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Whole mount in situ hybridization shows *Endo 16* to be a marker for the vegetal plate territory in sea urchin embryos

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We have used whole mount in situ hybridization to analyze the pattern of expression of the gene *Endo 16* in *S. purpuratus* embryos. The mRNA is first detectable at 18 h post-fertilization in the cytoplasm of blastomeres derived from the *Veg2* 6th cleavage tier. The number of *Endo 16* positive cells increases gradually through the beginning of gastrulation, and these cell numbers are in agreement with estimates of the number of cells that should be in the vegetal plate at these stages. We conclude that *Endo 16* expression is indeed an early vegetal plate marker and that this gene is expressed by all *Veg2* tier derivatives while they are part of the vegetal plate. The progressive regionalization of *Endo 16* expression that occurs in normal embryos is also seen in lithium chloride induced exogastrulae, leading to the conclusion that genetic regulation of endoderm differentiation is programmed into the vegetal plate cells once they have been specified. Finally, we report a reproducible phenomenon seen in cultures of LiCl exogastrulae, in which the tips of the everted archenterons fuse, followed by the induction of supernumerary pigment cells.

Vegetal plate; *Endo 16*; In situ hybridization; Sea urchin embryogenesis

Introduction

Of the five territories recognized in the early sea urchin embryo, the cells of the vegetal plate are the most pluripotent. This territory will not only produce the entire endoderm, but also most of the coelomic pouch founder cells and the true mesoderm (i.e., the secondary mesenchyme), which differentiates into pigment cells, muscle cells and larval mesenchyme or basal cells (Cameron et al., 1987, 1991). As the archenteron invaginates, the non-migratory small micromeres situated at its tip are carried to the area where they will take part in forming the coelomic pouches (Pehrson and Cohen, 1986). Moreover, experimental studies have shown that if primary (skeletogenic) mesenchyme cells are removed from the embryo, a subset of the secondary mesenchyme can regulate to replace this missing cell type and form a larval skeleton (Ettensohn and McClay, 1988). Thus, the vegetal plate is capable

of forming all the internal cell types of the pluteus larva, except those contributed by the small micromere descendants. The establishment of the vegetal plate territory is clearly a central event in sea urchin embryogenesis.

The eight blastomeres from which the vegetal plate is entirely descended arise at the sixth cleavage. They were termed the '*Veg2 tier*' by Hörstadius (1939), but in more recent cell lineage studies they are known as VAM1*l*, VAM2*l*, right and left VLM1*l*, right and left VLM2*l*, VOM1*l* and VOM2*l* (Cameron et al., 1991). As part of our effort at understanding founder cell specification during sea urchin embryogenesis, we are particularly interested in identifying territory specific molecular markers that are expressed soon after territorial founder cell segregation. The analysis of the regulation of such early territory specific genes affords an opportunity for identifying the regulatory factors and cellular processes directly involved in founder cell specification, since at least some of the transcription factors which regulate such early marker genes are likely to be directly responding to stimuli affecting cell-type specification (Davidson, 1989, 1991).

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In order to test the hypothesis that vegetal plate specification occurs in the *Veg2* tier cells, or their immediate descendents, we have undertaken to analyze the regulation of a molecular marker that is specific to the vegetal plate, and is expressed relatively early in embryogenesis. *Endo 16* is a gene encoding a protein found on the surface of archenteron cells, which possibly functions in gastrulation (Nocente-McGrath et al., 1989). The expression of this gene was first detected in the 20 h hatched blastula using RNA gel blot hybridization. Radioactive in situ hybridization on sectioned material indicated that the transcripts are present specifically in cells of the vegetal region in 28 h mesenchyme blastula (Nocente-McGrath et al., 1989). All cells of the invaginating archenteron were reported to contain transcripts in 36 h gastrulae, but by the 90 h pluteus stage transcripts were restricted to just the midgut (stomach). In this paper we reexamine *Endo 16* mRNA distribution patterns using an improved technique, a whole mount in situ hybridization method modified from Harkey et al. (1992). In undertaking this study we endeavored to analyze the expression pattern of *Endo 16* mRNA as completely as possible by using an approach that is easier, more versatile and more informative at the cellular morphology level than the traditional hybridization of radioactive probes to sectioned material followed by autoradiography. Our goal has been to determine whether this gene can be legitimately considered a molecular marker for the entire early vegetal plate, including the precursors of the various secondary mesenchyme cell types, or rather a marker only for endodermal precursors *per se*. To determine this we established the timing of productive *Endo 16* expression, and identified the number and location of cells containing *Endo 16* transcripts at early stages. We have also investigated the spatial distribution of *Endo 16* mRNA after lithium chloride treatment, since this agent is known to affect the number of cells that express *Endo 16* (Nocente-McGrath et al., 1991).

