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Implication of *HpEts* in Gene Regulatory Networks Responsible for Specification of Sea Urchin Skeletogenic Primary Mesenchyme Cells

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The large micromeres of the 32-cell stage of sea urchin embryos are autonomously specified and differentiate into primary mesenchyme cells (PMCs), giving rise to the skeletogenic cells. We previously demonstrated that *HpEts,* an ets-related transcription factor, plays an essential role in the specification of PMCs in sea urchin embryos. In order to clarify the function of *HpEts* in the gene regulatory network involved in PMC specification, we analyzed the zygotic expression pattern and the *cis*-regulatory region of *HpEts*, and examined the activity of the HpEts protein as a transcription factor. Intron-based PCR reveals that zygotic expression of *HpEts* starts at the cleavage stage, and that the rate of transcription reaches maximum at the unhatched blastula stage. A series of progressive deletions of the fragments from –4.2 kbp to +1206 bp of the *HpEts*, which directs PMC-specific expression, caused a gradual decrease in the specificity, implying that coordination of several *cis*-regulatory elements regulates the expression in PMCs. A minimum *cis*-element required for the temporal expression is located within a 10 bp from –243 bp to –234 bp. The HpEts protein remains in the cytoplasm of entire embryonic cells in the cleavage stage. At the unhatched blastula stage, the HpEts protein translocates into the nucleus in presumptive PMCs. Transactivation assays demonstrate that the HpEts protein activates a promoter of *Spicule Matrix Protein 50* (*SM50)*, which is a target of HpEts, which binds to the regulatory region of *SM50*.

Key words: Ets, sea urchin, primary mesenchyme cell, transcription, SM50, skeletogenesis

INTRODUCTION

In the sea urchin embryo, micromeres arise at the vegetal pole at the fourth cleavage. Micromeres function as a developmental organizer, inducing the overlaying embryonic cells to a vegetal plate fate (Davidson, 1989). When micromeres from the vegetal pole of a donor embryo are implanted into the animal pole of an intact recipient embryo, a complete respecification of cell fate occurs (Ransick and Davidson, 1993). The micromeres give rise to the large micromeres and the small micromeres at the fifth cleavage. The large micromeres are autonomously specified and differentiate into skeletogenic primary mesenchyme cells (PMCs) (Okazaki, 1975; Davidson, 1989, 1991), which express genes encoding spicule matrix proteins SM50 (Benson et al., 1987) and SM30 (George et al., 1991).

The gene regulatory network that underlies PMC specification and differentiation has been rigorously studied. It has been demonstrated that the translocation of β-catenin into nuclei of the micromeres is responsible for the initial specification of the large micromere lineage (Logan et al., 1999; Ettensohn and Sweet, 2000; Brandhorst and Klein, 2002; Angerer and Angerer, 2003; Ettensohn et al., 2004), and that *krl* (Howard et al., 2001) and *pmar1*/*micro1* (Oliveri et al., 2002; Nishimura et al., 2004) are the targets of β-catenin.

We previously demonstrated that the ets-related gene *HpEts* plays a key role in PMC specification and differentiation in the development of the sea urchin *Hemicentrotus pulcherrimus* (Kurokawa et al., 1999). Overexpression of *HpEts* enhances PMC specific *SM50* and represses ectoderm specific *HpArs* as well as endoderm-specific *HpEndo16*, causing differentiation of all the embryonic cells into PMC-like mesenchymal cells, whereas expression of a dominant negative *HpEts* represses *SM50* and the development of spicules. Thus, *HpEts* /*ets1* appears to directly activate promoters of genes such as a series of spicule matrix proteins responsible for PMC differentiation (Kurokawa et al., 1999; Zhu et al., 2001; Illies et al., 2002).

One recent study demonstrated that a double-negative

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regulatory system is involved in the activation of *ets1* in sea urchin embryos. A gene encoding a transcription factor HesC, which represses the transcription of *ets1*, is ubiquitously expressed in the cleavage stage. When the *pmar1*/*micro1* encoding a transcriptional repressor is activated specifically in micromeres, *ets1* is activated in the micromere descendants (Revilla-i-Domingo et al., 2007). However, the mechanism of regulation of *ets-promoter* and the function of Ets as a transcription factor remains elusive.

In a previous report (Kurokawa et al., 1999), we showed that a considerable amount of *HpEts* mRNA is maternally stored and present ubiquitously in cleavage stage embryos; however the significance of the accumulation of the *HpEts* mRNA in non-PMC lineage has been obscure. In the present paper, we show the involvement of HpEts in the gene regulatory network deployed in PMC specification. We report that zygotic expression of *HpEts* starts at the cleavage stage, cytoplasmic HpEts proteins in the presumptive PMCs translocate into the nucleus after the blastula stage, and binding of HpEts protein to the regulatory region of *SM50* enhances gene activity.

MATERIAL AND METHODS

Embryo culture

Adult *Hemicentrotus pulcherrimus* were collected from the seashore of Miura Peninsula or Boso Peninsula. Eggs and sperm were obtained by intracoelomic injection of 10 mM acetylcholine chloride. Eggs were washed several times and fertilized in Millipore filtered seawater (SW). Embryos were cultured in dishes of SW containing penicillin (50 mg/mL) and streptomycin (25 mg/mL) at 18°C.

