

# Micromeres are required for normal vegetal plate specification in sea urchin embryos

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## SUMMARY

**Vegetal plate specification was assessed in *S. purpuratus* embryos after micromere deletions at the 4th, 5th and 6th cleavages, by assaying expression of the early vegetal plate marker *Endo16*, using whole-mount in situ hybridization. After 4th cleavage micromere deletions, the embryos typically displayed weak *Endo16* expression in relatively few cells of the lineages that normally constitute the vegetal plate, while after 5th and 6th cleavage micromere deletions the embryos exhibited strong *Endo16* expression in larger fractions of cells belonging to those lineages. When all four micromeres were deleted, the embryos were severely delayed in initiating gastrulation and sometimes failed to complete gastrulation. However, if only one micromere was allowed to remain in situ throughout development, the**

**embryos exhibited strong *Endo16* expression and gastrulation occurred normally, on schedule with controls. Additional measurements showed that these microsurgical manipulations do not alter cleavage rates or generally disrupt embryo organization. These results constitute direct evidence that the micromeres provide signals required by the macromere lineages for initiation of vegetal plate specification. The specification of the vegetal plate is completed in a normal manner only if micromere signaling is allowed to continue at least to the 6th cleavage stage.**

Key words: sea urchin embryo, blastomere specification, cell interaction, vegetal plate territory, *Endo16*

## INTRODUCTION

The sea urchin embryo is representative of a diverse group of invertebrate embryos with an invariant early cell lineage. In embryos of this type, one embryonic axis is established by the time of fertilization, and initial cell specification processes occur during the cleavage stages to establish territories with different developmental fates. Zygotic transcription is activated in these embryos soon after fertilization, and the expression of territory-specific molecular markers begins almost immediately after segregation of lineage founder cells (Davidson, 1986, 1989, 1990, 1991). In the sea urchin embryo, 9-10 cleavage divisions follow an invariant pattern that is oriented with respect to the primordial animal/vegetal (A/V) axis, thus yielding fixed origins for the territory founder cells (Cameron et al., 1987, 1991). By the blastula stage, five polyclonal territories are distinguishable by the use of non-overlapping expression patterns of selected territory-specific molecular markers; they are the presumptive oral ectoderm and aboral ectoderm, the vegetal plate, the skeletogenic mesenchyme and the small micromeres.

Some aspects of the specification of the lineage founder cells that give rise to these territories must depend on utilization of localized maternal factors, given the primordial A/V axis of the embryo. However, there is also extensive experimental evidence that mid-cleavage stage blastomeres have an extensive capacity to change their states of specification in response to

cell interactions (reviewed in Davidson, 1986). The implication is that cell interactions between adjacent blastomeres are a required component of founder cell specification processes in normal embryogenesis. These developmental features have been incorporated into a predictive model for the mechanism of cell specification in sea urchin embryos (Davidson, 1989, 1991). Briefly, this model proposes that factors localized along the A/V axis of the egg are segregated to specific early blastomeres, where they result in the expression of specific inducing ligands that are presented, respectively, on the appropriate blastomere membranes. A global distribution of ligand receptors and maternal transcriptional regulatory factors in early cleavage stage blastomeres guarantees that all interacting cells have the capacity to mobilize the regulatory pathway appropriate for each set of intercellular signals. The model proposes that initial specification of the ectodermal and vegetal plate territory founder cells occurs as a consequence of such local cell interactions among mid-to-late cleavage stage blastomeres, while the skeletogenic mesenchyme territory is unique in being autonomously specified at the time that its founder cells, the large micromeres, segregate near the vegetal pole at 5th cleavage (refer to Fig. 1). The model specifically predicts that the micromeres produce intercellular signals required for initial vegetal plate specification in the neighboring macromere-derived cells and that the entire normal series of conditional specifications throughout the embryo is entrained by this initial interaction.

There is no doubt that the large micromeres have the capacity to induce vegetal plate specification, since implanting micromeres next to presumptive ectodermal cells evokes their respecification into ectopic vegetal plate cells (Hörstadius, 1939; Ransick and Davidson, 1993). In the study of Ransick and Davidson (1993), micromere transplantation to the animal pole region at the 8-cell stage induced ectopic vegetal plate cells which produced a complete second gut. The similarities in the morphological development of an induced gut forming from the animal pole region and the endogenous gut invaginating from the vegetal pole region were substantiated by whole-mount in situ hybridization assays for expression of the early vegetal plate marker, *Endo16* (Ransick et al., 1993), a gene encoding a gut-specific cell surface protein (Nocente-McGrath et al., 1989; Soltysik-Española et al., 1994). It was found that the induced and endogenous guts exhibit identical patterns of *Endo16* expression. Dramatic as were these results in demonstrating the sufficiency of the micromeres for inducing vegetal plate specification, this transplantation experiment does not directly address the issue of whether the micromeres, as predicted by the specification model, are in fact necessary in situ for specification of the normal vegetal plate founder cells. Moreover, it is frequently cited that the macromere progeny demonstrate, after prolonged periods of culture, an innate capacity to make archenteron and skeletal structures even in the absence of micromeres (Hörstadius, 1939). Here, a direct test of the necessity of micromeres for the normal specification of vegetal plate founder cells is accomplished by deleting the micromeres and applying the molecular level assay for *Endo16* as positive indicator of initial vegetal plate specification. The normal specification process is completed by the early blastula stage. Therefore, by assaying at this stage, the *Endo16* expression that we measure reflects the normal specification processes. This strategy effectively excludes marker expression resulting from regulative specification pathways that activate later in development.

