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Spatially regulated SpEts4 transcription factor activity along the sea urchin embryo animal-vegetal axis

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SUMMARY

Because the transcription of the *SpHE* gene is regulated cell-autonomously and asymmetrically along the maternally determined animal-vegetal axis of the very early sea urchin embryo, its regulators provide an excellent entry point for investigating the mechanism(s) that establishes this initial polarity. Previous studies support a model in which spatial regulation of *SpHE* transcription relies on multiple nonvegetal positive transcription factor activities (Wei, Z., Angerer, L. M. and Angerer, R. C. (1997) *Dev. Biol.* 187, 71-78) and a yeast one-hybrid screen has identified one, SpEts4, which binds with high specificity to a cis element in the *SpHE* regulatory region and confers positive activation of *SpHE* promoter transgenes (Wei, Z., Angerer, R. C. and Angerer, L. M. (1999) *Mol. Cell. Biol.* 19, 1271-1278). Here we demonstrate that SpEts4 can bind to the regulatory region of the endogenous *SpHE* gene because a dominant repressor, created by fusing SpEts4 DNA binding and *Drosophila* engrailed repression domains, suppresses its transcription. The pattern of expression of the *SpEts4* gene is consistent with a role in regulating *SpHE* transcription in the nonvegetal region of the embryo during late cleavage/early blastula stages. Although maternal transcripts are uniformly distributed in the egg and early cleaving embryo, they rapidly turn over and are replaced by zygotic transcripts that accumulate in a pattern congruent with *SpHE* transcription. In addition, in vivo functional tests show that the SpEts4 cis element confers nonvegetal transcription of a β-galactosidase reporter gene containing the *SpHE* basal promoter, and provide strong evidence that the activity of this transcription factor is an integral component of the nonvegetal transcriptional regulatory apparatus, which is proximal to, or part of, the mechanism that establishes the animal-vegetal axis of the sea urchin embryo.

Key words: SpEts4, Transcription factor, Sea urchin embryo, Polarity, Maternal regulation

INTRODUCTION

The establishment of polarities in the egg or early embryo that define embryonic axes are of fundamental importance in developmental biology. In sea urchin embryos, the animal-vegetal (AV) axis is established maternally during oogenesis, as shown by classic egg bisection experiments that revealed the different developmental potentials of animal and vegetal hemispheres (Boveri, 1901a,b; Hörstadius, 1939; Maruyama et al., 1985). During cleavage of the unperturbed embryo, a relatively invariant geometry of initial cell divisions is superimposed on this AV asymmetry, such that the fates of early blastomere tiers arrayed along it are largely predictable (reviewed by Davidson, 1989). These initial polarities provide foci of molecular information which produce gradients of gene regulatory activities and/or initiate cascades of cell-cell interactions that ultimately define and refine the positions of different embryonic anlagen in a three-dimensional coordinate system. Although embryos of a wide variety of taxa have maternally determined AV axes (Davidson, 1991), the molecular architecture of these axes is not understood for any such embryo.

Several years ago we identified a set of mRNAs that accumulate transiently at the very early blastula stage (VEB) (VEB messages; 12-15 hours; about 150 cells). The VEB genes are activated by the 8-cell stage and, at the 16-cell stage and thereafter, transcripts are detectable only in nonvegetal blastomeres (Nasir et al., 1995; Reynolds et al., 1992). The fact that the activation of the VEB genes is cell autonomous (Reynolds et al., 1992; Ghiglione et al., 1993; Nasir et al., 1995) indicates that it must be mediated by transcription factor activities that are also asymmetrically arrayed along the maternally specified animal-vegetal axis. Consequently, spatial regulators of VEB gene activity are likely to be part of, or closely linked to, the mechanism(s) that establishes the primary axis of sea urchin embryos.

Detailed analysis of the regulatory architecture of one of these genes, *SpHE,* which encodes the hatching enzyme, has revealed that cis-acting elements sufficient for high level, spatially correct expression are contained within a compact region 300 base pairs (bp) upstream of the transcription start site (Wei et al., 1995). This region is densely packed with at least nine sites for binding of six different proteins; most of

these confer positive promoter activity (Wei et al., 1995, 1997a). Furthermore, at least three nonoverlapping subsets of these elements sponsor expression only in nonvegetal cells, leading to the model that multiple positive gene regulators are partitioned, at least functionally, to the nonvegetal embryonic domain of the early embryo (Wei et al., 1997a). To begin to test this model, we isolated a gene, *SpEts4*, encoding one of the *SpHE* promoter binding proteins using a yeast one-hybrid screen (Wei et al., 1999), and demonstrated that the SpEts4 protein binds its target cis element in vitro with a specificity identical to that observed for proteins in early embryo nuclear extracts. Transactivation assays showed that SpEts4 contains an N-terminal activation domain and can mediate activation of *SpHE*/reporter transgenes both in yeast and in sea urchin embryos (Wei et al., 1999).

While our previous studies demonstrated that SpEts4 can bind the *SpHE* promoter in vitro and confer positive activity on *SpHE* transgene transcription in vivo, it is important to test whether it can also regulate transcription of the endogenous gene. Here we show by RNase protection that a chimeric protein containing the *Drosophila* engrailed dominant repression domain linked to the SpEts4 DNA binding domain suppresses transcription of the endogenous *SpHE* gene. Embryos expressing the chimeric repressor do not hatch, although extensive differentiation of all tissue types occurs. Since SpEts4 is one of the activators of *SpHE* transcription (Wei et al., 1999), we explored the possibility that it is also expressed in nonvegetal cells. *SpEts4* maternal transcripts, which are uniformly distributed in the egg and early cleaving embryo, disappear from the embryo between the 64-cell stage and 12 hours postfertilization, when *SpHE* transcription is maximal, and are replaced by embryonic *SpEts4* transcripts that accumulate only in the nonvegetal domain around the 6th cleavage. Furthermore, the SpEts4 protein appears to function only in nonvegetal cells, since the SpEts4 cis element linked to the *SpHE* basal promoter region is sufficient to drive detectable expression of a β-galactosidase reporter only in this region of the embryo. These data provide strong support for a model in which the animal-vegetal axis of the sea urchin embryo is defined by the separation of positive transcription factor activities into nonvegetal and vegetal domains.