Zygotic expression analysis by RT-PCR

Total RNA was extracted from *H. pulcherrimus* embryos at various developmental stages. cDNA was prepared from 5 μg of total RNA, and the following reverse transcriptase (RT) reaction was performed using a ThermoScript™ RT-PCR System (Invitrogen, USA) with random hexamers following the manufacturer's instructions. *HpEts* intron sequences were detected by the following polymerase chain reaction (PCR) with Ex Taq Polymerase (TAKARA, Japan) using 30 μM of gene specific primers for *HpEts* intron, intron Forward (5'-ATCTGCTGTGTGTGG-3'), and intron Reverse (5'-AACAGAGGGCGACAC-3') for 30 cycles (94°C 30 sec, 50°C 30 sec, 72°C 1 min) (Fig. 1A). As a control, *mitochondrial cytochrome oxidase subunit I* (*HpMitCOI*) mRNA, which is expressed constitutively at the same level during sea urchin development (Okabayashi and Nakano, 1983; Yamaguchi and Ohba, 1994; Fujiwara and Yasumasu, 1997) was detected by PCR using reaction mixtures containing 0.4 μM of *HpMitCOI*-specific forward primer (5'-GGCACAGCTATGAGTGTAATTATCC-3') and reverse primer (5'-GATAGTTCATCCAGTCCCTGCTC-3') for 22 cycles (94°C 30 sec, 58°C 30 sec, 72°C 1 min). The PCR products were separated on a 2% agarose gel, blotted onto a nylon membrane, and hybridized with the DNA fragment containing the first intron of *HpEts* or the coding region of *HpMitCOI* labeled with DIG DNA Labeling Mix (Roche). The signals were detected by antibody against anti-DIG conjugated alkaline phosphatase, and the chemiluminescence was captured on X-ray film (Fuji, Japan).

Screening and cloning of upstream region of *HpEts*

A *H. pulcherrimus* λ phage genomic library was plated on CES200 and hybridized with full-length *HpEts* cDNA labeled with digoxigenin (DIG)-11-UTP-labeled RNA. Following multiple rescreens using the same method, positive, isolated plaques were transferred to SM buffer (NaCl 5.8 g/L, $MgSO_4 \cdot 7H_2O$ 2 g/L, 2 M Tris-HCl 25 mL, 2% gelatin 5 mL), replated for amplification and the λ DNA was isolated using QIAGEN-tip20 (QIAGEN), following the manufacturer's instructions. A 13.5 kb DNA fragment was isolated following *Sal*I digestion, and subcloned into pBluescript II vector (pBSKII, Stratagene) to construct pBSK-13.5 (Figs. 3A and 5A). The presumptive transcription start site was determined by 5' RACE (Gene Racer, Invitrogen), following the manufacturer's instructions.

Reporter fusion gene constructs

A 13.5 kb *Sal*I fragment was obtained from the *HpEts* genomic clone (Figs. 3A and 5A), and then the fragment extending from –4.2 kb to +153 bp (accession number EU045842) was subcloned into the pBSKII vector (Stratagene, CA, USA). Progressive 5' deletion mutants were constructed by PCR with synthetic DNA primers (EXIGEN, Tokyo, Japan) using the –4.2 kb to +153 bp fragment as a template, and inserted into pGL2-Basic. Each of these fusion constructs was named according to the numbers of upstream base pairs of the inserted fragment, e.g., 525-LUC (–525 bp to +153 bp) and 313-LUC (–313 bp to +153 bp). For the analysis of *cis*-regulatory elements in the first exon and the first intron, the fragment from –525 bp to +688 bp was amplified by PCR, using pBSK-13.5 as a template, and the amplified DNA fragment was inserted into the pGLII-Basic vector. The structures of the deletion constructs are illustrated in Fig. 3.

GFP fusion constructs were obtained as follows: the fragment extending from –4.2 kb to +153 bp or –4.2 kb to +1206 bp was inserted into pGreenlantern-1 (GIBCO BRL) to produce 4K-GFP and D4K-GFP (Fig. 5B). Subsequent deletion constructs were created by PCR with synthetic primers using 4K-GFP as a template and named according to the number of the nucleotide of 5' end of the fragment from the transcription start site, e.g., 525-GFP, 313-GFP, etc.

Introduction of reporter fusion genes into the embryos and the *luciferase* reporter assay

Introduction of GFP fusion constructs was performed by injection, as described previously (Akasaka et al., 1995; Yajima et al.,

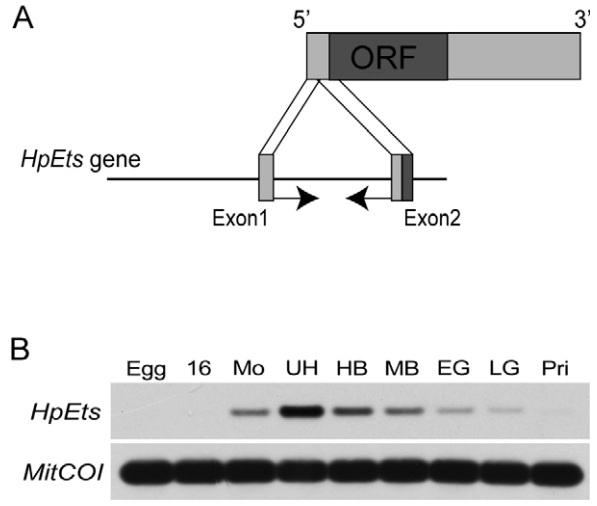

Fig. 1. Zygotic expression of *HpEts* mRNA during development. **(A)** Primers were designed within the first intron of *HpEts.* Arrows indicate the position of forward and reverse primers. Solid bar indicates the sequence of the fragment used as a probe detecting the amplified fragments. **(B)** Zygotic expression of *HpEts* represented by intron-based RT-PCR. Egg: unfertilized egg, 16 cell: 16-cell stage embryo, Mo: morula, UH: unhatched blastula, HB: hatched blastula, MB: mesenchyme blastula, EG: early gastrula, LG: late gastrula, Pri: prism larva.

2007). Expression of GFP was detected by fluorescence microscopy (TE300, Nikon, Japan). For the introduction of LUC fusion constructs into fertilized eggs, the particle gun method described by Akasaka et al. (1995) was used, and luciferase assays were conducted following the method described by Kurokawa et al. (1999). To normalize the luciferase activity, pRL-CMV (Promega) was co-introduced as a reference construct. Expression of constructs was determined using the Dual-luciferase Reporter system (Promega) in accordance with the manufacturer's instructions. Particle gun bombardment was carried out nine times for each construct. At the indicated periods after the fertilization, aliquots of bombarded embryos were collected by centrifugation, and stored at –70°C until used in luciferase assays.