The early vegetal plate territory can be uniquely distinguished in the 20 hour (approximately 400-cell stage) *Strongylocentrotus purpuratus* embryo by cell lineage and by *Endo16* expression. After ingress of the skeletogenic mesenchyme at around 22 hours, the definitive vegetal plate exists as a distinctly thickened region of the blastula wall arranged around the embryonic vegetal pole. At this stage, it is composed of about 60 cells entirely descended from eight *veg2* tier (Hörstadius, 1935) founder cells. These eight cells segregate at 6th cleavage from their sister cells, which form the overlying ring of cells known as the *veg1* tier. The ancestry of the *veg1* and *veg2* tier blastomeres, and their disposition in the 6th cleavage embryo, are shown diagrammatically in Fig. 1. Our earlier whole-mount in situ hybridization study (Ransick et al., 1993) demonstrated in detail that, at 18-24 hours postfertilization, the expression of *Endo16* is confined exclusively to the clones of cells derived from the *veg2* tier. Cell counts indicated that this remains the case up to the late mesenchyme blastula stage, 26-28 hours. Recent studies show that the domain of *Endo16* expression expands at some point between 28 and 48 hours to include clones of cells derived from the *veg1* tier, which are recruited to the invaginating archenteron late in gastrulation (our unpublished data; the contribution of *veg1* descendants to the archenteron was discovered by K. Logan and D. McClay in *L. variegatus*; personal communication). In pregastrular

embryos, the spatially regulated expression of *Endo16* serves as an excellent molecular marker for the initial specification of the lineages that constitute the vegetal plate. Complete vegetal plate specification is indicated when, in addition to *Endo16* expression, gastrulation is initiated by 28 hours and progresses to completion in a normal manner.

We now present results that support the hypothesis that intercellular signals from the micromeres are required to initiate vegetal plate specification processes in normal embryogenesis. We demonstrate that early removal of the micromeres results in weak or failed vegetal plate specification, and that prolongation of the period of contact between the large micromeres and the macromere descendants results in more extensive vegetal plate specification. The presence of only one micromere throughout development is sufficient for complete specification of an attenuated set of vegetal plate precursors.

## MATERIALS AND METHODS

### Culture and manipulation of embryos

Eggs of *Strongylocentrotus purpuratus* were fertilized in filtered sea water (FSW) in the presence of 1 mM 3-amino-1,2,4-triazole, which prevents hardening of the fertilization membrane. The fertilization membranes were stripped 1 hour postfertilization by aspirating the embryos through a glass pipette pulled out to the appropriate diameter. After third cleavage, about 100 synchronous 8-cell stage embryos with relatively equal cleavages were selected. Embryos with sub-equatorial 3rd cleavages were avoided because they yield macromeres of reduced size, which are disadvantageous for our microsurgical manipulations. For microsurgical deletion of micromeres, the first 10-15 minutes after 4th cleavage division was avoided because of the presence of the midbody (spindle remnant) between sister macromeres and micromeres (see Fig. 1). Thus, microsurgies on 16-cell embryos were carried out in a 45 minute period between the completion of the 4th cleavage and the beginning of the 5th cleavage.

Microsurgical operations were carried out under a dissecting microscope on 2% Noble agar coated glass dishes. Embryos were incubated in hyaline extraction media (HEM: 0.3 M glycine, 0.3 M NaCl, 0.01 M KCl, 0.01 M MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 M Tris, (pH 8.0) and 0.002 M EGTA) (Fink and McClay, 1985) until the blastomeres had rounded up (2-5 minutes); then a glass needle was used to dissect the micromeres away from the vegetal end of the embryo. The exposure of the embryos to the action of HEM was limited by operating on groups of 5 to 8 embryos in a dish chilled by an ice bath, then returning them to FSW within 10 minutes. For each experiment, a 'treated control' group was generated by incubating embryos in HEM for 5 minutes and then returning them to FSW.

Within minutes after returning to FSW, most of the embryos compacted tightly into the smooth spherical shape characteristic of normal embryos. At that time, any embryos visibly disorganized or damaged by the manipulations were removed. In early experiments, manipulated and control embryos in groups of 10 to 30 were cultured in 2-3 ml of FSW at 15°C in 24-well plastic dishes overlaid with mineral oil (equilibrated with 0.2 M Tris, pH 8.0) to prevent the relatively fragile swimming blastulae lacking the normal hyaline layer from being ripped apart by the surface tension at the air-water interface. In later experiments, an improved culture chamber was developed that contained the embryos inside an 'agar tunnel', which was made by pouring molten 2% Noble agar in distilled water over a glass filament laying in a plastic Petri dish, then removing the glass after hardening of the agar. Such agar-coated dishes require a 10 minute incubation in FSW prior to useage to equilibrate the salt concentration throughout the agar layer.

### Whole-mount in situ hybridization

To assay for expression of the gene *Endo16*, embryos were fixed and processed for whole-mount in situ hybridization according to the methods described in Ransick et al. (1993). Two significant modifications to the previously described whole-mount protocol, which tended to lower background staining, involved raising the hybridization temperature from 45° to 48°C, and switching from goat to sheep serum as a blocking agent during antibody staining. For each experiment, HEM-treated and untreated control embryos were processed in parallel with experimental embryos.

The mesenchyme blastula stage was the best stage to evaluate the experimental results by whole-mount in situ hybridization, because the 16 large-micromere descendants have by then ingressed out of the vegetal plate into the blastocoel. This establishes the definitive vegetal plate territory, consisting of the 64 direct descendants of the eight *veg2* tier founder cells surrounding the eight small micromere descendants. In normal control mesenchyme blastulae, all *veg2* tier descendants are expressing *Endo16* at levels easily detectable with in situ hybridization, while no other cells express the marker. The dynamic quality of the vegetal plate after the initiation of gastrulation makes later developmental stages much more difficult to analyze.