Results

Endo 16 expression during embryogenesis

Endo 16 transcripts can first be detected using the whole mount in situ hybridization method in 18 h embryos, in approximately 25 cells in the vegetal plate that are arranged in a ring pattern. At this stage, the staining is clearly restricted to the cytoplasm of cells in the vegetal plate, but is of varying intensity within the group of stained cells, and overall the staining is relatively weak. The typical pattern of *Endo 16* mRNA, as seen at several later landmark stages using the whole mount technique is shown in Fig. 1. At 20 h post-fertilization,

the staining intensity has increased and is more uniformly distributed throughout the cytoplasm of the vegetal plate cells. Fig. 1A,B shows clearly that *Endo 16* message is restricted to a ring of approximately 28 cells in 20 h embryos. The absence of staining in the center of the vegetal plate territory shows that the skeletogenic territory in the blastula (primary mesenchyme precursors) do not express *Endo 16* prior to ingression. Nor do they express this gene after their ingression in 30 h embryos (Fig. 1C). As the embryos approach gastrulation the number of *Endo 16* positive cells steadily increases, from approximately 50 cells at 23 h, to about 80 cells at 25 h and to about 100 cells at 29 h. Fig. 2 summarizes these cell count data.

During gastrulation, from 32 h until 48 h, the entire archenteron contains the *Endo 16* message (e.g., Fig. 1D). However, *Endo 16* expression is extinguished in the secondary mesenchyme as these cells become motile at the top of the ingressing archenteron, and begin to extrude filopodial extensions. No *Endo 16* mRNA is found in secondary mesenchyme that have crawled away from the tip of the archenteron (Fig. 1D). By 60 h the cells in the top third of the archenteron (prospective foregut) have also ceased to express *Endo 16* (Fig. 1E). At this stage the posterior two-thirds of the archenteron remain positive for *Endo 16* message. By 72 h, the differentiation of the endoderm into hindgut, midgut and foregut have progressed to the point that three distinct morphological regions are visible (Fig. 1F). *Endo 16* mRNA becomes restricted to the midgut (stomach) region at this stage, and this distribution pattern remains the same in plutei after seven days, which is the latest stage examined to date.

Endo 16 expression in exogastrulae

Fig. 3 shows *Endo 16* expression after the blastula stage in embryos that had been grown in the presence of 12 mM LiCl from the two-cell stage through 24 h post fertilization. At 30 h, which is the mesenchyme blastula equivalent, the morphology of the embryos and the distribution of *Endo 16* mRNA are both significantly altered from normal embryos (Fig. 3A). The embryos are distinctly flattened along the A-V axis, apparently as a consequence of a broader vegetal plate region. The pattern of *Endo 16* staining at this stage suggests an expansion of the vegetal plate, in that more cells have *Endo 16* mRNA compared to the normal 30 h embryo (compare Fig. 3A to Fig. 1C). Cell counts on lithium treated embryos at this stage indeed demonstrate an average increase of 37% over normal in the number of cells staining for *Endo 16* (Avg. 136 cells versus 99 cells in controls; see Fig. 2). The central region of the vegetal plate, which is the skeletogenic territory, remains negative for *Endo 16* after LiCl treatment just as in control embryos. The ingressed

