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Early mRNAs, spatially restricted along the animal-vegetal axis of sea urchin embryos, include one encoding a protein related to tolloid and BMP-1

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Summary

The cloning and characterization of cDNAs representing four genes or small gene families that are coordinately expressed in a spatially restricted pattern during the very early blastula (VEB) stage of sea urchin development are presented. The VEB genes encode multiple transcripts that are expressed transiently in embryos of *Strongylocentrotus purpuratus* between 16-cell stage and hatching, with peak abundance 12 to 15 hours post-fertilization (~150-250 cells). The VEB transcripts share the same spatial pattern in the early blastula embryo: they are asymmetrically distributed along the animal-vegetal axis but their distribution around this axis is uniform. Thus, the VEB transcripts are the earliest messages to reveal asymmetry along the primary axis in the sea urchin embryo. The temporal and spatial patterns of VEB transcript accumulation are not consistent with involvement of these gene products in cell division or in tissue-specific functions. Furthermore, VEB messages cannot be detected in either ovary or adult tissues, suggesting that these genes function exclusively during embryogenesis. We suggest that the VEB genes function in constructing the early blastula. Two VEB genes encode metalloendoproteases: one (SpHE) is hatching enzyme and the other (SpAN) is similar to bone morphogenetic protein-1 (BMP-1; Wozney et al., *Science* 242: 1528-1534, 1988) and the *Tolloid* gene product (tld) (Shimell et al., *Cell* 67: 459-482, 1991). Several lines of evidence suggest that the VEB genes are regulated directly by factors or regulatory activities localized along the maternally specificed animal-vegetal axis.

Key words: sea urchin embryo, sea urchin blastula, coordinate early gene expression, animal-vegetal axis, metalloendoprotease.

Introduction

The very early blastula (VEB, morula-hatching) period of sea urchin development is similar but not identical to the mid-blastula transition (MBT) of *Xenopus* embryos and the cellular blastoderm (CB) stage of *Drosophila* embryogenesis. In all three organisms, cell cycles, which are synchronous and rapid during cleavage, become asynchronous and the overall rate of cell division slows greatly (Hinegardner, 1974; Satoh et al., 1976; Edgar et al., 1986). In addition, similar changes in cell motility are observed in each of these embryos. Considerable pulsatile activity is apparent at the basal surface of VEB-stage sea urchin blastomeres (Wolpert and Mercer, 1963) and in cells of post-MBT *Xenopus* embryos (e.g., Newport and Kirschner, 1982a,b) and cell movement begins during the CB-stage of *Drosophila* embryogenesis (Foe and Alberts, 1983).

In the sea urchin embryo, several morphological changes are also evident at VEB stage. The blastocoel enlarges rapidly resulting in large increases in the apical and basal surfaces of blastomeres, and this is accompanied by vectorial deposition and modification of extracellular matrices both inside and outside the embryo (McCarthy and Burger, 1987; Alliegro et al., 1988; Bisgrove et al., 1991; for a recent review, see McClay et al., 1990). A single cilium forms on the apical surface of cells, nuclei are positioned in the basal cytoplasm, and desmosomes form at the apical ends of intercellular junctions (reviewed in Wolpert and Mercer, 1963).

The early sea urchin embryo differs significantly from *Xenopus* and *Drosophila* embryos with respect to onset of embryonic gene activity. The hallmark of the MBT and the CB stage is a 50-fold increase in transcription rate (e.g., Newport and Kirschner, 1982a,b; Edgar et al., 1986). While in these organisms, nuclei of cleaving embryos are essentially transcriptionally quiescent, nuclei of unfertilized sea urchin eggs and early cleavage stage embryos are as transcriptionally active as they are at blastula stage (reviewed by Davidson, 1976; Brandhorst, 1980). This difference arises in part from the fact that interphase during sea urchin embryo cleavage is considerably longer than in these other organisms.

During the VEB period, three general classes of embryonic transcripts that accumulate in spatially restricted patterns during sea urchin development begin to appear. Expression of the first class of mRNAs is correlated with cell division and future growth of specific regions of the embryo. They accumulate during oogenesis, decay in the egg and early cleavage stages and reaccumulate in all blastomeres at VEB stage. As development proceeds, these messages become progressively restricted to regions containing dividing cells, primarily oral ectoderm and endoderm of the pluteus larva (Kingsley et al., submitted). The second class of transcripts identify major tissue anlagen and presumably effect terminally differentiated phenotypes (e.g., Cox et al., 1986; Benson et al., 1987; Angerer et al., 1988; Harkey et al., 1988; Hardin et al., 1988; Yang et al., 1989; Angerer et al., 1989; Akasaka et al., 1990; Grimwade et al., 1991; Kingsley et al., submitted). Thus, the VEB period at the end of cleavage is a pivotal time when many genes whose functions are related to cell division and differentiation are significantly upregulated.

In this work we describe a third class of early, spatially restricted embryonic transcripts. These mRNAs are encoded by four genes or small gene families that are transiently expressed during the VEB stage of development and share surprisingly similar temporal patterns of expression. At least three of these genes encode mRNAs that accumulate in a similar, if not identical, spatially restricted pattern which reflects the maternally determined (Hörstadius, 1973; Maruyama et al., 1985) animal-vegetal embryonic axis. We show that mRNAs appear very soon after fertilization, at least by 8-cell stage, that transcripts accumulate preferentially in pluripotent 16-cell macromeres, and that VEB gene activation is independent of intercellular interactions. These observations lead to the hypothesis that activation of these genes is not a consequence of cell fate specification but is likely to be controlled by maternal regulatory activities. Sequence analysis identifes two of the VEB gene products as metalloendoproteases: *SpAN*, encodes a protein related to the developmentally regulated gene products, human BMP-1 and *Drosophila* tld, while *SpHE* encodes hatching enzyme, a member of the collagenase family.

Materials and methods

Embryo culture

Stronglyocentrotus purpuratus embryos were cultured at 15°C as previously described (Angerer and Angerer, 1981). When fertilization membranes were to be removed, fertilization was carried out in sea water containing 10 mM para-aminobenzoic acid and 0.1 mM ethylenediaminetetraacetic acid (EDTA) (Showman and Foerder, 1979; McCarthy and Spiegel, 1983). Any culture that did not produce morphologically normal pluteus larvae was discarded. Approximate times for major stages of development and cell numbers are: 5.5 hours, 16 cells; 12 hours, very early blastula, 180 cells; 18 hours, hatching blastula, 300 cells; 24 hours, mesenchyme blastula, 400 cells; 36 hours, mid-gastrula, 600 cells; 48 hours, late gastrula, 800 cells; and 72 hours, pluteus larva, 1800 cells.

Purification of mesomeres, macromeres and micromeres

Sixteen-cell embryos (>95%) from single pair matings were demembranated by passage through 54 μm Nitex cloth (Tetko Inc., Depew, NY; Showman and Foerder, 1979), washed at 4°C several times in calcium-magnesium-free sea water (CMFSW, Hynes and Gross, 1970) and dissociated to a single-cell suspension by repeated passage through 54 μm Nitex mesh according to Nelson and McClay (1988). The separated cells were fixed by adding an equal volume of ice-cold 95% ethanol over 5 minutes with constant slow stirring, resuspended in >50 volumes ice-cold 50% ethanol, 1.3% NaCl and then in ice-cold 95% ethanol. Fixed cells were either immediately prepared for elutriation or stored in 95% ethanol at 4°C for up to 4 months. Just prior to elutriation, any cell aggregates were removed by filtration and cells were separated by size using a modification of the long collection method (Keng et al., 1981) to be published elsewhere. Purity of blastomere types was greater than 90% and RNA extracted from separated cells was undegraded and representative as determined by RNA blot analysis of uniformly distributed mRNAs (Nasir et al., manuscript in preparation).

Dissociation and culture of separated cells

Demembranated 2-cell embryos were prepared as described above and washed at 4°C once by sedimentation at 2000 *g* in cell dissociation medium [CDM: 20% distilled water, 40% CMFSW, 40% 1 M dextrose (Harkey and Whiteley, 1985)], once in 50% CDM/50% CMFSW and three times in CMFSW. After removal of any clumps of cells by gentle filtration through 54 μm Nitex cloth, separated blastomeres were resuspended at approximately 1×10^4 cells/ml in calcium-free sea water containing 10 mM Tris-HCl, pH 8.0, 1 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), 50 μg streptomycin/ml, and 30 U penicillin/ml (Giudice and Mutolo, 1970) and cultured at 15°C with rapid stirring. Control embryos were held at 4°C during dissociation (< one hour) and did not undergo cytokinesis, but resumed cell division approximately one hour after addition of 10 volumes 15°C sea water containing 10 mM Tris-HCl and antibiotics (described above).

cDNA library construction and screening

Demembranated embryos, pooled from several cultures, harvested at either 9, 12 or 15 hours of development were lysed by 10-15 strokes in a Dounce homogenizer with a B pestle in polysome buffer [250 mM NaCl, 5 mM $MgCl_2$, 25 mM EGTA, 10 mM PIPES, pH 6.8, 250 mM glycerol, 110 mM glycine, 1 mM dithiothreitol (DTT), 1 mM phenylmethyl-

sulfonyl fluoride (PMSF) and 0.5% NP40 (Grainger and Winkler, 1987)]. Polysomal RNA was prepared by a modification of the gel filtration method of Calzone et al. (1982). The post-nuclear supernatant containing 1% sodium deoxycholate and 0.5% Triton X-100 was passed over a 3 × 43 cm Bio-Gel A-15m column (BioRad Lab., Richmond, CA), previously blocked with egg lysate and equilibrated in polysome buffer. Excluded fractions were pooled, brought to 50 mM EDTA, and RNPs sedimenting at <100 S on preparative exponential sucrose gradients containing 10 mM EDTA (Goldberg et al., 1973) were precipitated with isopropanol and RNA was purified by extraction with phenol/chloroform and precipitation with ethanol. Poly(A)$^+$ RNA was prepared by three consecutive passes of the RNA over an oligo(dT)-cellulose column as described by Maniatis et al. (1982), reextracted with phenol/chloroform and precipitated with ethanol.

An oligo(dT)-primed cDNA library was constructed from equal quantities of 9-, 12- and 15-hour polysomal poly(A)$^+$ RNA. Double-stranded cDNA was synthesized by the method of Gübler and Hoffman (1983), size-selected by the method of Huynh et al. (1986), inserted into λgt10 (Stratagene, La Jolla, CA) by means of *Eco*RI linkers and packaged in vitro with the Gigapack Plus packaging kit (Stratagene). For selection of additional cDNAs, a random-primed library was prepared from 9- and 12-hour RNA templates in λZap (Stratagene) via *Eco*RI/*Xmn*I adaptors (Huynh et al., 1986). These libraries contain 6.5 and 10 × 10^5 primary recombinants, respectively, and less than 2% mitochondrial sequences.

The oligo(dT)-primed library was screened by both competition and differential hybridization. For competition screening, an end-labeled VEB (9-, 12- and 15-hour) poly(A)$^+$ cytoplasmic RNA probe was prepared by partial hydrolysis to ~150 nt (Cox et al., 1984) and end-labeling with γ[^{32}P]-ATP and T4 polynucleotide kinase (Maniatis et al., 1982) to a specific activity of approximately 10^8 disints/minute/μg. For differential hybridization, a cDNA probe (4 × 10^8 disints/minute/μg) representing VEB (9- and 12-hour) poly(A)$^+$ RNA was depleted by hybridization with a 100-fold sequence excess of total pluteus RNA for three days at 50°C in 0.48 M sodium phosphate buffer, pH 6.8 (approximately 20 × $R_o t_{1/2}$ for rare mRNAs). After diluting the hybridization reaction to 50 μg RNA/ml, single-stranded cDNA (VEB-enriched) was separated from the cDNA/RNA hybrids (VEB-depleted) by hydroxyapatite chromatography at 50°C in 0.12 M sodium phosphate, pH 6.8, containing 0.2% sodium dodecyl sulfate (SDS). The bound hybrids were eluted at 95°C and RNA in both fractions was hydrolyzed with 0.3 M NaOH. Phosphate was removed by dialysis and cDNAs (VEB-enriched and VEB-depleted) were concentrated by ethanol precipitation in the presence of carrier *E. coli* DNA, and size-selected as described above, pooling fractions containing fragments greater than 100 nt.