### Methods of determining cell counts

To obtain accurate cell number counts (either of *Endo16*-expressing cells or total cells in the embryo), the cell nuclei were counter-stained after the whole-mount procedure with 40 µM Hoechst 33342 stain for 10 minutes. For counts of *Endo16* expressing cells, hybridized embryos were transferred gradually to 50% glycerol, then mounted on slides under supported coverslips in 'Vectashield' (Vector Labs) mounting media. The embryos were oriented to generate a video image of the vegetal end of the embryo then, under ultraviolet illumination, a mark was placed on a video monitor screen at each fluorescently labeled nucleus. Switching to transmitted light illumination revealed the area of alkaline phosphatase staining, which corresponded to locations of *Endo16* mRNA. The number of nuclei located within the alkaline phosphatase-stained area was a reliable measure of the number of *Endo16*-expressing cells. For counts of the total cell number, side views of slightly compressed, Hoechst-stained embryos were imaged with a video system under ultraviolet illumination, where each fluorescently labeled nucleus could be marked and counted.

DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes cat. #D-282) was introduced iontophoretically into the plasma membranes of macromere daughter cells (refer to Fig. 1) by holding a microneedle, containing 0.75% DiI dissolved in ethanol, against a hyalin-stripped cell membrane and applying a 10 nanoamp current for 2-3 minutes. The cell number in DiI-labeled clones in control versus experimental embryos was determined from photographic double exposures, which superimposed the fluorescent DiI signal upon a differential interference contrast image that showed cell borders on the surface of the embryo.

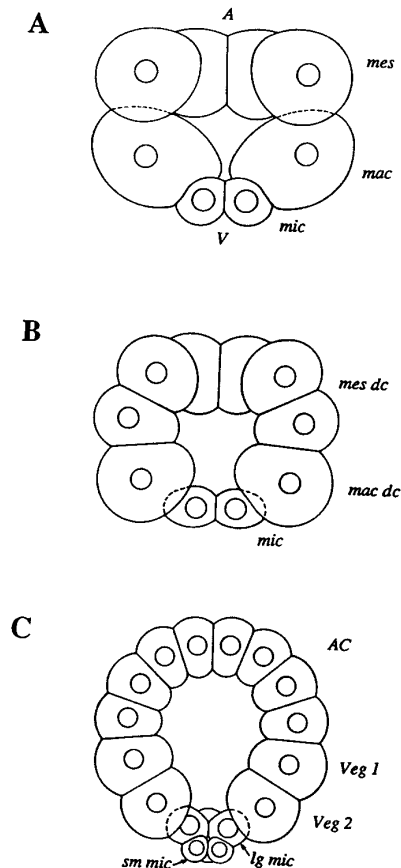
## RESULTS

### Vegetal plate specification after micromere deletions at 4th, 5th and 6th cleavages

The micromeres can be deleted without damage to the macromeres starting about 10 minutes after 4th cleavage. Removing the micromeres between 10 and 60 minutes after they are formed at 4th cleavage results in vegetal plates that develop after only a relatively short contact period between the micromeres and macromeres. This condition represents the baseline level of *Endo16* expression that we could obtain in these experiments. Deleting the micromeres after 5th cleavage

allows between 1 and 2 hours of contact with the macromeres, while their deletion after 6th cleavage allows between 2 and 3 hours of contact with the macromere progeny. The *Endo16*-positive cell counts that were obtained in four separate experiments are summarized in Fig. 2A-D and examples of stained embryos are shown in Fig. 3.

Consistent results were obtained in these four experiments. In each experiment, the average number of *Endo16*-positive cells after 4th cleavage deletions are ≤50% of control levels



**Fig. 1.** A schematic representation of mid-sagittal cross sections of *S. purpuratus* embryos at 4th (A), 5th (B) and 6th (C) cleavage stages, illustrating the relative size and position of the blastomeres, with labels as referred to in the text. All embryos are oriented as in A, with the original animal pole (A) upwards and the vegetal pole (V) downwards. (A) A 16-cell embryo at 4th cleavage stage, indicating the eight mesomeres (*mes*), four macromeres (*mac*) and four micromeres (*mic*). (B) A 28-cell embryo at 5th cleavage stage, indicating the 16 mesomere daughter cells (*mes dc*), the eight macromere daughter cells (*mac dc*) and the four micromeres (*mic*); the first division of the micromeres occurs a few minutes before the other cells undergo 6th cleavage. (C) A 56-cell embryo at 6th cleavage stage, indicating the animal cap (AC), comprised of 32 mesomere progeny, as well as eight *veg1* tier cells (*Veg 1*), eight *veg2* tier cells (*Veg 2*), four large micromeres (*lg mic*) and four small micromeres (*sm mic*). The large micromeres undergo division toward the end of 6th cleavage stage to produce a 60-cell embryo. The definitive vegetal plate, in the mesenchyme blastula stage embryo, is entirely descended from the eight *veg2* tier cells, which in more recent cell lineage analyses are known as VAM1l, VAM2l, right and left VLM1l, right and left VLM2l, VOM1l and VOM2l (Cameron et al., 1991).

and are consistently the lowest obtained from any experimental group. Specifically, in experiments A-D in Fig. 2, the average number of *Endo16*-positive cells after 4th cleavage deletions are 0, 19, 22 and 44, respectively. Thus each batch responded differently in absolute terms but similarly in relative terms. The average *Endo16*-positive cell numbers obtained after 5th and 6th cleavage deletions are consistently greater than the average levels obtained after 4th cleavage deletions. Sixth cleavage deletions yield embryos with *Endo16*-positive cell numbers closest to control levels. In fact, in three out of four experiments shown, there are individual cases from 6th cleavage deletions in which the number of *Endo16*-positive cells are equal to the controls.