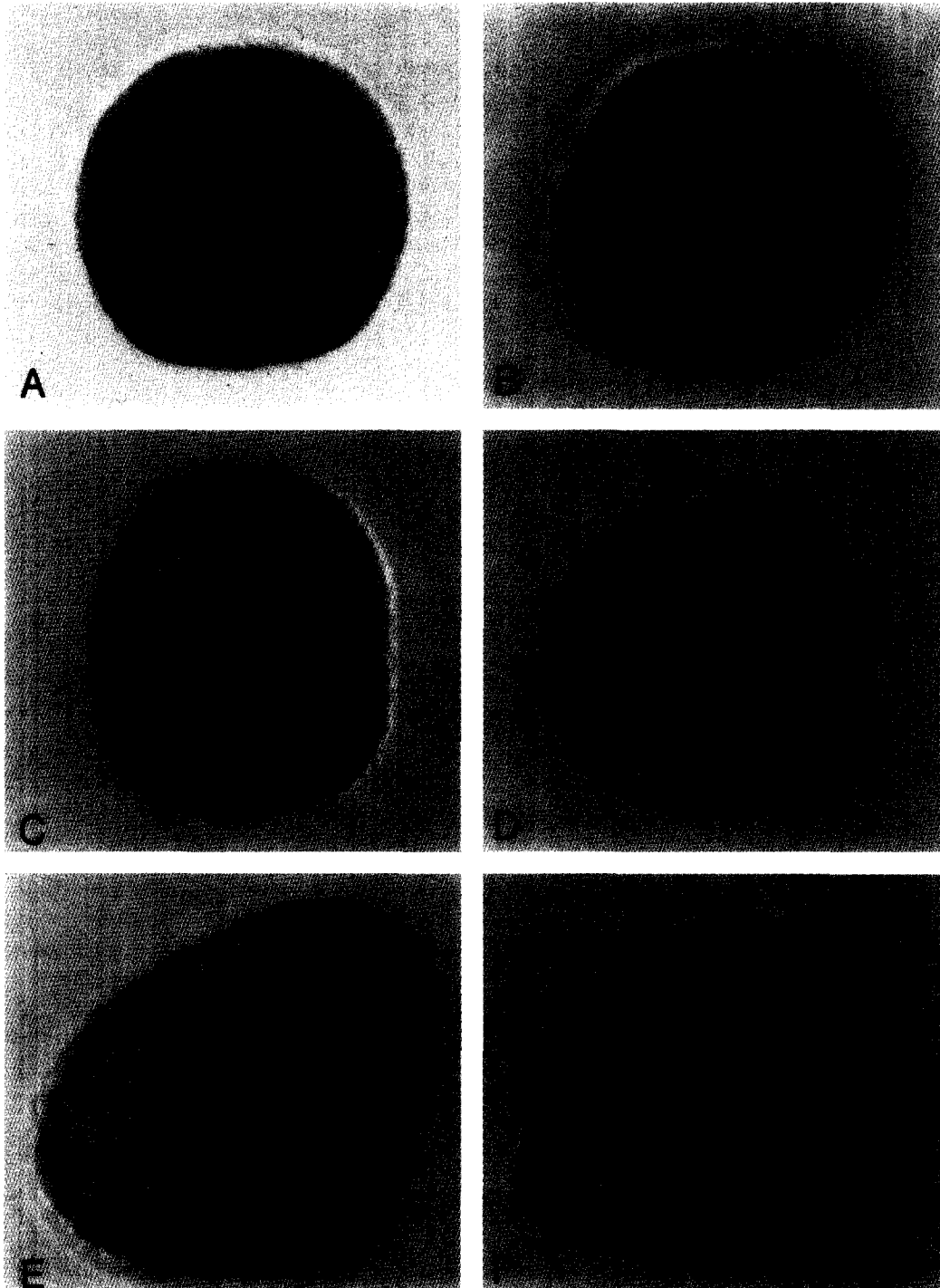


Fig. 1. Various stage *S. purpuratus* embryos illustrating the developmental pattern of expression of *Endo 16* transcripts (blue staining regions) localized by whole mount in situ hybridization. (A,B) 20 h embryos with a ring of *Endo 16* expressing cells in the vegetal plate. In B, the vegetal pole view shows the non-staining center of the vegetal plate, occupied by the skeletogenic mesenchyme and small micromeres. (C) 30 h mesenchyme blastula with *Endo 16* expressed in the vegetal plate, but not in the ingressed primary mesenchyme. (D) 48 h gastrula expressing *Endo 16* along the entire length of the archenteron; note that the secondary mesenchyme near the top of the archenteron are negative for *Endo 16*. (E) 60 h prism stage gastrula with *Endo 16* expression restricted to the midgut and hindgut regions of the archenteron. (F) 72 h early pluteus larva with *Endo 16* expression restricted primarily to the midgut region of the tripartite gut. Expression in the hindgut is weak at this stage and becomes undetectable soon after the stage shown. Scale bar = 20 μ m.

primary mesenchyme are also negative for *Endo 16* expression.

Most of the embryos exogastrulated after lithium treatment. At 48 h, *Endo 16* mRNA was present in cells along the entire length of the everted archenteron, with the exception of the tip where the secondary mesenchyme had begun to become motile (Fig. 3B). At 60 h *Endo 16* message was diminished in the foregut equivalent, but was still present in significant amounts in the posterior two-thirds of the archenteron (Fig. 3C). After approximately 90 h the everted archenteron had differentiated morphologically into a tripartite gut, with *Endo 16* mRNA only present in the middle region, the equivalent of the stomach.

