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Identification of a New Sea Urchin Ets Protein, SpEts4, by Yeast One-Hybrid Screening with the Hatching Enzyme Promoter

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We report the use of a yeast one-hybrid system to isolate a transcriptional regulator of the sea urchin embryo hatching enzyme gene, *SpHE*. This gene is asymmetrically expressed along the animal-vegetal axis of sea urchin embryos under the cell-autonomous control of maternal regulatory activities and therefore provides an excellent entry point for understanding the mechanism that establishes animal-vegetal developmental polarity. To search for transcriptional regulators, we used a fragment of the *SpHE* promoter containing several individual elements instead of the conventional bait that contains a multimerized *cis* element. This screen yielded a number of positive clones that encode a new member of the Ets family, named SpEts4. This protein contains transcriptional activation activity, since expression of reporter genes in yeast does not depend on the presence of the yeast GAL4 activation domain. Sequences in the N-terminal region of SpEts4 mediate the activation activity, as shown by deletion or domain-swapping experiments. The newly identified DNA binding protein binds with a high degree of specificity to a *SpHE* promoter Ets element and forms a complex with a mobility identical to that obtained with 9-h sea urchin embryo nuclear extracts. SpEts4 positively regulates *SpHE* transcription, since mutation of the SpEts4 site in *SpHE* promoter transgenes reduces promoter activity in vivo while *SpEts4* mRNA coinjection increases its output. As expected for a positive *SpHE* transcriptional regulator, the timing of *SpEts4* gene expression precedes the transient expression of *SpHE* in the very early sea urchin blastula.

The *Strongylocentrotus purpuratus* hatching enzyme gene, *SpHE*, is transcribed transiently only in nonvegetal blastomeres during the cleavage and very early blastula stages of sea urchin development (20). The activation of *SpHE* is early and cell autonomous and therefore is very likely to be regulated by localized maternal transcription-regulatory activities partitioned asymmetrically along the animal-vegetal (AV) axis. Consequently, the *SpHE* gene represents an excellent entry point for determining how the AV axis is established, through the identification of *trans*-acting factors that regulate its transcription.

Previously, we reported that a relatively compact region of the *SpHE* promoter, consisting of 300 bp upstream of the transcription initiation site, is sufficient to sponsor both high-level transcriptional activity and correct nonvegetal spatial expression (26, 27). Although this regulatory region is small, nine *cis*-acting elements have been defined within it, which can form complexes with at least six different proteins, as shown by in vitro DNase I footprinting and electrophoretic mobility shift assays (EMSAs) (26). Most of these *cis* elements were found to be occupied when the gene is active in vivo but to be unoccupied and in a nucleosome-like configuration when the gene is inactive (28). Extensive mutational dissection of the *SpHE* regulatory region supports a model in which all of the DNA binding proteins confer positive function in nonvegetal blastomeres (26, 27). This suggests that an important mechanism establishing AV polarity of developmental potential in the early sea urchin embryo is partitioning of positive maternal regulatory activities to nonvegetal blastomeres.

To test this model, we have begun to investigate the *SpHE* transcriptional regulators. Here we report the cloning of an *S. purpuratus* egg cDNA encoding a member of the Ets family, using a nontraditional yeast one-hybrid genetic screening approach in which a large portion of the *SpHE* promoter sequence instead of individual multimerized *cis* elements was used as bait. Using this method, we isolated 13 strong positive clones containing overlapping segments of the same sequence, which encodes a member of the Ets transcription factor family. Because this protein contains a conserved Ets domain that is much more similar to that of *Drosophila* Ets4 (6) than to any other Ets domain, we have named it SpEts4. We demonstrate that the *SpEts4* gene sequence encodes a protein that binds with the same specificity, and forms a complex of similar mobility in EMSA, as does the native protein found in nuclear protein extracts from very early blastulae. Using *SpHE* promoter transgenes, we show that the site to which SpEts4 binds confers positive regulatory activity and that exogeneously supplied recombinant SpEts4 augments promoter activity. Consistent with a positive function for SpEts4 in regulating *SpHE* transcription, *SpEts4* transcripts accumulate in the egg and early embryo transiently and just prior to the burst of *SpHE* mRNA expression.

MATERIALS AND METHODS

***S. purpuratus* egg cDNA library.** A total of 1 mg of total RNA from *S. purpuratus* eggs was used to extract poly(A)$^+$ RNA by using the FastTrack 2.0 kit (Invitrogen). The cDNA library was made by using a cDNA library construction kit supplied by Clontech (Matchmaker Two-Hybrid System). Briefly, 8-μg aliquots of poly(A)$^+$ RNA were reverse transcribed separately by using either random or oligo(dT) primers, and the cDNA products were combined and inserted into a shuttle vector, pGAD10, containing the GAL4 activation domain. Transformation by electroporation of *Escherichia coli* DH5α cells yielded >6 × 10^5 independent colonies. The library was amplified on 20- by 150-mm plates containing ~30,000 colonies/plate.

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Yeast one-hybrid screening of the sea urchin egg cDNA library. The *SpHE* promoter sequence from bp −324 to −143 was used as the bait to select DNA binding domains encoded in the sea urchin egg cDNA library. Within this promoter region are recognition motifs for Otx, Rel, and Ets as well as three additional binding sites defined by EMSAs and DNase I footprinting (26). The bait was inserted into reporter plasmids pHISi and pLacZi, and the recombinant plasmids were introduced sequentially into the genome of the yeast strain Y4271. These plasmids and control plasmids, containing either p53 *cis* elements, p53blue, or DNA binding sequences (pGAD53m), were supplied in the Clontech Matchmaker One-Hybrid System kit. The transformants were tested for growth on medium lacking His (His⁻ medium) in the presence of increasing concentrations of 3-aminotriazol (3-AT). Cells whose growth was inhibited by 5 mM 3-AT were selected as the host for the library screen. Transformation with the library was carried out by using LiCl-polyethylene glycol, and transformants grown on His⁻ and Leu⁻ selective medium were tested for β-galactosidase activity. Plasmids from putative positive clones were isolated from the yeast after homogenization with glass beads and then individually transferred into DH5α cells for amplification. To eliminate false positives, these plasmids were separately introduced into yeast cells containing either the *SpHE* bait or the p53 binding site, and the transformants were tested for β-galactosidase activity. The plasmids that conferred expression only in the *SpHE* host were chosen for further analysis. Inserts were sequenced by a combination of manual dideoxy sequencing and automated sequencing. DNA sequences were used to query the GenBank database, and sequence comparisons were made by using Clustal software (21).

