a transcription inhibitor (actinomycin D, AMD). When 10 μg/ml of AMD was added to the culture at 18 hpf (mid- to late gastrula, ca. 3 h after the activation of the gene at the early gastrula) and embryos were allowed to develop for another 2 or 7 h, the *EGIP* transcript ceased to increase in amount and kept its expression level even until 25 hpf. Two hours of pretreatment with 10 μg/ml of AMD before the onset of gastrulation was sufficient to completely suppress the accumulation of *EGIP* mRNA at the early gastrula stage, even though treated embryos continued swimming vigorously (data not shown), showing that 2 h is enough for AMD to suppress transcription in nuclei. Thus, the transcript of the *EGIP* gene is relatively stable at least during the 5-h culture in the absence of the transcription. Such stability of the *EGIP* mRNA would allow for continued accumulation of the transcript until the pluteus stage even at reduced levels of transcription. Taken together, the data suggest that temporal regulation of the *EGIP* gene is conducted at the transcriptional level.

Meanwhile, when embryos were injected with *EGIP-GFP* and allowed to develop until the prism stage, the *GFP* expression was observed predominantly in the ectoderm (Fig. 7). *GFP* expression was detected in 44% of injected embryos, and all the positive embryos express *GFP* in the ectoderm, but not in the endoderm or mesenchymal cells (Table 1). The expression was highly mosaic, though such mosaicism with respect to the expression of transferred gene is quite usual in sea urchin embryos (Hough-Evans *et al.*, 1988; Arnone *et al.*, 1997). Thus, the ectoderm-specific expression of the *EGIP* gene is conducted at the transcription level under the regulation of the 4.6 kb DNA region. For control, *SM50-GL*, a construct in which *GFP* is under the regulation of the regulatory region of *SM50* from *S. purpuratus,* was also introduced into embryos. This construct, which had been shown to be expressed specifically in primary mesenchymal cells (PMCs) of *S. purpuratus* embryos (Arnone *et al.*, 1997), was expressed in a PMC-specific manner in this experiment with *A. crassispina* as well, as was expected (Fig. 7 E,F, Table 1). In this case, most of the PMCs in respective positive embryos express *GFP* in contrast to *EGIP-GFP*. This apparent lack of mosaic expression was also noted before and attributed to diffusion of GFP proteins through the syncytial cables among PMCs (Arnone *et al.*, 1997).