Fused exogastrulae and induction of pigment production

In the course of these experiments on LiCl-treated embryos we observed a phenomenon that to our knowledge has not previously been described in the literature. We include it here in the hope that it might provide a useful experimental tool. After 72 h in cul-

ture clusters of LiCl-treated exogastrulae embryos can be found adhering to each other at a common junction point, that apparently corresponds to the tips of their everted guts (Fig. 4A). A specific cell type designation cannot unequivocally be assigned to this region in these abnormally developing embryos, but it corresponds to an area from which secondary mesenchyme are generated in normal embryos. At the stage when these embryo clusters form, this region normally would be involved in mouth formation. Projecting slightly ahead in development, the foregut region is where the coelomic pouches normally develop. In many cases, a large roundish cluster of pigmented cells appears at the site of the fusion within a day of the formation of the embryo clusters (Fig. 4B). Since these embryos contain a normal complement of pigment cells in their respective ectodermal regions, we conclude that the pigmentation that develops at the fusion sites is from supernumerary pigmented cells whose differentiation has been specifically induced, at least in part, as a result of a cell interaction occurring at that location. This phenomenon, which probably involves the ectopic induction of genes involved in pigment production, might provide a useful route in which to study specification of pigment cells or regulation of pigment related genes.

Discussion

Endo 16 is a marker for the vegetal plate territory

A careful analysis of the spatial pattern of *Endo 16* mRNA expression during the blastula period has shown that *Endo 16* is expressed by all derivatives of the *Veg2* tier while they are part of the definitive vegetal plate, which includes the precursors of the pigment cells and all other secondary mesenchyme cell types. Whole mount in situ hybridization provided a revealing method for examining the location and number of cells expressing *Endo 16* in blastulae between 18 and 30 h post-fertilization. Throughout this 12 h period, the vegetal plate viewed from the vegetal pole consistently shows a ring of *Endo 16* expressing cells encircling the prospective skeletogenic mesenchyme and small micromere territories, which are both negative for *Endo 16* mRNA. Counting stained cells in embryos at progressively later stages throughout this period clearly showed that the number of *Endo 16* positive cells is in agreement with estimates of the number of cells that are derived from the *Veg2* tier that should be present throughout this period (Burke, 1980; Davidson, 1986; Cameron et al., 1987). Thus *Endo 16* can legitimately be regarded as a molecular marker for the vegetal plate in pre-gastrula stage embryos. The detailed observations we have presented here provide an impor-

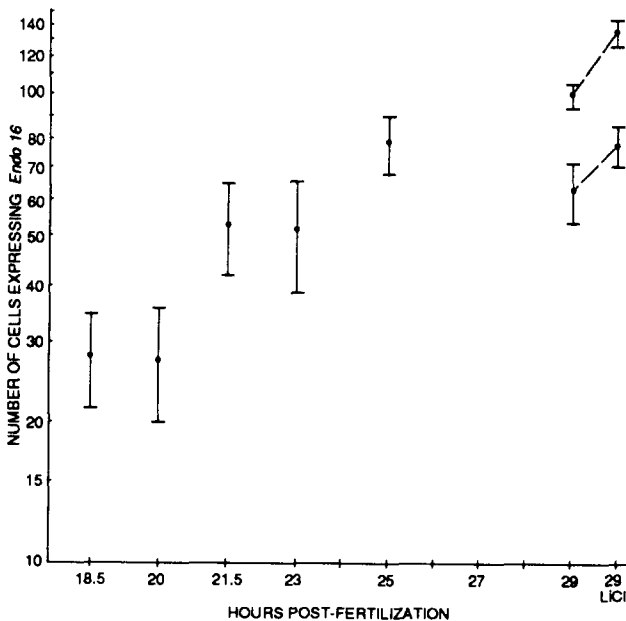


Fig. 2. Summary of the number of cells expressing *Endo 16* at various developmental time points prior to gastrulation. The horizontal axis denotes hours post-fertilization and the vertical axis (log scale) denotes the number of cells expressing *Endo 16* (see Experimental Procedures). The mean number of *Endo 16* positive cells at each stage is represented by a closed circle and the standard deviation by a vertical line. Note that for the 29 h embryos, both normal and LiCl treated, two mean cell numbers and two standard deviations are shown. The counts represented by the lower mean number correspond to embryos that are most likely a division cycle behind the majority of embryos at 29 h. Interestingly, however, after LiCl treatment such 'slowly developing' embryos still have an increase (dashed line) over normal in number of cells expressing *Endo 16*. The number of embryos examined for each stage are: 18 h = 24; 20 h = 30; 21.5 h = 15; 23 h = 13; 25 h = 10; 29 h = 20; 29 h LiCl = 7.