MATERIALS AND METHODS

Construction of EngEts4

The construct for expressing the dominant repressor EngEts4 fusion protein was made by adapting the SE-pXT7 plasmid (Fan and Sokol, 1997; gift from the authors). *Siamois* sequence in the plasmid was replaced by that encoding the SpEts4 DNA binding domain: a PCR fragment of *SpEts4* (encoding amino acids 269-363) was linked to the *engrailed* sequence (296 amino acids), which encodes the repression domain, and inserted into the plasmid at the *Hin*dIII and *Eco*RI sites. The control construct encoding only the engrailed repression domain was made by inserting the *engrailed* fragment at *Hin*dIII and *Bgl*II sites of pXT7. The constructs were verified by sequencing.

In vivo transcription activity assays

Measurements of promoter activity in vivo were made using constructs carrying the wild-type *SpHE* promoter driving a bacterial chloramphenicol acetyltransferase (CAT) reporter gene. These constructs (approx. 2500 copies) were microinjected into fertilized *S. purpuratus* eggs along with synthetic capped, polyadenylated mRNA (approx. 0.2 pg, equivalent to approx. 2×10^5 molecules) encoding either the engrailed repression domain alone (Eng) or a chimeric protein in which this domain was linked to the SpEts4 DNA binding domain (EngEts4). Assays of CAT activity and transgene DNA content were done exactly as described previously (Wei et al., 1995).

In vitro translation

The *SpEts4* cDNA was transferred to pGEM-T Easy plasmid (Promega) and protein was synthesized using 1 μg of linearized plasmid and the TNT Coupled Reticulocyte Lysate kit (Promega) in the presence of [^{35}S]methionine (40 pmol; 1000 Ci/mmol). The labeled products (1 μl of the 50 μl reaction) were assayed for size by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoretic mobility shift assay (EMSA)

EMSAs were conducted using proteins translated in vitro as described previously (Wei et al., 1999). Probes (base pairs −261 to −241 in the *SpHE* regulatory region) containing the Ets (CAATTCAAACC-<u>CGGAA</u>CTACG) motif (core sequence underlined) were end-labeled with [γ-^{32}P]ATP to a specific activity of approximately 2.0×10^6 cpm/pmol. For each reaction, approx. 4.5 fmol of probe was incubated on ice for 10 minutes with 0.5 μl of the in vitro translation reaction (see above) (Calzone et al., 1988). To test for specificity of binding, various competitor DNAs, each containing a different variant Ets binding site sequence, were added in 200-fold molar excess. The reactions were then fractionated by electrophoresis through nondenaturing 5% polyacrylamide gels.

RNase protection assays

Embryo RNA was prepared using the TRIzol protocol (Gibco-BRL) from both 9-hour-injected and uninjected embryos at selected developmental stages. Probes complementary to *SpEts4* mRNA (from −102 to +99 bp relative to the translation initiation site) and *SpHE* sequences (from +316 to +543 bp relative to the transcription start site and containing exon 1 and intron 1 sequences) were labeled with [α-^{32}P]UTP to specific activities of 6×10^8 cpm/μg and 0.75×10^8 cpm/μg, respectively. The *SpEts4* and *SpHE* probes protect 202-nucleotide (nt) and 110-nt fragments, respectively. 0.5 ng of each probe was hybridized with either a mixture of 8.5-hour and 12-hour embryo RNA (0.5 μg each), or RNA isolated as described above from 200 microinjected embryos. Hybridization, RNase digestion and electrophoresis of the protected products were conducted as previously described (Yang et al., 1989).

Spatial in vivo transcription assays

The SpEts4 cis element was tested for whether it mediates transcriptional activation in nonvegetal and vegetal blastula cells by microinjecting into fertilized *S. purpuratus* eggs constructs composed of the *SpHE* basal promoter region (−90 to +20 bp), either containing or lacking this site, and linked to the β-galactosidase reporter gene. At 30-32 hours, embryos were stained with X-gal and the primary mesenchyme cell (PMC)-specific 6e10 antibody (a gift of Dr C. Ettensohn, Carnegie Mellon) as described previously (Wei et al., 1995, 1997a). This double-staining approach distinguishes between true primary mesenchyme cells in the blastocoel and a few other abnormal cells that appear there as a result of microinjection.

RNA blots

RNA from selected developmental stages was isolated as described above for RNase protection assays. The quantity of RNA was determined by spectrophotometry and its quality was verified by gel electrophoresis on formaldehyde-containing agarose (1%) gels. Probes representing either the 5′ untranslated region (UTR) plus coding region sequence (−112 to +740 relative to the translation start site) or the 3′ UTR region (+1815 to +2894) were randomly primed with [α-^{32}P]dATP and dCTP to a specific activity of 2.1×10^8 cpm/μg.

Each probe (approx. 5×10^6 cpm) was hybridized to blots of embryo RNAs in the presence of 5× Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% BSA), 2× SSC (0.3 M NaCl, 0.03 M trisodium citrate), 1% SDS, 25 mM sodium phosphate, pH 7.5, 10% PEG (polyethylene glycol) and 50 µg salmon sperm DNA/ml at 60°C overnight. The blots were washed stringently in 0.1× SSC at 60°C.

In situ hybridization

In situ hybridization was carried out as described previously (Angerer et al., 1987) on 5 µm sections of embryos of selected stages, using riboprobes labeled with [α-^{33}P]UTP to specific activities of 1×10^8 cpm/µg (*SpHE* coding region), 3×10^8 cpm/µg (*SpEts4* 3′ UTR) and 1.5×10^8 cpm/µg (*SpEts4* coding region).