Western blotting

Sea urchin embryos were collected at different developmental stages and the protein samples (50 μg/lane) were separated by SDS-polyacrylamide gel (10%) (SDS-PAGE) electrophoresis and transferred to PVDF membrane (MILLIPORE, Immunobion-P Transfer Membrane). After blocking for 1 hour in the blocking buffer (5% nonfat dry milk, and 1% normal goat serum in TBST ([150 mM NaCl, 2 mM Tris-HCl, 0.05% Tween-20, pH7.4]), and washing in TBST 5 times for 10 min each, the membrane was incubated with affinity purified anti-HpEts antibodies (Fuchikami, 2002) for one hour. The membrane was then washed 5 times in TBST for 10 min each, and incubated with the secondary antibody (Anti-Rabbit IgG (H + L), Goat, Horseradish Peroxidase; diluted 10^6) for one hour. After washing in TBST 3 times, bound antibodies were detected by Super Signal West Dura Extended Duration Substrate (Pierce).

Indirect immunostaining

Embryos were collected at the unhatched or mesenchyme blastula stage from which the fertilization membranes were previously removed by shaking the fertilized eggs in Ca^{2+}/Mg^{2+}-free seawater (SW). Embryos were fixed with 2% para-formaldehyde in SW for 5 min at room temperature, replaced with 100% cold methanol (–20°C stocked) for 5 min., then washed with SW, and stored at 4°C until use. For the immunostaining, fixed embryos in SW were placed into SW containing 0.1% Triton, washed with a series of TBST starting from 10%, 25%, 50%, 75%, 90%, and 100% for 5 min each. TBST was replaced by blocking buffer (4% goat serum in TBST) and incubated for an hour, then reacted with affinity purified anti-HpEts antibodies (Fuchikami, 2002) in blocking buffer for one hour. Specimens were next washed with TBST for 5 min, reacted with the secondary antibody (Anti-Rabbit IgG(H+L), Goat, Oregon Green; diluted 10^6) for an hour, and then washed with TBST. The embryos were observed by fluorescence microscopy (TE300, Nikon, Japan).

Transactivation assay

The transactivation assay in sea urchin embryos was performed according to the method described by Kiyama et al. (2000). The fusion promoter of *SM50* (Makabe et al., 1995), which contains the GAL4-binding sites instead of the Ets-binding site, was generated by inserting a UAS fragment (5-tandem-repeated GAL4-binding sites; Clontech) into the HpEts core-binding site in the *SM50* promoter sequence (–437 bp to +126 bp). The *SM50-UAS* fusion promoter was inserted into pGL2-Basic, constructing SM50-UAS-LUC (Fig. 8A). *Gal4-HpEts* fusion expression construct was generated by ligating the sequence encoding the *GAL4* (1–147) DNA binding domain (Clontech) in the correct reading frame to *HpEts* cDNA. The *GAL4-HpEts* fusion cDNA was connected to the 3' end of the *SM50* promoter, constructing SM50-GAL4-Ets (Fig. 8A). SM50-UAS-LUC with or without SM50-GAL4-Ets was introduced into fertilized eggs using the particle gun method, and the luciferase assay was performed at mesenchyme blastula and gastrula stages. To normalize the luciferase activity, *pRL-CMV* (Promega) was co-introduced as a reference construct and expression of the constructs was determined by the Dual-Luciferase Reporter System (Promega), as described in the instruction manual. The firefly luciferase activity driven by activation of *SM50-UAS* promoter was normalized based on the activity of *Renilla* luciferase.

RESULTS

Zygotic expression of *HpEts mRNA*

To distinguish zygotically expressed *HpEts* from maternally stored mRNA, we performed intron-based RT-PCR. Introns of the maternally mature mRNA have been spliced out, while the newly synthesized zygotic RNA retains the intron for a short period. Thus, the signal of intron-based RT-PCR represents rate of the RNA synthesis. RT-PCR revealed that the zygotic expression of *HpEts* starts at the morula stage; however, the rate of HpEts-RNA synthesis remained low in the early stages. The expression level reached maximum at the unhatched blastula (Fig. 1B). These results suggest that accumulation of *HpEts* mRNA in the early cleavage stage embryo is due to storage of maternal mRNA. *De novo* expression of *HpEts* decreases after the hatched blastula stage, and then nearly disappears by the prism larval stage.

Regulatory region required for temporal *HpEts* expression

We deduced the transcription start site from the sequence information of the cDNA obtained by 5' RACE (Fig. 2). To survey the region required for proper *HpEts*

Fig. 2. Sequence of promoter region (–550 bp to +300 bp) of *HpEts*. The transcription start site is indicated by a bent arrow. Underline indicates the first exon of *HpEts*. Genbank accession number (EU045842). The regions responsible for temporal (–243 bp to –234 bp) and spatial expression (–525 bp to –393 bp), respectively, are shaded in gray. Putative HES binding site in the fragment (–525 bp to –393 bp) responsible for the PMC specific expression, CAAT box and TATA box are highlighted in bold.

expression, a series of deletion constructs (Fig. 3B) was introduced into fertilized eggs, and the embryos were collected at the mesenchyme blastula stage to assay luciferase activity. Promoter activity was almost constant until the deletion of the 4K-LUC reached –243 bp, although some fluctuation of activity was observed in each deletion construct. When a fragment between –243 bp and –233 bp, which contains a CAAT box (Fig. 2), was deleted, the promoter activity decreased dramatically to the background level (Fig. 4A). The CAAT box at -239 bp may be responsible for the minimum promoter activity. The expression pattern of the deletion constructs during development revealed that the genomic region from –243 bp to +153 bp (243-LUC) is sufficient for a proper temporal profile of *HpEts* (Fig. 4B), as seen for the endogenous expression pattern of *HpEts* identified by intron-based RT-PCR (Fig. 1). A nearly identical expression level and pattern was obtained using a 4K-LUC that included a longer upstream region of *HpEts* (Fig. 4B), implying that core *cis*-elements responsible for the proper temporal expression reside in the fragment from –243 bp to +153 bp. No element related to the temporal expression of *HpEts* seems to exist in the first intron, down to +688 bp (Fig. 4C), since the reporter activity of 525-LUC (–525 bp to +153 bp) and D525-LUC (–525 bp to +688 bp) is almost the same (Fig. 3C).