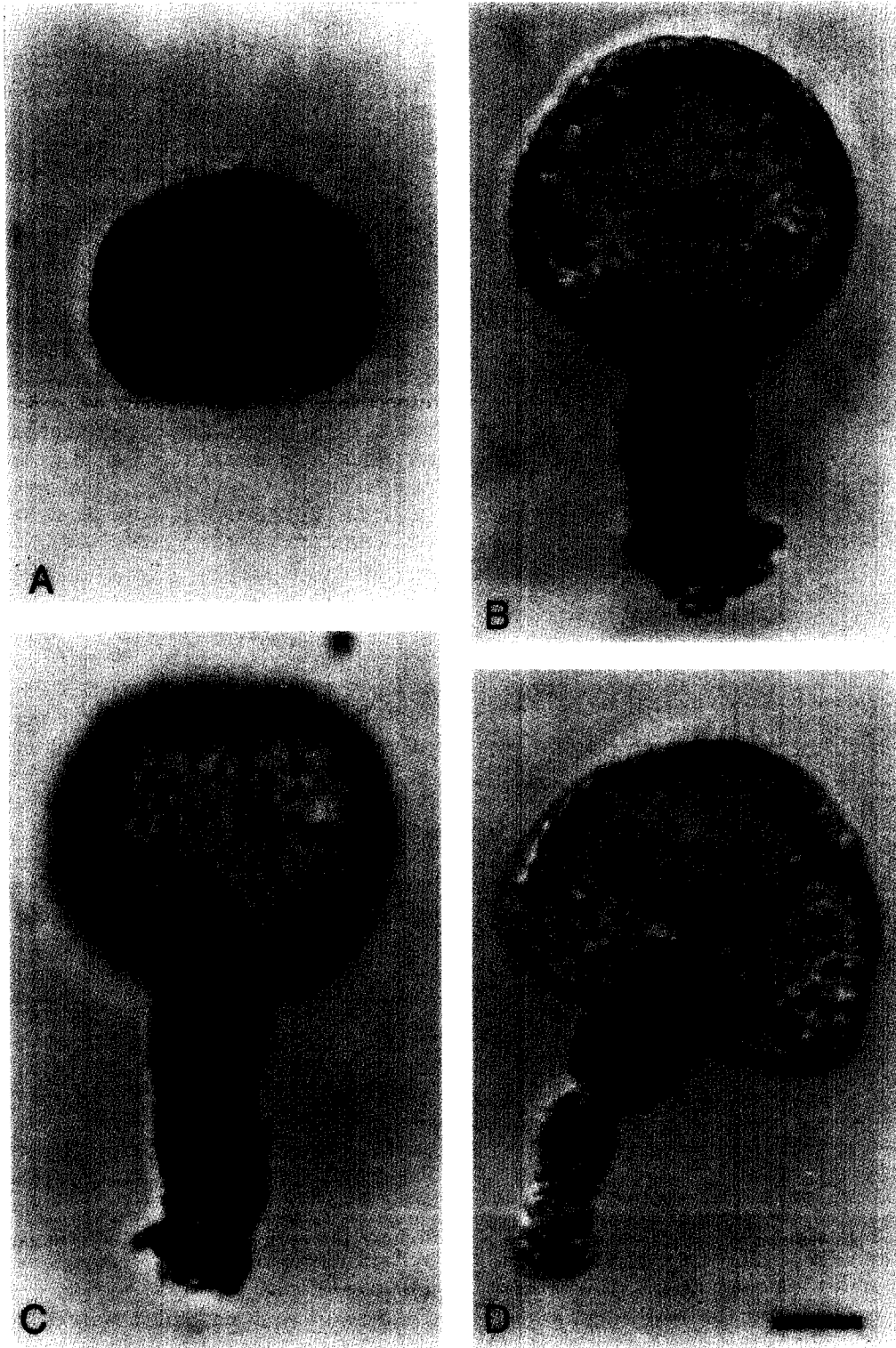


Fig. 3. *S. purpuratus* embryos in various stages of lithium-induced exogastrulation, and exhibiting the pattern of *Endo 16* expression seen after whole mount in situ hybridization. (A) 30 h mesenchyme blastula equivalent showing a distinct flattening along the A-V axis and a broadened region of *Endo 16* expression in the vegetal plate region. (B) 48 h exogastrula with *Endo 16* expression along the entire everted archenteron, excepting the tip where secondary mesenchyme cells are detaching. (C) 60 h exogastrula with *Endo 16* expression restricted to the proximal half of the everted archenteron corresponding to the midgut and hindgut of a normal embryo. (D) 90 h exogastrula with *Endo 16* expression restricted to the midgut equivalent in the everted archenteron, which has undergone regionalization into a tripartite structure. Scale bar = 20 μm .

