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REVIEW ARTICLE

Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms

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SUMMARY

An early set of blastomere specifications occurs during cleavage in the sea urchin embryo, the result of both conditional and autonomous processes, as proposed in the model for this embryo set forth in 1989. Recent experimental results have greatly illuminated the mechanisms of specification in some early embryonic territories, though others remain obscure. We review the progressive process of specification within given lineage elements, and with reference to the early axial organization of the embryo. Evidence for the conditional specification of the *veg*$_2$ lineage subelement of the endoderm and other potential interblastomere signaling interactions in the cleavage-stage embryo are summarized. Definitive boundaries between mesoderm and endoderm territories of the vegetal plate, and between endoderm and overlying ectoderm, are not established until later in development. These processes have been clarified by numerous observations on spatial expression of various genes, and by elegant lineage labeling studies. The early specification events depend on regional mobilization of maternal regulatory factors resulting at once in the zygotic expression of genes encoding transcription factors, as well as downstream genes encoding proteins characteristic of the cell types that will much later arise from the progeny of the specified blastomeres. This embryo displays a maximal form of indirect development. The gene regulatory network underlying the embryonic development reflects the relative simplicity of the completed larva and of the processes required for its formation. The requirements for postembryonic adult body plan formation in the larval rudiment include engagement of a new level of genetic regulatory apparatus, exemplified by the *Hox* gene complex.

Key words: Lineage, Conditional specification, Autonomous specification, Endomesoderm, Marker gene, Gene regulator, Rudiment, Larva, Echinoderm

INTRODUCTION

The sea urchin embryo has lent itself to the study of the role of the genome in embryonic development ever since the discovery of pronuclear fusion in these eggs by Fol (1877). Boveri's experiments on the developmental fate of polyspermic eggs and of aneuploid blastomeres isolated from them, following earlier leads from O. Hertwig and Driesch, proved that expression of the complete chromosome set is required for the process of embryogenesis (Boveri, 1902, 1904, 1907; reviewed by Baltzer, 1967; Sander, 1993). In more recent times, maternal mRNA was discovered in sea urchin eggs by A. Monroy, A. Tyler, P. Gross and others (reviewed by Davidson, 1968, 1986); and the first measurements of the complexity of gene expression in an embryo were carried out, showing that many thousands of genes are represented in the polysomal message population throughout early development (reviewed by Davidson, 1976, 1986). The sea urchin embryo is now a major system for studies on the mechanisms of gene regulation in early development. A separate intellectual tradition, first developed by Boveri (1901a), established portions of the early cell lineage of the sea urchin embryo and culminated in the spectacular blastomere recombination experiments of Hörstadius (reviewed 1939, 1973). Hörstadius' interpretations were devoid of any form of genetic causality and, instead, he and his school favored explanations couched in terms of intersecting 'gradients' of 'animalizing' and 'vegetalizing' potential. However, his experiments in themselves provided impressive (though usually preliminary) evidence for a widespread role of interblastomere interactions in establishing blastomere fate in the sea urchin embryo. A model for blastomere specification in this embryo (Davidson, 1989) reinterpreted Hörstadius' data, proposing that short-range, cleavage-stage signaling interactions cause activation of specific sets of territory-specific genes in the various lineage founder cells of the embryo (see also Davidson, 1986, Chapter

6 and Wilt, 1987 for prior discussions). Almost a decade later, it seems clear that this view is in essence correct.

Compelling and incisive new technologies have had an enormous impact on sea urchin embryology, as everywhere throughout the molecular biology of early development. The initial intent of this essay is to provide a current image of how cleavage stage specification occurs in the sea urchin embryo, based on the large amount of new experimental evidence that has now accumulated. We then take up developmental specification processes that are not completed until the blastula-early gastrula stage of development, in particular, the late phase of endoderm specification and mesoderm specification within the vegetal plate. Finally we consider the sea urchin embryo as the product of a process of indirect development, and briefly compare the level of genetic regulatory programming required for embryogenesis to that utilized in the very different process by which the adult body plan is formed in the postembryonic development of the larval rudiment.

EARLY TERRITORIES OF THE SEA URCHIN EMBRYO: LINEAGE AND SPECIFICATION

An important concept of the 1989 model is that the late cleavage- and blastula-stage sea urchin embryo can be considered to be composed of 'territories.' These are conceived as polyclonal assemblages of contiguous blastomeres, each of which will produce progeny that express a particular set of genes. The transcription of these genes would constitute the outcome of the initial processes of territorial specification. In embryos such as the sea urchin, such processes undoubtedly occur soon after fertilization, since by the end of cleavage various territorial marker genes are indeed already being differentially expressed (many examples are referred to in the following). Because the orientation of the cleavage planes is invariant through the 6th cleavage (Cameron et al., 1987, 1990, 1991), in the 1989 model and subsequent discussions (Davidson, 1989; Cameron and Davidson, 1991) we assumed that the lineage ancestry of each territory is also invariant. Subsequent lineage tracing experiments carried out in several laboratories, including ours, have shown that this assumption requires modification, as discussed below. It is true that some territories are indeed composed of invariant lineage elements, and some portions of all territories have the same lineage in all embryos of the species. However, this is not the case for the regions within which the boundary between endoderm and ectoderm forms, nor for the regions separating oral and aboral ectoderm. These boundaries are formulated only later in development by processes that are independent of lineage.

Fate map of the sea urchin embryo

The following discussion necessarily relies at certain junctions on details of cell lineage allocation which in some details are species specific. The diagrams in this paper refer to *Strongylocentrotus purpuratus*, the species studied in our laboratory. Except where otherwise noted, however, we have attempted to provide conclusions that should pertain to most of the regular indirectly developing sea urchins used for studies of embryology (which belong largely to the euechinoid orders Temnopleuroida and Camarodonta; Smith, 1984).

A fate map of the embryo is shown in Fig. 1. This indicates the topological relations of the territories, but such a map cannot actually be drawn to a cellular level of resolution until the late blastula stage. It is only then that each cell of the embryo can be allocated to one or another of the territorial fates that will be manifest morphologically in the immediately succeeding stage, the gastrula. At late blastula stage the