Transcription activation domain mapping. The SpEts4 activation domain was identified by using the yeast one-hybrid system. The GAL4 transcription activation domain in plasmids pGAD10 (for SpEts4) and pGAD424 (for p53) was removed by internal deletion of sequences from restriction site *Kpn*I to *Eco*RI, and SpEts4 sequences were inserted. To test for activation activity in SpEts4, constructs encoding proteins with deletions in three separate regions (EtsΔ36–123, EtsΔ126–184 [pointed domain], and EtsΔ184–274) were transformed into *SpHE* bait-containing yeast cells. To test whether SpEts4 sequences could mediate activation when linked to a heterologous (p53) DNA binding domain, four fusion protein constructs, p53E1–275, p53EΔ126–184, p53E1–123, and p53E184–275 were prepared and used to transform p53 bait-containing yeast. *SpEts4* transformants were tested for growth ability on His⁻ plates with 5 mM 3-AT and for β-galactosidase activity. p53 transformants were tested only for β-galactosidase activity by filter lift assay as described in the Clontech Yeast Protocols Handbook.

In vivo transcription assays. Measurements of promoter activity in vivo were made by using chimeric constructs carrying wild-type or mutated *SpHE* promoters linked to a bacterial chloramphenicol acetyltransferase (CAT) reporter gene. The mutation changed CGGAAC at bp −250 in the *SpHE* promoter to an *Eco*RI site, GAATTC. These constructs were microinjected into fertilized *S. purpuratus* eggs and assayed exactly as described previously (26).

For transactivation assays, capped, polyadenylated *SpEts4* mRNA was synthesized by using the Message Machine kit from Ambion and purified according to the manufacturer's instructions, and ~0.2 pg was coinjected with 2,500 copies of the *SpHE* promoter transgene.

Developmental RNase protection assays. RNA was prepared from eggs and embryos at selected developmental stages by using the TRIzol protocol (GIBCO-BRL). The quantity of RNA was determined by spectrophotometry, and its quality was verified by gel electrophoresis on denaturing formaldehyde-containing agarose gels. Probes complementary to *SpEts4* mRNA (from bp −102 to +99 relative to the translation initiation site) and *SpHE* sequences (from bp +316 to +543 relative to the transcription start site and containing exon 1 and intron 1 sequences) were labeled with [α-³²P]UTP to specific activities of 6×10^8 and 0.75×10^8 cpm/μg, respectively. The *SpEts4* and *SpHE* probes protect 202- and 110-nucleotide fragments, respectively. One-half nanogram of probe was hybridized with 1.5 μg of total RNA to kinetic termination at 50°C for 18 h. RNase protection and electrophoresis of the protected products were conducted as described previously (30).

In vitro translation of SpEts4 protein. The *SpEts4* cDNA was transferred to pGEM Easy plasmid (Promega). The protein was synthesized by using 1 μg of linearized plasmid and the TNT coupled reticulocyte lysate kit (Promega) in the presence of [³⁵S]methionine (40 μCi; 1,000 Ci/mmol). The labeled products (1 μl of the 50-μl reaction mixture) were assayed for size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Control luciferase protein encoded from a plasmid provided by the manufacturer was synthesized and assayed in the same way.

EMSA. EMSA was conducted by using in vitro-translated proteins and 9-h nuclear extracts as described previously (26). Probes (bp −274 or −261 to −241) containing only the Ets motif were end labeled with [γ-³²P]ATP to a specific activity of ~2.0×10^6 cpm/pmol. For each reaction, ~4.5 fmol of probe was incubated on ice for 10 min with either an aliquot of the in vitro translation reaction mixture (see above) or nuclear extract protein; the latter was prepared as described previously (5). To test for specificity of binding, various competitor DNAs, each containing a different variant Ets binding site sequence, were added in a 200-fold molar excess. The reaction mixtures were then fractionated by electrophoresis through nondenaturing 5% acrylamide gels.

RESULTS

Isolation of *trans*-acting factors that interact with the *SpHE* promoter sequence by using a yeast one-hybrid screen of an egg cDNA library. In order to identify the *trans*-acting factors involved in *SpHE* promoter activation, we used a nontraditional version of the yeast one-hybrid method. In general, this assay selects among cDNA sequences, fused to a vector-encoded transcriptional activation domain, for those whose protein products can bind target DNA and activate expression of a reporter gene in yeast cells (23). According to presently used methods, the one-hybrid approach is said to require a bait consisting of a multimerized *cis* element in order to increase the probability of protein-DNA interaction. However, because the *SpHE* promoter contains a large number of functional *cis* elements, and because the exact boundaries of some of these are difficult to define precisely, we used a single copy of the native promoter sequence from bp −324 to −143. This region includes all identified *cis* elements except two CCAAT sites that were excluded to avoid possible background resulting from known CCAAT transcription factors in yeast. Another reason for using this approach is that among the many *cis* elements regulating the *SpHE* promoter, none is detectably more important than the others for mediating either quantitative or spatial regulation (26–28). Finally, we wanted to increase the probability of selecting for strong protein-DNA interactions, which are likely to be favored by using the natural promoter sequence instead of multimerized elements. Because the *SpHE* gene is very likely activated by maternal transcription factors, we used RNA from eggs to build a cDNA library of fusions to the GAL4 activation domain in plasmid pGAD10.

The screening strategy consisted of two tests: (i) growth on His⁻ medium in the presence of 3-AT and expression of β-galactosidase and (ii) elimination of false positives by double screening of selected transformants with an unrelated promoter bait and with the bait containing *SpHE* promoter sequences. First, a test cell line that contained integrated copies of *SpHE* promoter bait linked to either the histidine gene or the β-galactosidase gene was selected for growth on His⁻ medium that was completely inhibited by 5 mM 3-AT. After transformation with the egg cDNA library, of 2×10^6 egg cDNA transformants of this cell, more than 100 colonies grew under 3-AT selection, and they all were positive for β-galactosidase activity, although at variable levels. The plasmid from each transformant was extracted and individually reintroduced into yeast cells containing either *SpHE* promoter bait or a p53 bait. Of these, 14 clones were *SpHE* specific and conferred the highest levels of β-galactosidase activity. Sequence analysis revealed that 13 clones shared a 1.8-kb sequence (Fig. 1). Of these 13 clones, 8 contained inserts of about 1.8 kb, and the remaining inserts ranged from 2.3 to 2.9 kb. Interestingly, many of these positive inserts were not linked in frame to a GAL4 activation domain, raising the possibility that the selected egg cDNA sequences encode a protein with its own transcription activation function as well as a DNA binding domain. Furthermore, several strong positive transformants contained plasmid inserts in opposite orientation with respect to the ADH1 promoter that drives transcription of fusion proteins. This observation suggests that sufficient transcription must have occurred from a downstream cryptic promoter.