Phage at 10^4 or 10^3 plaques per 100 mm Petri plate were used for competition or differential screening, respectively. Prior to hybridization, phage lifts were washed twice with 4 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M trisodium citrate), 1% SDS, 50 mM 2-mercaptoethanol at 68°C for 1 hour, and twice in 4 × SSC, 1% SDS at 68°C to reduce backgrounds. Competition hybridization to pinpoint plaques was carried out in 1.0 M NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, 1 mg yeast RNA/ml, 1 × Denhardt's solution (1 × Denhardt's is 0.02% each Ficoll, bovine serum albumin, fraction V, and polyvinylpyrrolidone) and 10% polyethylene glycol, (PEG; relative molecular mass, 6000-8000) at 45°C for three days using 1 × 10^7 cts/minute 150 nt RNA probe/ml (2 × 10^7cts/minute/filter) and 1.0 mg pluteus total cellular RNA/ml (2.0 mg/filter) as the competitor. Filters were washed by the method of Church and Gilbert (1984) at 50°C. In some instances, the background was reduced by treatment of the filters with RNAase A as previously described for in situ hybridization (Cox et al., 1984). Autoradiography was carried out for 1-2 days at −70°C using two intensifying screens. Screening by the differential method was carried out by hybridizing replicate lifts using 2-4 × 10^5 cts/minute VEB-enriched or VEB-depleted cDNA probes/ml (4-8 × 10^5 cts/minute/filter) in 0.825 M NaCl, 25% deionized formamide, 25 mM phosphate buffer, pH 6.8, 5 mM EDTA, 10 mM EGTA, 1 × Denhardt's solution, 1% SDS, 10% PEG, and 12.5 μg calf thymus DNA/ml at 37°C for three days. Filters were washed successively at 54°C in 4 × SSC, 2 × SSC, 1 × SSC and 0.1 × SSC, each containing 1% SDS. Autoradiography was carried out for 1-2 weeks at −70°C using two intensifying screens. To conserve probe, secondary screens were carried out on matrices consisting of five to ten plaques from each primary isolate selected from a low density intermediate plate.

Three independent screens, one by the competition method and two by the differential method, yielded 52 putative VEB cDNAs. To select those representing mRNAs absent from maternal RNA, these inserts were subcloned in pGEM3 or pGEM4 (Promega Biotec, Madison, WI), labeled with ^{32}P using the Klenow fragment of DNA polymerase I and random oligonucleotide primers and hybridized at approximately T_m−25°C to four-lane (egg, 9-hour, 12-hour, pluteus) RNA blots as described below. Eleven (Egg−, VEB+, Pluteus−) cDNAs representing four different genes or small gene families were identified. Two cDNAs representing the most abundant VEB mRNAs (*SpHE* and *SpAN*) were selected in the competition screen while two sets of rarer mRNAs (*VEB3* and *VEB4*) as well as 6 additional isolates of *SpHE* and one additional isolate of *SpAN* were identified in the differential screen. The remaining 41 clones selected in the initial screens hybridize to mRNAs that are expressed at similar levels in eggs and VEB-stage embryos and at variable levels in plutei.

RNA isolation

All RNAs used in this study, except those derived from total adult RNA or elutriated 16-cell blastomeres, were purified according to the method of Nemer et al. (1984). RNA used in the developmental RNA blots was obtained from a single pair mating. Ovaries were pooled from five female *S. purpuratus* that had shed the majority of their eggs. Pluteus RNA used as a competitor or subtractor was pooled from 3-5 cultures.

Total adult sea urchin RNA was isolated from a single adult male according the the method of Kingsley et al. (submitted). Briefly, the whole urchin was quick-frozen in liquid N_2, pulverized, and blended with 3 volumes lysis buffer (Nemer et al., 1984). RNA in the lysate was extracted with phenol/chloroform, ethanol precipitated, resuspended with a Dounce homogenizer in a buffered solution of guanidinium isothiocyanate (Chirgwin et al., 1979), and sedimented through 5.7 M CsCl, 0.1 M EDTA. After ethanol precipitation, the RNA was dissolved in diethylpyrocarbonate (DEPC)-water, precipitated with 2 M LiCl, reprecipitated with ethanol, and stored at −20°C until use.

RNA was isolated from elutriated blastomeres according to Kingston et al. (1988) modified as follows: cells in selected fractions were collected by centrifugation, resuspended in >10 volumes of 4 M guanidinium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM dithiothreitol, 0.5% N-lauroylsarcosine and vortexed vigorously until complete lysis occurred. Lysates were either used immediately or stored at

−70°C. RNA was purified by sedimentation through 5.7 M CsCl, 0.1 M EDTA, pH 8.0. All RNA preparations were analyzed for concentration, purity and quality by spectrophotometry and gel electrophoresis.

Probe sequences

VEB probes used in this study were from the coding regions of *SpHE* (550 nt for RNA blot analysis and RNAase protection, and 1.1 kb for in situ hybridization), *SpAN* (320 nt for RNA blot analysis and 320 nt or 450 nt for RNAase protection), *VEB3* (700 nt for RNA blot and in situ analysis, 300 nt for RNAase protection). The *SpAN* probe used for the in situ hybridization shown was 600 nt from the 3′ untranslated region of the message. The *VEB4* probe for all analyses was 400 nt of 3′ untranslated sequence. Control probes were *p782* (512 nt, Wells et al., 1982; Hurley et al., 1989) for mitochondrial 16S rRNA; *RTS* (360 nt, Angerer et al., 1992) for an internal transcribed spacer sequence of the ribosomal RNA primary transcript; *Spec1* (390 nt protein coding region, Hardin et al., 1988) and *Spec3* (1.74 kb cDNA, Eldon et al., 1987).

Hybridization analyses

RNA blots

RNA was fractionated by electrophoresis and blotted as described previously (Hurley et al., 1989). Blots were hybridized with random-primed DNA probes in a solution containing 2 × SSC, 1% SDS, 5 × Denhardt's solution, 0.025 M phosphate buffer, pH 6.8, 0.1% sodium pyrophosphate, 10% PEG, and 100 μg calf thymus DNA/ml. The temperature and formamide concentration were adjusted to approximately T_m−25°C. Blots were washed successively in 4 × SSC, 2 × SSC, 1 × SSC and 0.1 × SSC, each containing 1% SDS, at 5 to 10°C below the T_m.

RNAase protection assays

Probe preparation, hybridization and analysis of RNAase-resistant hybrids were carried out as described previously (Yang et al., 1989). A total of 10 μg of sea urchin RNA + yeast RNA were hybridized to kinetic termination with a sequence excess of labeled probe (1.0 ng). Probe specific activities and amounts of urchin RNA are indicated in the figure legends. Control hybridizations were carried out using 1 to 5 μg sea urchin RNA and either a *Spec1* or *p782* (mitochondrial rRNA) probe.

In situ hybridization

These experiments were carried out as described in Cox et al. (1984) and Angerer et al. (1987). All experiments included a high stringency wash step (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 10 mM DTT, 60-65°C, 10 minutes) prior to treatment with RNAase A. Other details are given in the figure legends.

Sequencing

SpAN, *SpHE* and *VEB3* cDNAs were either partially deleted by the exonuclease III-mung bean method (Stratagene, La Jolla, CA) or restriction fragments were subcloned into M13mp18, M13mp19 (Messing and Vieira, 1982), pBluescript [SKII+] or pBluescript [SKII−] (Stratagene, La Jolla, CA). Sequencing was carried out according to United States Biochemical instructions using [^{35}S]dATP and Sequenase (Version 1.0). DNA and deduced amino acid sequences were analysed by computer programs developed by Pearson and Lipman (1988) and the Genetics Computer Group Sequence Analysis Software Package (University of Wisconsin, Madison, WI; Devereux et al., 1984).

Results

Selection of very early blastula (VEB) cDNAs

The goal of this work was to identify mRNAs that are transiently expressed in spatially restricted patterns during very early sea urchin development. Our screen selected for mRNAs with the following characteristics. First, they are present at very early blastula (9-15 hours, 100-250 cells), but not at later stages of development, and thus do not encode gene products involved in differentiated cell functions. Second, they are absent from the maternal RNA pool and are therefore unlikely to have general functions in cell division. Third, they accumulate in spatially restricted patterns. Screening by this strategy yielded 11 cDNA clones representing mRNAs transcribed from 4 genes or small gene families. We refer to these collectively as the VEB genes. The inferred protein sequences of two mRNAs suggest that they encode metalloendoproteases. SpAN is related to Astacin, [Titani et al., 1987; BMP-1 (Wozney et al., 1988) and tolloid (tld; Shimell et al., 1991)], and SpHE appears to be the *S. purpuratus* homolog of the *Paracentrotus lividus* hatching enzyme (Lepage and Gache, 1990). *VEB3* and *VEB4* gene products have not yet been identified.

The VEB transcripts are coordinately expressed

RNA blot analyses shown in Fig. 1 demonstrate that the VEB mRNAs share very similar temporal patterns of accumulation and decay. By this assay, transcripts are first detectable during late cleavage, rapidly reach peak abundance 12 to 15 hours post-fertilization, and disappear (with one exception) by mesenchyme blastula stage (25 hours post-fertilization). As dictated by the screening strategy, the VEB messages are undetectable in eggs or in pluteus larvae. Hybridization with a probe for mitochondrial rRNA (MITO) which is present at equal concentrations at all developmental stages (Wells et al., 1982) verifies that these blots contain equal amounts of intact RNA from each stage.

The single exception to the VEB temporal pattern is the shorter *VEB3* transcript which accumulates with the VEB mRNAs but persists at much lower levels through pluteus stage (Fig. 1). Gene counting by reconstruction blots (Hardin et al., 1988) and sequence analysis of multiple cDNAs (data not shown) strongly support the idea that at least two different *VEB3* genes are transcribed in early blastulae and these presumably encode the different mRNAs detected in the RNA blot. However, gene-specific probes will be required to verify this conclusion.