Regulatory region required for PMC-specific *HpEts* expression

To identify the *cis*-regulatory regions of *HpEts* responsible for the PMC-specific expression, a fragment from –4.2 kb to +1206 bp was fused to a GFP reporter gene (Fig. 5A, 5B). A series of deletion constructs was injected into the fertilized eggs, and the resulting GFP expression was examined at late gastrula stage (Table 1). Approximately 90% of the embryos injected with D4K-GFP and 4K-GFP showed PMC-specific expression, suggesting that a fragment from –4.2 kb to +153 bp is almost sufficient for PMC-specific expression. The expression of GFP in the embryos injected with 4K-GFP was first observed in the presumptive PMCs at the hatched blastula stage (Fig. 6A, 6B), then in PMCs at the mesenchyme blastula stage (Fig. 6C, 6D), and remained detectable until the prism stage (Fig. 6E, 6F). When 2K-GFP or 1K-GFP was injected, the PMC-specific expression was decreased to approximately 80% (Table 1). PMC-specificity decreased slightly more in embryos injected with 525-GFP (66%). Significant decrease in the specificity was observed in the embryos injected with 393-GFP (28%), suggesting that *cis*-regulatory elements regulating PMC-specific expression exist between –525 bp and –393 bp (Table 1), although the subset of embryos showing PMC-specific expression retained nearly the same level as that of the longer fusion constructs. With shorter constructs (303-GFP), frequent ectopic expression was observed in ectoderm and endoderm at the late gastrula stage (Fig. 6G and H). *GFP* expression was almost imperceptible when 233-GFP or shorter constructs were introduced (Table 1), which is consistent with the results from the luciferase reporter assay described above. D4K-GFP (Fig. 5B) containing the first exon, the first intron, and the second exon showed similar specificity of expression compared to 4K-GFP (Table 1). These results suggest that a downstream

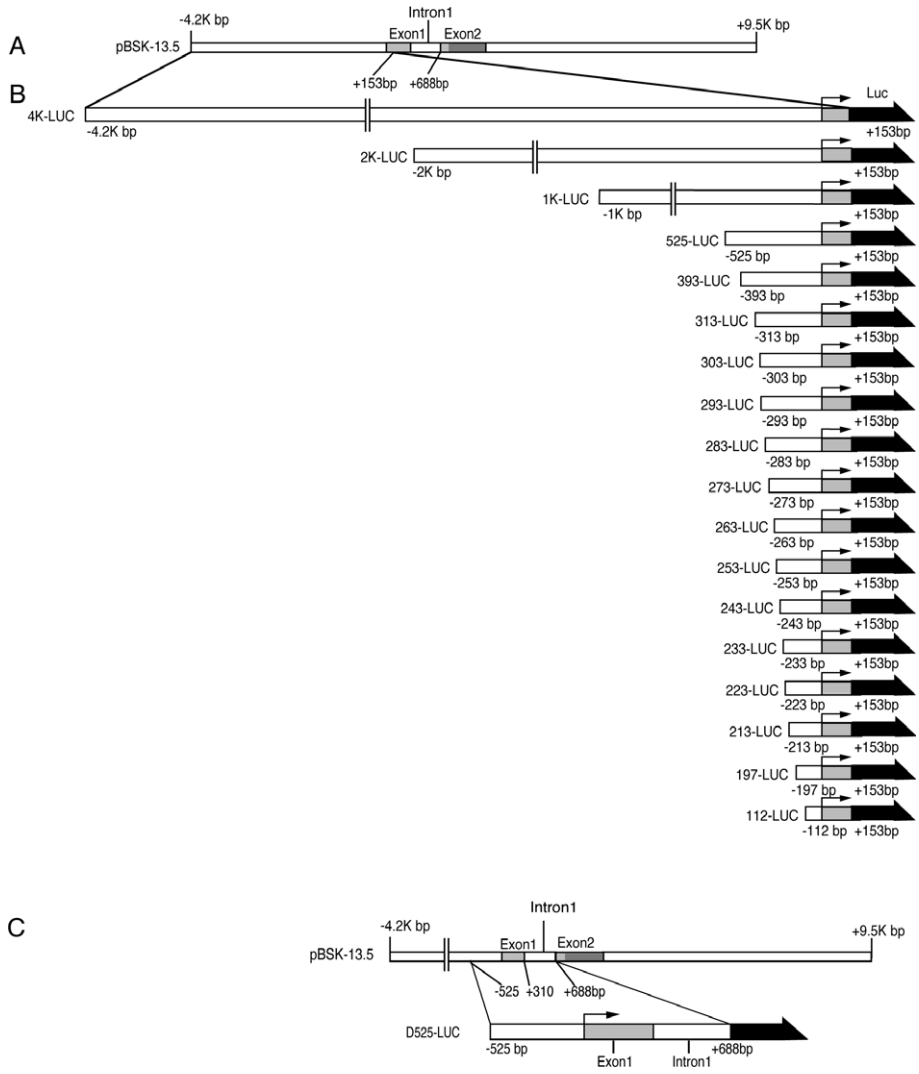

Fig. 3. Schematic diagrams of constructs used for the LUC reporter assay. **(A)** Fragment containing the transcription start site of *HpEts* (–4.2 kb to +9.5 kb) isolated from the genomic library. **(B)** Series of deletion constructs of the *HpEts-luc* fusion gene. Exons and *luc* are indicated in gray and black respectively. The coding region is indicated in dark gray. **(C)** Construct bearing the first exon and the first intron.