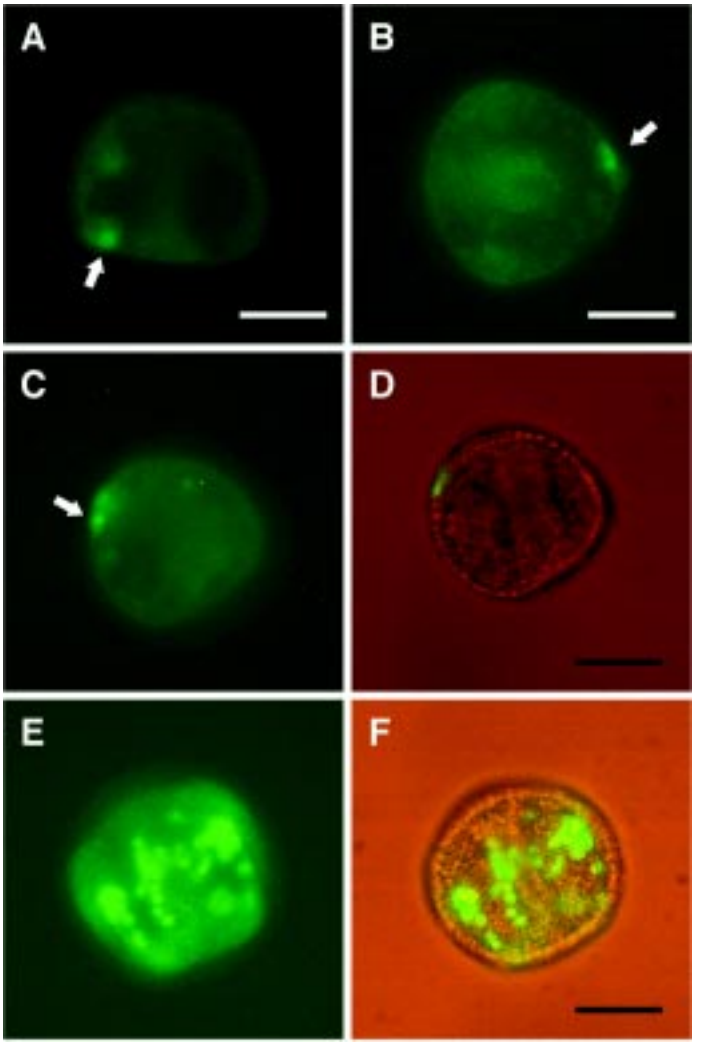

Fig. 7. Tissue-specific expression of the *EGIP-GFP* construct in prism embryos. (A-D) *Ectoderm-specific expression of EGIP-GFP in embryos. White arrows indicate the ectodermal expression of EGIP. A, B and C represent different embryos, while D shows the same embryo as C.* **(E,F)** *The same embryo injected with SM50-GL. A, B, C, E. Injected embryos were observed by epifluorescence microscopy. D, F. Embryos were observed by epifluorescence microscopy with faint illumination. Bars, 50 μm.*

TABLE 1

GERM LAYER-SPECIFIC EXPRESSION OF *EGIP-GFP* CONSTRUCT IN SEA URCHIN EMBRYOS[1]

Construct	Expression Rate[2]	Germ layer-specific expression[3]				
		Ectoderm	PMC	Endoderm	Aggregates[4]	Ectoderm+Aggregates[5]
EGIP-GFP	36/81 (44%)	25/36 (69%)	0/36 (0%)	0/36 (0%)	0/36 (0%)	11/36 (31%)
SM50-GL	100/131 (76%)	0/99 (0%)	55/99 (56%)	0/99 (0%)	20/99 (20%)	25/99 (25%)

[1]Prism embryos were collected at 24 hpf. Values represent data from at least three separate experiments carried out on independent batches of eggs.
[2]Positives versus total embryos are shown in addition to % expression in parentheses.
[3]Embryos positive in a given germ layer versus total positive embryos are shown in addition to % germ layer-specificity.
[4]Aggregates represent abnormal cell clusters that sometimes appear in embryos injected with plasmid DNA in some batches of embryos.
[5]Some injected embryos express *GFP* both in the ectoderm and abnormal cell clusters.
PMC, primary mesenchyme cells.

Discussion

Though the effects of exogenous EGIPs on the development of sea urchin embryos implicated the polypeptides in regulation of gastrulation, their exact roles in embryos are still elusive. To

address this issue, we have examined how expression of the *EGIP* gene is regulated in the process of the development. WMISH clearly showed that *EGIP* is expressed exclusively in the ectoderm. Most identified ectoderm-specific genes in sea urchin embryos are expressed rather evenly in either the oral or the aboral ectoderm, and their expression seems to be dependent on the lineage of the embryonic cells (Davidson, 1989). On the other hand, *EGIP* expression occurs in subsets of ectodermal cells in early gastrulae. The *EGIP* domain gradually expands in late gastrulae and prism embryos, and eventually the expression is seen in almost all the ectoderm in plutei, except the oral food and the tips of the postoral arms. Such dynamic changes in the expression region without fixed boundaries suggests that the expression of *EGIP* is not specified only by the cell lineage, but by influences from surrounding tissues. Vertical signals from PMCs, secondary mesenchyme cells, and the endoderm are candidate sources. Planar interaction is also possible within the ectoderm or with the endoderm considering the initial expression domain in early gastrulae. Recently such horizontal induction was suggested with regard to the differentiation of the oral ectoderm in sea urchin embryos (Yoshikawa, 1997).

EGIP expression proved to be sensitive to agents which disrupt the ECM. Thus, 5-*cis*-hydroxyproline suppresses the expression of *EGIP* effectively compared to other genes such as *AcEFP* and *AcSrc1*. Similar results were obtained with another ECM disrupter, β-aminopropionitrile, though not so significant (data not shown). These treatments suppress the endodermal differentiation (Wessel and McClay, 1987; Benson *et al.*, 1991), but do not significantly affect differentiation of the ectoderm (Wessel *et al.*, 1989). Thus it seems improbable that high sensitivity of *EGIP* to ECM disruption is due to the arrest of ectodermal differentiation. Rather, the present result suggests that *EGIP* expression requires external signal at least for its maintenance in addition to the differentiation of the ectoderm.