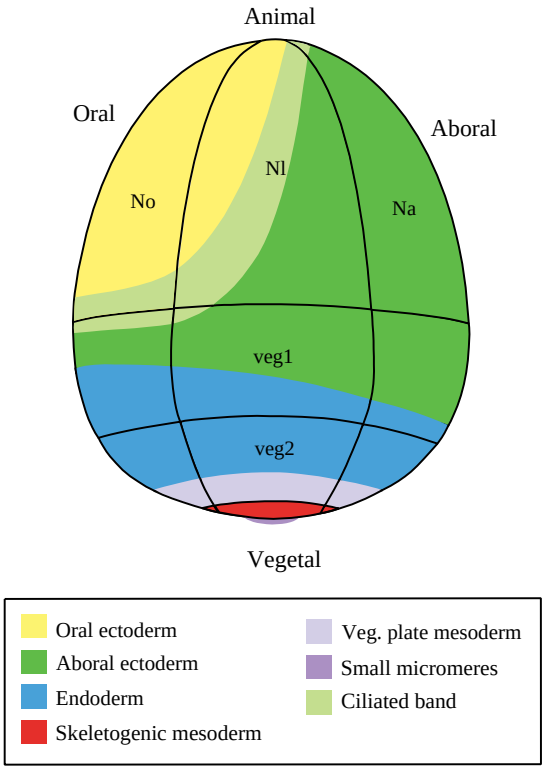

Fig. 1. Fate map of territories in the *S. purpuratus* embryo, projected as an external image of the hatched blastula-stage embryo of about 400 cells (~20 hours postfertilisation). Territories are indicated by the key at the bottom. The black lines show major lineage domains: No, Nl, Na indicate the clonal descendants of these 8-cell blastomeres, which give rise to oral and aboral ectoderm (Cameron et al., 1987). The positions of *veg*$_1$ and *veg*$_2$ lineage elements are shown explicitly in Fig. 2A at the 60-cell stage; in that figure Na and No domains occupy the central regions of the green and yellow portions of the drawing, respectively. The yellow-green stripe in this figure indicates the position of the future ciliated band, to which both oral and aboral ectoderm elements will contribute (Cameron et al., 1993). *Veg*$_1$ indicates the clonal descendants of the 6th cleavage ring of eight blastomeres, the upper granddaughters of the 4th cleavage macromeres, so named by Hörstadius (1939). At gastrulation, some *veg*$_1$ progeny will contribute to the hindgut and midgut, more on the oral than the aboral side in this species (Ransick and Davidson, 1998), and the remainder will contribute aboral ectoderm, except for the small ciliated band contribution on the oral side. *Veg*$_2$ indicates the descendants of Hörstadius' 6th cleavage ring of eight vegetal blastomeres of that name, the lower granddaughters of the 4th cleavage macromeres. On gastrular invagination, the *veg*$_2$ domain generates foregut, part of the midgut and all mesodermal elements except for the coelomic pouch constituents, that derive in part from the small micromeres, and the skeletogenic mesenchyme. The skeletogenic mesenchyme cells will soon ingress into the blastocoel and the small micromeres will be carried inward on invagination of the archenteron.

allocation of cells to the ciliated band is still indeterminate (indicated by the yellow-green area), as this structure forms at the boundary of the oral ectoderm only toward the end of embryogenesis.

Early specification of embryonic territories and lineage compartments

In Fig. 2A is shown a diagram of the 60-cell embryo, indicating in color five different polyclonal lineage elements, which at this stage have apparently already undergone specification. Note that commitment, or lockdown, of these states of specification is manifest only much later. In Fig. 2A, the small micromere territory (purple), the skeletogenic mesenchyme territory (red) and the vegetal plate territory (blue) each correspond exactly with a definitive polyclonal lineage compartment that has segregated by 6th cleavage. Clones at the aboral (green) and probably the oral (yellow) poles of the 2nd (oral-aboral) axis as well are specified, while the remaining regions, shown in white, are specified only later. Comparison of Fig. 2A-C with Fig. 1 distinguishes the state of specification in each region at the indicated stages from the ultimate fate that will be assigned to each region.

(1) The small micromere territory

The four small micromere founder cells arise at the unequal 5th cleavage (Okazaki, 1975; Pehrson and Cohen, 1986; Cameron et al., 1991), and they will divide only once more during embryogenesis, producing eight progeny which constitute about 40% of the cells initially constituting the coelomic sacs, which ultimately constitute much of the adult animal. They are not primordial germ cells (a reasonable but incorrect speculation), as we showed earlier (Ransick et al., 1996); in sea urchins, and also starfish (Inoue et al., 1992), the definitive germ cell precursor cells appear only during postembryonic development. No genes have yet been found that are expressed specifically at cleavage or blastula stages in the small micromeres and their state of specification prior to their inclusion in the coelomic sacs is thus not really known. The small micromeres can be considered to be precociously segregated mesodermal set-aside cells (Peterson et al., 1997), the postembryonic contribution of which