The probes used in the RNA blot analysis were synthesized at different specific activities and the autoradiographic exposures were chosen to reveal the presence of minor transcripts, or to resolve multiple transcripts of similar length (see below). Therefore, the relative abundance of the different sets of VEB mRNAs

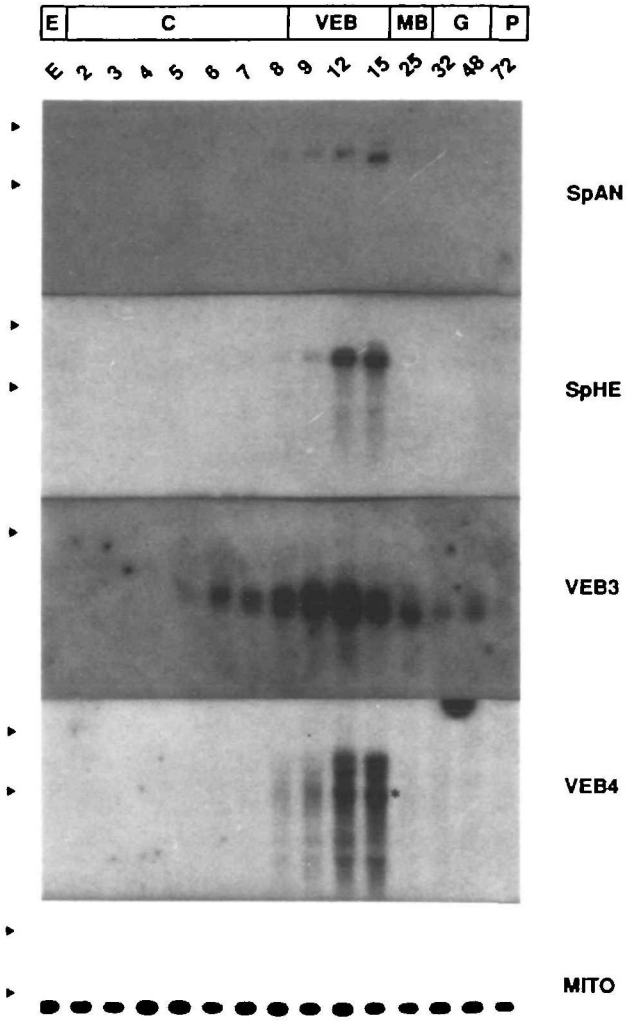


Fig. 1. VEB probes detect multiple messages that are coordinately expressed. VEB probes were hybridized to blots of total RNA prepared from embryos of the same single pair mating. The RNA loads were approximately equal as demonstrated by stripping the *SpHE* blot and rehybridizing with the mitochondrial rRNA probe, *p782*, which detects an RNA expressed at uniform levels throughout development (MITO). Times of development, in hours, are indicated above the lanes and are related to morphologically defined stages at the top of the figure and to cell number in *Materials and Methods*; E, Egg; C, cleavage; VEB, very early blastula; MB, mesenchyme blastula; G, gastrula; P, pluteus. Arrowheads at left indicate the positions of the 26 S (3800 nts) and 18 S (1800 nts) rRNAs. For the *VEB3* blot, only the position of the 18 S rRNA is indicated. The asterisk between the 12- and 15-hour lanes of the *VEB4* blot marks a band similar in length to transcripts detected by the *SpHE* and *SpAN* probes.

is not accurately reflected by the signal intensities. Solution titration indicates that there are approximately 150,000 *SpHE* transcripts and 25,000 *SpAN* transcripts per embryo at 12 hours (data not shown). The absolute values differ severalfold among cultures presumably as a result of the rapid accumulation and decay of VEB mRNAs in cultures of slightly different developmental age. *VEB3* and *VEB4* transcripts are approximately twentyfold less abundant than *SpHE* as determined by comparison of signals on RNAase protection autoradiograms (data not shown).

VEB gene families encode multiple transcripts

The RNA blot analyses (Fig. 1) indicate that each VEB probe detects transcripts of more than one length. *SpHE*, *SpAN* and *VEB4* transcripts present at 12 hours undergo similar slight decreases in size (~100 nt) by 15 hours. This probably results from shortening of the poly(A) tails related to mRNA turnover (reviewed by Bernstein and Ross, 1989), and is consistent with the observation that these mRNAs rapidly disappear from the embryo within the next several hours (data not shown). A similar phenomenon may apply to the larger *VEB3* transcript, but this cannot be resolved on the blot shown in Fig. 1. In addition, all probes detect multiple transcripts not attributable to polyadenylation differences. This is unlikely to result from cross-reaction of the probes with distantly related mRNAs (> 10% mismatch) because the stringency of the post-hybridization wash was T_m-5 to 10°C. *SpHE* and *SpAN* cDNAs hybridize to single resolvable species in 9- and 12-hour RNA, but to at least 1 larger transcript in 15-hour embryo RNA (Fig. 1). These new transcripts may be derived from different copies of the *SpHE* and *SpAN* genes since results of genomic DNA blots and gene counting are consistent with the presence of two copies of each of these genes per haploid genome (data not shown). Multiple transcripts have also been observed for the *Paracentrotus lividis* homolog of *SpHE* (Lepage and Gache, 1990). The transcripts detected by the *VEB4* cDNA probe probably derive from several different transcription units since this probe hybridizes to 5-8 fragments on genomic DNA blots even under moderately stringent conditions (data not shown). The most abundant RNA species (marked by an asterisk in Fig. 1) recognized by this 3′ untranslated region probe is similar in length to transcripts detected by *SpHE* and *SpAN* coding region probes, raising the possibility of a shared repetitive element. However, the lengths of the other *VEB4* transcripts distinguish them from *SpHE* and *SpAN* mRNAs.

VEB mRNAs are detectable only in embryos

To determine whether VEB genes are expressed during oogenesis or in adult tissues, we analyzed RNAs isolated from ovaries and from a whole male adult sea urchin by RNAase protection, shown in Fig. 2. Positive controls using RNA from 12-hour embryos (12) demonstrate that each probe is hybridizable, and the integrity of ovary (O) and adult (A) RNAs is illustrated by their ability to form RNAase-resistant hybrids with a ribosomal transcribed spacer *(RTS)* probe (Fig. 2, right panel). Despite intentional over-exposure of the autoradiograms, none of the VEB messages is detectable in either ovary or adult RNA. All RNAase-resistant

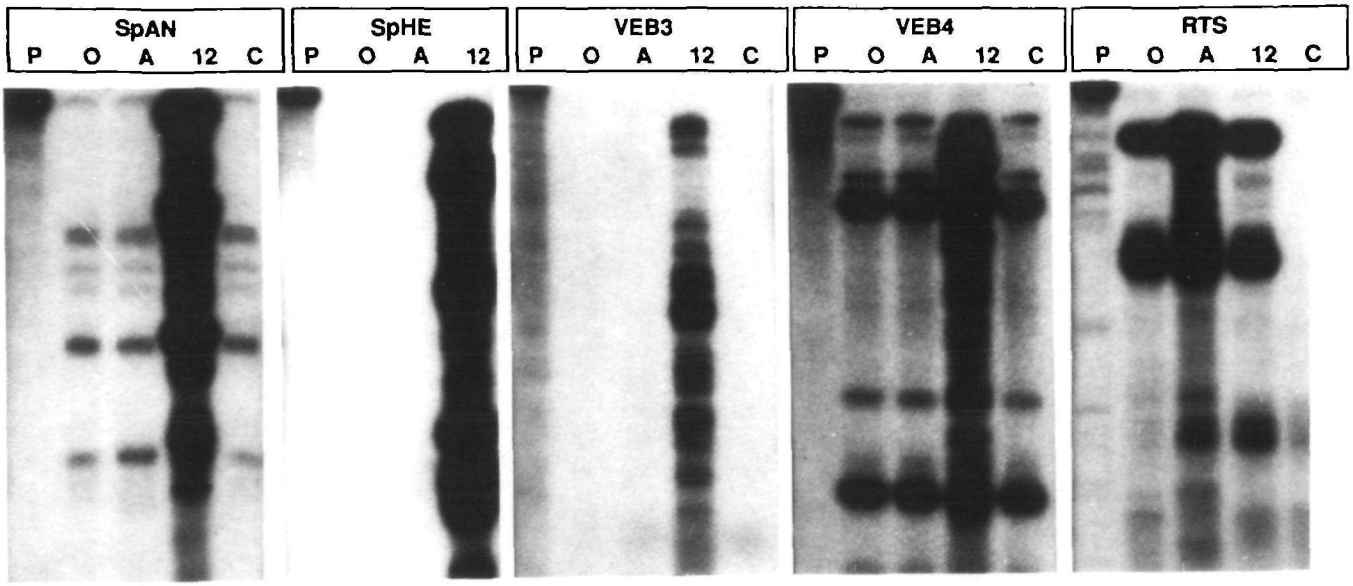


Fig. 2. VEB mRNAs are expressed only during embryogenesis. Antisense probes for *SpAN*, *SpHE*, *VEB3* and *VEB4* mRNAs (10^8 disints/minute/μg) were used in RNAase protection assays with 10 μg RNA from ovary (O) or a single whole adult male sea urchin (A). *SpHE* and *SpAN* probes were also hybridized with 0.5 μg 12- hour embryo RNA and 9.5 μg yeast tRNA (12); *VEB3* and *VEB4* probes were hybridized with 10 μg 12-hour embryo RNA. Hybridization of the *RTS* probe which is complementary to the internal transcribed spacer of the ribosomal RNA primary transcript confirms our previous observation (Angerer et al., 1992) that this sequence is present at approximately equal concentrations in ovary and 12-hour RNA. In this assay, 0.5 μg ovary and 12 hour RNA and 1.0 μg adult RNA were used and the total mass of RNA was brought to 10 μg with yeast tRNA. P, unhybridized probe; C, yeast tRNA (10 μg) was substituted for sea urchin RNA as a control for self-hybridization of the probe. The presence of RNAase-resistant fragments that are shorter than completely colinear duplexes results from the presence of related transcripts (Fig. 1) and/or polymorphism among alleles. The *SpAN* probe includes sequences of the Cys-rich and first C1r/s domains (Fig. 10A). Autoradiographic exposures were carried out with two intensifying screens for 6 days for *SpAN*, *SpHE* and *RTS* or 13 days for *VEB3* and *VEB4*.

duplexes present in these lanes are attributable to probe secondary structure as shown by hybridizations of these probes with yeast tRNA (lanes marked C, Fig. 2). Since the ovaries used were depleted of eggs and therefore consisted primarily of oocytes at various stages of development, it is unlikely that the VEB genes are expressed during oogenesis. Although the VEB genes could be expressed in only one or a few adult somatic cell types, a limit on the possible levels of expression can be set. The RNAase protection assay can detect approximately 100 molecules per embryo (Grimwade et al., 1991). Therefore, assuming the average RNA content of an adult sea urchin cell is similar to that of a pluteus cell, of which there are about 1800/embryo, then there is less than 1 VEB transcript per 15-20 adult cells (or, for example, 5 copies/cell in 1% of the cells). We conclude that it is very likely that VEB gene expression is confined to embryogenesis.

Early activation of VEB genes

To determine more precisely when the VEB genes are activated, we used probes of ten-fold higher specific activity in RNAase protection assays. This assay can detect as few as 10 transcripts per embryo, based on extrapolation from similar analyses of mRNAs of known concentration (Grimwade et al., 1991). The fact that VEB transcripts are undetectable in maternal RNA provides a zero baseline against which the first new transcripts are detected. In these hybridizations, the ability of egg and early stage RNA to form RNAase-resistant duplexes and the equivalence of RNA concentrations at each developmental stage were demonstrated by detection of the rare maternal *Spec1* transcript (Fig. 3, bottom panel). This temporal pattern, including the slightly higher level of *Spec1* message in egg RNA, has been previously documented (Gagnon et al., 1992). The results in Fig. 3 clearly show that the *SpAN*, *SpHE*, and *VEB3* genes are active by 8-cell stage, and signals above background are detectable in the original autoradiograms for *SpHE* and *VEB3* at 4-cell stage. While transcription may begin at 4-cell stage, we cannot exclude the possibility that these first few transcripts are derived from early 8-cell embryos which might have been present at low frequency in this culture.