The *Endo16* staining seen in 4th cleavage deletion embryos after whole-mount in situ hybridization is typically relatively weak (Fig. 3A-C), and in some individual cases is entirely absent. The stained cells are located at the vegetal end of the embryo, either as a single large patch or as separate smaller patches with nonstaining cells interspersed between the stained patches. Staining is consistently stronger in embryos after 5th and 6th cleavage deletions, as shown in Fig. 3 D-F; G-I. Non-staining cells occur less frequently than in 4th cleavage deletions, but when observed they are distributed in patches, suggesting that entire clones derived from *veg2* tier cells are negative for *Endo16* expression.

Two additional measurements showed that the observed decrease in *Endo16*-positive cell numbers in experimentally manipulated embryos was not a result of cleavage retardation. The first measurement determined the total cell number in embryos after introducing a fluorescent nuclear stain (see Materials and Methods). The cell counts, presented in Table 1, show that experimental and control embryos have about the same total number of cells. The second measurement labeled the plasma membranes of individual macromere-derived blastomeres with DiI at 5th cleavage, and determined the number of cells in the DiI-labeled clones at the blastula stage, similar to the example shown in Fig. 4B. These results are presented in Table 2 and they confirm that the labeled clones contain exactly the same number of cells in the experimental as in the control embryos. Thus the rate of cleavage in the macromere lineage is not altered by the microsurgical removal of blastomeres. The results in Tables 1 and 2 show that the experimental embryos develop at the same overall rate as the HEM-treated controls. These data can be interpreted directly by reference to our previous analysis of normal vegetal plate specification (Ransick et al., 1993), which demonstrates a highly reproducible progression in *Endo16* cell number relative to total cell number. In addition, we observe that the micromere-less embryos become ciliated and begin swimming normally at the same time as controls. As the example in Fig. 4A shows, the epithelial layer in blastula stage micromereless embryos has a normal appearance, with typical differences in thickness between the animal and vegetal regions. Therefore, we interpret the lower *Endo16*-positive cell number after micromere deletions as a direct result of eliminating intercellular signals normally provided by the micromeres.

The results of the four experiments presented in Fig. 2 above are typical of the results obtained in many other micromere deletion experiments carried out during the course of this study. However, in one exceptional batch of eggs the number of *Endo16*-expressing cells obtained after 4th-6th cleavage

micromere deletions was not lower than control levels (data not shown). This result serves as a potent reminder that the duration of contact required between micromeres and macromeres for initial specification of the entire vegetal plate can vary greatly from one batch of eggs to the next. It suggests that individual components of the specification process are variable, such as the relative strength of the inductive signals or the efficiency with which the signals are received and processed by macromere derived cells.

### **Vegetal plate specification with one micromere**

A variation on the original micromere deletion experiment that demonstrates a clear positive effect on vegetal plate specification is to delete three micromeres at 4th cleavage, and thus allow the embryo to develop with one micromere remaining in place. This remaining micromere produces four progeny by the time of primary mesenchyme cell ingressión. The *Endo16*-positive cell counts that were obtained in two separate experiments of this sort are summarized in Fig. 2, and examples of the stained embryos are shown in Fig. 3J-L. The average counts of *Endo16*-positive cells in one-micromere embryos are significantly higher than those obtained after deletions of all four micromeres at 4th cleavage. The average counts of *Endo16*-positive cells either equal (Fig. 2D), or exceed (Fig. 2C), those obtained after micromere deletions at 5th and 6th cleavages. The *Endo16* staining seen in one-micromere embryos after whole-mount in situ hybridization is consistently strong and unstained cell patches are not present.

Aside from clearly demonstrating that the micromeres produce a positive inductive effect, this variation on the micromere deletion experiment serves a second purpose. One conceivable cause of the poor vegetal plate specification after deleting the micromeres could be that the physical gap at the vegetal end of the embryo disrupts the vegetal plate specification processes. The one-micromere embryos are generated under identical microsurgical conditions and they also develop through 6th cleavage with a physical gap at their vegetal ends, since one micromere can not immediately fill the space normally occupied by four micromeres. The relatively strong *Endo16* expression that one-micromere embryos exhibit argues that vegetal plate specification processes are not disrupted by the presence of this physical gap.

As a related experiment, all four micromeres were deleted at 4th cleavage, then the physical gap was plugged with one mesomere transplanted next to the macromeres. Such transplantations produce no positive effect on vegetal plate specification (6 cases; data not shown). Whole-mount in situ hybridization shows that the *Endo16*-positive cells are distributed around the periphery of the cell clone derived from the transplanted mesomere. The *Endo16*-positive cell counts are in the range typically produced by 4th cleavage micromere deletions. This result shows that eliminating the physical gap does not rescue vegetal plate specification processes, which supports the conclusion that the gap is not the cause of the negative effects.