Dissociation of embryos after fertilization abolished the *EGIP* expression, while the same treatment only moderately suppressed the expression of other genes such as *AcEFP* and *AcSrc1*. Moderate sensitivity of *AcEFP* to cell dissociation in contrast to its refractoriness to ECM disruption seems to be ascribed to a more severe, though not so highly toxic, condition in terms of disruption of cell-cell interaction. It is likely that expression of *AcEFP* (and *AcSrc1*) also depends on the presence of neighboring cells, but not so strictly as *EGIP*. Similar dissociation experiments have been done before (Hurley *et al.*, 1989; Stephens *et al.*, 1989), showing that most ectoderm-specific genes are less sensitive to cell dissociation than *EGIP* expression (*CyIIIa*, *Spec1*, *Spec2a*, *Spec3*; 11-33% inhibition) in a similar way to *AcEFP* and *AcSrc1*. The only exception was a homeobox gene, *SpHbox1*, which is highly sensitive to cell dissociation similarly to *EGIP* (Hurley *et al.*, 1989). Interestingly, *SpHbox1* expression is also confined to subsets in the ectoderm just like *EGIP*. Taken all these together, *EGIP* expression seems to be regulated by external signals provided by ECM and/or surrounding cells. Our present data also suggest that the intercellular signaling is essential for *EGIP* expression especially after hatching. Our preliminary result showed that protein synthesis during gastrulation is indispensable for *EGIP* expression (data not shown), which is consistent with the idea that *EGIP* expression is regulated by cell-cell interaction, and not maternally programmed in the egg.

Recently variations in the expression level within single germ layers were reported for several tissue-specific genes in sea urchin embryos; *SM30*, *SM50*, and *msp130* in PMCs (Guss and Ettensohn, 1997), *SpEGFII* (Grimwade *et al.*, 1991) and *SpHbox1* (Angerer *et al.*, 1989) in the ectoderm, and *CyIIa* in the vegetal plate (Miller *et al.*, 1996). Especially, the level of expression for PMC-specific genes (*SM30*, *SM50*, and *msp130*) varies extensively depending on the localization of PMCs within the embryo, raising the possibility that signals from the ectoderm contributed to this variation of expression levels (Guss and Ettensohn, 1997). In this context, it is worthy of note that zygotic expression of *EGIP* in gastrulae seems to roughly coincide with the place of spiculogenesis. This coincidence might suggest a possibility that EGIPs from the ectoderm are involved in some aspects of spiculogenesis, though. further study will be needed to test this possibility.

The expression of another *EGIP*-like sea urchin gene, *SpEGFII*, was examined by *in situ* hybridization on sections (Yang *et al.*, 1989; Grimwade *et al.*, 1991). Exact comparison of expression between *EGIP* and *SpEGFII* is impossible due to the difference in the method of *in situ* hybridization (whole mount vs. section); however, the expression of both genes does not seem much different because their expression is restricted to subdomains of the ectoderm in plutei. On the other hand, there are a number of differences in the expression patterns of *EGIP* and *SpEGFII* (Grimwade *et al.*, 1991). 1) *EGIP* is expressed in ovaries and unfertilized eggs, while *SpEGFII* is not. 2) *EGIP* starts its zygotic expression at the onset of gastrulation and its transcript continues to increase in amount until the pluteus stage. In contrast, *SpEGFII* is zygotically expressed already in early blastulae, and show maximal expression around the early gastrula stage, then declines thereafter. 3) The expression of *SpEGFII* is more ubiquitous in earlier stages. In blastulae the expression of *SpEGFII* is seen as a broad band around the embryo, and in gastrulae it was detected in a generally uniform manner except for the ciliary band, where the gene expression is absent. Structural similarities, dynamic changes in expression pattern, and the restriction of expression to similar subsets of ectodermal cells make it probable that there are common functions of the two genes. Meanwhile, differences in the pattern of gene expression might suggest some functional diversification between the two genes in evolution. It remains to be examined if the two genes are orthologous or paralogous.

It is common that the spatial and temporal regulation of a given gene in sea urchin embryos is conducted at the transcriptional level (Davidson, 1986, 1989), though post-transcriptional regulation is also possible (Gagnon *et al.*, 1992). In the present study we examined whether the upstream sequence of 4.6 kb, including the 5'-untranslated region and 5'-terminal sequence of the coding region, was sufficient for the temporal and spatial regulation of *EGIP*. Initiation of the reporter gene expression during the development slightly precedes the initial increase in the level of *EGIP* mRNA, showing that the regulatory region directs the activation of the gene at the onset of gastrulation. Furthermore, the present result shows that the discrepancy of the accumulation profile of endogenous mRNA and the temporal pattern of *CAT* expression on a template basis in later phases can be explained by the stability of *EGIP* mRNA. Therefore, we assume that the regulatory region examined here is basically sufficient for the temporal regulation of the *EGIP* gene.