VEB mRNAs share a very similar pattern of spatially restricted expression

The spatial pattern of SpHE gene expression during development was determined by in situ hybridization as shown in Fig. 4. All VEB mRNAs show similar distributions at the time of peak accumulation (see below), but the higher signals provided by the abundant SpHE message allowed us to examine modulations in the pattern during the accumulation and decay periods. In agreement with the RNA blot analysis described

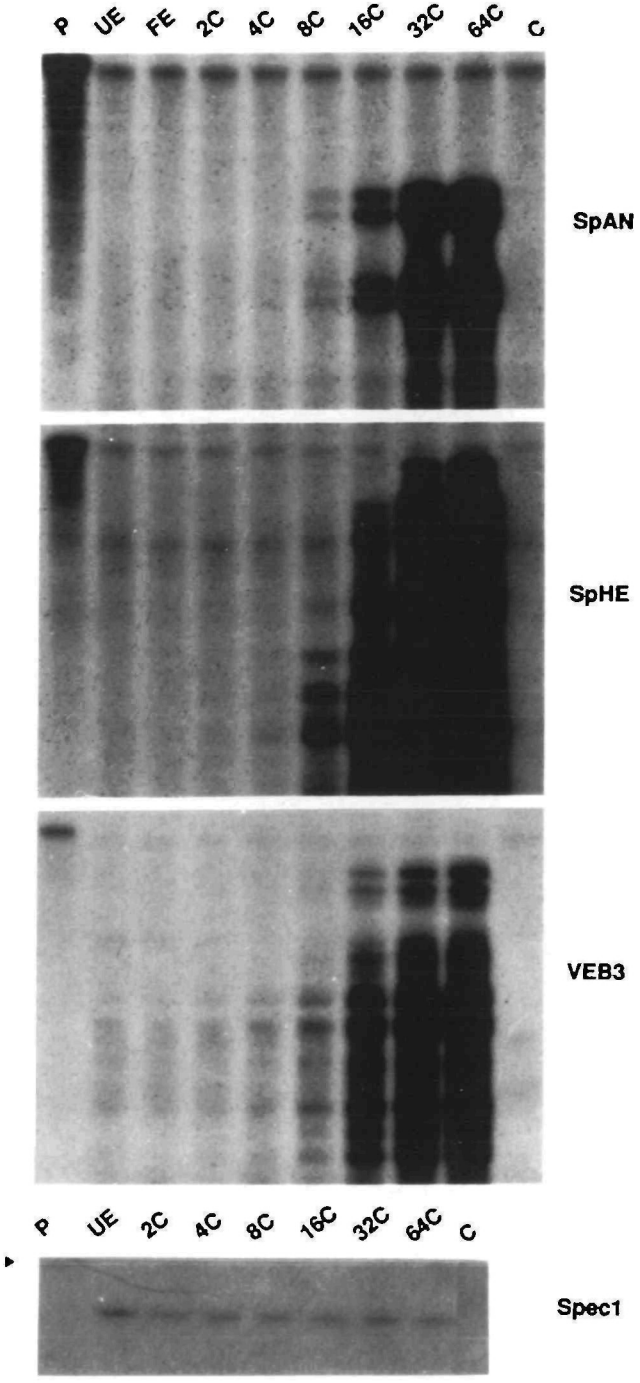


Fig. 3. Transcription of the VEB genes is activated by 8-cell stage. Antisense probes for *SpHE*, *SpAN* and *VEB3* mRNAs (10^9 disints/minute/μg) were used in RNAase protection assays with 10 μg RNA from embryos at the cleavage stages indicated above the lanes. The origin of multiple bands is discussed in the legend to Fig. 2. The *SpAN* probe lies downstream of the probe fragment used in Fig. 2 and includes sequences in the Thr-rich domain and the N-terminal half of the second C1r/s domain (Fig. 10A). The RNA samples used in this experiment were also used for RNA blots shown in Fig. 1. Equality of the loads and hybridizability of all RNAs was also verified through detection of the rare maternal *Spec1* transcript by hybridizing a probe (1×10^8 disints/minute/μg) with 5 μg sea urchin RNA plus 5 μg yeast RNA. The position of the unhybridized *Spec1* probe which was too faint to reproduce is indicated by the arrowhead to the left of the *Spec1* panel. P, unhybridized probe; UE, unfertilized egg; FE, fertilized egg; 2C etc. indicate cell number; C, yeast RNA (10 μg) was substituted for sea urchin RNA as a control for self-hybridization of the probes. Autoradiographic exposure times with two intensifying screens were 9.5, 9.5, and 7 days for *SpAN*, *SpHE* and *VEB3*, respectively.

above (Fig. 1), *SpHE* mRNA is undetectable in eggs and gastrulae and reaches maximum levels 9 (128 cells) to 15 hours (250 cells) post-fertilization. At the time of peak accumulation, this mRNA is present in a large region encompassing the majority of the embryo volume but is undetectable in a single, smaller unlabeled region. This message is first detectable at 64-cell stage when it accumulates in a nonuniform pattern which is consistent with that observed one to two cleavages later (9 hours). Although SpHE mRNA is virtually undetectable by RNAase protection assays in the 20-hour hatched blastula (data not shown), a few cells in many of these embryos still retain relatively high concentrations of this message (Fig. 4).

In situ hybridizations carried out with probes representing all the VEB mRNAs reveal that, near the time of peak accumulation, these messages are distributed in patterns that are quite similar. Although the VEB mRNA spatial patterns appeared to be the same, the morphological symmetry of the early embryo prevented us from identifying the labeled region and from concluding that it is the same for all probes in all embryos. To determine this, the spatial patterns of VEB mRNAs were compared to each other and to that of a well-characterized marker mRNA, *Spec3*. This latter message, which is not a member of the VEB group, initially accumulates throughout the embryo, but decays detectably in cells at the vegetal pole by 15 hours (Eldon et al., 1987). Sections in the upper row of Fig. 5 show a comparison of *SpHE* and *Spec3* mRNA distributions on adjacent 5 μm sections of 15-hour blastulae, while the lower two rows show similar comparisons between *SpHE* and *SpAN* or *SpHE* and *VEB3*. This analysis confirms that these three VEB mRNAs accumulate in the same region of the embryo and demonstrate that the unlabeled region is at the vegetal pole. The hybridization pattern obtained with the *VEB4* probe is similar but is not shown here because the signals are biased toward the abundant target RNA which could include one or more of the other VEB mRNAs as discussed above. Thus, transcripts from at least three of the VEB genes are asymmetrically distributed along the maternally specified animal-vegetal axis. In contrast, these mRNAs are present at uniform concentration around this axis and therefore do not reflect the oral-aboral axis.

The distribution of VEB mRNAs along the animal-vegetal axis is variable

The finding that VEB mRNAs are present in all cells

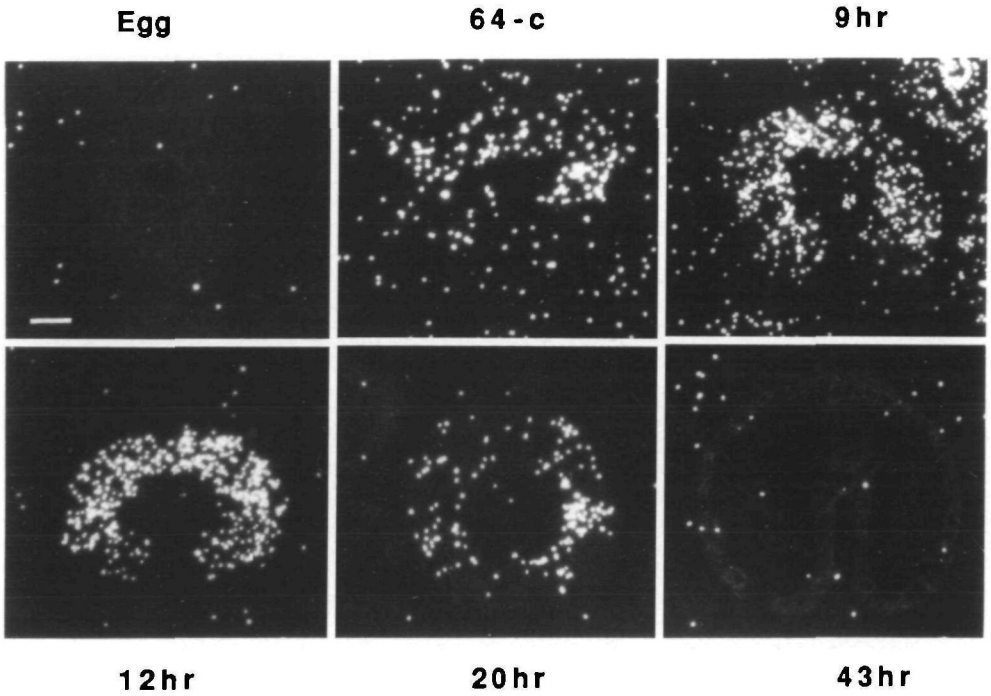


Fig. 4. Spatial expression of *SpHE* during development. *SpHE* antisense riboprobes were hybridized to sections of egg, 64-cell (~7.5 hour) embryos, 9-hour, 12-hour, and 20-hour blastulae and 43-hour gastrulae. All hybridizations shown were carried out with ^{3}H-labeled (1×10^{8} disints/minute/μg; 1.1 kb coding region) probe to 1 μm sections, except for that at 64-cell stage. To increase sensitivity approximately 7-fold (including difference in exposure time), hybridization at this early stage was done using ^{35}S-labeled probe (2.2×10^{8} disints/minute/μg) and 5 μm sections. Exposure times were 3 weeks for ^{3}H- and 2 weeks for ^{35}S-labeled hybrids. The bar in the top left panel represents 10 μm.

except those at the vegetal pole of the blastula suggested that they might be confined to specific tissue anlagen. The origin and fates of different lineages in the sea urchin embryo have been determined from classical dye marking experiments (reviewed by Hörstadius, 1973) and contemporary lineage analysis (Cameron et al., 1987, 1989, 1990). The regions of presumptive ectoderm, endoderm, secondary mesenchyme, primary mesenchyme and the small micromeres are arranged, in this order, along the animal-vegetal axis as diagrammed in Fig. 6. The approximate volumes of the embryo corresponding to these anlagen are 70%, 10%, 10% and 10%, respectively. To determine whether the unlabeled region corresponds to specific tissue anlagen, a *SpHE* probe was hybridized to serial 4 μm sections of 12-hour embryos and the size of the unlabeled region was estimated as follows. For each embryo, the section containing the largest unlabeled region was identified and the angle, α, formed by two lines drawn from the center of the embryo along the borders of the unlabeled region was measured, as diagrammed in Fig. 7A. Since the unlabeled region is radially symmetric around the animal-vegetal axis, the fraction of the total embryo volume it includes is equal to $0.5(1-\cos\alpha/2)$. Measurements of 29 embryos, represented by individual lines in Fig. 7B, yielded an average value of 12.7%, illustrated by the example in Fig. 7D. However, values ranged from 4.5% (Fig. 7C), which is less than that corresponding to micromere derivatives (about 10%), to 25% (Fig. 7E), which is sufficiently large to include most of the derivatives of the vegetal plate (presumptive secondary mesenchyme and endoderm). These data indicate that

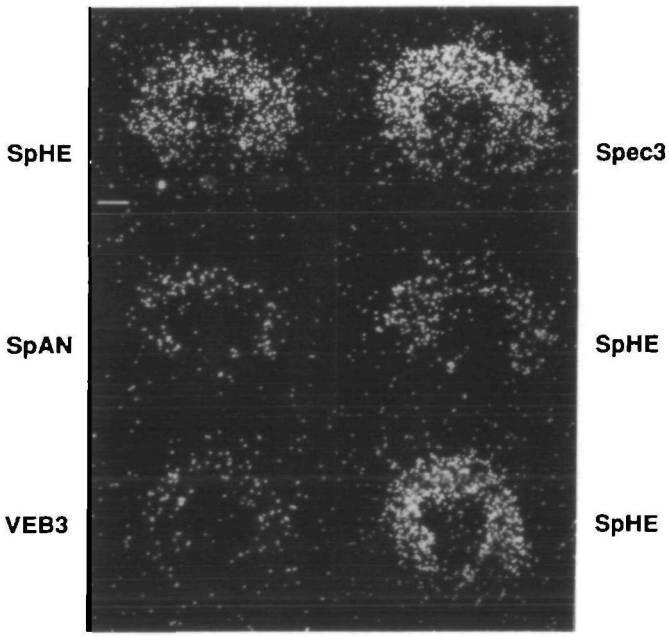


Fig. 5. Three VEB mRNAs accumulate in non-vegetal pole cells of early blastula stage embryos. At 15 hours the vegetal pole is marked by reduced levels of *Spec3* mRNA. Adjacent 5 μm sections of 15-hour early blastulae were hybridized with radioactively labeled antisense riboprobes complementary to *Spec3*, *SpHE*, *SpAN*, and *VEB3*. *Spec3*, *SpHE*, and *VEB3* probes used in the hybridizations shown in the top and bottom rows were labeled with α[^{35}S]-UTP to a specific activity of 1×10^{8} disints/minute/μg; for the hybridizations shown in the middle row, *SpHE* and *SpAN* antisense riboprobes were labeled with [^{3}H]-UTP and [^{3}H]-CTP to 1×10^{8} disints/minute/μg. *Spec3* hybridizations were exposed for 1 week, *SpHE* and *VEB3* for 2 weeks and *SpAN* for 3 weeks. All sections are oriented with the vegetal pole down as determined by the *Spec3* pattern and are shown photographed with dark-field illumination. Examination with bright-field illumination showed that all sections are intact (data not shown). The bar in the top left panel represents 10 μm.