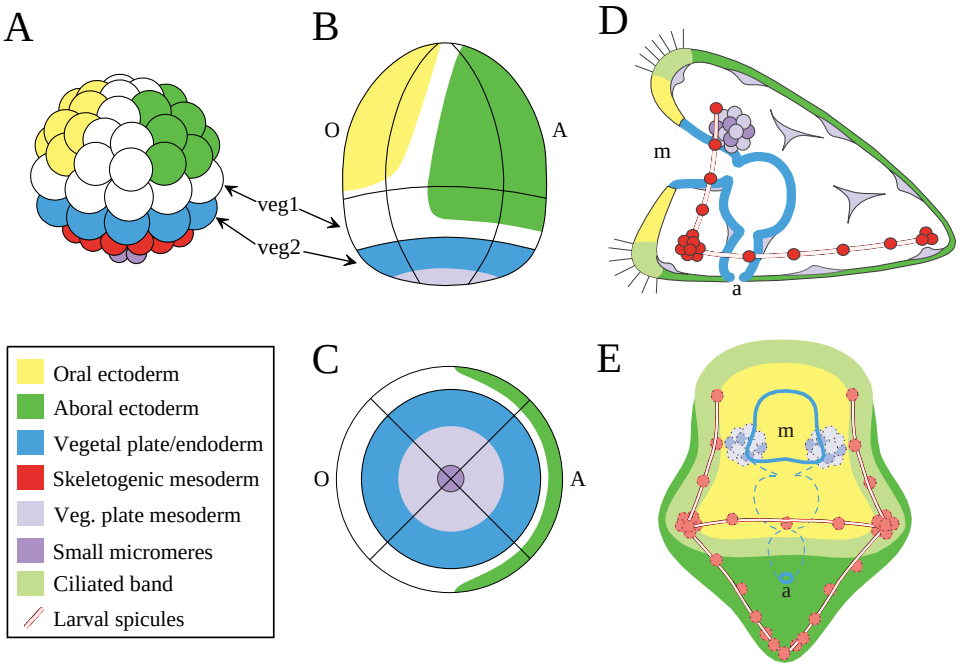

Fig. 2. Proposed specification maps for *S. purpuratus* at mid cleavage and blastula, and final disposition of cell types of pluteus stage. (A) 60-cell stage, 6th cleavage (except that division of small micromeres has not yet occurred). Eight large micromeres, descendants of the four founder cells of the skeletogenic mesenchyme are present (shown in red). The skeletogenic founder cells are autonomously specified and all descendants will begin to express skeletogenic genes by late cleavage. Beneath them are the four small micromeres (shown in purple). Though their progeny will contribute exclusively to the coelomic pouches, their actual state of specification at 6th cleavage is not known. The eight blastomeres of the *veg*$_2$ ring are also specified and their progeny will shortly express vegetal plate marker genes. As discussed in text, the *veg*$_1$ domain, here shown in white, is not yet specified, since its progeny will be allocated by subsequent, lineage-independent specification processes, to three different fates (see Fig. 1). Nor are the lateral ectodermal domains of Nl origin on either side of the embryo (white) yet specified. Some progeny of these blastomeres will form the boundary regions between oral and aboral ectodermal territories. The polar clones of the aboral ectoderm territory, namely, the progeny of Na (green) are already specified, and these clones will soon begin to express aboral markers. The polar clones of the oral ectoderm territory deriving from the No blastomere (yellow) may also be specified (see text for markers, genes and discussion). Some Na progeny contribute to the ciliated band, but these were earlier aboral in specification, since they retain *CyIIIa* mRNA even after incorporation in the band (Cameron et al., 1993). (B) Mesenchyme blastula, about 500 cells, external lateral view. Lineage compartments and territorial designations are as in Fig. 1. Regions where specifications have still not yet occurred are shown in white. (C) Mesenchyme blastula, vegetal view. The central region of the vegetal plate is now divided radially into a central mesodermal territory (shown in light purple), consisting of cells destined to give rise to secondary mesenchyme and coelomic pouches and an endodermal territory that will produce foregut and midgut (shown in blue). The skeletogenic cells have already ingressed and are not shown. The small micromere descendants are located in the center (darker purple). The aboral ectoderm territory has recruited components of the *veg*$_1$ lineage domain on the aboral and lateral faces of the embryo (green). (D) Final state of specification in early pluteus-stage larva, about 1500 cells (65 hours postfertilisation); shown in lateral view; (E) same stage, oral ('facial') view. Both D and E are shown as if they were projections of a collapsed 'Z-series' of images, so that structures at several different planes of focus are superimposed. Mesodermal cell types derived from the early territories include the skeletogenic mesenchyme, shown secreting the bilaterally organized skeletal structure (red) and various secondary mesenchyme cell types (see text). Coelomic pouches are depicted as circular arrays of purple cells at the side of the foregut, pigment cells are shown embedded in the aboral ectoderm, and fusiform blastocoelar cells are illustrated. Esophageal and duodenal sphincter muscles mark the divisions of the archenteron. The ciliated band territory is indicated in yellow-green, since it is constructed of cells previously belonging to both oral and aboral ectodermal components. Neurons (not shown) appear in regions of both the oral ectoderm and the ciliated band.

is to form mesodermal components of the future adult body plan (see below).

(2) The skeletogenic territory

Extensive evidence demonstrates that the four 5th cleavage skeletogenic lineage founder cells are autonomously specified, as discussed in some detail below. They provide a remarkable example of asymmetric distribution of fate in early development, in that they are the sister cells of the small micromeres. All progeny of the skeletogenic micromeres execute exclusively skeletogenic functions during embryogenesis. For this territory, there is perfect correspondence between the polyclonal lineage compartment and the ultimate embryonic fate.

(3) The vegetal plate territory

This early embryonic territory is also synonymous with a polyclonal lineage component, the 6th cleavage, eight blastomere ring termed *veg*$_2$ by Hörstadius (1939; see Figs 1, 2). Certain transcription factors and a well-studied gene encoding a terminal differentiation protein of the midgut, *Endo16*, are expressed in all the cells of this territory by blastula stage or earlier, as reviewed in a later section. During late blastula stage, these cells form a particular morphological structure, the vegetal plate. This consists of a disc of tall epithelial cells, which constitutes the vegetal wall of the embryo and which initiates invagination as gastrulation begins. Thus in terms both of expression of territory-specific molecular markers and of morphological disposition, the *veg*$_2$ lineage compartment constitutes a legitimate territory, the identity of which is specified by late cleavage. But it is only a transient territory of the embryo and the later embryonic fate of its cells is complex. As shown in Fig. 2B,C, by late blastula stage, a central mesodermal domain has arisen within the vegetal plate and, during the gastrula stage, a variety of differentiated mesodermal cell types derives from this domain. The peripheral region of the original vegetal plate becomes endodermal, and from it derives the cells that constitute the foregut and most of the midgut.

(4) The aboral ectoderm territory

The aboral ectoderm founder cells arise at one pole of the second, or oral/aboral (O/A) axis. The polar blastomeres of this territory produce progeny all of which transcribe aboral ectoderm markers, a number of which are now known. Only one cell type arises from the aboral ectoderm, a squamous epithelium that forms the wall of the late embryo and the larva, except for the oral and ciliated band regions (see Fig. 2D). The *CyIIIa* cytoskeletal actin gene serves as an aboral ectoderm marker. Sensitive run-on measurements show that this gene is transcribed as early as the 60- to 120-cell stage (Hickey et al., 1987; Lee et al., 1992); transcripts are detectable at 10 hours, and when these transcripts can first be seen by in situ hybridization at 18 hours they are confined to the future aboral side of the embryo (Cox et al., 1986; Lee et al., 1986). We infer that at least some of the clones of cells constituting this territory are specified by the time this gene turns on, as indicated in Fig. 2A. However, at 5th cleavage, some blastomeres at the interface between oral and aboral domains still produce progeny that will contribute to both oral and aboral domains (Cameron et al., 1993), and it is not clear exactly when the clones in these lateral ectodermal regions become specified. Furthermore, after 6th cleavage, the cleavage pattern in this region loses its spatial regularity, and mechanisms not dependent on lineage are clearly required for the formation of the oral/aboral boundary. Hence these lateral regions are shown in white in the 60-cell specification map of Fig. 2A.

(5) The oral ectoderm territory

No early embryo molecular markers for this territory have yet been uncovered, and its state of specification early in development remains speculative. Lineage labeling studies suggest that much the same may be stated about this territory as for the aboral territory. The polar clones may be specified very early, since all progeny of these clones invariably become part of the oral ectoderm, while this will not be true for the lateral clones, as indicated in Fig. 2A. The oral ectoderm territory is of particular interest because of the complexity of its ultimate fate. It produces a variety of structures and cell types and in this it contrasts greatly with the aboral ectoderm. The larval mouth or stomodaeum derives from oral ectoderm (see Fig. 2D,E), as does the specialized oral epithelium and several neurogenic structures including the oral hood and ciliated band elements. In addition, as we discuss later in this essay, the oral ectoderm harbors cells, which, after feeding begins, generate the ‘vestibule,’ a fundamental component of the adult rudiment. The aboral ectoderm, in contrast, disintegrates at metamorphosis.

INITIAL STATE: ORGANIZATION OF THE EGG ALONG THE A/V AXIS AND AUTONOMOUS SPECIFICATION IN EARLY CLEAVAGE

Primordial polarity of the egg

The sea urchin egg is irrevocably polarized along the animal/vegetal (A/V) axis at time of fertilization. This maternal feature was recognized clearly by Boveri (1901a,b) who used a naturally occurring canal in the egg jelly at the animal pole to mark the surface of the egg with ink (the animal pole is classically that at which the polar bodies are extruded). The mark was always found at the opposite end of the egg from that where the micromeres arise. Working with *Paracentrotus lividus*, Boveri also noted a subequatorial band of pigment granules in the unfertilized egg, which at gastrular invagination are carried inward, whereafter they can still be discerned lining the archenteron. These observations were extended using *Arbacia* eggs by Schroeder (1980a,b) who also described in detail a cortical contraction moving outward from the vegetal pole following fertilization. This can be followed by observing the upward movement of pigment granules, which are distributed globally in the unfertilized egg. These cortical movements generate a vegetal-most region free of granules that will be included in the skeletogenic and small micromere territories, i.e., in the 4th cleavage micromeres (reviewed by Davidson, 1986, pp. 494-497).