### **Effects on gastrulation**

The early events in vegetal plate development culminate with the beginning of morphogenesis, the initiation of gastrulation at 26-28 hours. We refer to gastrulation here specifically as the processes leading to the invagination of the vegetal plate and formation of the archenteron. Gastrulation occurs over a

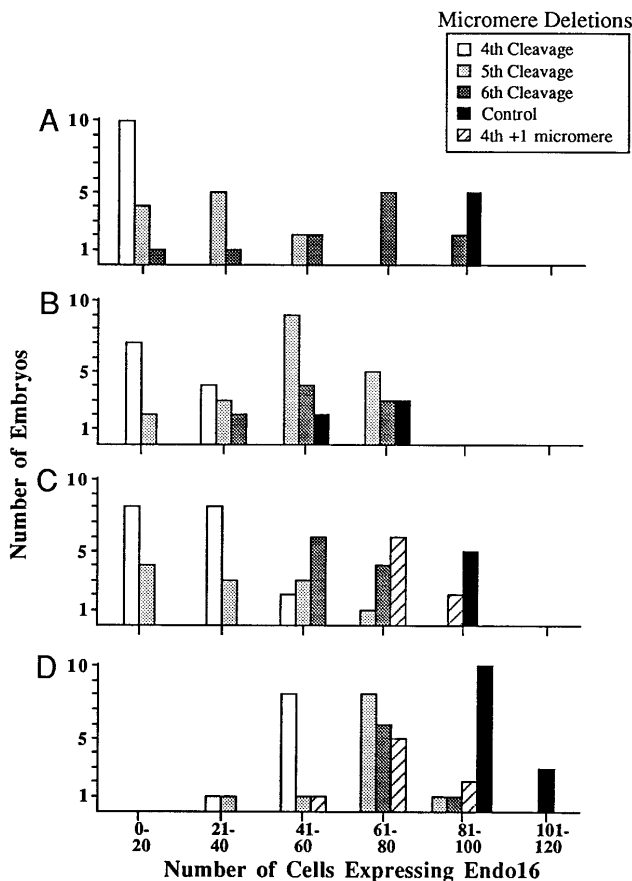
20 hour period of development, during which the vegetal plate is transformed into a dynamic group of cells that are motile and undergo convergent extension. The cells involved in archenteron formation constitute a changing population, as pigment cell precursors and secondary mesenchyme migrate out of the central region and *veg1* tier lineage cells are recruited along the periphery.

In the experiments presented above, our focus was on the early molecular marker *Endo16* as a measure of initial vegetal plate specification. Therefore, the experimental embryos were routinely processed for whole-mount in situ hybridization relatively early, i.e., after 9th cleavage and after primary mesenchyme cell ingress, but never later than the initial phase of gastrulation. However, in evaluating a living embryo, the initiation of gastrulation is the first morphogenetic process

involving the definitive vegetal plate cells that can be monitored as a measure of vegetal plate specification. If complete vegetal plate specification is a prerequisite to gastrulation, the effects of micromere deletions should be reflected in the early morphogenetic behavior of the vegetal plate.

A few experiments were carried out specifically to observe any effects on gastrulation. We found that embryos developing from 4th, 5th or 6th cleavage deletions are consistently delayed in initiating gastrulation, from a few hours up to 10 hours. Since we know from our in situ results that the vegetal plates are relatively poorly organized after micromere deletion, we interpret the delayed entry into gastrulation as a downstream consequence of interfering with the early vegetal plate specification processes. Remarkably, a significantly different efficiency in gastrulation is observed in one-micromere embryos. Even though the cell counts in Fig. 2C and D show that most of these one-micromere embryos have only 75% of the normal control number of *Endo16*-positive cells in their vegetal plates, the examples in Fig. 3J-L illustrate temporally normal gastrulation. Fig. 4G and H show later stages of the development of one-micromere embryos, in which gastrulation and gut differentiation proceed normally.

Most embryos lacking all micromeres eventually gastrulate, or at least attempt to gastrulate; however, the number of successful cases varies from experiment to experiment (i.e., female to female). Some examples are shown in Fig. 4. One remarkable group of embryos was carried through larval development to metamorphosis, and 8 out of 15 metamorphosed juveniles remain alive after two years. It is important to stress that the manner in which experimental embryos develop beyond the initial phase of gastrulation is subject to the remarkable regulative capacities for which this embryo is well known (for regulation in later embryogenesis, see recent review of McClay et al., 1992). These embryos, after a considerable lag time, apparently initiate compensatory developmental programs leading to the reconstruction of the missing vegetal plate-derived structures. Although fascinating in their own right, these late regulative phenomena are not directly relevant to the *normal* processes of initial blastomere specification, and are not considered further here.



**Fig. 2.** A graphic representation of the number of *Endo16*-expressing cells in 24-28 hour blastula stage *S. purpuratus* embryos after micromere deletions. (A-D) Summary of the data obtained in four separate, but similarly executed experiments. The key at the upper right of the figure provides an explanation of the coding used to represent the various experimental conditions. The x-axis of each plot gives the number of *Endo16*-expressing cells for each condition and the Y-axis gives the number of embryos at each level of expression. The data from all four experiments consistently show that the early micromere deletions (4th cleavage) resulted in embryos with the smallest number of *Endo16*-expressing cells. Embryos resulting from the later micromere deletions (5th and 6th cleavage) and the one-micromere group displayed numbers of *Endo16*-expressing cells at levels that are intermediate between the early deletion groups and the control groups. Fig. 3 shows photographs of embryos from the experiment summarized in Fig. 2C.