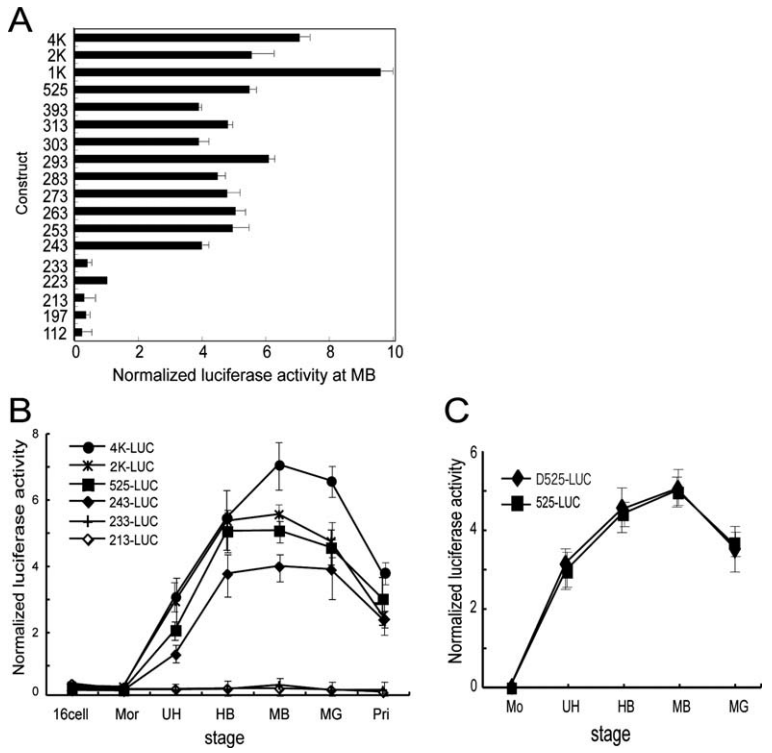

Fig. 4. Expression of the *HpEts-luc* fusion constructs. **(A)** Expression of series of the deletion constructs at mesenchyme blastula stage (MB). The digits indicated on the vertical axis of the graph show the deletion constructs. **(B)** Temporal expression patterns of the deletion constructs. **(C)** Comparison of temporal expression patterns between 525-LUC and D525-LUC, which contains the first exon and first intron. Bars indicate standard errors of nine independent experiments. 16 cell: 16-cell stage embryo, MO: morula, UH: unhatched blastula, HB: hatched blastula, MB: mesenchyme blastula, MG: mid gastrula, Pri: prism larva.

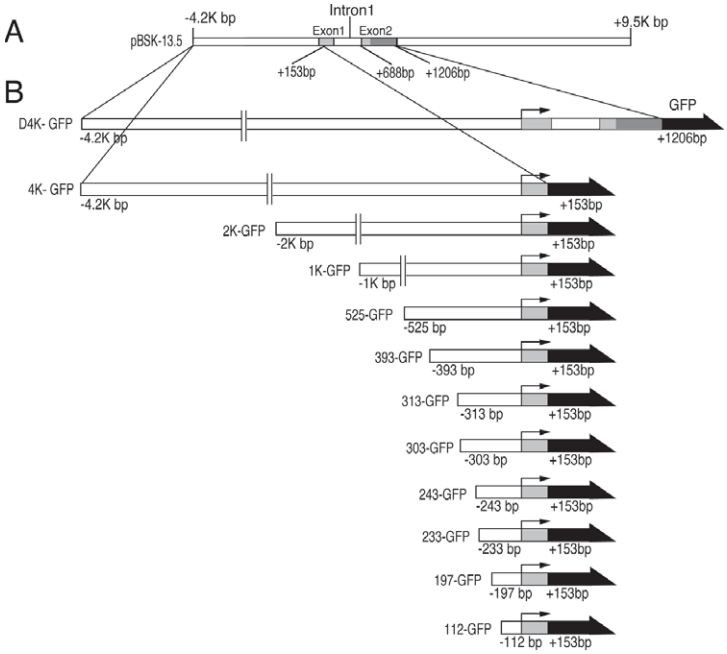

Fig. 5. Schematic diagrams of constructs used for the GFP reporter assay. **(A)** Fragment containing transcription start site of *HpEts* (–4.2 kb to +9.5 kb) isolated from the genomic library. **(B)** Series of deletion construct of the *HpEts-GFP* fusion gene. Exons and *GFP* are indicated in gray and black respectively. The coding region is indicated in dark gray.

Table 1. PMC-specific GFP expression in a series of deletion mutants

Construct[1]	PMC-specific expression (%)[2]	Total expression (%)[3]	N[4]
4K-GFP	91	52	229
2K-GFP	82	88	170
1K-GFP	78	67	232
525-GFP	66	73	349
393-GFP	28	62	165
313-GFP	35	62	178
303-GFP	27	89	165
243-GFP	28	66	196
233-GFP	25	26	110
197-GFP	11	25	256
112-GFP	4	27	160
D4K-GFP	89	23	310

1. Deletion constructs injected into fertilized eggs.
2. Percentage of embryos showing PMC-specific expression of GFP at late gastrula stage. The ratio of embryos showing PMC-specific GFP expression to total embryos expressing GFP, including ectopic expression, is shown.
3. Percentage of embryos showing GFP expression at the late gastrula stage. The ratio of embryos showing GFP expression including ectopic expression to total embryos injected with HpEts-GFP constructs is shown.
4. Total number of injected embryos.

region from +153 bp to +1206 is not involved in the PMC-specific expression.

Subcellular localization of HpEts protein

Ontogenic changes in the amount of HpEts protein was examined by western blotting using affinity purified anti-HpEts antibodies (Fuchikami et al., 2002). The HpEts protein is detected as a single band, and is relatively constant in abundance throughout development, although it decreases slightly at unhatched and hatched blastula stages (Fig. 7A). This is contrasted by the accumulation pattern of HpEts mRNA, which changes dramatically throughout development; the amount of HpEts mRNA is constantly abundant during early development until the unhatched blastula stage, and then decreases rapidly at hatching (Kurokawa et al., 1999).