The distribution of pigment in these species is a manifestation of prelocalized differences that clearly affect the states of specification that the animal and vegetal portions of the egg are able to support. The animal blastomeres (mesomeres) are conveniently defined as those lying above the horizontal 3rd cleavage plane. At 4th cleavage, the vegetal blastomeres below this plane divide horizontally, producing four large blastomeres (macromeres) and the four micromeres. The most simple and direct test is to separate and culture mesomeres as opposed to

macromeres and their progeny. This experiment was done by numerous experimentalists between the 1890's and Hörstadius' review of 1973. Complete mesomere tiers or isolated mesomeres were observed to produce only hollow ciliated epithelial balls, while the vegetal halves produce somewhat deformed larvae that contain clearly visible archenteron and skeletal elements. These different outcomes directly reflect the primordial A/V organization of the egg even before cleavage begins. Thus Hörstadius (1937) dissected unfertilized *Arbacia* eggs equatorially and obtained the same results: on fertilization, animal half eggs formed only ciliated epithelial balls, while vegetal halves produced micromeres on time, eventually gastrulated, and then generated archenteron and skeletal elements. Similar experiments with similar results were carried out by Maruyama et al. (1985) on *Hemicentrotus* eggs, and by A. Ransick (unpublished) on *S. purpuratus* eggs. The prelocalized A/V differences revealed by all these experiments extend to two different euechinoid orders (Echinoida: *Strongylocentrotus*, *Paracentrotus* and *Hemicentrotus* and Physomatoida: *Arbacia*; phylogeny of Smith, 1984). However, maternal A/V polarity may be a much more broadly distributed, basal echinoderm character. For example, the same kind of primordial A/V organization has been demonstrated for starfish by fusing fragments of unfertilized egg cytoplasm with the animal pole region of activated oocytes (Kiyomoto and Shirai, 1993). Only egg fragments of vegetal origin confer the capacity to generate an archenteron (see also Zhang et al., 1990, and other data reviewed by Kiyomoto and Shirai, 1993).

Some modern sea urchin embryo blastomere isolation experiments are summarized in Table 1. These are distinguished from classical studies by use of molecular markers of specification (as well as by more conscientious attention to reproducibility and quantitation). Isolated pairs of mesomeres, or the whole 3rd or 4th cleavage mesomere tier, display relatively little ability, and sometimes none, to generate endomesodermal structures or cell types, or to express gut markers such as alkaline phosphatase, or skeletal markers such as the spicule matrix protein gene *SM50* (Table 1, isolates 1 and 2). As almost universally reported earlier, the great majority of surviving embryoids that derived exclusively from mesomeres, and all of those formed from intact mesomere tiers, develop only as hollow ciliated epithelial balls. In contrast, when isolated 4th cleavage macromeres are cultured together with their micromere sister cells (that is, the two daughters of one vegetal 3rd cleavage blastomere), they express endomesodermal markers with very high frequency (Table 1, isolate 3). The position of the 3rd cleavage plane is crucial for these results, so that if mesomere pairs are obtained from embryos in which this plane is unusually low on the A/V axis, much larger fractions of the isolates display endomesodermal markers (Henry et al., 1989). Thus the relevant constituents of the egg are organized with respect to its A/V coordinates, not with respect to the 3rd cleavage plane per se.

Autonomous activation of VEB genes

Autonomous specification processes in early development are those in which the fate of the blastomere depends exclusively on the maternal cytoplasmic constituents that it inherits, as discussed earlier (Davidson, 1990). Two autonomous specification systems have now been uncovered in these embryos and have been analyzed at the *cis*-regulatory level. One of these, a set of genes expressed at the very early blastula stage ('VEB' genes), is activated in all blastomeres that inherit egg cytoplasm above the vegetal-most region of the egg. Four VEB genes have thus far been characterized, of which the two best known encode metalloendoproteases. These are the *HE* gene, which encodes hatching enzyme (Lepage and Gache, 1990; Reynolds et al., 1992; Nasir et al., 1995) and the *SpAN* gene, which encodes a protein similar to BMP-1 and tolloid (Reynolds et al., 1992; Lepage et al., 1992a). At blastula stage, expression of the VEB genes extends over the whole embryo, excepting only a variable region at the vegetal pole. In *S. purpuratus*, the non-expressing domain at its smallest lies within or near the margin of the skeletogenic territory and, at its largest, includes most of the veg_2 territory as well (Reynolds et al., 1992; see Figs 1, 2); in *P. lividus* the non-expressing domain extends up into the veg_1 region (Lepage et al., 1992b). Expression of these genes begins remarkably soon after fertilization. Reynolds et al. (1992) found transcripts of the *SpHE* and the *SpAN* genes as early as the 8-cell stage. No maternal transcripts are present, but it would seem almost certain that the transcription factors activating these genes so early in cleavage are of maternal origin. The *SpAN* gene is transcriptionally silenced at the end of cleavage, as measured with intron probes (Kozlowski et al., 1996) and expression of other VEB genes is similarly extinguished (Ghiglione et al., 1993; Grimwade et al., 1991). The mRNAs encoded by these genes have disappeared before the mesenchyme blastula stage.

Cis-regulatory analyses of the *SpAN* gene (Kozlowski et al., 1996) and the *SpHE* gene (Wei et al., 1995, 1997a,b) have revealed control systems that are similar in architecture. For both genes, the necessary and sufficient regulatory elements required for the spatial and temporal expression pattern are confined within compact domains a few hundred base pairs long. These control modules are densely packed with transcription factor target sites, though the factors to which the two genes respond are largely dissimilar. A significant aspect is that neither *cis*-regulatory system appears to contain any negatively acting regulatory elements; these are apparently 'positive-only' systems, and this is indeed what might be expected of genes that are activated by autonomous processes (Arnone and Davidson, 1997).

The image that emerges is that the VEB genes are transcriptionally activated by maternal factors that are absent or rendered inactive in the vegetal-most region of the egg. The activation of these genes is independent of the mid-late cleavage territorial specification processes summarized in Fig. 2A, since they are already being transcribed at 3rd cleavage in blastomeres that will later give rise to elements of several dissimilar territories (Reynolds et al., 1992). Furthermore, blastomeres maintained in a disaggregated state express VEB genes on schedule (Reynolds et al., 1992) and transcribe them at just the normal rate (Ghiglione et al., 1993) though, in the absence of cell contact, they are not later downregulated. In addition, isolated animal and vegetal half embryos express VEB genes just as would be predicted from the normal expression patterns in the intact embryo (Ghiglione et al., 1996). The latter work also showed that transplantation of micromeres to the top of the 8-cell-stage embryo, which induces a second vegetal plate (see below), does not affect the autonomous expression of the hatching enzyme gene in the respecified animal pole blastomeres.

The strictly autonomous activation of the VEB genes thus provides very strong evidence of a specific difference in transcriptional activity along the A/V axis. This almost certainly depends on an A/V distribution of activity of maternal transcription factors, reflecting the primordial polarization of the egg in this axis.