RESULTS

The dominant repressor, EngEts4, binds with the same specificity as SpEts4 to the *SpHE* Ets cis element

Previously we used the *SpHE* regulatory region and a yeast one-hybrid screening approach to isolate a gene encoding a new sea urchin Ets family member. This gene was named *SpEts4* because its closest relative in the conserved DNA binding (Ets) domain is the *Drosophila Ets4* gene (Chen et al., 1992). In EMSAs, recombinant SpEts4 translated in vitro binds specifically to an Ets recognition motif in the *SpHE* promoter, previously called Site IC (Wei et al., 1995), and the complex formed is competed differentially with variants of Ets motifs with relative efficiencies identical to those observed for a protein in 9-hour embryo nuclear extracts. Although the interaction of SpEts4 with DNA appears to be highly specific in these in vitro assays, it is important to test whether SpEts4 can also regulate the *SpHE* promoter in vivo. Although we were previously able to detect transactivation by injected SpEts4 mRNA of *SpHE* promoter transgenes that are present at several thousand copies in the cells that inherit them, we reasoned that it might be difficult to transactivate the two copies of the endogenous gene if the endogenous SpEts4 concentration were high enough to be in excess, as we suspected, because *SpEts4* mRNA is relatively abundant in embryos (see below). Furthermore, the contribution of SpEts4 to the output of the *SpHE* regulatory region is relatively modest because many different trans-factors provide positive activity. Therefore, we tested the converse approach of using the *Drosophila* engrailed repression domain (Fan and Sokol, 1997) to create a dominant repressor of *SpHE* transcription acting *via* the SpEts4 cis element.

The construction of the chimeric gene, designated *EngEts4*, which encodes the SpEts4 DNA binding domain linked to the engrailed repression domain, is shown in Fig. 1A and described in Materials and methods. The DNA binding domain was placed at the C terminus as in the native SpEts4 protein and the engrailed repression domain constitutes the N-terminal region. We also prepared a construct encoding only the repression domain, and synthesized both of these proteins and SpEts4 in vitro using a coupled transcription/translation method. These constructs were verified by analyzing the [^{35}S]methionine-labeled products by SDS-PAGE (Fig. 1A), which shows that the size of the largest species in each case is in close agreement with values predicted from their respective amino acid sequences. These translation products were further tested by EMSA for their ability to bind specifically to the *SpHE* Ets4 site (Fig. 1B). As expected, the engrailed repression domain does not interact detectably with the *SpHE* Ets4 site, whereas the chimeric protein forms a complex. Although EngEts4 is larger than SpEts4, its greater negative charge results in a complex that migrates more rapidly in this native gel. To check whether EngEts4 binds with the same specificity as SpEts4 itself, a series of competition assays was performed with variant Ets site motifs (*Drosophila* E74; PU.1 mammalian

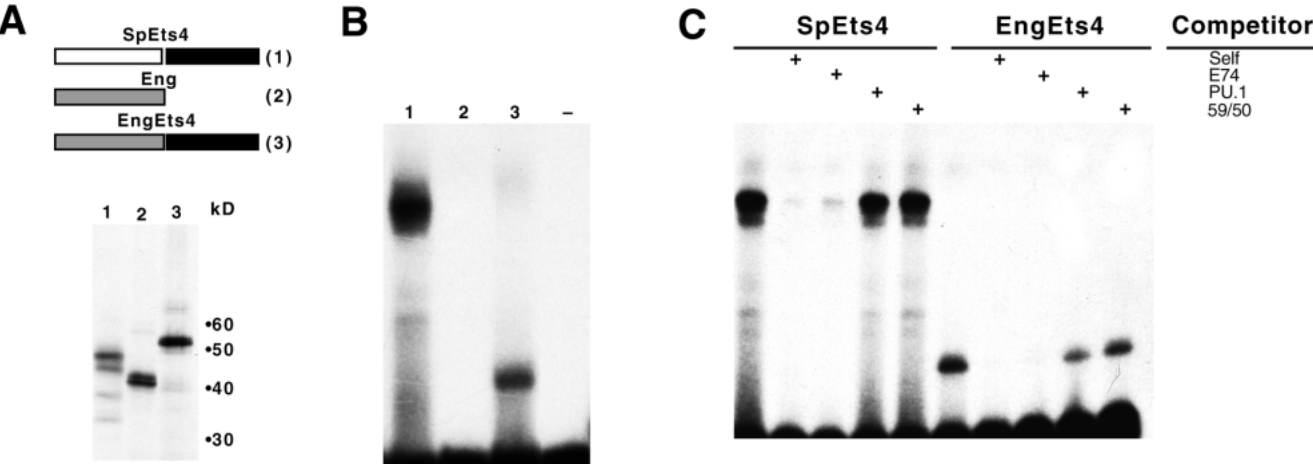

Fig. 1. Characterization of recombinant proteins Eng and EngEts4. (A) The upper diagram shows the structures of the recombinant proteins. SpEts4 is the complete protein where the DNA binding domain is indicated by a black box; the SpEts4 N-terminal region is represented by an open box and the engrailed repression domain by a shaded box. The lower panel shows an autoradiogram of ^{35}S-labeled proteins synthesized in vitro and after separation by SDS-PAGE. The positions of marker proteins (kD) are shown. (B) EMSA was carried out by using a labeled probe representing the SpEts4 binding motif from *SpHE* and the indicated recombinant proteins 1-3 translated in vitro; –, no added protein.
(C) EMSA was carried out as in B using either SpEts4 (left five lanes) or EngEts4 (right five lanes). Binding was carried out in the absence of competitor or in the presence of a 200-fold molar excess of unlabeled probe sequence (self) or of three different variants of Ets binding sites (E74, PU.1 or 59/60).