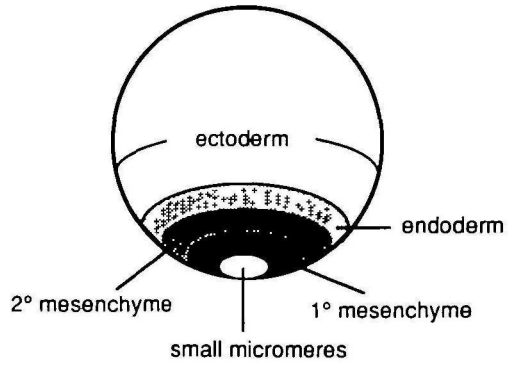


Fig. 6. Fate map of the sea urchin embryo showing anlagen arrayed along the animal-vegetal axis. The equatorial line illustrates the position of the third cleavage plane which separates the embryo into animal and vegetal hemispheres. The animal pole is up, and the vegetal pole is down.

the VEB transcripts always accumulate in presumptive ectoderm but to a variable extent in cells of the endoderm and mesenchymal lineages. The embryos used for this analysis were taken from two separate cultures. Sixteen measurements (indicated by asterisks in Fig. 7B) were derived from embryos of a single-pair mating which develop at a more uniform rate. Both the average and range of values were essentially identical for the two cultures, showing that the variability in the size of the unlabeled region is not due to differences among embryo batches. Furthermore, because the range of VEB mRNA distributions along the animal-vegetal axis is unchanged in 15-hour embryos from these same cultures (data not shown), it is unlikely that differences in mRNA stability and/or the time of down-regulation of the VEB genes among embryos contributes significantly to variability in the 12-hour pattern. Because analysis of *SpHE* and *SpAN* mRNA distributions on adjacent one μm sections show that their patterns are identical for all embryos (data not shown), we conclude that there is the same degree of variability in the distribution of SpAN messages.

A second type of variability among embryos was observed in the distribution of *SpHE* transcripts within the labeled region. In some embryos, signals were essentially uniform over this region (Fig. 7D) while, in many embryos, the mRNA distribution forms a distinct animal-vegetal gradient (Fig. 7C,E,F). These patterns were reproducible on adjacent sections. We conclude that, around the time of peak abundance, the concentration of *SpHE* transcripts shows a polarized variability along the animal-vegetal axis and that the boundary between labeled and unlabeled regions (Fig. 7B) does not coincide with a specific border between tissue anlagen. The variable accumulation of VEB transcripts by cells of different anlagen representing all three germ layers raises the possibility that VEB gene

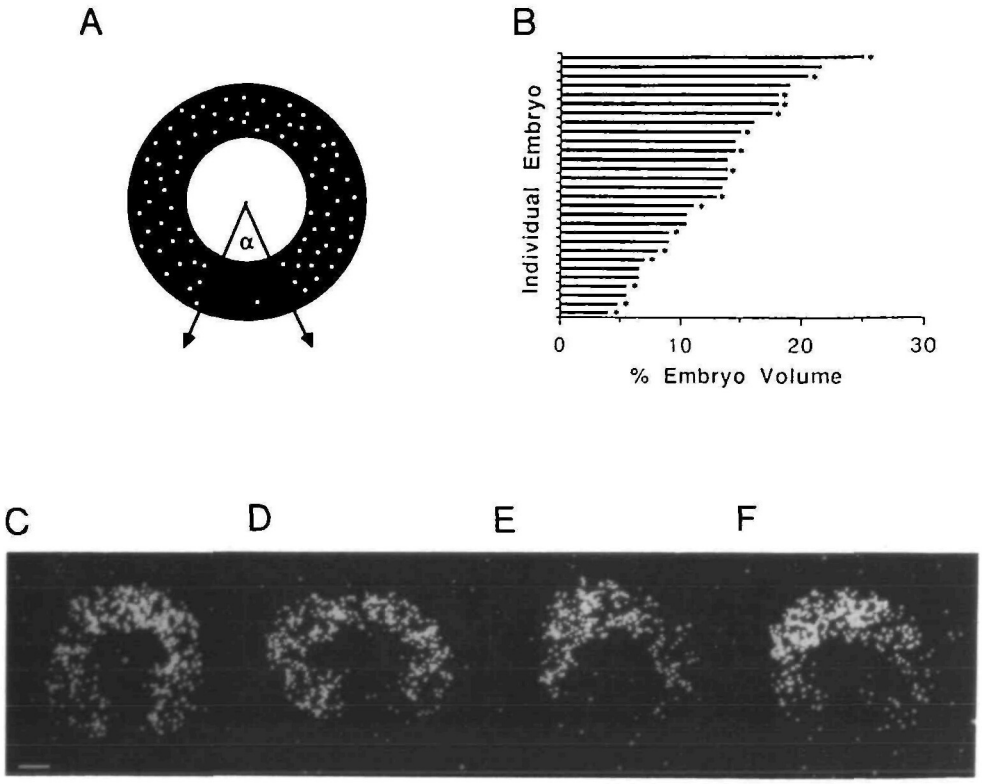


Fig. 7. The distribution of VEB mRNAs in 12-hour embryos is variable and does not coincide with borders between different tissue anlagen. Serial 4 μm sections of 12-hour embryos were hybridized with a probe for *SpHE* mRNA, and the section with the largest unlabeled area at the vegetal pole was identified for each. (A) The angle, α, was measured for each embryo, and the volume of the embryo that was unlabeled by the *SpHE* probe was calculated as $0.5(1-\cos\alpha/2)$. These values for 29 embryos are plotted in (B). Measurements marked by an asterisk (*) were obtained from embryos of the same single pair mating. Examples of embryos with the smallest, average and largest unlabeled regions observed are shown in C, D and E, respectively. The sections in C, E, F show embryos in which *SpHE* mRNA is distributed in a gradient along the animal-vegetal axis. Sections in C-F are oriented with the vegetal pole at bottom. The bar in C represents 10 μm.

expression is not a consequence of cell fate specification.

VEB genes are activated in pluripotent macromeres of the 16-cell embryo

We were able to test critically whether VEB gene activation is independent of tissue specification by analyzing specific blastomere types of the 16-cell embryo. At this stage, blastomeres with distinctive fates are arrayed in tiers along the animal-vegetal axis. At fourth cleavage, cells of the animal hemisphere divide symmetrically to form a ring of 8 mesomeres, while asymmetric cell division in the vegetal hemisphere produces 4 macromeres and 4 micromeres. Micromeres are still bipotent at 16-cell stage and will give rise to the primary mesenchyme cell lineages and the small micromeres at the next cell division. Macromeres are the most pluripotent cell type. Their descendents produce ectoderm, secondary mesenchyme and endoderm (reviewed by Davidson, 1986), and have a latent capacity to produce primary mesenchyme (Fukushi, 1962; Hörstadius, 1973; Ettensohn and McClay, 1988). At 16-cell stage, the only founder cells (i.e., those whose fates have been specified; Davidson, 1989) are four of the eight mesomeres, two of which give rise to oral ectoderm, and two which contribute to aboral ectoderm (Cameron et al., 1987). If the VEB genes were activated only after founder cells are established, then the first VEB transcripts would accumulate only in some of the mesomeres at this stage.

RNAase protection assays employing probes of very high specific activity were used to measure the abundance of VEB transcripts in equal amounts of RNA isolated from mesomeres, macromeres and micromeres. Highly purified blastomere fractions were generated by counterflow centrifugal elutriation of ethanol-fixed cells (see Materials and Methods). As a result of the increased resolution of this method, greater than 90% of the RNA purified from each cell population is derived from the indicated cell type. Control RNA blots verify that the concentration and quality of RNAs isolated from these blastomere fractions are equivalent (A. Nasir et al., manuscript in preparation). Fig. 8 demonstrates that all three VEB mRNAs tested are not only expressed in macromeres, but are significantly enriched in RNA of this blastomere type. Normalization of these signals for the approximate relative amount of RNA per cell (assuming that the total RNA content of each blastomere is proportional to its fraction of the embryo volume), converts these signals to the relative number of transcripts per nucleus. This normalization is appropriate since the assay detects the first few transcripts synthesized in each cell and it further increases the difference in signals for macromeres versus micromeres or mesomeres by factors of 4 and 1.6, respectively. In this experiment, cellular debris, which derives primarily from larger blastomeres, was a minor contaminant (< 5%) of the micromere fraction, while the mesomere fraction was slightly contaminated (< 3%) with intact macromeres. Thus, the signals observed for micromere

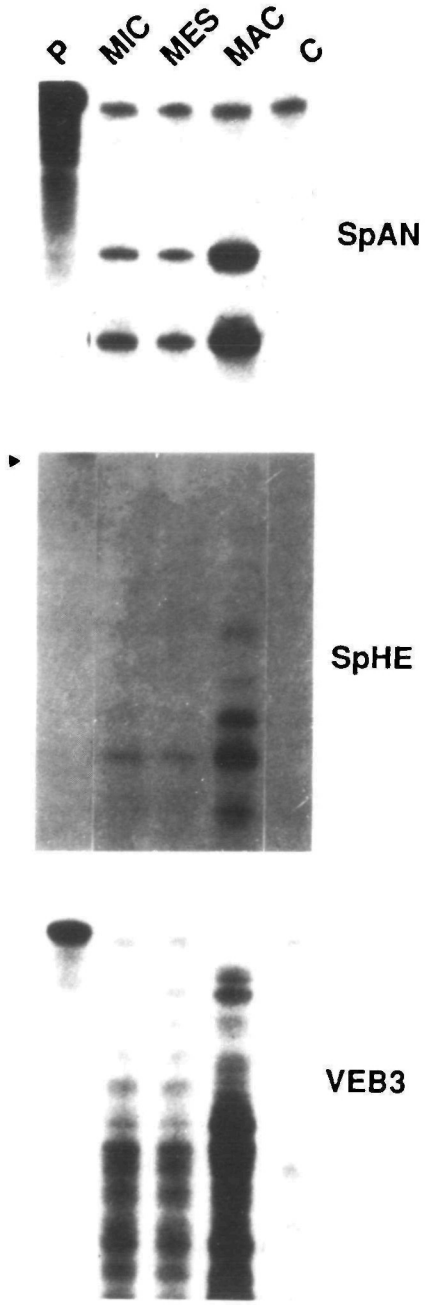


Fig. 8. VEB mRNAs accumulate first in macromeres of 16-cell embryos. Probes for *SpAN*, *SpHE* and *VEB3* mRNAs (10^9 disints/minute/μg) were used in RNAase protection assays with 2.5 μg of total RNA isolated from cell populations enriched for micromeres (MIC), mesomeres (MES) or macromeres (MAC). Yeast RNA (7.5 μg) was included in the hybridization. The origin of multiple bands is discussed in the legend to Fig. 2. The *SpAN* probe used in this experiment is the same as that used in Fig. 3. The position of the unhybridized *SpHE* probe is indicated by the arrowhead at left. P, unhybridized probe; C, yeast RNA (10 μg) was used as a control for self-hybridization of the probe. Autoradiographic exposure times with two intensifying screens were 9.5, 2.5, and 8 days for *SpAN*, *SpHE* and *VEB3*, respectively.

and mesomere RNA are at least partially attributable to contamination with macromere RNA. Activation of VEB genes in macromeres was confirmed with RNAs obtained from three other cultures. However, differences in signals for RNAs of the three blastomere types were not as great. Preferential accumulation of VEB transcripts in macromeres is not due to a higher general rate of transcription in these cells, because total RNA synthesis rates are similar in the three blastomere types (Hynes et al., 1972; Spiegel and Rubinstein, 1972; Czihak, 1977) and greater than 90% of the RNA synthesized at this stage is transcribed by RNA polymerase II (reviewed by Davidson, 1976). Therefore, we conclude that, at least in the case of the macromeres, VEB gene activation precedes founder cell specification.