Autonomous specification of the skeletogenic territory

Evidence for the autonomous specification of the skeletogenic lineage descendant from the four 5th cleavage micromeres has been reviewed extensively (Davidson, 1986, pp. 498-500; Davidson, 1989; McClay et al., 1992; Ettensohn, 1992). Essentially, this evidence consists of numerous experimental demonstrations that (1) isolated micromeres give rise to skeletogenic cells in vitro, beginning with Okazaki's (1975) observation, and (2) no other fate is ever observed for micromeres on transplantation to ectopic locations in the embryo or in any other blastomere recombination experiments. Wherever they are placed, all skeletogenic micromere progeny express skeletogenic genes on schedule and display the stereotypic behavior of their lineages: they divide a set number of times to produce a total of 32 skeletogenic mesenchyme cells in *Strongylocentrotus* (Cameron et al., 1987; Arnone et al., 1997) or 64 in *Lytechinus* (Ettensohn, 1990), they ingress, they attain mobility, they fuse and they carry out spiculogenesis. While their specification and differentiation are clearly autonomous functions, the three-dimensional pattern in which the skeleton is ultimately laid down during and after gastrulation, and the timing with which this occurs, evidently depend on signals that the skeletogenic mesenchyme cells encounter on the inner ectodermal wall of the blastocoel (Ettensohn, 1992; Ettensohn and Ingersoll, 1992; McClay et al., 1992; Armstrong et al., 1993; Armstrong and McClay, 1994).

Table 1. Developmental fates and marker gene expression for sea urchin blastomere isolates and chimeric recombinants[1]

			Structures formed and marker expression (%)					
			Embryonic domain:	Endomesoderm			Oral ectoderm	Aboral ectoderm
			Structure:	Gut	Spicules	Pigment cells	Stomodaeum	Cb[2]	
Isolate	Cleavage[3]	Species[4]	Marker:	Endo 1 (1) AP[5] (2)	SM50 (1)		EctoV (pattern)[6]		Specific gene [7]
(1) Mesomere pair	3 4 5	*Lp*	Henry et al., 1989	22 (2) 10 4	16 3 0	4 0 0	0 2 0	0 1 0	
	4	*Lp*	Livingston and Wilt, 1990	4 (2)	2	1	(uniform)		
	4	*Sp*	Livingston and Wilt, 1989	4 (2)	6 (1)				
(2) Mesomere tier	3 4	*Lp*	Henry et al, 1989	0 0	0 0	0 0	0 18	0 68	
	3	*Lp*	Wikramanayake et al., 1995	a few	a few		0 (uniform)	0	0
	3	*Sp*		a few	a few		0 (uniform)	0	~100
(3) Macromere + micromere pair	4	*Lp*	Henry et al., 1989	75	83				
	4	*Lp*	Livingston and Wilt, 1990	98 (2)	50	90	(polarized)		
	4	*Sp*	Livingston and Wilt, 1989	85	83 (1)				
(4) Mesomeres + micromeres	4	*Lp*	Khaner and Wilt, 1990	50 (1)	62	12	(partially polarized)		
	4	*Sp*		66 (2, 1)	85	62	(partially polarized)		
(5) Animal half embryo + veg_1 tier	6	*Lv*	Logan and McClay, 1998	≥50% small invagination; expressing (1)			(polarized)		
(6) Animal half embryo + veg_2 tier	6	*Lv*		100% tripartite gut (1)			(polarized)		
(7) Mesomere tier + vegetal half	3	*Lp*	Wikramanayake et al., 1995	normal	normal	normal	normal	normal	normal expression

[1]Numerals in Table refer to morphological features listed at the top. Markers are indicated by numbers in parentheses if they were included.
[2]Ciliated band is formed at the intersection of oral and aboral ectoderm and in cellular constitution is a product of both (Cameron et al., 1993).
[3]3rd cleavage isolated here refer to animal half blastomeres.
[4]Abbreviations: *Lp*, *Lytechinus pictus*; BioEssaysLv, *Lytechinus variegatus*; *Sp*, *Strongylocentrotus purpuratus*; cb, ciliated band; AP, alkaline phosphatase.
[5]AP, expression of alkaline phosphatase, a gut-specific enzyme.
[6]Polarization, as opposed to uniform expression, of EctoV expression is an indication of oral/aboral ectoderm specification.
[5]Spec1 in *S. purpuratus*; LpS1 and LpC2 in *Lytechinus pictus*.

Table 2. Some genes expressed in endodermal and mesodermal compartments of the sea urchin embryo

Gene[1]	Detection	Pattern of expression		Reference
Endoderm		*Initial (stage)*[2]	*Final (stage)*[3]	
Hphnf-3β	mRNA	Whole vegetal plate (h. blastula)	Blastopore lip (gastrula)	Harada et al., 1996
Spfkh1	mRNA	Archenteron (early gastrula)	Blastopore lip (late gastrula)	Luke et al., 1997
SpKrox1	mRNA	Macromeres (16-cell stage) Whole vegetal plate (h. blastula)	Blastopore lip (prism)	Wang et al., 1996
Notch (apical)	Ab	Endodermal domain of the vegetal plate but not 2° mesenchyme (m. blastula)	Archenteron, not 2° mesenchyme (gastrula)	Sherwood and McClay, 1997
Endo16	mRNA	Whole vegetal plate (h. blastula)	Midgut (pluteus)	Nocente-McGrath et al., 1989; Ransick et al., 1993
CyIIa actin	mRNA	Skeletogenic mesenchyme (h. blastula) secondary mesenchyme (early gastrula)	Hindgut/midgut (pluteus)	Arnone et al., 1998
Mesoderm[4]				
Brachyury	mRNA	Whole vegetal plate (m. blastula)	Archenteron tip and 2° mesenchyme (late gastrula)	Harada et al., 1995
SpHMX	mRNA	Archenteron (gastrula)	Pigment cells (pluteus)	Martinez and Davidson, 1997
Snail	mRNA	Archenteron tip (early gastrula)	Arm tips (prism)	Hardin, 1995
SUM-1 (MyoD)	mRNA	Archenteron tip (prism)	Esophageal muscles (pluteus)	Venuti et al., 1991
Sp1	Ab	8 cells of the vegetal plate (blastula)	Pigment cells (pluteus)	Gibson and Burke, 1985
S9	mRNA	Whole vegetal plate (m. blastula)	Pigment cells (pluteus)	Miller et al., 1996
Sp12	Ab	Mesenchyme cells (blastula)	Mesenchyme cells (pluteus)	Tamboline and Burke, 1989
profilin	mRNA	Archenteron tip (early gastrula)	Pigment cells and gut-associated mesenchyme (pluteus)	Smith et al., 1994
SM50	mRNA	Skeletogenic mesenchyme (blastula)	Skeletogenic mesenchyme (pluteus)	Sucov et al., 1987 Benson et al., 1987
SM30	mRNA	Skeletogenic mesenchyme (blastula)	Skeletogenic mesenchyme (pluteus)	George et al., 1991
MSp130	Ab	Skeletogenic mesenchyme (blastula)	Skeletogenic mesenchyme (pluteus)	Anstrom et al., 1987
Pm27	mRNA	Skeletogenic mesenchyme (blastula)	Skeletogenic mesenchyme (pluteus)	Harkey et al., 1995
SM40	mRNA	Skeletogenic mesenchyme (blastula)	Skeletogenic mesenchyme (pluteus)	Lee and Davidson, 1998

[1]Transcription factors are shown in bold face.
[2]h. blastula, hatching blastula; m. blastula, mesenchyme blastula.
[3]When final stage is not pluteus, expression disappears by pluteus stage.
[4]Mesodermal assignment refers to final locus of expression.