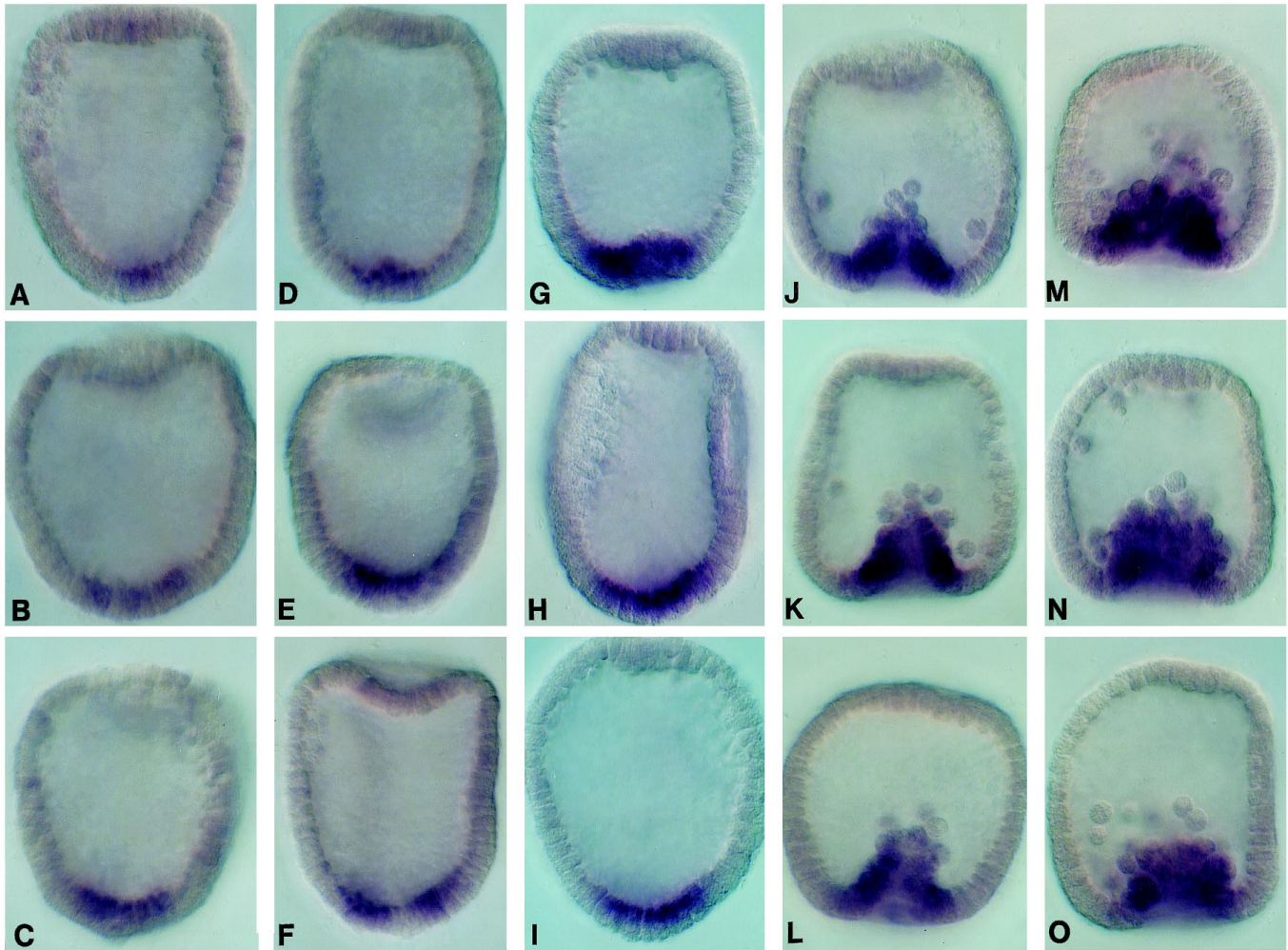
## DISCUSSION

We show here that deletion of the micromeres after a minimal contact period with the macromeres typically leads to a significant reduction in the number of cells that express *Endo16*, an early vegetal plate marker. The number of cells expressing this marker gene approaches control levels if a prolonged period of contact between micromeres and macromere descendants is allowed, or if only one micromere is permitted to remain in situ throughout development. These results provide direct evidence that the micromeres normally provide inductive signals to the macromere lineage, and that the macromere lineage normally requires these inductive signals to initiate vegetal plate specification.

### Conditional specification of the vegetal plate

It is important at the outset to make a distinction between the activation of a gene that serves as a marker of initial vegetal plate specification and the processes that indicate complete