Immunohistology reveals low-level accumulation of HpEts proteins in the basal side of the cytoplasm in the entire embryo before hatching (Fig. 7B). The HpEts protein is not detected in the apical side of the cell, in which the nucleus is localized in the cleavage and the early blastula stages. After hatching, HpEts protein signal disappears in the cytoplasm of all embryonic cells except for presumptive mesenchyme cells (Fig. 7C), and the HpEts protein is translocated into the nucleus of the presumptive PMCs (Fig. 7C and Fig. 7D). At mesenchyme blastula stage, the HpEts protein localizes specifically in PMCs (Fig. 7E) as previously reported (Fuchikami et al., 2002).

HpEts activates the *SM50* promoter

The *SM50* gene, encoding spicule matrix protein, is specifically expressed in PMCs in the

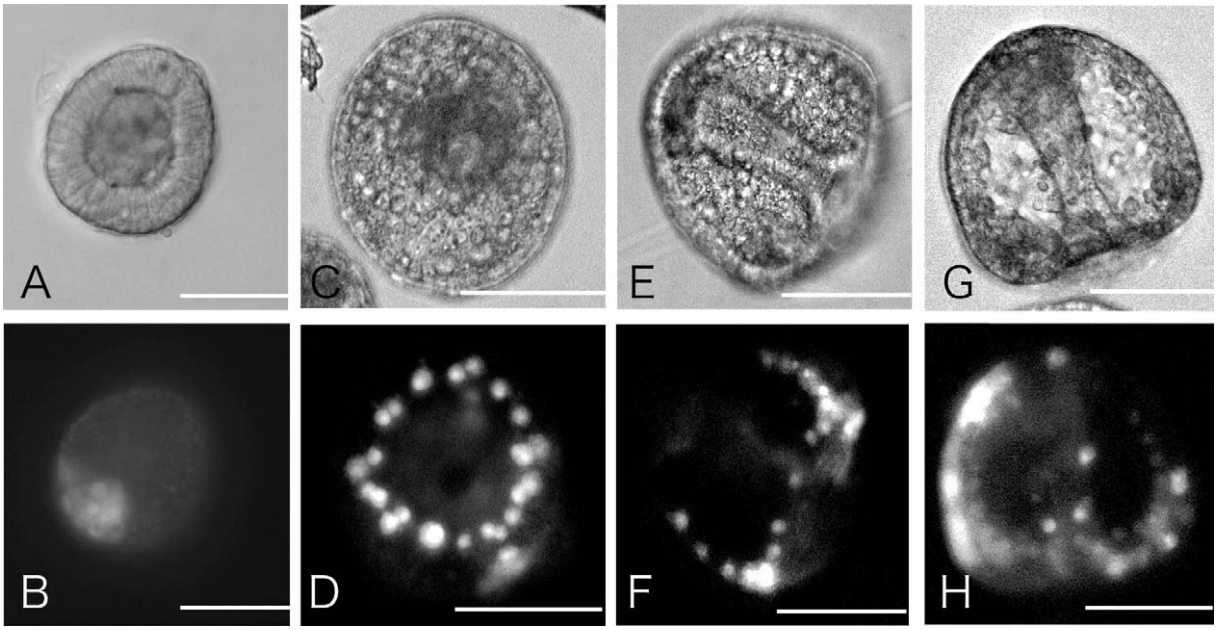

Fig. 6. Spatial expression pattern of GFP in the embryos injected with *HpEts-GFP* fusion genes. Embryos injected with 4K-GFP **(A, B, C, D, E, F)** or 303-GFP **(G, H)**. A and B: hatched blastula, C and D: mesenchyme blastula, E and F: prism larva. Ectopic expression of 303-GFP is shown in G and H. Bright field (A, C, E, G): fluorescence of GFP (B, D, F, H).

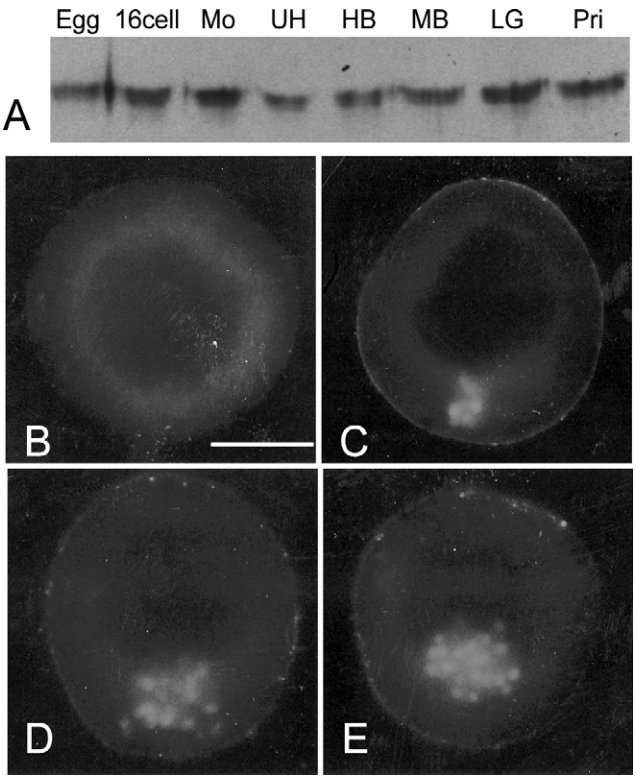

Fig. 7. Ontogenic change of HpEts protein in the amount and the localization. **(A)** Western blot detecting HpEts protein. Egg: unfertilized egg, 16 cell: 16-cell stage embryo, Mo: morula, UH: unhatched blastula, HB: hatched blastula, MB: mesenchyme blastula, LG: late gastrula, Pri: prism larva. **(B–E)** Indirect immunostaining of embryos with anti-HpEts antibody. Bar indicates 50 μm. B: unhatched blastula. C: hatching blastula. D: blastula. E: mesenchyme blastula.