The selected cDNAs encode a novel sea urchin Ets family transcription factor. The longest open reading frame obtained from the 13 selected egg cDNAs predicts a protein of 363 amino acids (Fig. 1). The translation start site is defined by the codon for the first methionine residue downstream from an in-frame stop codon, whose local context conforms to the

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                                                          GATCTCGCTTTGG
TGAACAACTCACGATTCAAGCTTAGGAATTATAACACAAGACAACTGCCTCTACAGGTGT
AATTTTAACCTGAATCTCAGCTTGATTTATTTAAATCACAACATCACACCTTCAAGCAAC
ATGTTCTCAACTGGCGAAGTTTGCATTGTTTCACCCACACCAATGAACGATCCCGACGCA
 M  F  S  T  G  E  V  C  I  V  S  P  T  P  M  N  D  P  D  A
ATTCATCTAGCTGAAATCAGCATGGGGAGCACGCTTCTAGACCTCGATCTTATCCAACAA
 I  H  L  A  E  I  S  M  G  S  T  L  L  D  L  D  L  I  Q  Q
CAGCAACGCAACGGCCCCTCCCCCAGTCCCTTCAGCGGCGGCTTCGGACTCCCCCCTTCA
 Q  Q  R  N  G  P  S  P  S  P  F  S  G  G  F  G  L  P  P  S
CCAGACATGCAAGGGTCCTTCTATCCAGAGCAGAACTTCTTTCCTACACAGCAGATGGGC
 P  D  M  Q  G  S  F  Y  P  E  Q  N  F  F  P  T  Q  Q  M  G
TCTACCATTCAGCAGCAGGGGGGGAAACCAGATCTCAGAGATCGAGGAGAACGAGATGATG
 S  T  I  Q  Q  Q  G  G  N  Q  I  S  E  I  E  E  N  E  M  M
CGATTACTAATGAGGGAGATGATTCCTGATATCCTCGAGGATTGCCACAAACTCAACTTG
 R  L  L  M  R  E  M  I  P  D  I  L  E  D  C  H  K  L  N  L
ATTCCAAATGTTTACTGCTGGACGCCGGAAGATGTCCAGAAGTGGTTGTTATGCGTCGCC
 I  P  N  V  Y  C |W  T  P  E  D  V  Q  K  W  L  L  C  V  A
AATCGCTTTGAGCTAGGGGAACTCGAGATGGGCCACTTTTACATCAATGGACCAACCCTT
 N  R  F  E  L  G  E  L  E  M  G  H  F  Y  I  N  G  P  T  L
GCAACACTCCAGGACGTGGATTTCAGGCACAGGGCGCCGAAATGCGGCGGACATCCTTTAC
 A  T  L  Q  D  V  D  F  R  H  R  A  P  K  C  G  D  I  L  Y
AGCGTTGTATGTCTTCTTAAATCAAGTATTCACTTTGACGTAGCACCCCAGGTCGTACCT
 S  V  V| C  L  L  K  S  S  I  H  F  D  V  A  P  Q  V  V  P
CAAGCACAACAGATGATGCCCAATATTCCCAACCCTCGCACTTCATTCATTACCGGGGCACCC
 Q  A  Q  Q  M  M  P  N  I  P  Q  S  P  H  F  I  T  G  A  P
ACCACAACCATGATGCCACCCAAGTACACCTACACGCCCGTCTCACCGGTAGCAACGCCA
 T  T  T  M  M  P  P  K  Y  T  Y  T  P  V  S  P  V  A  T  P
GGGAGCGAGAGCGGTAGCACTGGCTCGCCAAGCGATGACGAGATCTCAATCCCATCACCC
 G  D  E  S  G  S  T  G  S  P  S  D  D  E  I  S  I  P  S  P
GCCCCCAGCGGACCCAACACCTCCATGACCCCGAACCACACAGGAGGTATCCAGCTCTGG
 A  P  S  G  P  N  T  S  M  T  P  N  H  T |G  G  I  Q  L  W
CAGTTCTTGAAGGAGCTCCTCCTCCAGCCCAACTCCTACAGCTACTGCATCAGGTGGATA
 Q  F  L  K  E  L  L  L  Q  P  N  S  Y  S  Y  C  I  R  W  I
GACAGAACACAAGGTATCTTCAAGATCGAGGACTCTGTCGAGGTCGCCAGACTTTGGGGC
 D  R  T  Q  G  I  F  K  I  E  D  S  V  E  V  A  R  L  W  G
TTGAGGAAGAACAGGCCGGCCATGAACTACGACAAGCTCTCTCGCTCCATCAGGCAGTAC
 L  R  K  N  R  P  A  M  N  Y  D  K  L  S  R  S  I  R  Q  Y
TACCGCAAAGGCATCATGAAGAAGACCGAGGTTTCGCAGCGACTCGTCTACCAGTTTGTC
 Y  R  K  G  I  M  K  K  T  E  V  S  Q  R  L  V  Y  Q  F  V|
ACCCCCTGTTAAAGAATGGAATAGTCTAAAAACTCTCGCGATATGAACACGCACACAGAG
 T  P  C
GGAGAGAGAAGCAGAATGTACTCTACGAAAGACATTGCCAATTACTATTGCCTATCAAAG
TGGAACTAGTTTCCAACTTGAAAGGGCGAAATGGACTTTTCACTTGTTATGCATCGCACGA
GTTTTATGTGTATTTAATATTTAAAGACTTTTAAACAATCTCATTCTTGCCCGAAGAGACA
TGGTTTCCATGGTTACTAACTAATCGCAGTACTAGGGTTGAATAACATGTAGTATTTTAAA
CTCTGATTGTGTTATAATCAACAATTTTGTAGCAAATATCTTGCAATTGTATCATAATAAA
GTCGAGGAGGTGGGGGTTAGGTGAACATTCTTTTCTATGGATTGTGAGAAAAGCAGACATG
ATGCCTTATTGGTATTTTTTTAATGGTATGTCTTTTAACGGTACTGTTTTTCTAGGAATGAT
TTTATGCATTGTTTTCTTGATGTTATTAATTCTCATGTGATTTTTTGTTGTACATATTCTT
ATACTTCTACTATCATGATGATTTTTCGAGAGGAAGAAGATATTTTTAAATTTAAAATAAT
TAAAAAAAAA
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FIG. 1. Sequence of the *SpEts4* cDNA and its predicted translation product. Stop codons upstream of the translation initiation site and at the end of the open reading frame are underlined. The region in the C-terminal quarter of the protein that is similar to the conserved DNA binding domains of Ets factors is shown boxed and in boldface, and a region in the middle of the protein with similarity to the pointed domain and thought to be involved in protein-protein interactions is also boxed. Transcriptional activation is mediated by sequences upstream of amino acid 123 (see Fig. 3).

translation consensus sequence (17). Comparison of the following open reading frame to sequences in the GenBank database shows that the C-terminal amino acid region comprises a conserved domain of about 85 amino acids that is related to the DNA binding domain of the Ets family of transcription factors (Fig. 1). The consensus core sequence recognized by Ets factors is C/AGGAA/T, which appears in the *SpHE* −300 promoter at bp −250 and −107. Only the −250 site was included in the bait sequence, suggesting that this Ets site can bind the Ets family member selected in our screen.