Transcription of VEB mRNAs is independent of cell-cell interactions

Specification of cell fates in the region of the embryo that accumulates VEB mRNAs is mediated by cell-cell interactions (reviewed by Hörstadius, 1973; Wilt, 1987; Davidson, 1989). However, evidence presented above argues that activation of the VEB genes does not require specification and predicts therefore that it might also be independent of cell-cell interactions. To test this hypothesis, we dissociated embryos at the 2-cell stage, which is at least one cleavage before the first VEB transcripts are detectable, and cultured them in calcium-free sea water so that daughter cells separate at subsequent divisions. VEB mRNA levels were assayed by RNAase protection at times between 9 and 27 hours. As a control, intact embryos were held at 4°C during the one hour required for embryo dissociation. The results, shown in Fig. 9, demonstrate that cells denied intercellular contacts accumulate essentially normal levels of VEB transcripts. Accumulation in separated cells is slightly delayed, as illustrated by comparison of embryo and separated cell samples at nine hours. This delay is probably due to a post-separation recovery period. The higher level of VEB transcripts in separated cells relative to controls at 15 hours may reflect a corresponding delay in the time of their peak accumulation. In contrast, RNAase protections carried out with a probe for *Spec1* mRNA (Fig. 9, bottom panel) confirmed previous demonstations that the concentration of this mRNA begins to increase in control embryos during mesenchyme blastula stage (21-27 hours), but is severely reduced in separated cells (Hurley et al., 1989; Stephens et al., 1989). This result as well as RNA blot analysis of these same RNA samples using a mitochondrial rRNA probe (data not shown) verify that the quantity of hybridizable embryo RNA is equivalent among samples. We conclude that

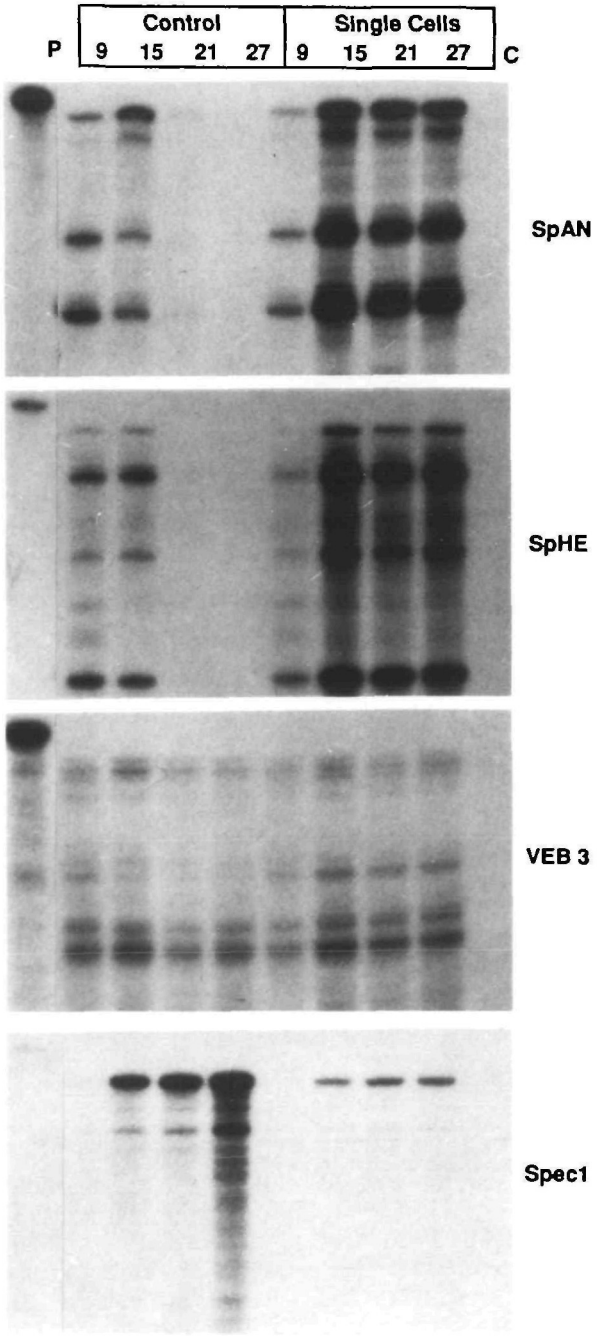


Fig. 9. Accumulation of VEB mRNAs is independent of cell-cell interactions. Embryos were dissociated at 2-cell stage as described in Materials and Methods. RNAase protection assays are shown in which *SpAN*, *SpHE* and *VEB3* probes (10^8 disints/minute/μg) were hybridized to 2 μg total RNA from separated cells or from control embryos at the hours post-fertilization indicated above the lanes. The origin of multiple bands is discussed in the legend to Fig. 2. The *SpAN* probe used in this experiment was the same as that shown in Figs 3 and 8. The temporal pattern for control embryos indicates that many bands obtained with this *VEB3* probe derive from hybridization to the shorter message(s) which persists later in development (see Fig. 1). Control hybridizations with a *Spec1* probe (bottom) confirm previous measurements (see text) and verify that the quantity of hybridizable RNA was equivalent among samples. The length of the unhybridized *Spec1* probe is indicated by the arrowhead at left. Autoradiographic exposure times were 4.5 days, 19 hours, and 7 days for *SpAN*, *SpHE* and *VEB3*, respectively. P, unhybridized probe; C, yeast RNA (10 μg) was used as a control for self-hybridization of the probe.

activation of the VEB genes is cell autonomous and does not require intercellular communication via direct cell contacts or diffusible molecules that act over short distances.

In intact embryos, VEB mRNA abundance decreases sharply between 15 and 21 hours (Fig. 9, left). In contrast, high levels of *SpHE* and *SpAN* mRNAs are maintained at least through 27 hours in separated cells (Fig. 9, right). A similar phenomenon probably also occurs for *VEB3* mRNAs, but is partially obscured by the fact that one VEB3 transcript, unlike true VEB mRNAs, continues to be present in later stages of development in intact embryos (Fig. 1). The persistence of the VEB mRNAs in separated cells suggests that down-regulation of VEB gene transcription and/or turnover of the transcripts may require cell-cell interactions.

SpAN encodes a metalloendoprotease related to tld and BMP-1

The entire open reading frame of *SpAN* has been determined by sequencing a 2300-nt cDNA. Searches of the SwissProt and NBRF data banks revealed striking similarities of SpAN to three known peptides: Astacin (Titani et al., 1987), an 198-amino acid metalloendoprotease with a novel specificity purified from the crayfish *Astacus fluviatilis*; human bone morphogenetic protein-1 (BMP-1, Wozney et al., 1988), which is capable of participating in cartilage formation in vivo; and Tolloid (tld, Shimell et al., 1991), which is involved in dorsal-ventral axis formation in the early *Drosophila* embryo. In addition to the Astacin domain (described below), the C-terminal halves of SpAN, BMP-1 and tld contain domains similar to the putative Ca^{2+}-dependent protein interaction domains of the complement cascade serine proteases, C1r and C1s (Leytus et al., 1986; Journet and Tosi, 1986; Mackinnon et al., 1987; Tosi et al., 1987). The organization of various domains in members of the Astacin family and complement proteins is presented in Fig. 10A. These domains are aligned to facilitate comparison and this alignment does not necessarily imply functional or evolutionary relationships among these proteins.

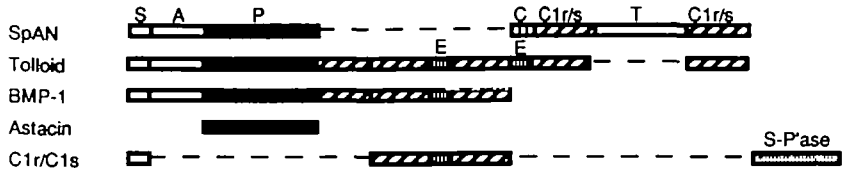
The 200 amino acids between and including Ala_{94} and Cys_{293} of SpAN exhibit 33% identity with Astacin, and 41% and 40% identity with the inferred protease domains of BMP-1 (Ala_{121}-Cys_{319}) and tld (Ala_{127}-Cys_{327}), respectively (Fig. 10B). Stöcker and coworkers (1988) have established that the active site of Astacin includes the sequence HELMHAIGFH and that a Zn^{2+} coordinated to His_{92} and His_{96} is required for hydrolysis. The active site of Astacin is highly conserved in SpAN, BMP-1 and tld (>60%, dashed line, Fig. 10B). Of five Glu residues in Astacin that may be involved in catalysis, two (Glu_{38} and Glu_{93}; double underlines) are in positions conserved in all four proteins. In addition, all of the Astacin-related proteins have four similarly positioned Cys residues, suggesting that these domains have a comparable secondary structure. Similarity among SpAN, BMP-1 and tld extends an additional five amino acids in the N-terminal direction past the Astacin domain (not shown) to include a set of two to five basic amino acids that may serve as a cleavage site for activation of these putative metalloendoproteases (Furie and Furie, 1988; Matrisian, 1990; Nagase et al., 1990). Although the sequences of the putative activation peptides are divergent, cleavage in the basic region would generate active proteases with similar N termini.

The presence of a hydrophobic sequence (Perlman and Halvorson, 1983; von Heijne, 1984) at the N terminus of SpAN (data not shown) suggests that this protein is secreted. Cleavages that remove the putative N-terminal signal sequence and activation peptide of the SpAN preproprotein would generate an active protease of M_r 57.9×10^3 with a net charge of -30 at neutral pH. All members of the Astacin family appear to be secreted peptides and BMP-1 and tld, like SpAN, are probably synthesized as preproproteins (Wozney et al., 1988; Shimell et al., 1991).

The Astacin-related proteases all share domains related to the putative protein interaction domains (C1r/s) of the complement cascade proteases, C1r and C1s, although the order and number of these domains are variable. SpAN has two C1r/s domains which are 37% identical to each other and each contains four Cys residues with conserved spacings as indicated by the alignment presented in Fig. 10C (rows 1 and 5). The sequence identities among the eight C1r/s domains aligned in Fig. 10, including those of SpAN, are all between 30 and 38%. C1r and C1s, as well as BMP-1 and tld, contain the CEC supermotif (Shimell et al., 1991) in which C1r/s domains flank an EGF-like domain. SpAN lacks this central EGF-like domain, but it does contain a Cys-rich (C) region (aa 299-328; $CysX_5CysX_9CysXCysXCysX_8Cys$), immediately N-terminal of the first C1r/s domain (See Fig. 10A). Despite the unusual spacing of the Cys residues, other sequence features suggest this region could resemble an EGF-like domain (see Discussion).