embryo (Sucov, 1987). The *cis*-regulatory region of *SM50* contains a typical Ets-binding motif at –272 bp. As we reported previously, mutation into the core sequence of the motif causes a significant decrease in its transcriptional activity (Kurokawa et al., 1999). Thus, *SM50* is considered to be a target of HpEts. In order to examine the effect of binding of HpEts protein to the regulatory region of *SM50* on transcriptional activity, we performed a transactivation assay using the GAL4/UAS system, as described previously (Kiyama, 2000) (Fig. 8A). The Ets-binding motif in the *SM50-Luc* fusion construct was mutated by inserting the UAS sequence to avoid contamination by endogenous HpEts transcription factors in the embryonic cells. The GAL4-HpEts fusion protein, which contains the GAL4-DNA binding domain and entire HpEts polypeptides, was produced in the embryos introduced with *SM50-GAL4-HpEts*, driven by the *SM50* promoter. When the *SM50-GAL4-HpEts* and *SM50-UAS-Luc* were co-introduced into the embryos, GAL4-HpEts fusion protein activates the expression of the luciferase reporter construct driven by the fusion promoter *SM50-UAS* by one-and-a–half fold at mesenchyme blastula stage and over twofold at gastrula stage (Fig. 8B). These results suggest that *SM50* is a direct target of HpEts.

DISCUSSION

Maternal and zygotic expression of *HpEts*

HpEts plays a major role in PMC specification after the blastula stage, yet its mRNA is present at high levels in eggs and cleavage stage embryos. It has not been determined whether zygotic expression of HpEts is responsible for the accumulation of HpEts-mRNA in early stage embryos, or whether maternal HpEts is involved in PMC specification. We previously reported that HpEts-mRNAs, which are prom-

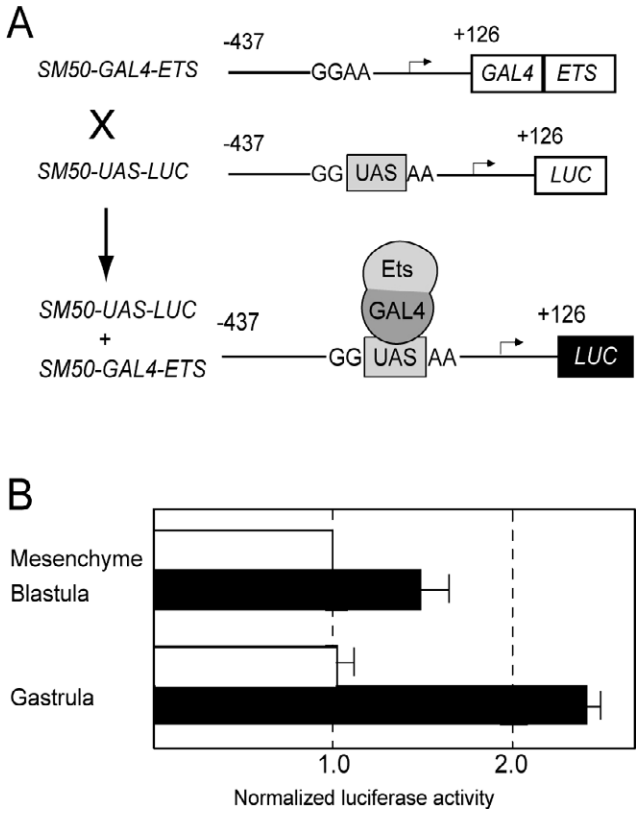

Fig. 8. HpEts protein activates *SM50* promoter. **(A)** Schematic diagrams of the effector and the reporter constructs. Fusion gene encoding GAL4-DNA-binding domain; HpEts was ligated to the *SM50* promoter. In order to construct the reporter gene, the *SM50* promoter, in which the HpEts binding site was destroyed by inserting *UAS* fragment, was ligated to the luciferase gene. The reporter construct was introduced into sea urchin fertilized eggs by particle gun with or without the construct *SM50-GAL4-ETS*, which produces Gal4-HpEts fusion proteins in the embryos. **(B)** Promoter activities represented as luciferase activities relative to that of *SM50-UAS-LUC* at the mesenchyme blastula stage. Luciferase activities in the embryos introduced with SM50-UAS-LUC alone (open bar) or co-introduced with SM50-UAS-LUC and SM50-GAL4-Ets (filled bar) were determined. Bars indicate standard errors of nine independent experiments. The activity of the open bar at mesenchyme blastula stage was set as a standard to 1.0.

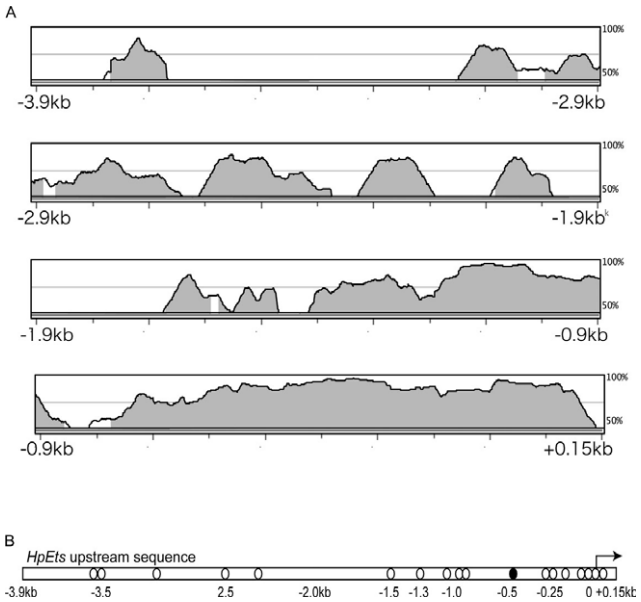

Fig. 9. Sequence comparisons of upstream regions between *HpEts* and *SpEts1*. **(A)** VISTA outputs were based on a 100-bp sliding window and a sequence identity range of 50–100%. Regions of similarity greater than 50% are shown in the diagram. **(B)** Putative HesC-binding sites in the regulatory region of *HpEts*. Circles indicate putative HesC-binding sites. Closed circle indicates putative HesC-binding site in the region, which plays major role in the PMC-specific expression of *HpEts*.