Comparison of Ets (DNA binding) domain sequences in the database to the recovered sea urchin Ets sequence indicates that the closest relative is *Drosophila* Ets4 (6). In the Ets domain, the amino acid residues are 82% identical, while the identity to all other Ets domain sequences is much lower (35 to 54%) (Fig. 2). The newly cloned sea urchin Ets protein is distinct from the Ets2 factor previously reported for these embryos (52% identical in the DNA binding domain) (7, 19). Ets transcription factors contain a second conserved sequence

in the N-terminal region, called the pointed domain. Corresponding sequence is not available for *Drosophila* Ets4. Of the available sequences, the pointed domain of the vertebrate Ets protein, Tel (11), is most closely related to a region of SpEts4 (amino acids 127 to 183) (Fig. 1), having 40% identity and 57% similarity; this is comparable to the level of identity between the Ets domains of SpEts4 and Tel, which is only 44%. The function of the pointed domain is not well understood, but it is thought to mediate dimerization of Tel proteins. This is because Tel-ABL tyrosine kinase fusion proteins, which are created as a result of a chromosomal translocation, dimerize, causing constitutive activation of the kinase and an oncogenic transformation (11). From these comparisons and the currently available data, we conclude that *Drosophila* Ets4 is the closest known relative of the newly identified sea urchin Ets factor, and we accordingly have named the sea urchin factor SpEts4.

SpEts4 contains a transcription activation domain. SpEts4 appears to have transcription activation activity, since, as discussed above, linkage of the GAL4 activation domain to SpEts4 sequence was not required for strong expression of reporter genes in the yeast one-hybrid screen. To eliminate possible activation via GAL4 peptides binding in *trans* to SpEts4, positive clones lacking GAL4 sequences were tested for β-galactosidase expression (not shown) and growth in 3-AT-containing His⁻ medium (Fig. 3C; compare sector 2 [+GAL4] with sector 3 [−GAL4]). Both assays show that transcription activity was retained after deletion of GAL4 sequence. In the case shown in Fig. 3C, GAL4 sequence was in frame with SpEts4 in the parent clone; after GAL4 deletion, a slight but detectable reduction in activity was observed, consistent with both GAL4 and SpEts4 sequences' providing activation activity. In cases in which GAL4 and SpEts4 sequences were not linked in frame, removal of GAL4 had no effect (data not shown).

To map the general location of transcription activation activity in the SpEts4 protein, constructs encoding proteins with deletions of three separate regions upstream of the Ets domain were transfected into yeast cells containing the *SpHE* promoter bait. These constructs, diagrammed in Fig. 3A, constitute a series of internal deletions that either flank or include the pointed domain. Deletion of the pointed domain (EtsΔ126–184) did not reduce activity (Fig. 3C, sectors 8 and 9), while EtsΔ36–123 was completely inactive when tested for growth under His⁻ selection (Fig. 3C, sectors 4 and 5) or for β-galactosidase activity. These results suggest that the upstream region, but not the pointed domain, supplies a transactivating function. The activity of the Δ184–274 protein was lower than that of either the full-length protein or pointed-domain deletion mutant (Fig. 3C, sectors 6 and 7). However, it is unlikely that this reflects the presence of a second independent activation region, because the Δ36–123 protein, which contains it, is entirely inactive. Instead, sequences between amino acids 184 and 274 may be required for optimal activity of the upstream domain by providing either a coactivation or structural function.

Since deletion of amino acids 36 to 123 resulted in loss of transcriptional activation, it was important to determine whether this sequence was sufficient to provide positive activity when linked to a heterologous DNA binding domain, p53. Plasmids encoding this fusion protein as well as those encoding other SpEts4 sequences (diagrammed in Fig. 3B) were transformed into yeast containing the p53 promoter bait linked to the reporter β-galactosidase and grown to approximately equal densities (Fig. 3D). Transcription activation mediated by SpEts4 sequence is reflected by both the time of appearance

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                                                                                              identity
                                                                                                  %
SpEts4   GG..IQLWQFLKELLLQPNSYSYCIRWIDRTQGIFKIED..SVEVARLWGLRK.NRPAMNYDKLSRSIRQYYRKGIMKKTEVSQRLVYQFV      -

DEts4    GGSHIHLWQFLKELLASPQVNGTAIRWIDRSKGIFKIED..SVRVAKLWGRRK.NRPAMNYDKLSRSIRQYYKKGIMKKTERSQRLVYQFC     82

SpEts2   SG.PIQLWQFLLELLTDK.TCQHIISWTG.DGWEFKLSD..PDEVARRWGKRK.NKPKMNYEKLSRGLRYYYDKNIIHKTA.GKRYVYRFV     52

HEts2    SG.PIQLWQFLLELLSDK.SCQSFISWTG.DGWEFKLAD..PDEVARRWGKRK.NKPKMNYEKLSRGLRYYYDKNIIHKTS.GKRYVYRFV     54

E74      EGSTTYLWEFLLKLLQDREYCPRFIKWTNREKGVFKLVD..SKAVSRLWGMHK.NKPDMNYETMGRALRYYYQRGILAKVD.GQRLVYHFV     48

Tel      ....RLLWDYVYQLLSDS.RYENFIRWEDKESKIFRIVD..PNGLARLWGNHK.NRTNMTYEKMSRALRHYYKLNIIRKEP.GQRLLFRFM     44

PU.1     SKKKIRLYQFLLDLLRSG.DMKDSIWWVDKDKGTFQFSSKHKEALAHRWGIQKGNRKKMTYQKMARALRNYGKTGEVKKVK..KKLTYQFS     37

              *.... **           * *       *         . .** * *. * *.. * .* *       . *      ... *
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FIG. 2. Sequence comparisons of the DNA binding domains of selected members of the Ets family. The percent identity between the Ets domain of each Ets factor and that of SpEts4 is given at the right. Asterisks indicate residues that are identical in all seven proteins. Dots represent conserved similar amino acids.

and intensity of the blue reaction product (Fig. 3E). By each of these criteria, p53E1–275 (sector 2) and p53EΔ126–184 (sector 3) are strongly positive and comparable to the positive control plasmid, pGAD53m, containing the GAL4 activation domain (sector 4). Further, sequences between amino acids 1 and 123 (p53E1–123; sector 6) also confer promoter activity at a somewhat reduced level compared to the wild type. A requirement for downstream sequences (amino acids 184 to 275) for full activity was also observed in the deletion analysis (cf. growth in sectors 6 and 7 with that in either sectors 8 and 9 or sectors 2 and 3 in Fig. 3C). In contrast, the activity of the fusion containing only amino acids 184 to 275 (p53E184–275; sector 5) is similar to background levels obtained for untransformed p53 bait cells (sector 1). These results confirm the deletion analysis and lead to the conclusion that transcriptional activation is mediated by sequences upstream of the pointed domain in SpEts4.