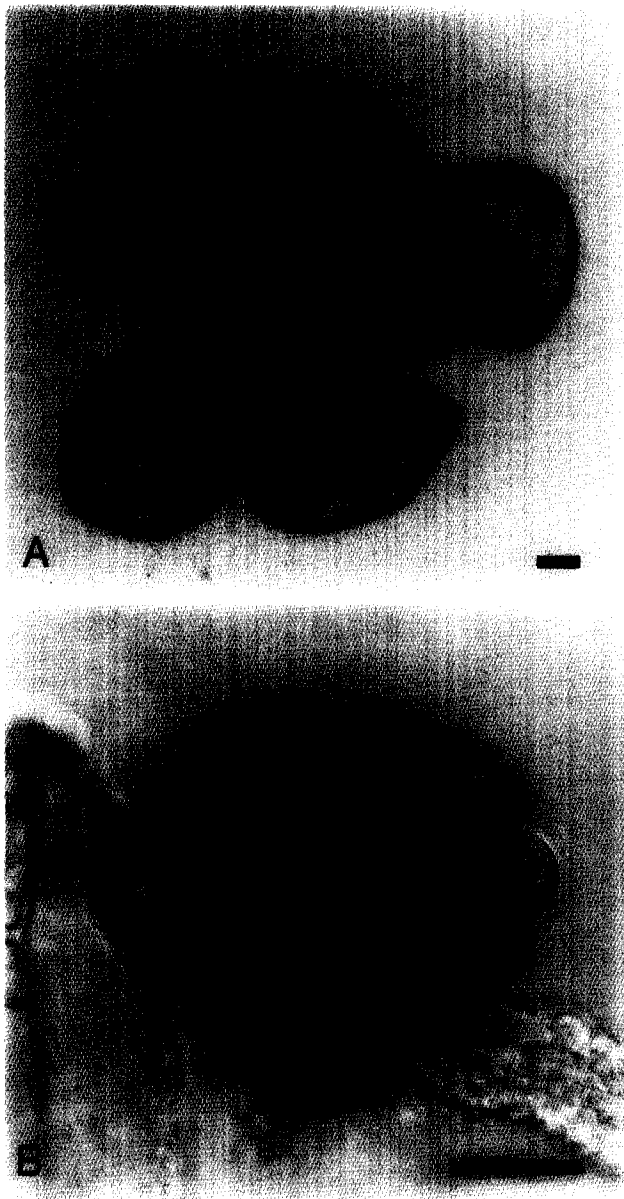


Fig. 4. Cluster of 4-day old *S. purpuratus* exogastrulae which have fused at the tips of their respective everted archenterons. (A) Lower magnification view of exogastrulae cluster with six plutei fused at their archenteron tips. Reorganization of the cells in the fusion site produces a ball of cells that becomes heavily pigmented and seems to enlarge. (B) Higher magnification view of the pigmented ball of cells shown in A. Scale bars = 20 μ m.

tant argument concerning the sequence of molecular level decisions within the vegetal plate. The expression of *Endo 16* throughout the early vegetal plate is consistent with the concept that the early specification events are responsible for generating territories, each of which can be characterized as a domain of cells enabled to express a particular battery of region specific molecular markers. The enabling of such a territory-specific gene battery requires that all essential regulatory factors are present and functional within the specified domain of cells. It is not unreasonable to

speculate that one of the first gene regulation pathways affected by 'vegetal plate' specification involves production, or modification, of one or more of the factors which regulates *Endo 16*.

Interestingly, *Endo 16* is quickly turned off in any *Veg2* tier derived cells that ingress from the vegetal plate, i.e. pigment cell precursors, or any cells that initiate motile behavior and migrate away from the archenteron, i.e. definitive secondary mesenchyme. The termination of *Endo 16* expression in the various secondary mesenchyme cells types appears to be a discrete change at the transcriptional level. While these cells are part of the vegetal plate territory they actively transcribe *Endo 16*. But apparently simultaneously with becoming motile, a switch in transcriptional activity occurs that most likely involves the inactivation of vegetal plate specific genes and activation of secondary mesenchyme specific genes. In recent studies we have identified a gene that is activated in secondary mesenchyme cells at precisely this point in time (C. Smith et al., unpublished data). Thus, monitoring the down-regulation of *Endo 16* expression, as well as the up-regulation of specific gene(s) in secondary mesenchyme, provides molecular markers through which it will be possible to focus on the precise timing of the switch from vegetal plate gene batteries to secondary mesenchyme specific gene batteries. This switch indicates that within a subset of vegetal plate cells a new cell specification has been superimposed on the original territory specification. Analysis of the transcriptional regulators of *Endo 16* is therefore likely to provide an avenue for identifying the molecular mechanisms underlying this dramatic switch in gene activity, and will also illuminate the mechanism by which pigment cells and secondary mesenchyme are specified within the vegetal plate territory.