Though expression of the reporter *GFP* gene was highly mosaic, as is know with the present method (Hough-Evans *et al.*, 1988; Arnone *et al.*, 1997), the expression of the reporter gene was observed specifically in the ectoderm. We note that the numbers of expressing cells seem to be smaller for the *EGIP* transgene compared to those for other ectoderm-specific genes of the sea urchin (Arnone *et al.*, 1997; Hough-Evans *et al.*, 1988; Gan *et al.*, 1990). This is probably because the expression domain of *EGIP* is much smaller than these ectoderm-specific genes examined thus far, which are expressed uniformly throughout the entire aboral ectoderm. Because of the high mosaicism of the expression, however, it is impossible from the present data to conclude if the expression of the *EGIP-CAT* transgene is restricted to the same subdomain of the ectoderm as that of the endogenous gene. However, it is certain that at least the ectoderm specificity of the expression is provided by the 4.6 kb upstream region of *EGIP*.

Regulatory regions of gene are known to contain multiple *cis*-elements that are responsible for responses to a variety of *trans*-acting factors or different signals. Thus the regulatory DNA integrates such diverse information to generate on-off responses of the gene during the development of embryos (Kirchhamer and Davidson, 1996; Davidson, 1999). Now we have shown that the 4.6 kb upstream region of the *EGIP* gene contains *cis*-element(s) that are responsible for the temporal and spatial regulation of the gene. It will be interesting to test if the same regulatory region is sufficient for responding to external signals from the ECM or surrounding cells.

Materials and Methods

Preparation and culture of embryos

Gametes of the sea urchin *A. crassispina* were obtained, eggs fertilized, and embryos cultured at 23-24°C in filtered seawater (FSW; Yamasu *et al.*, 1995). 5-*cis*-hydroxyproline (Sigma) was used for disrupting the integrity of ECM (Saito and Yamasu, 1999).

Cell dissociation

Eggs 3 min after fertilization or pre-hatching blastulae at 8 h post-fertilization (hpf) were allowed to settle 3 times in filtered calcium/magnesium-free sea water (CMFSW), and cultured for appropriate times with gentle stirring (60 revs min^{-1}) in calcium-free sea water (CFSW). This procedure results in embryos which are completely dissociated to single cells with no cell clumps (Fig. 4A). When necessary, dissociated cells were transferred to normal FSW and allowed to develop further. After the transfer, dissociated cells reaggregate and formed embryoids with guts, pigment cells, and spicules (data not shown).

Northern blot analysis

Total RNA from staged embryos or adult tissues was purified by acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Five μg of RNA per lane was separated on a 1% formaldehyde gel unless specified otherwise, and the blotted membrane was hybridized with the 1.7 kb insert of the *EGIP* cDNA (pEG32; Yamasu *et al.*, 1995) labeled with [α-^{32}P]dCTP, washed, and subjected to autoradiography. When expression of more than one gene was compared, single blots were used repeatedly after removal of previously used probes.

Whole mount in situ *hybridization (WMISH)*

WMISH was performed essentially as described previously (Onodera *et al.*, 1999). Digoxigenin labeled RNA probe was prepared from *EGIP* cDNA in pBluescript SK(-) using T3/T7 RNA polymerase (Stratagene) and Dig Labeling Mixture (Boehringer Mannheim).

Expression plasmid constructs

The genomic clone for *EGIP* was obtained from a genomic library of *A. crassispina* constructed with EMBL3 (to be published elsewhere). From this clone, a *Pst*I fragment of 4.6 kb was obtained that includes at its proximal end 68 bp 5'UTR and 5'-terminal 27 bp of the coding region of the *EGIP* cDNA clone (pEG32) in addition to the upstream DNA of the *EGIP* gene (Fig. 5). This fragment was cloned into the *Pst*I sites of pCAT-basic (Promega) or pGreen Lantern-I (Gibco BRL) in the correct orientation in order to construct expression plasmids with *CAT* or *GFP* as a reporter gene, respectively.

Microinjection

Collection of gametes and microinjection into eggs were performed essentially as described by McMahon *et al.* (McMahon *et al.*, 1985) with slight modifications (Yamasu and Wilt, 1999). Plasmid constructs were linearized with *Nde*I or *Bam*HI. A five-fold excess of sperm DNA was included as carrier which had been digested with the same restriction enzyme used for digesting the constructs. The expression of the gene for CAT usually reached saturation above 8000 molecules of construct per egg; hence, 6000-8000 molecules of construct per egg were introduced throughout the present experiments. Experiments were repeated several times employing independently prepared reporter constructs and carrier DNA with basically the same results.

CAT assay

Injected embryos were collected and suspended in 0.25 M Tris-HCl (pH7.8). CAT assays were carried out as described by Gorman *et al.* (Gorman *et al.*, 1982) and McMahon *et al.* (McMahon *et al.*, 1985). Quantification of the enzyme reaction was performed on a Molecular Imager (BioRad) after separation by thin layer chromatography, and standardized by the incorporated plasmid DNA as was described before (Yamasu and Wilt, 1999).

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