SpEts4 protein translated in vitro binds specifically to the −250 Ets site in the *SpHE* promoter. To test whether SpEts4 protein can bind specifically to the *SpHE* Ets site, a plasmid containing the SpEts4 open reading frame was transcribed in vitro, and the RNA was translated in the presence of [³⁵S]methionine by using a rabbit reticulocyte lysate. As shown in Fig. 4A, several labeled products were obtained; the predominant one of these was about 47 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This value is reasonably close to the size predicted from the inferred amino acid sequence, which is 41 kDa. These proteins formed a complex with a *SpHE* probe (bp −274 to −241) containing the Ets CGGAA core and flanking sequences, as shown by the EMSA results in Fig. 4B. In contrast, no complex was formed when a template encoding luciferase was used in parallel reactions.

To help determine whether SpEts4 corresponds to the endogenous embryo protein that binds the −250 Ets *cis* element, complexes formed with in vitro-translated protein and nuclear extract protein were compared (Fig. 4C). Two characteristics of the complexes formed with factors from these two sources strongly suggest that they are the same protein. First, the complexes have the same mobility. Second, when each of these complexes was competed by a series of different Ets motif sequence variants, both were competed equally effectively by the *SpHE* Ets and E74 motifs, but neither was competed as well by the PU.1 or the sea urchin Ets2 site (59/60) (8). The sequences of these competitors are listed in Fig. 5. Although all of the sequences contain the same GGAA core motif, as expected, residues both 5′ and 3′ to the core in the −250 *SpHE*

Ets site and E74 are more similar to each other than those in elements to which binding is weak or undetectable. We conclude that SpEts4 and a factor in 9-h nuclear extracts bind to different Ets elements with similar relative affinities.

We also tested the other putative Ets motif located at bp −107 in the *SpHE* promoter for binding to in vitro-translated SpEts4 and 9-h nuclear extracts. In neither case was a complex detected (data not shown), leading us to conclude that the upstream site is the only site in the *SpHE* promoter to which SpEts4 binds. As shown in Fig. 5, this result undoubtedly reflects the fact that the sequences surrounding the Ets core sequence at bp −107 differ significantly from those found at the −250 *SpHE* Ets4 or E74 sites to which SpEts4 binds.

SpEts4 positively activates the *SpHE* promoter-containing transgenes. Our previous studies led to the conclusion that most, and probably all, *cis* elements in the *SpHE* promoter bind positively acting factors (27). Relevant to the present studies is the demonstration that an Ets site-containing partial promoter from bp −240 to −310 fused to the basal promoter region (bp −90 to +20) retained significant, spatially correct transcriptional activity (27). We tested whether SpEts4 behaves as a positive regulator of *SpHE* promoter-driven transgenes in vivo by using three independent assays: by mutation of the Ets *cis* element, by linking the Ets site to the basal promoter, and by in vivo transactivation via coinjected SpEts4 mRNA.

Because EMSAs showed that the −240 to −310 region tested previously contains binding sites for SpOtx and an unidentified factor as well as SpEts4 (26), we prepared constructs that specifically test the contribution of the SpEts4 element to *SpHE* promoter activity by replacing the CGGAAC core sequence. Linearized constructs containing *SpHE* promoter elements driving CAT expression were microinjected into fertilized *S. purpuratus* eggs. The resulting embryos were analyzed for CAT activity and for exogenous template DNA content as described previously (10, 26). As shown in Fig. 6A, replacement of the Ets core binding site in the context of the −310 promoter results in reduced CAT enzymatic activity. When the −240 to −310 region is tested directly linked to the −90 to +20 basal promoter region, promoter activity resulting from replacement of the Ets site also is decreased to close to the basal promoter levels that we have observed in previous experiments (26). In a second set of experiments, we found that a 20-nucleotide fragment containing the Ets site linked to the *SpHE* basal promoter could reproducibly mediate a low level of activation (Fig. 6B). As shown in the transactivation experiment described below, one likely reason that this effect is
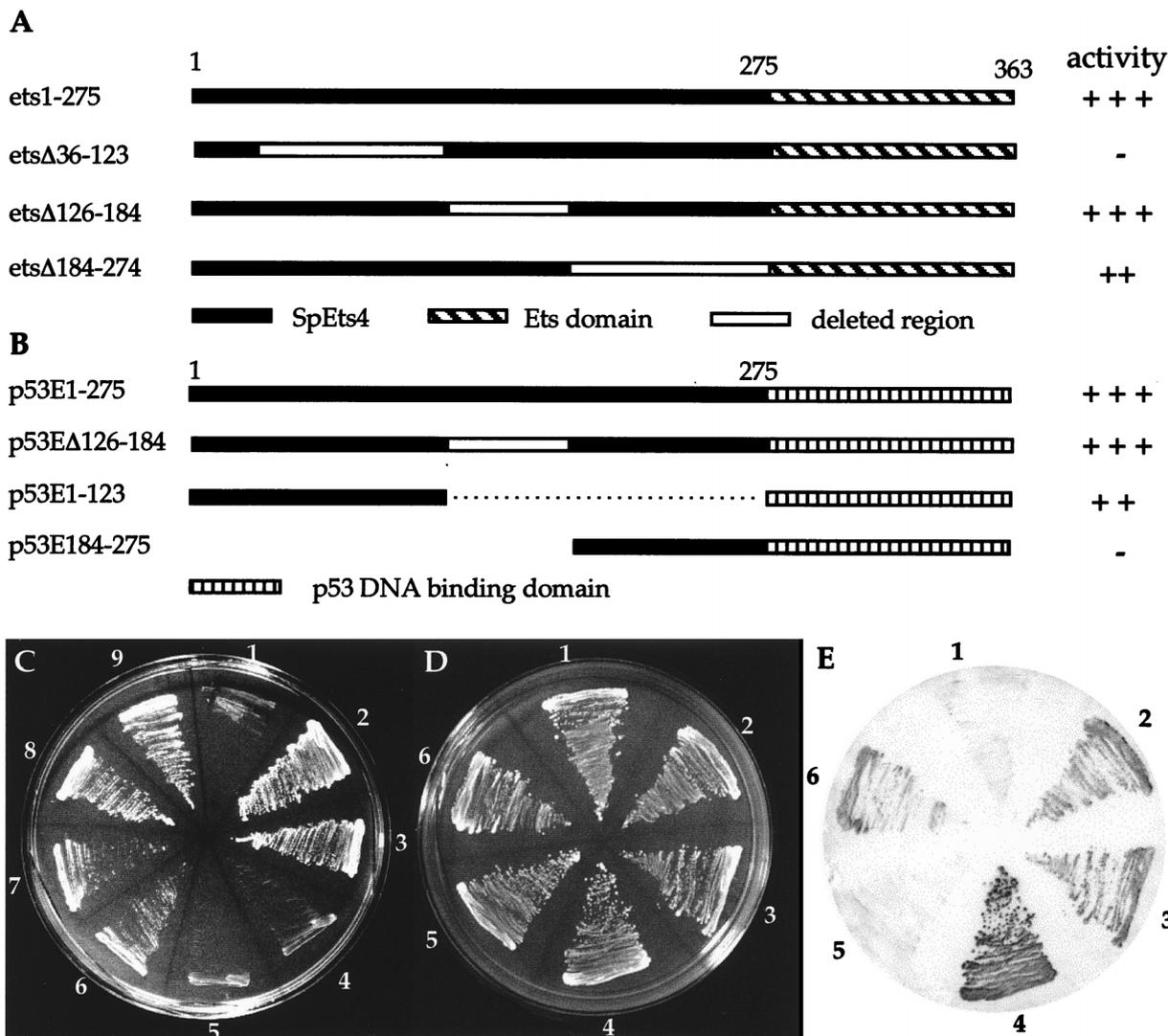
FIG. 3. Mapping of the SpEts4 transcription activation domain by the yeast one-hybrid assay. (A) Constructs encoding SpEts4 mutated by internal deletions. Each of these constructs lacks the GAL4 activation domain. (B) Constructs encoding fusion proteins of SpEts4 sequences linked to the p53 DNA binding domain. Numbers refer to SpEts4 amino acid sequences. Relative activities of reporter gene expression as measured by growth under His⁻ selection in 5 mM 3-AT or β-galactosidase expression are shown at the right. (C) Growth of yeast cells under His⁻ selection transformed with plasmids expressing wild-type or mutant SpEts4 proteins. 1, negative control transformed with nonrecombinant plasmid only; 2, positive control with wild-type SpEts4 in pGAD10, containing the GAL4 activation domain linked in frame; 3, wild-type SpEts4, lacking GAL4 sequences; 4 and 5, EtsΔ36–123 in forward and reverse orientations, respectively; 6 and 7, EtsΔ184–274 in forward and reverse orientations, respectively; 8 and 9, EtsΔ126–184 in forward and reverse orientations, respectively. (D and E) Transcriptional activation provided by p53-SpEts4 fusion proteins. (D) Growth of yeast containing an integrated copy of the p53 bait and transformed with plasmids encoding the p53-SpEts4 fusion proteins diagrammed in panel B. (E) Yeast colonies from panel D were analyzed for β-galactosidase expression. 1, negative control (untransformed cells); 2, p53E1–275; 3, p53Δ126–184; 4, positive control, containing the GAL4 domain; 5, p53E184–275; 6, p53E1–123.