Between the C1r/s domains, SpAN contains a novel Thr-rich (T) region which consists of a series of perfect repeats of the 8-amino acid sequence, STTTLQTT (Fig. 10D, left). Of seven cDNAs sequenced through this region, four had four repeats and three had three repeats. Individual repeats can be distinguished from one another by their bordering N- and C-terminal amino acids (Fig. 10D, left) and/or by their nucleotide sequences (Fig. 10D, right). Those cDNAs with three repeats lack either the second or third repeat. This difference among cDNAs does not result from a cloning artifact since RNAase protection assays demonstrate that mRNAs containing both the 4- and 3-repeat motifs are present in early blastulae (data not shown). PCR analysis of 13 sperm samples and 9 *SpAN* genomic clones using primers that flank the T-rich region, failed to amplify any other fragment containing this sequence (data not shown). Together with genomic DNA blot and sequence data (not shown), these results verify that the 4- and 3-repeat sequences are not generated by alternative splicing. Furthermore, since DNA from some sperm samples contains only the 3- or 4-repeat

A Domain Structure of the Astacin-Related Proteins



B. Sequence Comparison of Protease Domain

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                                                                *
SpAN    .ATIYESQRW SYKIIPYVIE SS.SSGQSSL IRSAMDHWEQ NTCLRFEPLT SSHSSRLGHT SYISF.FRGN
BMP-1   A||SRPERV| PDGV||F||G GNFTGS|RAV F|Q||R|||K H||VT|LER| DE.......D |||V|TY|PC
Tld     AV|VRKERT| D|GV|||E|D TIF|GAHKA| FKQ||R|||N F||IK|VERD PNL.....|A N||Y|TVKNC
Ast.    AAILGDEYL| |GGV|||TFA GVSGAD||AI L.|G|QEL|E K||I||V|R| TE.......S D|VEIFTS|S
Cons.   AAT---E--W --GVIPY-I- ------Q-A- ---AM-HWE- -TC--F--RT ---------- -YI-F-----

        *                       *
SpAN    GCWSHVGRSF TNQQQISIGP QCGYFGTIVH EIGHAIGFHH EQSRPDRDEY INVHFENVQS GREHNFAKYT
BMP-1   ||C|Y|||RG GGP|A|||||K N|DK||IV|| |L||VV||W| |HT|||||RH VSIVR||I|P |Q|Y||L|ME
Tld     ||C|FL|KNG NGR|P|||||R N|EK||I|I| |L||T|||| |HA|G|||KH |VINKG|IMR |Q|Y||DVLS
Ast.    ||||Y|||IS GA||VSLQAN G|V|H|||I| |LM|||||Y| |HT|M|||N| VTINYQ||DP SMTS||DID|
Cons.   GC-S-VGR-- -G-Q-ISIG- -C--FG-I-H EIGH-IGF-H EH-R-DRD-- --I---NI-- G-E-NF---T
                              ------------------------------

                                                                              *
SpAN    WGSVTSSNVE YDVGSIMHYG GYGFSSN... .GRPTITTID PRLNSRLGQR TALSAADIEL ANRIYE..C
BMP-1   PQE|E|LGET ||FD|||||A RNT||RG.IF LDTIVPKYEV NGVKPPI||| |R||KG||AQ |RKL|K..|
Tld     PEE|DLPLLP ||LN|||||A KNS||KS.PY LDTI|PIG|P |GTHLE|||| KR||RG||VQ ||LL|K..|
Ast.    YSRY..VGED |QYY|||||| K|S|||QWGV LETIVPLQNG ID|TDPY.DK AHMLQT|ANQ I|NL|TNE|
Cons.   ---V-S---- YD--SIMHY- ---FS----- L-TI-P---- --L----GQR --LS--DI-Q AN-LY---C
```

C. Sequence Comparison of C1r/s Domain

```
        *                                   *                                   *
SpAN-1  C..SYRFTEM ...TGEITSP NYPSN.YEDN TACVYEIEGP YG.STIELTF ....LDMEIE TET.LCRYDA
TLD-4   C..KFEITTS ...YGVLQSP NYPED.YPRN IYCYWHFQTV LG.HRIQLTF ....HDFEVE SHQ.ECIYDY
C1r-2   C..SSELYTE ..ASGYISSL EYP.RSYPPD LRCNYSIRVE RG.LTLHLKF LE.PFDIDDH QQV.HCPYDQ
C1s-2   C..SGDVFTA ..LIGEIASP NYP.KPYPEN SRCEYQIRLE KGFQVVVTLR RE.DFDVEAA DSAGNCLDSL
SpAN-2  C..GGTFVGV ...EGRVASP NYPND.YDNS LQCDYVIEVD DG.RRVELIF ....EDFGLE DET.TCRWDS
TLD-5   C..GGYLRAT NHSQTFYSHP RYGSRPYKRN MYCDWRIQAD PE.SSVKIRF ....LHFEIE YSE.RCDYDY
C1r-1   CRAGGSIPIP QKLFGEVTSP LFP.KPYPNN FE.TTTVITV PTGYRVKLVF ..QQFDLEPS E...GCFYDY
C1s-1   CIVLFILLAVVVAIL PTMYGEILSP NYP.QAYPSE VE.KSWDIEV PEGYGIHLYF ..THLDIELS E...NCAYDS
Cons.   C---G----- ----GEI-SP NYP---YP-N --C-Y-I--- PG---V-L-F -----D-E-E -----C-YD-