Ets subclass; and a sea urchin Ets site found in the early histone H3 promoter, 59/60). Previously we have demonstrated that sea urchin 9-hour embryo nuclear protein (Wei et al., 1995) and in vitro-translated SpEts4 (Wei et al., 1999) each bind to these different sites with similar relative affinities, providing a sensitive test of binding specificity. In vitro-translated SpEts4 and EngEts4 exhibit the same pattern of competition, as shown in Fig. 1C, left five lanes and right five lanes, respectively, indicating that the presence of the engrailed domain does not detectably alter the interaction between the SpEts4 DNA binding domain and the *SpHE* SpEts4 site.

EngEts4 acts as a dominant repressor of SpHE transcription in sea urchin blastulae

To test whether EngEts4 can repress transcription of a *SpHE* promoter transgene, we introduced the −300 nt *SpHE* promoter linked to the chloramphenicol acetyl transferase (CAT) reporter gene into sea urchin 1-cell zygotes by microinjection, as described previously (Wei et al., 1995, 1997a), along with synthetic capped, polyadenylated mRNA encoding either EngEts4 or the control engrailed domain (Eng) protein. Groups of either 25 or 75 embryos were assayed for CAT activity and each set of samples provided the same results (Fig. 2, right). The engrailed repression domain alone has no effect on transgene transcription (cf. activity levels when no mRNA was injected, −). In contrast, the EngEts4 repressor reduces activity to almost undetectable levels. Slot blot analysis of DNA eliminated the possibility that this difference resulted from differences in transgene levels among samples (Fig. 2, left). This result shows that EngEts4 efficiently suppresses transgene transcription and complements our previous finding that injection of synthetic SpEts4 mRNA results in the activation of a transgene containing the SpEts4 cis element.

To test whether EngEts4 can also repress endogenous *SpHE*

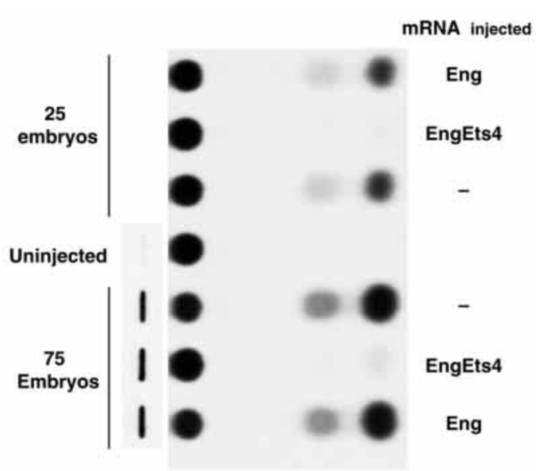

Fig. 2. The EngEts4 chimeric protein blocks activity of the *SpHE* promoter on transgenes. A construct consisting of the −300 bp *SpHE* promoter coupled to the CAT structural gene was microinjected into 1-cell sea urchin zygotes alone (−) or in combination with synthetic mRNA encoding the engrailed repression domain alone (Eng) or linked to the SpEts4 DNA binding domain (EngEts4). At 20 hours postfertilization embryos, harvested in sets of 25 or 75, were assayed for CAT enzyme activity (right). Samples of the lysate from the 75-embryo pool were processed for DNA slot blots for content of the transgene construct (left).

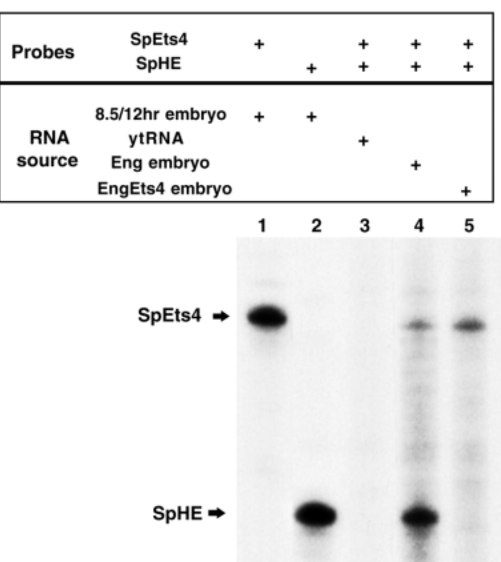

Fig. 3. The EngEts4 chimeric protein represses expression of embryonic *SpHE* genes. Levels of *SpHE* mRNA were analyzed by RNase protection. (Lanes 1 and 2) *SpEts4* and *SpHE* probes were separately hybridized with a mixture of RNAs from uninjected 8.5- and 12-hour embryos, the stages in which *SpEts4* and *SpHE* mRNAs accumulate to maximum levels, respectively. (Lane 3) Negative control in which only yeast tRNA was used. (Lanes 4 and 5) RNA from 200 embryos injected with mRNA encoding either Eng or EngEts4 hybridized simultaneously with both *SpEts4* and *SpHE* probes.

transcription in vivo, we microinjected synthetic mRNA (0.2 pg, or approx. 2×10^5 molecules, which is tenfold below lethal doses, see below) encoding either EngEts4 or Eng and used RNase protection to assay the levels of *SpHE* transcripts at 10 hours postfertilization, when the *SpHE* transcription rate is maximal in the embryo (Fig. 3). As an internal control for RNA recovery in these samples, a probe for endogenous *SpEts4* mRNA was combined with the *SpHE* probe. Hybridization to RNA from either control (uninjected) or mRNA-injected embryos was specific since all the RNase-resistant fragments were full length. Control *SpEts4* levels were equivalent in the Eng and EngEts4 samples. In contrast, *SpHE* transcripts were undetectable in embryos expressing the chimeric protein, demonstrating that transcription of the endogenous genes was strongly repressed.