modest is that the amount of SpEts4 protein in the embryo is limiting with respect to the transgenes, which are present at about 2,500 copies/expressing cell (18). It is also possible that the position of the Ets site with respect to the basal promoter is not optimal in the context of this artificial promoter. In any case, these results support the Ets site mutation data, which suggest that the *SpHE* SpEts *cis* element binds a factor with a transcriptional activating function. To test directly for transactivating activity of SpEts4, *SpEts4* mRNA was coinjected with a *SpHE* promoter transgene into sea urchin one-cell zygotes. As shown in Fig. 6C, 0.2 pg of mRNA coinjected with the Ets site-basal promoter transgene increased CAT activity several-fold compared to that in embryos containing the transgene alone. This result indicates that endogenous SpEts4 protein is

not sufficient to saturate the transgene target sites. These observations indicate that SpEts4 supplies a transcriptional activating activity in sea urchin embryos, as it does in yeast one-hybrid assays.

The early expression of the *SpEts4* gene is appropriate for its encoding a *SpHE* regulator. To determine whether expression of *SpEts4* is consistent with its encoding a *SpHE* regulatory protein, an RNase protection assay was performed with combined probes representing *SpEts4* and *SpHE* transcripts and total RNA from unfertilized eggs and selected embryo stages. To avoid possible cross-reaction and the resulting production of additional protected fragments, we used an *SpEts4* probe representing sequence outside the conserved regions and likely to be gene specific. As shown in Fig. 7, fragments of the size
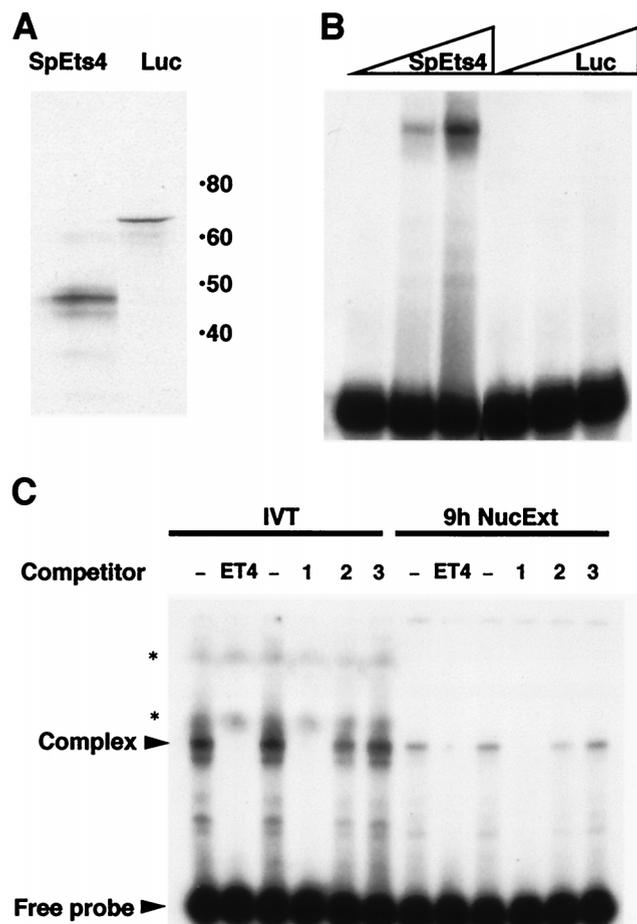
FIG. 4. SpEts4 protein synthesized in vitro binds the *SpHE* −250 promoter element. (A) SpEts4 and luciferase (Luc) (control) proteins were synthesized in vitro in the presence of [^{35}S]methionine by using a coupled transcription-translation system. The autoradiograph of products separated by polyacrylamide gel electrophoresis shows a major band at 47 kDa for SpEts4, which is similar to the size of the predicted translation product of SpEts4 cDNA. Numbers on the right are molecular masses in kilodaltons. (B) Aliquots of the translation reaction mixtures (0.04, 0.2, and 1 μl) were used in EMSAs with a ^{32}P-labeled DNA probe containing the Ets *cis* element (bp −274 to −241) from the *SpHE* promoter. (C) EMSA was carried out (as described for panel B) with either 9-h sea urchin embryo nuclear extract (NucExt) (2 μg) or in vitro-translated (IVT) SpEts4 protein (0.5-μl translation reaction mixture). The probe represented *SpHE* promoter sequence from bp −261 to −241, and, where indicated, a 200-fold molar excess of unlabeled competitor DNA fragments was included. Lanes: −, no competitor; ET4, same as the probe sequence; 1, 2, and 3, E74, PU.1, and 59/60, respectively (three different Ets *cis* elements for which the factor in sea urchin embryo nuclear extracts has different affinity). Bands marked with asterisks represent signal from the IVT protein, labeled with [^{35}S]methionine.