Implications of the dynamic pattern of Endo 16 expression within endoderm

Our results confirm that *Endo 16* is a marker gene for endoderm regionalization, from gastrulation through larva formation, with the pattern of expression changing with the state of differentiation of the endoderm. Monitoring these changes in expression is made easy by the use of the whole mount in situ method, whereby we can examine a large number of embryos at any stage of embryogenesis. Section in situ hybridization would have been prohibitively tedious for examinations of the long everted archenterons of exogastrulae. The whole mount method proved particularly useful in the present context for visualization of *Endo 16* expression in the entire gut of normal (or exogastrulated) early plutei. Previous studies reported the difficulty of visualizing the entire gut in sectioned material, in which consecutive sections were required to recon-

struct the pattern of expression (Nocente-McGrath et al., 1989). The whole mount hybridizations demonstrate at once that *Endo 16* mRNA is found exclusively in the stomach of the mature pluteus. The function of *Endo 16* protein in the stomach of the pluteus is unknown, but its persistence there demonstrates that its function is unlikely to be limited to a role in the morphogenetic cell rearrangements of archenteron cells, as proposed earlier.

One way to address the question of what regulates regional expression of *Endo 16* within the endoderm is to induce exogastrulation by treatment of early embryos with LiCl, and then examine the pattern of *Endo 16* expression in the everted archenterons that have been deprived of the blastocoelic environment and all lateral cell contacts. The spatial distribution of *Endo 16* message in exogastrulae has not been examined previously, although antibodies to *Endo 16* fusion protein have been used to localize the distribution of *Endo 16* protein in exogastrulae (Nocente-McGrath et al., 1991). We found that during exogastrulation the distribution of *Endo 16* mRNA within the cells of vegetal plate territory follows a dynamic pattern, similar to that seen in the corresponding areas during normal development. Comparison of whole mount in situ hybridizations of normal and LiCl-treated embryos at 30 h clearly shows that more cells express *Endo 16* after lithium treatment, which is consistent with the notion of recruitment of extra cells to the vegetal plate territory (Nocente-McGrath et al., 1991). Nevertheless, during the subsequent stages of exogastrulation the same relative proportion of archenteron cells contain *Endo 16* mRNA as in normal development. For example, at 48 h the entire evaginated archenteron is positive for *Endo 16* mRNA, while by 60 h there is no expression in the 'foregut' region and by 90 h expression is restricted to the midgut equivalent. The morphological regionalization of the everted archenterons of echinoid exogastrulae is well known (Moore, 1927; Hardin and Cheng, 1986). By observing transcript prevalence in situ, we confirm a normal progression in the differential expression of the *Endo 16* marker gene in the everted archenterons. It follows that the genetic regulatory processes that lead to regionalized *Endo 16* expression in normal embryos are entirely autonomous with respect to any lateral cell contacts or contacts with blastocoelic fluid. The responsible regulatory program must therefore be installed and activated within the vegetal plate archenteron precursors, though signals may also be required from the ectoderm cells surrounding the base of the gut.

The distributions of *Endo 16* mRNA and protein overlap throughout the period of expression of this gene in both normal embryos and in lithium induced exogastrulae (Nocente-McGrath et al., 1991). The lack of *Endo 16* message in secondary mesenchyme and the

increasingly restricted range of expression within the endoderm support the notion that the turnover rate of *Endo 16* message is high, so that the mRNA prevalence is under immediate transcription level control. It is worth noting that the morphological boundary that develops between the foregut and midgut also represents the spatial limit of expression of other genes expressed by endoderm cells. For example, *LvN1.2* mRNA accumulates throughout the archenteron during primary invagination and later becomes restricted to hindgut and midgut (Wessel et al., 1989). Conversely, two mRNAs have been found that accumulate exclusively in the top third of the archenteron (foregut) during gastrulation (Kingsley et al., 1993). Interestingly, *Endo 16* is the first endoderm gene analyzed whose expression pattern is spatially defined by the hindgut-midgut boundary, and so becomes confined exclusively to the midgut. *Endo 16* and *LvN1.2* could share the same mechanism for negatively regulating their expression in the foregut, but they clearly have distinct control mechanisms for regulating hindgut expression.

In summary, we have used whole mount in situ hybridization to extend observations on the expression of the gene *Endo 16* in *S. purpuratus* embryos. We definitively demonstrate that *Endo 16* is a specific vegetal plate marker gene that begins transcription at an early stage in development, and is expressed by all cells comprising this territory. Expression of this gene is rapidly terminated in any cells that leave the vegetal plate prior to or during gastrulation. Later expression is restricted as the archenteron differentiates into a tripartite gut. Analysis of the *Endo 16* regulatory domain will clearly provide a molecular insight into the mechanisms by which vegetal plate founder cells are initially specified, as well as those by which other cell types derived from the vegetal plate are specified.