Introduction of EngEts4 into sea urchin embryos completely inhibits their hatching, for at least 5 days postfertilization, consistent with *SpHE* transcriptional repression. This inhibition occurred at EngEts4 mRNA doses from 6×10^4 to 2×10^5 injected mRNA molecules/embryo, which do not prevent differentiation of embryonic cell types. At the upper end of this range, more than 95% of the embryos did not hatch, but post-hatching cell differentiation occurred in nearly half of the embryos. For example, primary mesenchyme cells (PMCs) ingressed and secreted spicules, archentera formed and pigmented mesenchyme cells appeared. At the lower doses, more than half of the embryos failed to hatch but virtually all contained differentiated cells and tissues characteristic of the fully developed pluteus larva. Examples of these phenotypes are illustrated in Fig. 4. Fig. 4A,B shows DIC images of control

Eng or EngEts4 embryos, respectively, at 26 hours post-fertilization. Although this is about 8 hours after the normal time of hatching, EngEts4 mRNA-injected embryos fail to hatch. Staining with an antibody that recognizes an epitope expressed by primary mesenchyme cells after their ingression (about 4 hours after hatching of the normal embryo) (Fig. 4C,D) illustrates that at least some of the cells in the blastocoel are bona fide differentiating PMCs. EngEts4-containing embryos can survive for at least 5 days postfertilization. Many contain overly large guts, thicker than normal spicules and have fewer, larger pigmented cells (Fig. 4E,F). Although organization of embryonic tissues is clearly disrupted, these advanced embryos are surprisingly healthy. When the fertilization membrane is manually punctured (Fig. 4F), the ciliated embryos swim away within several minutes (Fig. 4G,H). Collectively, these observations clearly indicate that the inhibition of hatching and suppression of *SpHE* expression do not result from toxic effects of the repressor protein or general developmental arrest.

Several other changes in embryo morphology are apparent, suggesting that EngEts4 also suppresses the activity of some other genes during embryogenesis. At mesenchyme blastula stage, the number of cells is reduced about 1.5- to 2-fold and the blastocoel is smaller but contains greater than the normal number of cells. By examining these embryos in real time, it is clear that these extra cells result from precocious ingression of cells from the vegetal plate. At later stages, skeletal patterning, which is regulated by ectodermal cues in the normal embryo, is abnormal. In addition, pigment cells are larger than normal, presumably by premature cessation of cell division, and fail to distribute throughout the ectoderm. The gut is also enlarged, sometimes being subdivided into as many as five sections rather than the normal three. However, examination of these phenotypes as a function of EngEts4 dose shows that none is more sensitive to expression of EngEts4 than is hatching. From these observations we conclude that the Ets domain of SpEts4 mediates suppression of *SpHE* transcription by EngEts4, to the extent that hatching can be completely prevented at doses that do not inhibit the cell differentiation characteristic of post-hatching development.

Early *SpEts4* expression includes uniformly distributed maternal transcripts and nonvegetally restricted zygotic transcripts

We previously showed that SpEts4 is a positive regulator of *SpHE* transcription since it can transactivate a *SpHE* promoter transgene, it contains a strong activation domain, and *SpEts4* transcripts are present at the correct time to sponsor *SpHE* transcription (Wei et al., 1999). Since *SpHE* transcription is confined to nonvegetal cells, we wanted to test the hypothesis that SpEts4 is expressed specifically in these cells. As a first step, we asked whether *SpEts4* mRNAs accumulate in the same nonvegetal cells that transcribe the *SpHE* gene. To maximize in situ hybridization sensitivity, we used a probe that includes a major portion of the SpEts4 coding sequence but lacks the conserved Ets domain sequence. To verify the specificity of this hybridization probe, it was hybridized to blots of RNAs isolated from selected embryonic stages. Unexpectedly, two relatively abundant *SpEts4* transcripts were detected (Fig. 5A). The shorter 1.8 kilobase (kb) transcript, found in the egg, persists through early cleavages and then is rapidly degraded after about 6th cleavage. The postfertilization sequential increase and decrease in the length of this species undoubtedly indicate cytoplasmic polyadenylation and deadenylation (Wilt, 1977), which is observed for most maternal mRNAs (Kingsley et al., 1993). The longer 2.9 kb transcript is extremely rare in egg RNA, rapidly increases in abundance after 6th cleavage and persists through 12 hours postfertilization, several hours after the maternal transcripts have disappeared. Both long and short transcripts are present at similar levels in late-stage embryos. These results confirm our earlier RNase protection data (Wei et al., 1999), which demonstrated the same biphasic expression of *SpEts4* mRNAs.

Both transcripts are derived from the same transcription unit. Sequencing of the cDNAs representing both species reveals that they contain identical open reading frames and differ only by the presence of an additional 3′ UTR sequence in the longer RNA. This is illustrated by the experiment in Fig. 5B: when this 3′ UTR extension was used to probe the same RNA blot, only the longer species was detected. Since comparison of 3′ UTR sequences to genomic sequence rules out the possibility that the additional sequence results from alternative splicing