expected for perfect duplexes were protected for each probe, indicating that these assays are specific for each mRNA, since, under the conditions used, RNase will cleave the hybridized probe at a single mismatched base pair. *SpEts4* transcripts are relatively abundant and present at nearly equivalent levels in the unfertilized egg and in embryos through the first six cleavages (8.5 h; 64-cell stage). *SpHE* transcripts were first detectable in this assay at 8.5 h, reached peak levels at the very early blastula stage (12 h), and then rapidly decayed. In previous similar assays with probes that had higher specific activity, *SpHE* transcripts could be detected in the four- to eight-cell embryo (3 to 4 h) (20) and, as observed here, rapidly turned over after about 15 h postfertilization. We conclude that the relative patterns of accumulation of these mRNAs are consis-

tent with *SpEts4's* encoding a positive regulator of *SpHE* transcription.

SpEts4 is also expressed at much later stages, after gastrulation is complete and when *SpHE* is no longer transcribed. This observation implies that SpEts4 is also involved in the regulation of other genes in later stages of morphogenesis and histodifferentiation.

DISCUSSION

In this study we used a modification of the yeast one-hybrid system to clone a *trans*-acting factor that can activate the sea urchin *SpHE* gene. The one-hybrid approach has been successfully used to isolate other transcription factors, but virtually all of those studies utilized as bait a specific multimerized *cis* element. That kind of construct requires relatively precise mapping of the *cis* element by mutation and deletion, which is time-consuming. Furthermore, the *SpHE* regulatory region contains many positively acting sites, and multiple combinations of elements are able to drive expression to high levels in the correct cells (26, 27). Because of this functional redundancy, it was not possible for us to assign primary significance to any individual element. Therefore, we chose to use a major portion of the *SpHE* regulatory region, containing six different *cis* elements. To our knowledge, a similar approach has been used only once previously (16); however, in that case, the *cis* element was essentially naturally multimerized, since it appeared in the promoter several times. A related but converse approach, which utilized expression of DNA binding proteins in yeast to select single target *cis* elements from genomic DNA, has been successful (29).

Although the bait contained at least six *cis* elements (26), we only recovered clones encoding the one protein binding at an Ets motif, previously called site IC. Potential reasons for this include the possibilities that some proteins require interaction with other cofactors lacking in yeast for strong DNA binding, that mRNAs encoding some factors may be of relatively low abundance in eggs or underrepresented in our egg library, and that some factors may require posttranslational modification for binding that does not occur in yeast. However, an alternative explanation is that SpEts4 was more easily selected in the yeast screen because of its own transcriptional activation activity that resides in the N-terminal region. Furthermore, this activity appears to be quite strong, since it is nearly equivalent to that obtained with the positive control GAL4-p53 fusion protein.

The activating region of SpEts4 is characterized by a relatively high density of residues that are also found in the activation domains of other transcription factors, which include glutamine, serine, proline, and acidic residues. Different members of the Ets family also contain mapped activation domains that lack a conserved activation sequence but are characterized by similar enrichments for these amino acids (reviewed in reference 9). Which of these are responsible for transcriptional activation by SpEts4 is not known yet.

The selected protein, SpEts4, that binds to a *SpHE* promoter *cis* element is a member of the Ets transcription factor family, based on strong conservation in the DNA binding domain as well as similarity to a pointed domain outside this region. Based on the available sequences of the conserved Ets domain, this transcription factor is most closely related to *Drosophila* Ets4. Although sequence outside the *Drosophila* Ets4 DNA binding domain is not yet available, it is likely that it also will be more similar to SpEts4 than to other Ets proteins. Phylogenetic comparison of Ets domain sequences suggests that *Drosophila* Ets4 cannot be assigned to any of the nine known

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SpHE -250                    TTCAACCCGGAACTACG
E74                 CTTCTCTAGCTGAATAACCGGAAGTAACTCATCG
PU.1               TGAAATAACCTCTGAAAGAGGAACAAGGTTAGGT
59/60              TTGTTTAGCGCGTGACGGGAAGGGGGCTTGGCGATC
SpHE -107                  TACGCGTACGGAACAGGC