                    *
SpAN-1  VEV.RKD.DI NSI..GEKFC GNTLPPVQIS SS..... NQMMVSFTSD PSITRRGFKA TYVI.II
TLD-4   VAI.YDGRSE NSSTLGIY.C GGRE.PYAVI AST..... NEMFMVLATD AGLQRKGFKA TFVSE..
C1r-2   LQIYANGKNI .....GEF.C GKQRPPDLDT SS..... NAVDLLFFTD ESGDSRGWKL RYTTEII
C1s-2   VFV.A.GDRQ F....GPY.C GHGFPGPLNI ETKS..... NALDIIFQTD LTGQKKGWKL RYHGDPM
SpAN-2  LMI.NLG.NG IKV..GMKMC GREYPAASLV SIG..... NRMELKLKTD GSVNDRGFVA SYRAIDL
TLD-5   LEITEEGYSM NTIH.GRF.C GKHKPPIIIS NS..... DTLLLRFQTD ESNSLRGFAI SFMAVDP
C1r-1   VKISADKKSL .....GRF.C GQLGSPLGNP PGKKEFMSQG NKMLLTFHTD FSNEENGTIM FYKGFLA
C1s-1   VQIIS.GDTE E....GRL.C GQRSSNNPHS PIVEEFQVPY NKLQVIFKSD FSNEERFTGF AAYYVAT
Cons.   V-I---G--- -----G---C G---PP---- -S-------- N-M-L-F-TD -S---RGFK- -Y-----
```

D. T-rich domain of SpAN-M

Amino Acid				Nucleotide		
F	STTTLQTT	P	Repeat 1	TT	CTCAACGACCACATTACAGACGACA	CCT
P	--------	N	Repeat 2	CC	------------------------	AA-
P	--------	N	Repeat 3	CC	------T-----------------	AA-
P	--------	D	Repeat 4	CC	----------TC-G--------C	GA-

Fig. 10. Sequence comparison of SpAN. (A) Comparison of domain structure and organization in members of the Astacin family of proteases. (S) signal peptide, (A) activation domain, (P) protease domain, (C) Cys-rich domain, (C1r/s) domain similar to domains I and III of C1r and C1s, (T) Thr-rich domain, (E) EGF-like domain, (S-P'ase) serine protease domain. The dashed lines represent gaps introduced to maximize alignment of domains in the proteins. (B) Sequence comparison of protease domains. SpAN, aa 94-293; tld, aa 127-327; BMP-1, aa 121-319; Astacin, aa 1-198. Vertical lines (|) indicate identity with SpAN, dots (·) indicate gaps introduced to maximize alignment of the sequences, (Cons.) represents a consensus sequence obtained when a residue is identical in 3 of 4 peptides. Cys residues are marked with an asterisk (*) above the SpAN sequence. Solid and dashed lines beneath the Cons. sequence indicate the active site of Astacin and the highly conserved region surrounding the active site. Double underlines indicate conserved glutamyl residues, of which at least one is the likely catalytic amino acid (see text). (C) Sequence comparison of C1r/s domains. SpAN-1, aa 340-451; tld-4, aa 787-899; SpAN-2, aa 503-616; tld-5, aa 900-1018; C1r-2, aa 193-307; C1s-2, aa 175-292; C1r-1, aa 13-136; C1s-1, aa 3-130. Proteins are aligned with respect to Cys residues marked with an asterisk (*) above the SpAN sequence. A consensus (Cons.) on the last line indicates residues found in at least four of the eight sequences presented. (D) Sequence of the T-rich repeats. Repeat 1, aa 457-466; Repeat 2, aa 467-476; Repeat 3, aa 477-486; and Repeat 4, aa 487-496. The amino acid and nucleotide sequences of the four Thr-rich repeats are presented on the left and right, respectively. The repeats can be distinguished by the bordering N- and C-terminal amino acids and/or their nucleotide sequence. Nucleotide sequence variations among different repeats were verified by sequencing seven different cDNAs and one genomic DNA on both strands. Dashes (-) represent identity with Repeat 1.

motif, this difference must be allelic. If these motifs characterized different alleles of the same gene, which were present in the population at equal frequency, then a maximum of 50% of sperm samples would contain both forms of this motif. However, we observed a significantly higher frequency (11/13) consistent with the possibility that that there is more than one *SpAN* gene in the haploid genome of *S. purpuratus*.

SpHE is hatching enzyme, a metalloendoprotease in the collagenase family

The partial *SpHE* cDNA sequence is very similar to the *Paracentrotus lividis* cDNA HE6, which encodes sea urchin hatching enzyme (Lepage and Gache, 1990). Several features of the HE6 protein sequence, particularly in the activation and catalytic sites, identify HE6 as a member of the collagenase family of metalloendoproteases. The sequenced region of *SpHE* predicts a protein sequence that corresponds to Ala_{203}-Val_{289} of HE6 and spans the C terminus of the activation site and the first 10 amino acids of the active site. The overall identity between SpHE and HE6 is 80% (86% similarity) at the amino acid level and increases to 100% in the active site (data not shown). In addition to their sequence identity, *SpHE* and *HE6* mRNAs are similar in their temporal patterns of accumulation and in their number and lengths (Fig. 1; Lepage and Gache, 1990). These facts strongly suggest that SpHE is the *S. purpuratus* hatching enzyme, a member of the collagenase family and, like SpAN, a metalloendoprotease.

VEB3 and VEB4

Although entire open reading frames of several *VEB3* cDNAs have been determined, no similarity to any other protein sequence has been found (J. Palis, unpublished observations). The conceptual protein product has a putative signal sequence and Thr, Ser and Pro constitute 45% of the residues, indicating that VEB3 may be a secreted protein which could be highly O-glycosylated (Wilson et al., 1991; O'Connell et al., 1991). Analysis of the protein coding sequences of the multiple *VEB4*-containing mRNAs (Fig. 1) is in progress.

Discussion

We identified the VEB genes in a temporal screen that eliminated only mRNAs present in either eggs or pluteus larvae. The expression of this unique set of genes shares a surprising number of properties. The mRNAs that they encode accumulate transiently and coordinately in the early sea urchin embryo, reaching maximum concentrations at the transition from cleavage to blastula stage. Although we expected this screening strategy to identify multiple sets of early mRNAs with different times of peak accumulation and/or decay rates in the long interval between VEB and pluteus stages (15 to 72 hours), only a single temporal pattern was uncovered. Furthermore, at least three of the gene families share similar, if not identical, spatially restricted patterns of expression. Current evidence suggests that the additional mRNAs complementary to the *VEB4* probe share this spatial pattern, although generalization of this conclusion to all these messages will require gene-specific probes. The uniformity of temporal and spatial expression suggests that the VEB genes represent a major pattern of expression for genes active during early embryogenesis but not during oogenesis or in the pluteus larva.

Although the VEB genes begin to be transcribed very early in embryogenesis, it is likely that the activities of the corresponding proteins are most important around the time of peak accumulation of the messages or even later. The spatial and/or temporal patterns of VEB mRNA expression differ from those of the large number of messages whose accumulation is related to cell growth and division (Angerer et al., 1992; Kingsley et al., submitted). These mRNAs are present in oocytes, reaccumulate uniformly in embryos beginning around VEB stage, persist in dividing cells of the pluteus larva (mainly oral ectoderm and endoderm), and are also found in adult tissues. In contrast, the VEB gene products are embryo-specific and their temporal and spatial patterns are not consistent with general cellular functions.

The VEB gene products are not associated with the terminal differentiated functions of specific cell types. The spatial distributions of a large number of such cell-type specific messages conform to borders between tissue anlagen in the early embryo and to histological borders between tissue types in the later embryo. In contrast, the distribution of VEB mRNAs does not align with cell type. These messages accumulate in all cells of the presumptive ectoderm, as well as in variable portions of presumptive endoderm and mesenchyme. Furthermore, cell-type-specific messages continue to accumulate during tissue differentiation whereas the VEB transcripts disappear before mesenchyme blastula stage.

Some or all of the VEB gene products may instead function to support early events in the formation of the blastula. During the VEB stage of sea urchin development, the embryo undergoes a number of intracellular and extracellular changes: cell cycle times begin to lengthen, the first morphological changes associated with formation of the blastula are evident and tissue-specific programs of gene expression are initiated (see *Introduction* for references). DNA sequence analysis shows that the *SpHE* gene encodes a protein with sequences nearly identical to the *P. lividis* hatching enzyme (Lepage and Gache, 1990), a collagenase-like metalloendoprotease. This protease is required by the early blastula but not in a cell-type-specific role and could have functions in addition to digestion of the fertilization membrane. SpAN contains a domain that is very similar to another metalloendoprotease, Astacin (Titani et al., 1987) and a putative signal sequence suggesting that it is also secreted. The highly coordinate expression of the *SpHE* and *SpAN* genes in both time and space raises the possibility that these proteases are secreted together. Consistent with this possibility, a proteolytic activity in addition to that of hatching

enzyme has been detected in the culture medium of hatched blastulae (unpublished observations cited in Lepage and Gache, 1990; W. Lennarz, personal communication).

The multiple domain structure of SpAN has interesting similarities to two proteins which are known to have important developmental functions: human BMP-1 plays a role in cartilage formation (Wozney et al., 1988) and tld functions in dorsal-ventral patterning of the *Drosophila* embryo (Shimell et al., 1991). All three Astacin-related proteases appear to be secreted and they share variable numbers of C1r/s domains (Wozney et al., 1988; Shimell et al., 1991). Two of the C1r/s domains of BMP-1 and tld are arranged in a CEC (C1r/s-EGF-like-C1r/s) supermotif (Shimell et al., 1991) which, by analogy with C1r and C1s, could mediate interactions with other proteins. There is biochemical and genetic data that BMP-1 and tld interact with members of the TGF-β family (reviewed in Shimell et al., 1991). While it appears that an EGF-like domain and the N-terminal C1r/s domain are necessary to mediate Ca^{2+}-dependent dimer and tetramer associations among complement proteins (Thielens et al., 1990), the requirements with respect to the number and arrangement of these domains or the role of Ca^{2+} in interactions of BMP-1 and tld with other proteins are not yet established.

SpAN has two C1r/s domains that are immediately C-terminal of a Cys-rich region that is clearly related to EGF. This Cys-rich region contains several additional residues that are conserved among members of the EGF family (the tetrapeptide, Leu_{306} Asn_{307} Gly_{308} Gly_{309}, as well as Tyr_{323} and Gly_{325}; Appella et al., 1988). Folding of the Cys-rich domain using the standard EGF disulfide linkages would generate a structure similar to that of EGF-like domains with an abbreviated second (B) loop containing a single residue. Such a structure would contain in the first (A) loop the conserved tetrapeptide, which is known to be important in the binding of urokinase-type plasminogen activator to a receptor (Appella et al., 1987, 1988). Unlike the EGF-like domains in C1r/s (Rees et al., 1988), tld (Shimell et al., 1991) and BMP-1 (Wozney et al., 1988), the Cys-rich sequence of SpAN lacks the β-hydroxylation consensus sequence (Stenflo et al., 1987), making it unlikely that this region of SpAN forms a high-affinity Ca^{2+}-binding site.

The presence of C1r/s domains and a region resembling an EGF-like domain in SpAN raises the distinct possibility that these domains could regulate its interaction with another protein(s). The T-rich region between the C1r/s domains could also influence the association of SpAN with other molecules. This region consists of short repeats extremely rich in Thr, and Pro residues between these repeats could allow them to fold back on each other. Thus, this region has an extremely high density of hydroxyl groups that could be sites for O-glycosylation (Wilson et al., 1991; O'Connell et al., 1991) which might serve to protect SpAN against proteolysis or to regulate intermolecular interactions. The likelihood that SpAN is secreted suggests that it could interact with protein(s) of the extracellular matrix. In this regard, it is interesting that one such protein, fibropellin, contains a partial C1S domain and many EGF-like domains (Delgadillo-Reynoso et al., 1989; Bisgrove et al., 1991) and that the structure of the fibropellin network changes at VEB stage, around the time when SpAN is probably secreted. Defining the role SpAN plays at blastula stage will require demonstration of its proteolytic activity and identification of its target(s).

Several lines of evidence suggest that the initial expression of VEB genes is controlled by maternal factors asymmetrically arrayed in the egg rather than by regulatory cascades initiated indirectly by inductive interactions that are required to specify fates of most different blastomeres of the sea urchin embryo (reviewed by Davidson, 1989). We refer to these molecules as factors for convenience, realizing that localized regulatory activities might be achieved by mechanisms other than localization of the regulatory proteins themselves. First, the distribution of VEB transcripts strikingly reflects the animal-vegetal axis which is known to be established by prelocalized maternal determinants (reviewed by Hörstadius, 1973; Maruyama et al., 1985). In contrast, the distribution of these mRNAs is symmetric around the animal-vegetal axis and thus gives no indication of the oral-aboral axis, which is believed to be established between fertilization and 2-cell stage in *S. purpuratus* embryos (Cameron et al., 1989). Second, the activation of VEB genes soon after fertilization is consistent with control by factors already resident in the egg. Although we cannot yet exclude the possibility that messages for an intervening set of regulatory molecules are transcribed and translated after fertilization before the first VEB transcripts are detected, this seems unlikely in view of the short time (3-4 hours) and the small number of gene copies ($\leq$8) available. Third, the VEB genes are activated in early pluripotent blastomeres (the 16-cell-stage macromeres). Fourth, and consistent with the observation that VEB mRNAs accumulate in pluripotent cells, the distribution of these messages during their period of peak abundance does not align with borders between different tissue anlagen. Thus, VEB gene activation is not the result of a tissue-specific program of differentiation; it neither reflects nor requires commitments of cells to specific fates. Fifth, VEB mRNAs accumulate when blastomeres are deprived of all cell-cell interactions (except the transient contacts at the time of formation of individual daughter cells) beginning at first cleavage, and thus before any cell-cell interaction can take place along the animal-vegetal axis. Such cell-autonomous mRNA accumulation strongly suggests that VEB gene expression can be initiated by activities that are passively inherited via the egg cytoplasm and that at least the initial temporal regulation is independent of regulatory cascades requiring inductive interactions among blastomeres. Whether the spatial regulation of these genes depends on intercellular interactions will require measurements of VEB transcript accumulation in the progeny of separated cells.

Variability in the distribution and abundance of the VEB transcripts along the animal-vegetal axis is reminiscent of the inferred variability among embryos in the graded distribution of maternal determinants along this axis (Driesch, 1900; Hörstadius, 1973; Henry et al., 1989; Livingston and Wilt, 1990). In particular, the distribution of the vegetal pole determinants among cells of the vegetal hemisphere is known to vary among embryos (Hörstadius, 1937). Thus, the variable spatial patterns observed during the period of peak VEB transcript abundance could be the consequence of similar variability among embryos in the distribution of maternal regulatory activities.

An intriguing observation from experiments with interspecies hybrids also supports the idea that VEB genes are maternally regulated. Barrett and Angelo (1969) demonstrated that in reciprocal hybrids between *S. purpuratus* and *S. franciscanus*, only the maternal-type hatching enzyme (i.e., SpHE or its homolog) is synthesized. This result is not attributable to purely maternal expression of the hatching enzyme gene, since it is clear that this gene is active only during embryogenesis [Fig. 2; Lepage and Gache, 1990; and experiments utilizing interspecies hybrid andromerogones (Showman and Whiteley, 1980)]. Since the paternal gene has been shown to be expressed in homologous androgenetic merogones (Showman and Whiteley, 1980), the exclusive expression of the maternal gene in reciprocal hybrid embryos is most easily explained by species specificity in the interaction between maternal regulators and this VEB gene.

The differential spatial regulation of VEB gene activity among blastomeres is first evident in the 16-cell embryo in which a significantly higher number of transcripts accumulates in the macromeres. In the subsequent two cleavages, the mature spatial pattern emerges and all but a variable number of vegetal cells express these genes. Although a variety of complex models involving sequential blastomere-specific activation could be invoked, it is possible to account for these observations through the following simpler model. In this scenario, transcription of the VEB genes is activated equally in all 8-cell embryo nuclei, but at low levels due to limiting positive factor concentration. At 16-cell stage, VEB transcription rates are higher in the macromeres as a result of unequal cleavage which generates a larger number of limiting positive factors per gene corresponding to the relatively greater macromere volume. A similar mechanism has been suggested for other genes of this embryo (Cutting et al., 1990). During the subsequent cleavages, the supply of active factors increases by either translation of maternal mRNA, modification of maternal proteins or zygotic gene expression. These factors then become nonlimiting and during this time, VEB mRNAs accumulate at a very high rate in the progeny of both mesomeres and macromeres. To account for the lack of expression by vegetal blastomeres, we postulate either the existence of a negative factor or the exclusion of the source of positive factors from this region at 16-cell stage. In this regard, the vegetal pole cytoplasm of sea urchin embryos is clearly a special domain of the egg (reviewed by Davidson, 1976) which is known, for example, to lack 25% of maternal RNA complexity (Rodgers and Gross, 1978; Ernst et al., 1980).

The VEB messages are a unique set of embryo-specific messages whose expression is coordinate and spatially restricted in the early sea urchin blastula. Activation of the VEB genes monitors changes in the transcriptional capacities of nuclei as they are exposed to different regions of the egg cytoplasm arrayed along the animal-vegetal axis. Thus, these genes will provide tools for identifying the localized regulatory activities that are asymmetrically distributed along this axis.

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Note added in proof

Recently the inferred protein sequences of several additional metalloendoproteases related to astacin have been reported (Dumermuth et al., 1991, *J. Biol. Chem.* **266**, 21381-21385. The nucleotide sequence of *SpAN* is in the Genbank database under accession number M84144.