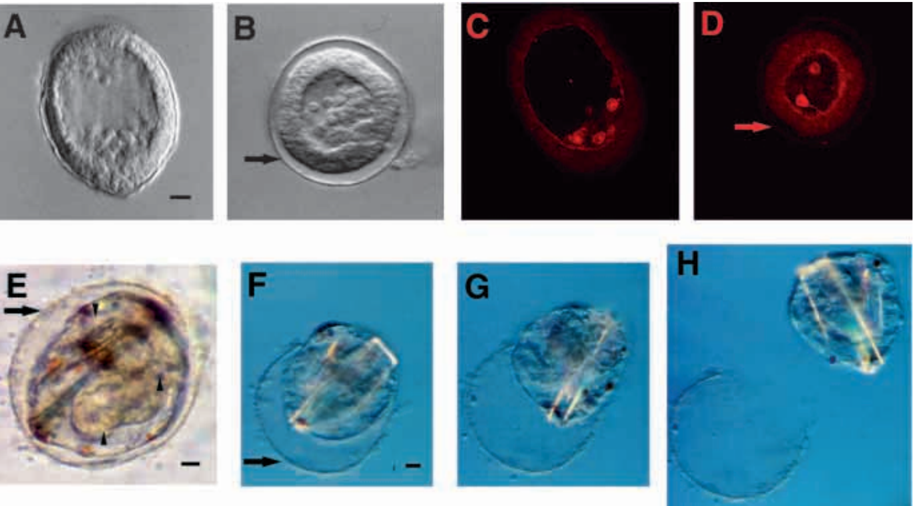

Fig. 4. SpEts4 prevents hatching but not tissue differentiation of embryos. (A,B) DIC images of a control 26-hour embryo injected with Eng mRNA (A) and a 26-hour embryo injected with EngEts4 mRNA (B). The arrows in (B,D,E,F) indicate the intact fertilization envelope. In B, note the abnormally large number of cells in the blastocoel. (C,D) Confocal images of the same embryos after immunostaining with the 6e10 antibody, which recognizes an epitope expressed in differentiating primary mesenchyme cells after ingression. Because only three 1 μm optical sections are stacked in these images, only a subset of labeled cells in the embryo are visible here. (E-H) EngEts4 mRNA-injected embryos 5 days postfertilization. The embryo in E illustrates an enlarged gut (arrowheads), abnormally thick spicules, large pigment cells and a lack of ectodermal oral/aboral patterning. (F-H) A series of photographs of another embryo during a 2-minute interval showing its escape after the fertilization envelope is manually ruptured. Bars, 10 μm.

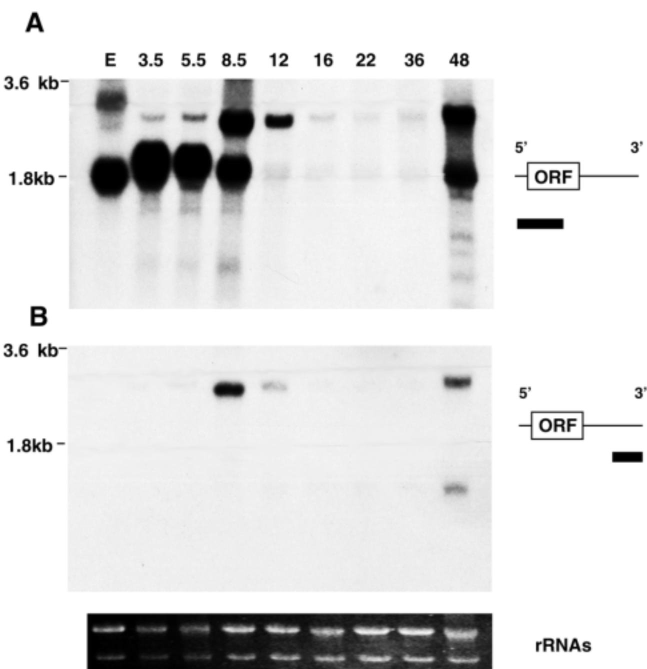

Fig. 5. RNA blot analysis of *SpEts4* transcript accumulation during development. Identical blots of RNAs from unfertilized eggs or embryos at the indicated hours post-fertilization were probed with sequences (indicated by black bars at right) from either the open reading frame (ORF) common to both long and short transcripts (upper panel) or the 3′-terminal untranslated extension specific for the larger species (middle panel). The bottom panel shows a photograph of the ethidium bromide-stained gel, illustrating that the loads of intact RNAs were similar.

(data not shown), it is likely that the two species result from use of different 3′ polyadenylation signals, although further experiments are required to verify this conclusion. The RNA blot data indicate that the *SpEts4* RNAs present in the early embryo derive from both maternal and zygotic transcription.

To determine the distribution of maternal and zygotic transcripts, we carried out in situ hybridization with ^{33}P-labeled riboprobes representing either the coding region that recognizes both species or a 3′ UTR sequence probe that hybridizes only to zygotic transcripts (Fig. 6). However, because of the much larger quantity of shorter transcripts in unfertilized eggs and early cleavage stages (see Fig. 5A), the coding region probe is essentially specific for shorter maternal transcripts during this period. These RNAs are uniformly distributed in the egg and early embryo (Fig. 6A,B and C,D, respectively). In contrast, accumulation of zygotic transcripts is restricted to about 85% of the embryo (Fig. 6E,F) in a pattern indistinguishable from that of *SpHE* mRNA accumulation (Fig. 6G,H), as shown by probing adjacent sections with *SpHE* and *SpEts4* probes.

The SpEts4 cis element from the *SpHE* promoter is sufficient to regulate nonvegetal transcription in embryos

To explore further the idea that SpEts4 functions in the nonvegetal domain, we tested whether the *SpEts4* cis element to which SpEts4 binds is sufficient to activate transcription in nonvegetal cells. The construct was composed of 20 bp containing the SpEts4 site linked directly to the *SpHE* basal promoter (–90 to +20 bp) upstream of the β-galactosidase gene, as illustrated in Table 1. Chemical modification interference and DNase I footprinting assays show that the length of DNA with which other Ets factors interact is 18 nts (Nye et al., 1992), so this sequence is likely to be close to the minimal size required for good interaction. If the SpEts4 cis element is sufficient to elicit spatially correct nonvegetal expression, then we expect that, as observed in our previous studies on wild-type and mutated *SpHE* promoters (Wei et al., 1995, 1997a), approximately 80% of the embryos will contain β-galactosidase-expressing nonvegetal cells. On the other hand, if active SpEts4 protein is present in vegetal cells, then