E74/Elf-1 consensus        AACCCGGAAGT
                                T  A
                                A
```

FIG. 5. Sequences of *SpHE* Ets sites and Ets competitors.
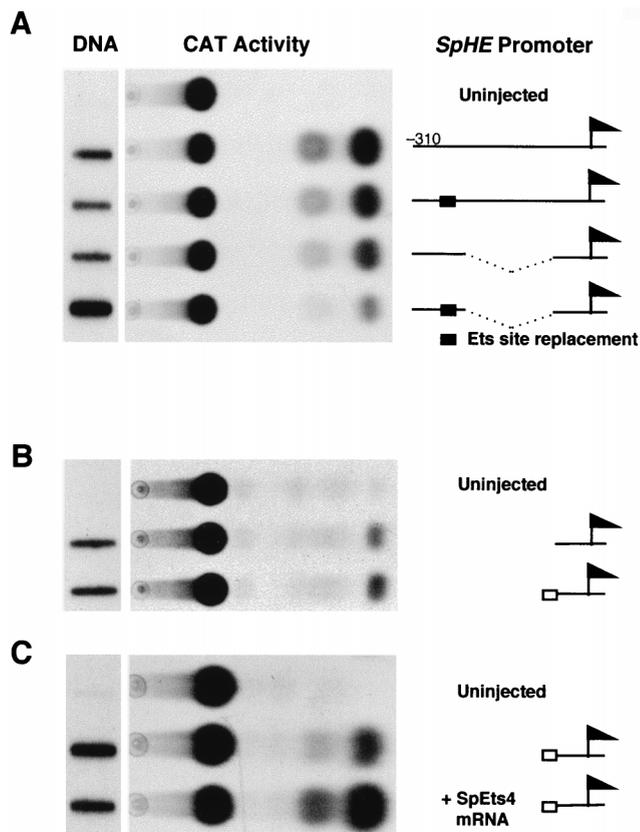

FIG. 6. SpEts4 activates the *SpHE* promoter via the Ets element. (A) The effect of the Ets element on *SpHE* promoter activity in vivo was determined in the context of the intact −310 promoter (upper two constructs) and with a mutated version from which sequences between bp −90 and −240 had been deleted (lower two constructs). CAT reporter activity expressed from microinjected constructs from 75 embryos was assayed at the blastula stage. (B) CAT activity elicited by addition of the *SpHE* −250 Ets site (open box) to the *SpHE* basal promoter region (line) (bp −90 to +20). (C) Transactivation of the *SpHE* Ets site transgene by coinjection of *SpEts4* mRNA. Levels of construct DNA were determined by slot blotting (left), using aliquots of the same embryo batches as assayed for CAT activity.

groups, including those with both invertebrate and vertebrate members (13). The identification of SpEts4 suggests that SpEts4 and *Drosophila* Ets4 constitute a new group within the Ets family. It is interesting that both factors are expressed in oocytes and early embryos (6), but determining whether they mediate conserved developmental functions will require identification of target genes in both species. It would be interesting to know if this group, currently with two invertebrate members, is conserved in vertebrates, as are some other groups of Ets factors. SpHE is a Zn^{2+}-dependent metalloprotease of the stromelysin/collagenase family. Interestingly, Ets transcription factors have also been shown to be important regulators of genes encoding mammalian stromelysin (4, 24) and collagenase (14).

SpEts4 binds effectively to an Ets site in the *SpHE* promoter and to the *Drosophila* E74 site from the E74 promoter (22) but not to motifs recognized by the PU.1 Ets class or a sea urchin Ets2-like factor (8). The exact sequence features that determine binding affinities of different Ets factors are not known. However, consistent with our in vitro binding data is the observation that in the sequences immediately flanking the GGAA core, the *SpHE* SpEts4 site is more similar to the *Drosophila* E74 competitor sequence than to those of the PU.1 and Ets2 binding site competitors. Random oligonucleotide site selection assays with either E74 (22) or its mammalian homolog, Elf-1 (15), generated a consensus recognition site, AAC/TCC/AGGAAGT, which is an excellent match (10 of 11) to the *SpHE* SpEts4 site (AACCCGGAACTA) (Fig. 5), although closely related, but somewhat more degenerate, consensus sites have also been obtained for other Ets subfamily members (reviewed in reference 13). As is the case for all Ets factors, binding of SpEts4 to the *SpHE* promoter results in DNase I hypersensitivity near the core recognition motif (12, 26, 28).

Many Ets proteins interact with other transcription factors, and in some cases (e.g., AP1, SRF, CBF, Sp-1, and myb), this interaction facilitates binding and/or activity (reviewed in references 9 and 25). Candidate regions for binding interacting proteins reside on either side of the SpEts4 binding site in the *SpHE* promoter. About 15 nucleotides 5′ of the SpEts4 site in the *SpHE* promoter is a consensus motif for binding the NF-κB class of transcription factors (GGGTAATCC) (3), which are known to interact with Ets family members (2). Immediately downstream of the Ets site is a large DNase I-protected region produced by a *cis* element(s) and factor(s) that remain to be defined (26, 28).

The site with which SpEts4 interacts confers positive activity in the *SpHE* promoter, as demonstrated by assays in vivo on wild-type and mutated *SpHE* promoter-driven transgenes. Mutation of the Ets site in a partial promoter containing only two other identified upstream *cis* elements reduces activity nearly to basal levels, indicating that it is at least essential, if not sufficient, for promoter activity in this construct. That the func-

tion conferred by the SpEts4 *cis* element is positive is strongly supported by the observation that transcription activated through the *SpHE* Ets site could be significantly upregulated by coinjected SpEts4 mRNA. The finding that SpEts4 protein activates *SpHE* transcription agrees well with our previous in vivo genomic footprinting assays. There is within the *cis* element to which SpEts4 binds a strong dimethylsulfate-sensitive site at early stages when the *SpHE* promoter is active but not at later stages when it is inactive (28). Since this site maps to the exact nucleotide position observed in similar assays of an Ets-DNA interaction (1), it likely reflects an in vivo SpEts4-DNA interaction that occurs when the *SpHE* gene is active. The demonstration that SpEts4 confers positive activity is consistent with our model that it is one of a set of positive factors regulating *SpHE* transcription.

The timing of *SpEts4* gene expression is consistent with its serving as an activator of *SpHE* transcription. *SpEts4* transcripts, which accumulate during oogenesis, persist only until about the 64-cell stage (8 h postfertilization) and turn over rapidly during the next several hours, corresponding to one or two cleavages. *SpHE* transcription begins in the 4- to 8-cell embryo, mRNA levels are maximal at about 12 h postfertilization (170-cell stage), and then transcription is repressed and
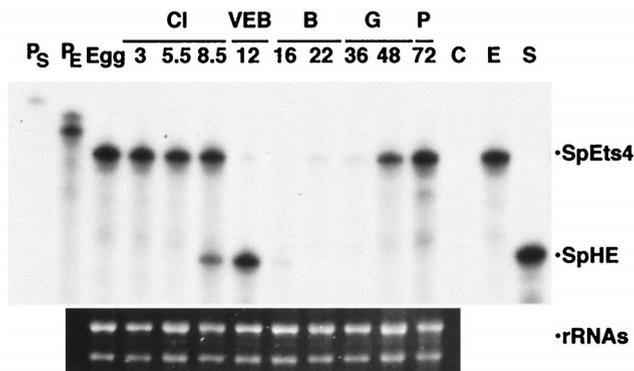
FIG. 7. Accumulation of *SpEts4* message precedes activation of the *SpHE* gene. Levels of *SpEts4* and *SpHE* mRNAs were assayed by RNase protection with both probes combined in the same reactions and hybridized with total RNA isolated from eggs or embryos at the indicated stages of development. Hours postfertilization are indicated, as are the corresponding developmental stages (Cl, cleavage; VEB, very early blastula; B, blastula; G, gastrula; P, pluteus). Samples in the last two lanes serve as markers and are the RNase-resistant fragments for each single probe (E, *SpEts4*; S, *SpHE*) after hybridization to early-blastula-stage RNA. The left two lanes show unhybridized *SpHE* and *SpEts4* probes (P_S and P_E, respectively). Lane C, negative control with both probes hybridized with yeast tRNA. The lower panel shows the ethidium bromide-stained gel of egg and embryo RNA samples, demonstrating that equal quantities of intact RNAs were used in these reactions.

mRNA levels decay within the next several hours. *SpHE* is not transcribed at late embryonic stages when SpEts4 mRNAs reappear, implying that SpEts4 protein regulates other genes during sea urchin embryogenesis.

In the context of a partial *SpHE* promoter transgene, this Ets *cis* element mediates binding of an essential positive activity. The same partial promoter also drives expression of a reporter gene appropriately only in nonvegetal cells, as can several different subsets of *cis* elements in the *SpHE* regulatory region (27). These results have led us to propose that restriction of *SpHE* transcription to nonvegetal cells of the early blastula reflects the partitioning of multiple positive activities to this early embryonic domain. The results presented here suggest that SpEts4 is one of those activities. Experiments to characterize the expression patterns of *SpEts4* during sea urchin embryogenesis and to test whether SpEts4 function is restricted to cells in nonvegetal blastomeres of the early sea urchin embryo are under way.

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