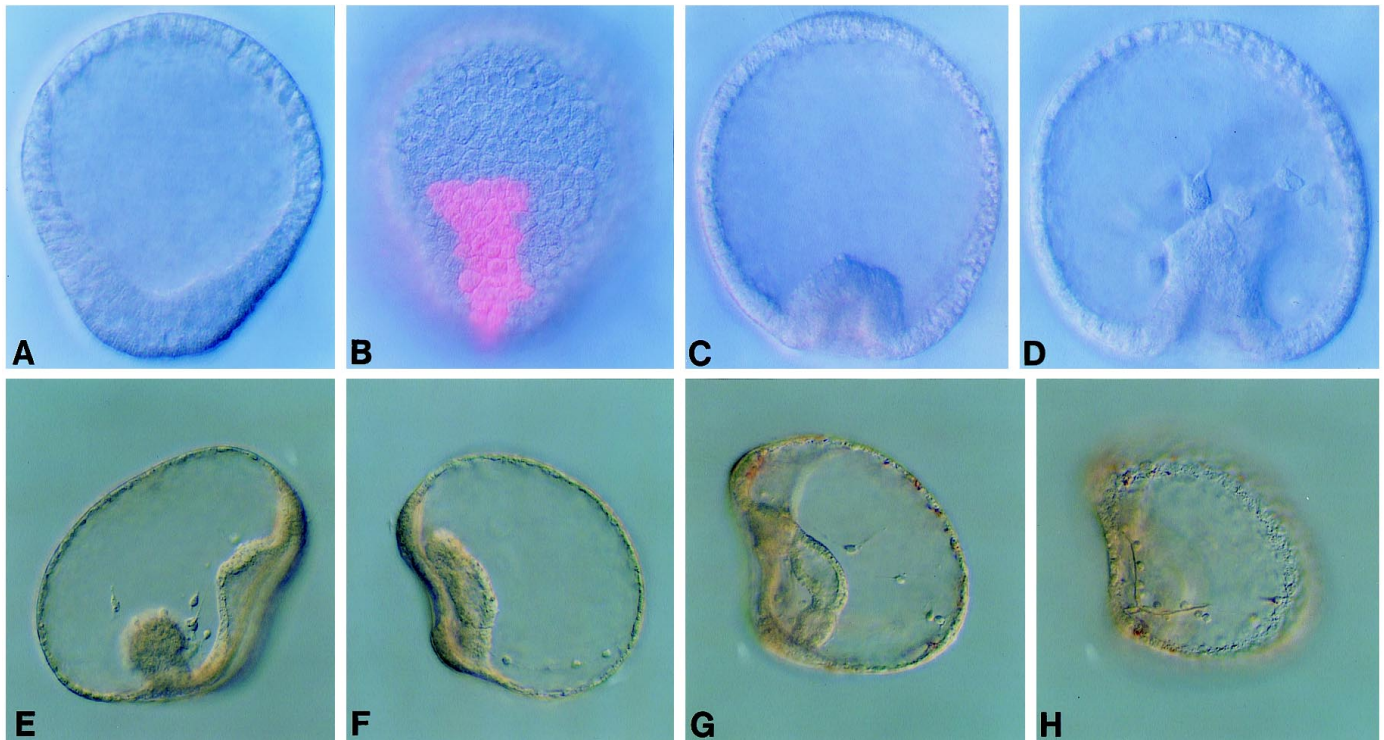
**Fig. 3.** Expression of *Endo16* in 28 hour *S. purpuratus* embryos. All of the embryos shown are the same age and derived from the same fertilization. They differ only in the number of micromeres that they retain. Either all four micromeres were deleted after 4th (A-C), 5th (D-F) or 6th (G-I) cleavages, or three micromeres were deleted after 4th cleavage (J-L), or no micromeres were deleted (i.e., treated controls; M-O). All embryos are oriented similarly, with the vegetal plate down, and have been processed for whole-mount in situ hybridization with a digoxigenin-labeled antisense *Endo16* probe, visualized here as purple staining. Weak staining in the fewest cells is visible after early micromere deletions, while stronger staining in more cells is visible after the later deletions. Allowing one micromere to remain gives consistently strong staining in even more cells, and dramatically rescues gastrulation. The three examples of each experimental condition that are shown serve to illustrate the degree of consistency obtainable with each type of manipulation. Fig. 2C presents the quantitation of *Endo16* expression obtained in this experiment. Other than the invaginations of the stained vegetal plates seen in G, and J-O, all deformations of the blastulae walls are postfixation artifacts of the whole-mount protocol and were not present in the living embryos.

specification. Our indicator of normal initial vegetal plate specification is expression of *Endo16* at 20-24 hours in development in all cells of the definitive vegetal plate, that is, all 60-90 cells derived from the *veg2* tier. The first indicator of complete specification of the vegetal plate territory is the initiation of gastrulation by 28 hours, followed by the morphogenesis of the archenteron, on schedule with control embryos.

Our evidence shows the micromeres present inductive signals from the time of their formation. These micromere signals, probably in the form of membrane-bound molecules, specifically trigger initial vegetal plate specification in the receiving cells. The macromeres and their progeny, and indeed, probably all cells in the cleavage stage embryo (Ransick and Davidson, 1993), are capable of receiving these signals, and

they are probably transduced via transmembrane receptors. It is likely that this interaction begins as soon as the micromere and macromere membranes are in close apposition at 4th cleavage stage. In most batches of embryos, if the signals are eliminated at an early point, the embryos do not complete the process of specification normally, while if the inductive signals are maintained for a further 2-2.5 hours (i.e., through 6th cleavage), nearly all of the vegetal plate lineage is initially specified, as defined above. However, these *Endo16*-positive cells are not completely specified, as indicated by their greatly delayed and morphologically abnormal gastrulation.

Complete vegetal plate specification may depend on signalling among the initially specified vegetal plate cells as well as on the inducing signals from the micromeres. The cells of the micromere lineage remain in direct contact with all of the



**Fig. 4.** Development of *S. purpuratus* embryos after 4th cleavage micromere deletions. (A) A blastula-stage embryo from which the micromeres were deleted at the 4th cleavage. This experimentally manipulated embryo looks identical to control embryos of the same stage, except for the lack of skeletogenic mesenchyme cells. (B) Surface view of blastula in (A), revealing a 'DiI' clone (in red), produced by labeling a macromere daughter cell after 5th cleavage. The 36 labeled cells in this clone are equivalent in number to those in similar control clones. Thus, micromere deletion did not affect the cleavage rate of the macromere lineage. (C) A micromereless embryo in the initial phase of its delayed gastrulation. (D) A micromereless embryo at midgastrulation that is generating secondary mesenchyme. (E) A retarded 72-hour, micromereless embryo with a partially invaginated archenteron. Secondary mesenchyme cells are forming and there is pronounced ectodermal differentiation, but the interaction between the archenteron and the oral ectoderm is atypical. Nevertheless, this embryo eventually completed gastrulation. (F) 72-hour, micromereless embryo that has completed gastrulation. Note that the mesenchyme cells have arranged themselves in a linear array, but are not yet forming spicules. (G,H) Two views of a 72-hour, one-micromere embryo, with a complete and differentiating gut (left panel) and advanced spicule development (right panel).