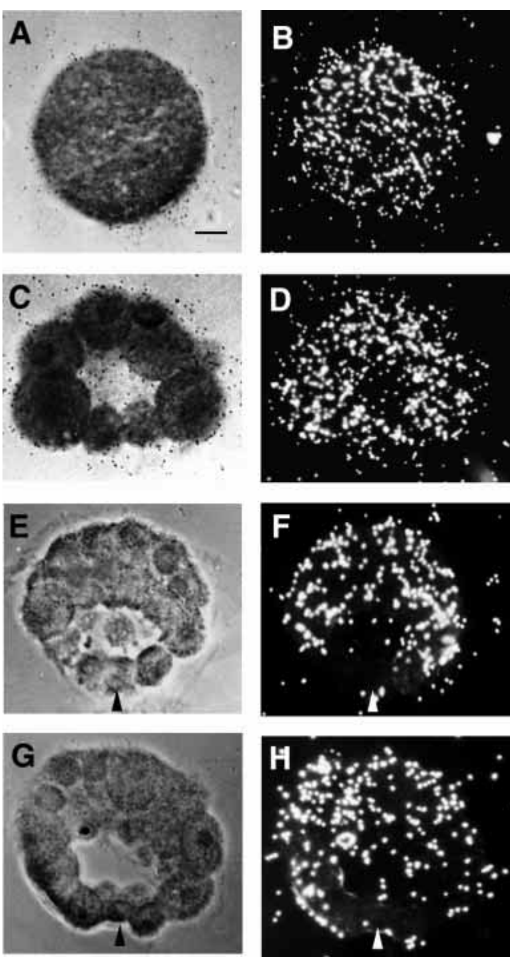

Fig. 6. Maternal SpEts4 transcripts accumulate throughout the embryo, whereas zygotic transcripts are restricted to the nonvegetal domain. Sections of unfertilized eggs (A-B) or 16-cell embryos (C-D) were hybridized with a probe representing the open reading frame of SpEts4, which essentially is specific for the predominant shorter maternal transcripts in this assay (see Fig. 5). Adjacent sections of 9-hour embryos (E-H) were hybridized with a 3′ UTR probe specific for the longer zygotic *SpEts4* transcript (E,F) or a probe for *SpHE* message (G,H). (A,C,E,G) and (B,D,F,H) show pairs of images of the same sections photographed in brightfield and darkfield illumination, respectively. The arrowheads in E-H indicate the vegetal region of these embryos, which lacks hybridization signals for both *SpEts4* and *SpHE* probes. Bar, 10 μm.

Table 1. The SpEts4 site is sufficient to confer nonvegetal expression

	n	NV	V
Ets4 BP	130 (2)	102 (78%)	0
BP	140 (2)	4 (3%)	0

BP, basal promoter region.
n, number of embryos analyzed. The number of egg batches is given in parentheses.
NV, number of embryos in which expression is nonvegetal.
The percentage of labeled embryos is given in parentheses.
V, number of embryos in which expression is vegetal.

we expect that about 20-25% of embryos will contain β-galactosidase-positive PMCs that are identifiable at early mesenchyme blastula stage by staining with the 6e10 antibody. These values of approx. 80% and 20% for nonvegetal and vegetal expression, respectively, take into account the fact that microinjected DNA is incorporated in single blastomeres, usually between the 2nd and 4th cleavages, and the relative contributions of blastomeres to nonvegetal and vegetal domains at this time. Such values have been obtained in previous assays of *SpHE* promoter function (Wei et al., 1995, 1997a) and that of SM50, which is transcribed specifically in PMCs (Makabe et al., 1995; Wei et al., 1997a).

We analyzed a large number of embryos microinjected with β-galactosidase constructs containing either the SpEts4 cis element linked to the basal promoter (Ets4/BP) or the basal promoter alone (BP) (Table 1). Also included in these experiments, for comparison, was a construct containing the wild-type −300 *SpHE* regulatory region (data not shown). Although, as expected, the enzymatic reaction time required to obtain blue signals was significantly longer for the Ets4/BP than for the wild-type construct (18 versus 2 hours, respectively), the percentage of embryos expressing β-galactosidase was similar and close to 80%. Transcription of the Ets4/BP construct was restricted to nonvegetal cells: no PMCs expressed β-galactosidase activity, just as is the case for the wild-type promoter (Wei et al., 1995, 1997a). As expected, the basal promoter region had extremely low activity, such that only a few percent of the embryos were labeled and within these only a small number of positive cells were observed. Since the basal promoter can function well in both nonvegetal and vegetal cells (Wei et al., 1997a), we conclude that the Ets site at −250 in the *SpHE* promoter is sufficient to activate transcription in the nonvegetal region.

DISCUSSION

The initial maternal patterning of sea urchin embryos along the AV axis establishes the nonvegetal and vegetal transcriptional domains that are largely segregated by the asymmetric cleavage of the vegetal four blastomeres of the 8-cell embryo. At the 16-cell stage, the nonvegetal transcriptional regulatory domain has been demonstrated by accumulation of transcripts of four different VEB messages in animal macromeres and mesomeres and their progeny, but not in vegetal micromeres (Reynolds et al., 1992; Nasir et al., 1995). Several cleavages later, appearance of *SM50* transcripts signals activation of the vegetal-specific program in the micromere-PMC lineage (Sucov et al., 1988). Extensive mutational analysis of the regulatory regions of two VEB genes, *SpHE* (Wei et al., 1995; Wei et al., 1997a,b) and *SpAN* (Kozlowski et al., 1996) and of that of *SM50* (Makabe et al., 1995) has led to a model in which initial spatial regulation along the animal-vegetal axis is achieved by functional partitioning of positive transcription regulatory activities. Since both the VEB genes and *SM50* are activated cell-autonomously, these positive activities are most likely to be maternal proteins, either encoded by localized maternal mRNAs or activated post-translationally by other localized maternal molecules. Alternatively, but less likely, the regulators could be encoded by upstream genes under similar primary maternal control.