Genes encoding proteins later found in the spicule biomineral begin to be transcribed in skeletogenic lineages long before skeletogenesis begins, while the embryo is yet a ball of morphologically undifferentiated cells. The cardinal example is the *SM50* gene (Benson et al., 1987; Sucov et al., 1987; Katoh-Fukui et al., 1991). The expression and regulation of this gene has been studied in several species in some detail (Killian and Wilt, 1989; Livingston et al., 1991; Makabe et al., 1995; Guss and Ettensohn, 1997). Three other skeletogenic genes that behave similarly are known, i.e., *MSp130* (Anstrom et al., 1987), *Pm27* (Harkey et al., 1995) and *SM37* (Y.-H. Lee and E. H. Davidson, unpublished data). In the case of *SM37*, skeletogenic regulation appears to depend on interactions with the same transcription factors as motivate *SM50* expression. Sensitive measurements show that *SM50* transcripts appear as early as 10 hours postfertilization, i.e., in late cleavage (Killian and Wilt, 1989), not long after sequestration of the lineage founder cells. Expression of *SM50* and of *MSp130* continues at equal levels in all skeletogenic mesenchyme cells until the climax period of skeletogenesis in late development, when it is modulated according to the spiculogenic activity of the individual cells at each moment (Guss and Ettensohn, 1997). Some other skeletogenic genes are expressed differently. An example is the *SM30* gene, which encodes a major matrix protein constituent (George et al., 1991; Frudakis and Wilt, 1995). *SM30* is activated much after *SM5*0, and its activity shows more dependence on extrinsic factors (Guss and Ettensohn, 1997). *SM50* expression takes place in isolated skeletogenic mesenchyme cells in culture whether or not fusion, which is required for spiculogenesis, has been allowed to occur (Kitajima et al., 1996), but this is not true of *SM30*. In summary, activation of the *SM50* gene serves as an excellent index of skeletogenic mesenchyme specification: it is cell autonomous, it is an exclusive and uniform early character of the cells of all the skeletogenic lineage elements, and it begins very shortly after definitive segregation of these lineages.

The *cis*-regulatory system controlling *SM50* expression (Sucov et al., 1988) was analyzed in detail by Makabe et al. (1995). Like those controlling expression of the VEB genes, the necessary and sufficient *SM50* regulatory system is confined to a single cluster of target sites only a few hundred base pairs in extent. The key spatial regulator is a member of the *cut* homeodomain transcription factor family (K. Makabe and E. Davidson, unpublished data), which interacts at a site also present upstream of the *SM37* gene. An oligonucleotide copy of this target site suffices to confer exclusively skeletogenic expression on injected reporter constructs. Again, no negative regulatory elements are present (in contrast to the *SM30* control system; Frudakis and Wilt, 1995). These parallels with the organization of the VEB gene *cis*-regulatory elements begin to define the characteristics of genomic control systems which mediate autonomous specification processes at the outset of embryonic development.

The most elemental view of the mechanism underlying the

autonomous specification of the skeletogenic territory is that a set of key transcription factors, including the *SM50 cut* domain factor, is precisely localized at the vegetal pole of the egg and is thus uniquely inherited by the skeletogenic micromeres. The prediction, not yet tested, would be that such factors will be found physically confined to the polar region of the A/V axis. However, it seems more likely that the cortical contraction and consequent cytoarchitectural reorganization defining the domain occupied by the skeletogenic founder cells actually constitutes part of the mechanism by which the micromeres are made distinct from the remainder of the embryo. That is, this reorganization could mediate key upstream steps in the specification process, causing the regionally confined release or activation of the relevant maternal transcription factors (or their cofactors). In this case, these factors might initially have a less precise, though still vegetal, localization (Table 1). It may be relevant that interference with the integrity of the cortical cytoskeletal reorganization by application of mild detergent solutions prevents skeletogenesis (in *Hemicentrotus*; Tanaka, 1976; Dan, 1979), though the argument is weak because it consists of negative evidence.

It is worth noting that the precocious early ingression of a specifically skeletogenic mesenchyme is probably a special, recently evolved mechanism, added during the divergence of the euechinoid sea urchins. The sister group of the euechinoids, the cidaroids or 'pencil urchins,' lack this feature. Their embryonic skeleton is formed instead by mesenchyme cells delaminating only at gastrulation (Wray and McClay, 1989). In euechinoids such as *Strongylocentrotus*, the secondary mesenchyme that delaminates at gastrulation in fact also retains skeletogenic capacity, which is normally repressed, but which can be elicited by depletion of the primary skeletogenic cells descendant from the micromeres (reviewed by Ettensohn, 1992).

In summary, there is solid experimental evidence for the primordial developmental polarization of the sea urchin egg along the A/V axis. Two sets of *cis*-regulatory spatial control systems of maternal origin; those activating the VEB genes and those activating skeletogenic mesenchyme genes have been described. These both reflect and illuminate the autonomous specification processes that result from the primordial polarization of the egg. A remaining mystery is the mechanism by which the adjacent small micromeres are protected from or deprived of the endogenous components required for skeletogenic specification.

THE ORAL/ABORAL AXIS OF THE EMBRYO

Initial specification

Many classical as well as recent experiments demonstrate that each blastomere of a 2- or 4-cell-stage sea urchin embryo is competent to produce a pluteus larva that manifests a clear O/A axis (Hörstadius, 1973; Davidson, 1989; Henry et al., 1992; Cameron et al., 1996). Therefore this axis cannot be prelocalized in the egg before fertilization as is the A/V axis, since were there a specific oral or aboral pole primordially positioned at a given spot on the equatorial circumference of the egg, at least two and probably three of four 2nd cleavage blastomeres would lack it. Nor is there any convincing experimental evidence of even a tendency toward O/A polarization in the unfertilized eggs of indirectly developing sea urchins, such as are treated here. It cannot be excluded that such prelocalizations exist (cf. Vlahou et al., 1996), but if so they are subject to immediate and complete revision, entirely unlike the primordial A/V axis.

Direct developing species such as *Heliocidaris erythrogramma* may, however, define both axes ab initio (Henry et al., 1990). But here there is a crucial difference: the second axis of a directly developing form is the dorsoventral (D/V) axis of the adult body plan, the construction of which is initiated immediately; while, in indirectly developing forms, the O/A axis has nothing whatsoever to do with the adult D/V axis. The O/A axis of indirectly developing forms such as the *Strongylocentrotus, Paracentrotus, Lytechinus* and many other species commonly used in research on embryogenesis are in fact orthogonal to the adult D/V axis, which in the advanced larva arises in the imaginal rudiment (as described in a subsequent section). There is no homology and no simple developmental relationship between the structures of the embryo which are formed along the O/A axis and the structures of the adult body plan formed along its D/V axis. It is therefore an inappropriate usage to apply the terms 'dorsal' and 'ventral' to the O/A axis of indirectly developing echinoid forms.