Experimental Procedures

Culture and manipulation of embryos

Adult *Strongylocentrotus purpuratus* were collected along the southern California coast and maintained in sea water tables at the Caltech Kerchoff Marine Lab. Gamete handling, fertilization and embryo culturing were carried out according to standard methods. Counts of cells expressing *Endo 16* were performed on embryos after they had been processed through the in situ protocol by gently flattening them under a coverslip in a minimal amount of mounting medium. By sliding the coverslip very slightly using the tip of a jeweler's forceps, while observing through a compound microscope, the individual cells of the embryo were dissociated without being macerated and those cells containing the blue-colored precipitate resulting from

the alkaline phosphatase reaction were counted as positive for *Endo 16* mRNA.

The protocol for lithium treatment followed established procedures (Nocente-McGrath et al., 1991; Hardin and Cheng, 1986). Two-cell embryos were cultured in filtered sea water (FSW) in the presence of 12 mM LiCl for 24 h (until the mesenchyme blastula stage), and the LiCl was then washed out and the embryos cultured in FSW.

Whole mount in situ hybridization

The whole mount in situ hybridization protocol used here is based on the method described by Harkey et al. (1992). With the exception of the modifications noted below, their protocol was followed. An *Endo 16* genomic clone was obtained by screening a genomic library with a probe derived from an *Endo 16* cDNA clone (pCNM4; Nocente-McGrath et al., 1989).

A modification worth noting specifically is our use of an RNA probe rather than a single stranded DNA probe as in Harkey et al. (1992). RNA probes are easier to produce in large quantities and they form more stable hybrids with mRNA. An antisense RNA probe labelled by incorporation of digoxigenin-11-UTP (Boehringer-Mannheim Biochem.) was prepared with Ambion's MEGASCRIPt kit utilizing the following conditions. A 20 μ l reaction was incubated for 7 h at 37°C, containing 1 μ g of template DNA (CsCl-purified subclone of *Endo 16* coding region from the genomic clone, linearized in order to produce a 650 nucleotide antisense probe); 40 U T7 RNA polymerase; 1 U placental ribonuclease inhibitor; 7.5 mM each of ATP, CTP and GTP; 4.85 mM UTP; 2.9 mM dig-11-UTP, 20 μ Ci ³⁵S-UTP (spec. act.: 650 Ci/mmol); and Ambion's optimized transcription buffer (patented). This reaction yielded 15 μ g of probe after precipitation and resuspension, and it was demonstrated by acrylamide gel stained with ethidium bromide and by autoradiography to be essentially pure full length transcript. Aliquots of concentrated probe at 50 ng/ μ l were stored at -70°C in water plus RNase inhibitor. Working stocks of probe at 1 ng/ μ l in hybridization buffer were useable for several consecutive experiments. Concentrated probe was stable at -70°C for more than a year, and the working stocks were stable for at least a month when stored at -20°C.

Fixation, pre-hybridization, post-hybridization washes, alkaline phosphatase staining and dehydration were carried out in individual wells of round bottom Falcon 3911 flexible plastic 96-well plates. Manipulations were carried out while observing through a dissecting microscope. Embryos were allowed to settle at 1 \times gravity and solutions were added and removed with a mouth pipette constructed from a pulled out pasteur pipette inserted into latex tubing. It was extremely important at all steps to thoroughly mix solu-

tions in the wells. Mixing was accomplished either by blowing air toward the side of the well to create a swirling vortex, or by aspirating the solution in and out of an automatic pipettor adjusted for 100 μ l volume. Individual embryos, or up to several hundred embryos per well, were effectively processed using this method.

The highest signal and lowest background staining were obtained using a probe concentration of 0.02 ng/ μ l. Probe concentrations above 0.05 ng/ μ l produced unacceptable levels of background staining due to non-specific probe binding. A third 1 \times SSC wash was added post-hybridization and all three 1 \times SSC washes were extended to 30 min at 60°C. During the staining procedure, the blocking and antibody incubations were carried out in the presence of 5% goat serum. To avoid production of a precipitate between the phosphate-containing rinsing buffer and the pH 9.5 alkaline phosphatase staining buffer (APB), two washes were carried out in APB at pH 8 before transferring the embryos to APB at pH 9.5. Control procedures (which omitted the antibody step) showed there was no detectable endogenous alkaline phosphatase enzyme activity in embryos at any stage after they had been processed through this in situ protocol.

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