inent in cleavage stage embryos, diminish rapidly in the whole embryo with the exception of presumptive PMCs at the hatched blastula stage (Kurokawa et al., 1999). In the present study, using intron-based PCR, we demonstrated that zygotic expression of *HpEts* starts at the morula stage (Fig. 1B). The rate of HpEts-RNA synthesis remains low at the morula stage and reaches maximum at the unhatched blastula stage, suggesting that the prior high level of HpEts-mRNA is maternal in origin. It has been shown that nuclear accumulation of β-catenin (Logan et al., 1999; Ettensohn and Sweet, 2000; Brandhorst and Klein, 2002; Angerer and Angerer, 2003; Ettensohn et al., 2004), *pmar/micro1* (Oliveri et al., 2002; Nishimura et al., 2004), and *HesC* (Revilla-i-Domingo et al., 2007) are involved in the expression of *HpEts/ets1* in sea urchin embryos. Micromere-specific expression of *pmar/micro1*, which represses repressor HesC at morula stage, seems to induce the onset of zygotic expression of *HpEts*.

Highly conserved sequences in the regulatory region of *HpEts* and *Sp-ets1*

Luciferase reporter assays demonstrate that there are one or more *cis*-regulatory elements responsible for proper temporal expression of *HpEts* between –243 bp and –233 bp, in which a typical CAAT box (CCAAT) is located at –239 bp. The CAAT box is also conserved in the same position of the upstream region of *ets1* in the *Strongylocentrotus purpuratus* genome, supporting the idea that the CAAT box functions as a *cis*-regulatory element in both species. A reporter assay using a series of deletion constructs of *HpEts-GFP* fusion gene indicated that shorter constructs show more ectopic expression in deleted-length-dependent manner, suggesting that multiple repressor binding sites involved in the restriction of the expression to PMCs are located along the upstream region from –4.2 kb to –393 bp. In particular, the deletion of the region from –525 bp to –393 bp causes a significant reduction in the specificity of the expression in PMCs, suggesting that the fragment contains the major spatial *cis*-regulatory element(s). Comparison of the sequence of upstream regions of *HpEts* and *S. purpuratus ets1* reveals that the sequence from –3.9 kb to +150 bp is highly conserved (Fig. 9A) between the two species, supporting our hypothesis that multiple *cis*-elements responsible for *HpEts* spatial transcription are located along the upstream region. It has been reported that HesC restricts the expression of *ets1* to PMCs in *S. purpuratus* embryos

(Revilla-i-Domingo et al., 2007). A number of putative binding sites (CACNNG) of HES (Sasai et al., 1992; Tietze et al., 1992; Oellers et al., 1994; Ohsako et al., 1994; Van Doren et al., 1994) are found along the entire 4.2 kb upstream region of *HpEts* (Fig. 9B), and in the upstream regions *S. purpuratus ets1* as well. It should be noted that the fragment from –525 bp to –393 bp, which plays a major role in the PMC-specific expression of *HpEts*, contains an HES binding motif at –465 bp, and that the HES binding motif is conserved in *S. purpuratus ets1* at the same position. It is possible that the multiple HES binding motifs, especially the binding motif at –465 bp, function cooperatively to restrict the expression of *HpEts*/ *ets1* to PMCs.

Translocation of HpEts protein into the nucleus

In this study, we showed that the amount of HpEts proteins remains nearly steady throughout development, and that the HpEts protein is localized in the cytoplasm in the cleavage stage (Fig. 7B). It is likely that the maternally-stored prominent HpEts-mRNA is either not translated or the translation level is very limited. Regulatory systems involved in cytoplasmic localization of HpEts proteins at the cleavage stage, degradation of maternal HpEts-mRNA and the protein at the hatching blastula stage, and the zygotic expression of *HpEts* in presumptive PMCs may cooperate in the specification of PMCs. Localization of HpEts proteins in the cytoplasm at the cleavage stage suggests that the HpEts protein does not function as a transcription factor before the hatching blastula stage.

It has been reported that a transient and highly localized activation of the MAP kinase ERK occurs in the micromere lineage and that the Raf/MEK/ERK signaling pathway is involved in the specification of PMCs and their subsequent transition from an epithelial to a mesenchymal state (Rottinger et al., 2004). They suggested that Ets1 is a putative target of phosphorylation by ERK. In future experiments, relationships between the translocation of the HpEts protein and Raf/MEK/ERK signaling should be examined.

Expression of *Ets* in the secondary mesenchyme cells (SMCs) at later stages has been reported (Rizzo et al., 2006; Duboc et al., 2010). We also confirmed expression of *HpEts* in SMCs in *H. pulcherrimus* at the gastrula stage (data not shown). However, we did not detect a GFP signal in SMCs in embryos injected with 4K-GFP (Fig. 6D, 6F). It appears that the expression of 4K-GFP in SMCs does not reach a detectable level at the gastrula and prism stages, or that 4K-GFP does not include any *cis*-regulatory elements that drive its expression in SMCs. Since we focused on the regulation of the expression of *HpEts* in PMCs at early stages, we did not analyze the regulation of *HpEts* expression or the localization of HpEts protein in SMCs in the present study.

HpEts directly activates transcription of *SM50*

A spicule matrix protein SM50 (Benson et al., 1987) is involved in biomineralization, which occurs in PMC progeny in sea urchin embryos (Wilt, 1999; Wilt et al., 2008). We have reported that the Ets-binding site located at –272 bp in the *SM50* transcriptional regulatory region is essential for its expression (Kurokawa et al., 1999). The transactivation assay clearly demonstrates that the HpEts transcription factor directly activates the *SM50*-promoter (Fig. 8B). The present study has revealed the role of *HpEts* in this regulatory cascade, beginning with the translocation of β-catenin into the nucleus in the 16-cell stage embryo through to the target structural gene, *SM50*.

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