precursors of the definitive vegetal plate only through the 7th cleavage cycle. Specifically, they contact 4 macromeres at 4th cleavage; 8 macromere daughter cells at 5th cleavage; the 8 *veg2* cells at 6th cleavage; and 16 *veg2* tier daughter cells at 7th cleavage. It is not known whether the embryo follows a precise cleavage pattern at 8th cleavage and beyond; however,

if the rule of alternating spindle orientations is followed, the 16 daughter cells of the *veg2* tier divide into two tiers of cells at this cleavage, with only the more vegetal tier remaining in direct contact with the micromere lineage. The arrangement of the vegetal plate cells at this stage could be taken as evidence that direct signaling from the micromere lineage is no longer

**Table 1. Total number of cells in blastula stage embryos after 4th cleavage micromere deletions**

Developmental stage	Experimental embryos	Control embryos
20 hour blastula	468±20 (n=7)	480±24 (n=4)
22 hour blastula	602±8 (n=3)	635±39 (n=4)
27 hour blastula	572±30 (n=10)	604±30 (n=3)

The total number of cells were counted in embryos at three blastula stages after deletions of the micromeres at 4th cleavage (left column), compared to controls at the same stages (right column). The mean total cell number is shown ( $\pm 1$  standard deviation), and the number of cases examined (*n*) is given in parentheses. Removal of the micromeres reduces the expected cell number at the mesenchyme blastula stage by approximately 40 cells. The 20 and 22 hour blastula data are from one experiment, and the 27 hour blastula data are from a second experiment. These data show there is no significant alteration in the rate of cell division after deleting the micromeres at 4th cleavage.

**Table 2. Average number of cells in DiI-labeled clones at the blastula stage**

	Average number of cells in DiI-labeled clones	
	Control embryos	Micromere deleted embryos
Experiment 1	28±0 (n=2)	28±0 (n=3)
Experiment 2	33±1 (n=3)	34±2 (n=8)
Experiment 3	–	35±1 (n=6)

The average numbers of cells ( $\pm 1$  s.d.) present in DiI-labeled clones at mesenchyme blastula stages (22–26 hours) after deletion of the micromeres at 4th cleavage (right column), compared to control embryos (left column) are shown for three separate experiments. The number of cases examined (*n*) is shown in parentheses. DiI was introduced iontophoretically into the membrane of a single macromere daughter cell at 5th cleavage. These data show that there is no significant alteration in the cleavage rate in the macromere lineage after 4th cleavage micromere deletions.



required by the vegetal plate precursors. However, the micromere signals may continue to be produced and received at late cleavage stages, but the *veg2* lineage cells may also express *intraterritorial* signaling functions that generate and maintain specification throughout the vegetal plate. The one-micromere experiments that we report directly imply such homotypic interactions amongst vegetal plate cells, since the progeny of the one remaining micromere (i.e., 4 cells by the time of ingression) forms far fewer than the normal set of contacts with macromere progeny at least through 6th cleavage. Yet the result is topologically uniform initial specification, that includes at least 75% of the *veg2* progeny.

The variability that we encountered in response to micromere deletion, within and between batches of eggs, is interesting and informative. Initial specification is not completed in most embryos even if the micromeres are left in place to 6th cleavage (Fig. 2), suggesting that contact between macromere and micromere descendants must normally be required beyond 6th cleavage. Yet, as we report in Results, there are exceptional batches of embryos that can complete this initial phase of specification after much shorter induction periods (i.e., <1 hour). Clearly the duration of contact required to complete some step(s) in the intercellular interaction mechanism differs in some embryos as compared to others. Since a strong component of this variation depends on egg batch, some of the molecular species required for the signal presentation, reception and/or transduction are undoubtedly maternal, and may exist in different eggs in different quantities or different states of activation.

We find it remarkable that retention of a single micromere completely rescues the timing and the morphology of gastrulation. Two different (nonexclusive) explanations can be envisaged: (1) The interactions necessary for *complete* vegetal plate specification require contact with descendants of the micromere lineage over an extended period of time, extending beyond 6th cleavage. (2) Complete specification, as monitored by gastrulation, requires some other function of micromere descendants, in addition to cleavage-stage signaling. For example, gastrular invagination may be potentiated in some way by the prior ingression through the vegetal plate of the skeletogenic mesenchyme precursors that descend from the micromeres. In any case the one-micromere experiments tell us that normal gastrulation, or complete specification, is independent both of the number of micromere descendants, and of the exact number of vegetal plate cells that have been specified, so long as the number of cells specified is at least half the normal number (Fig. 2).

### Current model of sea urchin development

Although there is ample experimental evidence that mid-cleavage stage blastomeres have an extensive capacity for inductive cell interactions, the results presented here provide a direct confirmation that the sea urchin embryo makes use of such interactions in a normal specification process. These findings are consistent with our current working model of early sea urchin development, and specifically with its predictions regarding vegetal plate specification (see Introduction; Davidson, 1989). The parts of this model for which there is now direct evidence include: (1) that the skeletogenic mes-

enchyme territory is autonomously specified early in development as the micromeres segregate at the vegetal pole (see reviews of Davidson, 1986, 1989, 1990); (2) that the micromeres present specific signals that induce vegetal plate specification in neighboring cells (Ransick and Davidson, 1993; and this work); (3) that a capacity to receive and respond to these signals is a global property of the embryo (Ransick and Davidson, 1993; Hörstadius, 1935) and (4) that the macromere lineage normally requires these signals to initiate vegetal plate specification (this work).

Another prediction of the model is that the entire series of conditional blastomere specifications is entrained by the micromere induction described here. We now have a means of testing this prediction, since we can generate embryos that have failed to proceed normally with initial vegetal plate specification. Thus we would predict that the specification of the ectodermal territories, especially their boundaries, will be affected in embryos where the vegetal plate specification process has been interrupted by 4th cleavage micromere deletions.

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