We have previously shown that one of the positive activators of a *SpHE* promoter/*CAT* reporter transgene in sea urchin embryos is an Ets family transcription factor (Wei et al., 1999). This observation is consistent with genomic footprinting assays of the endogenous *SpHE* gene in vivo, which detect occupancy of this Ets site only at times during development when the gene is active (Wei et al., 1997b). That binding is by an Ets factor is strongly supported by the fact that the nucleotide position of the strong DMS hypersensitive site at the Ets element border corresponds precisely to that described previously for binding of an Ets factor that regulates the chicken lysozyme gene (Ahne and Stratling, 1994; Wei et al., 1997b). Here we show that a 20-bp fragment from the *SpHE* regulatory region that contains an Ets recognition motif is sufficient to drive nonvegetal expression of the β-galactosidase reporter when placed upstream of the *SpHE* basal promoter. Since the basal promoter region alone has little activity and can function in both nonvegetal and vegetal domains (Wei et al., 1997a), the factor binding to this short cis regulatory region must function positively in nonvegetal blastomeres.

An excellent candidate for the Ets factor that regulates nonvegetal *SpHE* transcription is SpEts4. We have demonstrated that it is a strong transcriptional activator in yeast (Wei et al., 1999). Recombinant SpEts4 translated in vitro forms a complex with the same mobility in EMSAs as that obtained with nuclear extracts from 9-hour embryos, the stage of peak *SpHE* transcriptional activity, and both complexes are competable to similar relative extents with an array of different Ets motifs. An important extension of this work is provided here by demonstration of an interaction of SpEts4 with the promoter of the endogenous *SpHE* gene. The engrailed dominant repression domain linked to the SpEts4 DNA binding domain suppresses transcription of the endogenous *SpHE* gene. This can be achieved with EngEts4 RNA microinjected into 1-cell zygotes at levels corresponding to mRNAs of moderate to low abundance (<0.1% of embryo mRNA). Finally, the accumulation of *SpEts4* zygotic transcripts is appropriate in both time and space to regulate *SpHE* during late cleavage when most transcripts accumulate. The concentration of these transcripts increases dramatically just prior to a corresponding increase in *SpHE* transcription rate (Wei et al., 1999) and they are restricted to nonvegetal blastomeres in a pattern that is identical with that of *SpHE* RNAs.

The congruence of the *SpEts4* spatial transcription pattern

in embryos with those of the *SpHE* target gene and other VEB genes shows that transcription of *SpEts4* itself during cleavage is also regulated by nonvegetal positive transcription activities. These activities are probably members of the same nonvegetal cohort that regulate the VEB genes. We have made a similar observation for a different factor that binds to an essential site in the *SpAN* promoter: transcripts encoding this protein are also uniformly distributed in the cytoplasm of eggs and early cleavage-stage embryos, but rapidly become restricted to nonvegetal cells through turnover in vegetal cells and possibly continued transcription in the others. Together these observations indicate that the nonvegetal domain of positive transcription activity regulates not only strictly zygotic VEB mRNAs such as those isolated in our original temporal screen (Reynolds et al., 1992), but also new zygotic transcription of genes expressed earlier during oogenesis. In addition, the fact that the latter set includes genes that themselves encode regulators of the nonvegetal pattern shows that there exists an amplification mechanism in which new zygotic transcription reinforces the initial maternal nonvegetal regulatory domain established in the early embryo.

The zygotic pattern of *SpEts4* transcription is consistent with its nonvegetal regulatory activity. However, the presence of uniformly distributed maternal transcripts was unexpected because nonvegetal expression of *SpHE* and the other VEB genes that have been analyzed is established by the 16-cell stage, as shown by the lack of VEB transcripts in micromeres (Reynolds et al., 1992; Nasir et al., 1995). There are several possible explanations for why *SpHE* is not transcribed in micromeres, despite their containing maternal SpEts4 mRNA, which encodes a functional SpEts4 transcription factor (Wei et al., 1999). First, nonvegetal *SpHE* transcription may be primarily driven by other factors at early cleavage stages. We have previously shown that multiple positive cis elements can confer the nonvegetal *SpHE* transcriptional pattern (Wei et al., 1997). Second, maternal SpEts4 mRNAs may not be translated. However, two lines of evidence suggest that maternal transcripts are translated. (1) They undergo changes in length, characteristic of cytoplasmic polyadenylation after fertilization and deadenylation shortly before they decay, as is observed for translated maternal mRNAs (Wilt, 1973, 1977). (2) Microinjected full-length synthetic mRNAs representing either the maternal or zygotic transcripts encoding SpEts4 tagged with green fluorescent protein are translated to similar levels in early sea urchin embryos (our unpublished results). A third possible explanation is that SpEts4 maternal mRNAs may not be translated in micromeres or SpEts4 protein itself may not be stable there. Fourth, there may be blastomere-specific regulation of entry of SpEts4 protein into nuclei. Whether endogenous maternal *SpEts4* transcripts provide significant levels of SpEts4 protein in the nuclei of vegetal blastomeres awaits acquisition of antibody reagents.

Other possible mechanisms for spatial regulation of *SpEts4* activity do not require either spatially regulated synthesis or activation of the protein. First, SpEts4 protein derived from maternal transcripts might be localized in the egg. Second, because asymmetric cleavage at the 16-cell stage distributes much more cytoplasmic volume to mesomeres and macromeres than to the micromeres, the latter would be expected to inherit correspondingly less uniformly distributed maternal SpEts4. Third, as embryos enter the fourth cleavage the cortical cytoplasm retracts from the vegetal pole, conferring a qualitatively different cytoplasmic domain on the micromeres (Schroeder, 1980). This retraction visibly sweeps pigment granules from the vegetal pole, and its possible function in a large-scale localization event is also suggested by the observation that micromeres lack about 25% of the maternal RNA sequence complexity (Rodgers and Gross, 1978; Ernst et al., 1980).

SpEts4 is the first example of a set of multiple genes encoding factors whose activities establish and maintain an early and specific pattern of gene expression in the animal 80-90% of the sea urchin embryo. The target genes of this set of regulators include effectors of diverse early blastula-stage developmental processes and include the hatching enzyme and a tolloid-related astacin protease with demonstrated morphogenetic potential (Wardle et al., 1999). With the first gene encoding a VEB transcription regulator now in hand, we can explore the possible cellular and molecular mechanisms underlying the transfer of maternal AV axial positional information to the zygote.

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