In *S. purpuratus* specification of the O/A axis must have begun by 3rd cleavage at the latest. Evidence for this assertion includes the following. (1) By the 8-cell stage in *S. purpuratus*, there have arisen polar blastomeres all of the progeny of which contribute either to oral or to aboral lineages, i.e., the Na and No blastomeres (Fig. 2; Cameron et al., 1987). (2) As indicated above, the *CyIIIa* actin gene is transcriptionally activated by 6-7th cleavage and, when first observed by in situ hybridization in late cleavage, the transcripts are confined to aboral blastomeres (Cox et al., 1986; Lee et al., 1986, 1992; Hickey et al., 1987). Other aboral ectoderm-specific markers, particularly the *Spec1*, *Spec2A* and *arylsulfatase* genes achieve this definitive pattern of expression following initial expression throughout the prospective ectoderm (Kingsley et al., 1993; Yang et al., 1993). (3) A gene encoding a homeodomain transcriptional regulator has been discovered in *Paracentrotus* embryos which is zygotically activated in about half of the blastomeres located on one side of the embryo, presumably the oral or aboral side, parallel to the A/V axis. This polarized zygotic expression pattern begins as early as the 4-cell stage (Di Bernardo et al., 1995).

How the initial asymmetry in blastomere fate along the future O/A axis is initially established remains a mystery. By 3rd cleavage, the blastomeres at the future oral pole display enhanced cytochrome oxidase (Czihak, 1963), suggesting a differential distribution of mitochondria across the future O/A axis. A second suggestive observation is that, in several sea urchin species, the position of the future O/A axis can be predicted from the orientation of the vertical plane of 1st cleavage. Thus in *S. purpuratus* the O/A axis normally arises 45° clockwise with respect to this plane, as viewed from the animal pole (Cameron et al., 1989a; Henry et al., 1992) while, in *Lytechinus pictus*, *Heliocidaris tuberculata* and *Strongylocentrotus droebrachiensus*, the plane of 1st cleavage corresponds with the plane of bilateral symmetry (Henry et al., 1992). In *Paracentrotus lividus* (Hörstadius and Wolsky, 1936), *Hemicentrotus pulcherrimus* (Kominami, 1988) and *Lytechinus variegatus* (Summers et al., 1996), no fixed relation between early (1st-3rd) cleavage planes and the future O/A axis can be discerned. However, the positive correlations observed for some species imply that the cytoskeletal reorganization that accompanies the initial spindle formation could be utilized as

a source of asymmetry in the formulation of the O/A axis, as discussed earlier (Cameron et al., 1989a).

From axial specification to differential gene expression on the O/A axis

The *cis*-regulatory architecture of the aboral ectoderm-specific *CyIIIa* gene indicates at least one mechanism by which axial specification is translated into a spatially confined pattern of gene expression. The blastular phase of *CyIIIa* expression is determined by a proximal regulatory module that mediates both positive and negative regulatory interactions (Kirchhamer and Davidson, 1996). Positive regulators binding within this module promote expression in the progeny of both oral and aboral blastomeres, so that correct spatial expression requires repression of transcription on the oral side. This is the function of a negatively acting transcription factor called SpP3A2, which also binds at specific sites within the proximal module (Hough-Evans et al., 1988, 1990; Calzone et al., 1991; Kirchhamer and Davidson, 1996). Deletion of either of two SpP3A2 target sites from the proximal module causes ectopic expression of *CyIIIa•CAT* expression constructs in the oral ectoderm (Kirchhamer and Davidson, 1996). The same result occurs on inactivation of the P3A2 factor in vivo, and such inactivation causes ectopic oral expression of the endogenous *CyIIIa* gene as well (L. D. Bogarad, M. I. Arnone, C. Chieh and E. H. Davidson, unpublished data). SpP3A2 is initially a maternal factor (Zeller et al., 1995a). At fertilization, maternal SpP3A2 must be present and potentially active everywhere, since any point on the circumference of the egg may become the oral pole of the embryonic ectoderm. It follows that a direct consequence of the initial specification of the O/A axis must be activation of the SpP3A2 repressor in the future oral blastomeres. In this case, the O/A axis is translated into a differential spatial readout at the gene level by means of transcriptional repression. A prediction is that the axial polarization results in covalent modification of SpP3A2 on the future oral side of the embryo.

Late specification of the interface between oral and aboral territories

As Fig. 2A indicates, the lateral boundary between oral and aboral domains remains unspecified until after 6th cleavage, and this may be true even at blastula stage (Fig. 2B). Furthermore, the state of specification remains labile for a long time in that the whole of the ectoderm can be converted to oral fate if the embryos are treated with $NiCl_2$, as indicated by greatly expanded expression of the oral-specific antigen EctoV (Coffman and McClay, 1990). The sensitive period for this treatment is the blastula stage. After gastrulation is complete the ciliated band arises at the border between the oral and aboral ectoderm. Not until this has occurred is the O/A boundary entirely fixed, since oral and aboral ectoderm cells both contribute to it in a process that is dependent on position rather than lineage (Cameron et al., 1993).

CONDITIONAL SPECIFICATION OF TERRITORIAL FOUNDER CELLS DURING CLEAVAGE

The effect of micromeres on vegetal plate specification affords the clearest example of conditional specification in the sea urchin embryo. We begin with a description of this case, and then proceed to other less-well-defined interactions.

Specification of the *veg$_2$* lineage compartment, the definitive vegetal plate, by signals from the micromeres

Among the functions that the autonomously specified skeletogenic micromere lineages execute is signaling to the adjacent macromeres and their daughters and granddaughters, the *veg$_2$* blastomeres. This interaction is required for the normal specification of the prospective vegetal plate, and it is at present the best known of all such interactions occurring in the cleavage-stage sea urchin embryo. Modern evidence was obtained by Khaner and Wilt (1991), who combined mesomeres with skeletogenic micromeres, and showed with the aid of markers that the mesomeres in these recombinants generate gut elements that express alkaline phosphatase, as well as pigment cells, a secondary mesenchyme cell type (Table 1, isolate 4). We showed that 4th cleavage micromeres emit signals that suffice for the ectopic induction of a complete and normally functional vegetal plate even in the context of the whole embryo (Ransick and Davidson, 1993). Sets of micromeres were transplanted to the animal poles of intact recipient embryos, at 3rd cleavage stage, causing the formation of a second vegetal plate from descendants of the mesomere directly apposed to the transplanted micromeres. The ectopic vegetal plates express the endoderm specification marker *Endo16* and, at the appropriate time, they invaginate, ultimately creating a complete second gut. This fuses with the endogenous gut in the foregut region. Examples of these remarkable results are illustrated in Fig. 3A-D. The transplanted micromeres, which in these experiments are labeled with a lineage tracer, can be seen to ingress and participate in the formation of a second skeletal apparatus that is apparently positioned by the interface between the ectopic vegetal plate and the oral ectoderm. In a second set of experiments, results from which are reproduced in Fig. 3E-I, the micromeres were removed after 4th, 5th or 6th cleavage (Ransick and Davidson, 1995). These experiments addressed the necessity of micromere signaling for normal specification of the overlying *veg$_2$* blastomeres. Ablation at 4th cleavage causes a sharp attenuation of *Endo16* expression, which in some of these embryos fails almost completely (Fig. 3E). However, ablation at 6th cleavage, i.e., after 2-3 hours of contact between micromeres and macromeres permits almost normal *Endo16* expression (Fig. 3G). The low level of residual *Endo16* expression observed even after 4th cleavage ablation could indicate that only the few minutes of micromere-*veg$_2$* blastomere contact prior to ablation suffices, or it could reveal a marginal level of autonomous capacity for activation, which in normal embryos is greatly stepped up by the micromere signal. Micromere ablation at 4th-6th cleavage severely delayed gastrulation and in some embryos gastrulation fails completely. On a much delayed schedule, an archenteron is generated by a late regulative event and these embryos are eventually capable of producing complete feeding larvae, which in turn can give rise to normal adults (Ransick and Davidson, 1995; Ransick et al., 1996). Remarkably, a single micromere left in place significantly rescues vegetal plate specification including timely gastrular invagination (Fig. 3H). These experiments demonstrate clearly that micromere-to-*veg$_2$* signals beginning at mid-cleavage are required for the normal specification of the *veg$_2$* lineages, and hence for the process by which the vegetal plate is normally formed in the embryo.

Current model for signaling interactions in the mid-cleavage to mid-blastula-stage embryo

Most of the blastomeres of the embryo are specified conditionally. Fig. 4 indicates the minimum diversity of signaling interactions required by present data, up to about the hatching blastula stage. Modern evidence summarized in this diagram is in many respects consistent with predictions based on Hörstadius' early blastomere recombination experiments (Davidson, 1989). The rationale for each of the signaling interactions represented by the sets of arrows in Fig. 4 is as follows.

Micromere-to-*veg*$_2$ signal

See previous section.

veg$_2$-to-lower-*veg*$_1$ signal

The function of this signal is specification of endodermal *veg*$_1$ components, a matter that we take up in detail below. The late specification of these *veg*$_1$ endoderm precursors may occur by *veg*$_2$-to-*veg*$_1$ signaling, though the necessity of such a signal has not been directly demonstrated. However, a potent *veg*$_2$ signaling capacity has been shown to exist by Logan and McClay (1998) in the experiments summarized for isolates 5 and 6 in Table 1 (compare isolate 2). These experiments show that *veg*$_2$ + mesomere combinations generate a complete tripartite gut, which includes mesomere progeny that have been induced to express endodermal fates; in contrast, *veg*$_1$ cannot similarly recruit mesomeres and, when combined with mesomeres, it at best produces a small invagination rather than a complete gut. Even a single *veg*$_2$ blastomere combined with the mesomere tier generates a complete gut in a fraction of embryos (Logan and McClay, 1998).

veg$_2$-to-*veg*$_2$ signal

We believe that the function of this signal is to promote endoderm gene expression within the *veg*$_2$ domain. This interaction is directly indicated by the experiment illustrated in Fig. 3H, in which a single micromere suffices to endow 75-100% of *veg*$_2$ progeny with the capacity to express *Endo16* at a normal level and to form a normal, functional vegetal plate. That is, although the remaining micromere is of a size such that it can make contact with only one or two of the *veg*$_2$ blastomeres, they all respond. Therefore they must communicate with one another. By a similar argument, there may also exist *veg*$_1$ to *veg*$_1$ signaling within the *veg*$_1$ endodermal domain, after this is specified (not shown). Thus, in the experiments of Logan and McClay (1998), a single *veg*$_2$ blastomere combined with a *veg*$_1$ tier in *veg*$_1$-mesomere recombinants suffices, in some embryos, for the formation from the *veg*$_1$ cells of a complete archenteron. The *veg*$_1$ cells must thus communicate amongst themselves.

Negative interactions across the future endoderm-ectoderm boundary

These interactions would contribute to the ectodermal state of specification above the endoderm boundary. The evidence derives from experiments on the *Endo16 cis*-regulatory system (Yuh and Davidson, 1996; Yuh et al., 1996; unpublished data). *Endo16* expression constructs are transcribed only within the vegetal plate and later the endoderm proper. Repressive interactions that occur within certain elements of the *Endo16 cis*-regulatory system prevent ectopic ectoderm expression (Yuh and Davidson, 1996; Yuh et al., 1996). Two observations reveal that this repression system lies downstream of a signal

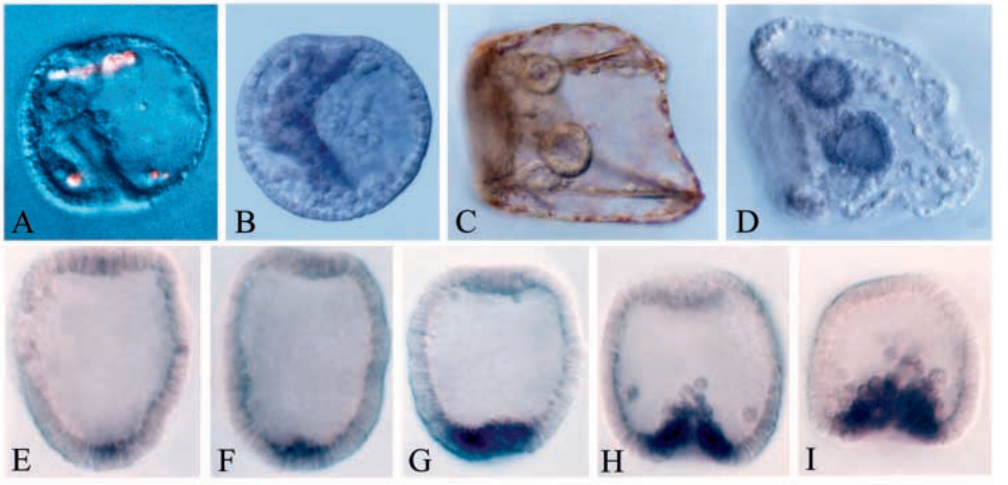

Fig. 3. Conditional specification of *veg*$_2$ cells by signals from micromeres. (A-D) Development of experimental embryos with induced vegetal plates. (A) Processed video image of a 40-hour gastrula of *S. purpuratus* with two archenterons, which developed after transplantation of a second set of labeled micromeres (red) to the animal pole region. The primary mesenchyme cells are arranged in rings around the base of each invagination. Most labeled mesenchyme cells remain around the induced archenteron (top), but a few have incorporated into the endogenous population around the true archenteron (bottom). (B) Photograph of the same embryo as shown in A after whole-mount in situ hybridization with an antisense probe for *Endo16*. Both archenterons are expressing *Endo16* (purple stain) along their entire length at this stage. (C-D) Two photographs of the same *S. purpuratus* 72-hour pluteus stage equivalent. (C) The living specimen with mirror image symmetry for gut and spicule development. (D) Same embryo after whole-mount in situ hybridization with an antisense probe for *Endo16*. Both archenterons have become regionalized and the expression of *Endo16* is restricted to the stomach regions (from Ransick and Davidson, 1993). (E-I) Effect of micromere deletion on 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 (E), 5th (F), or 6th (G) cleavages, or three micromeres were deleted after 4th cleavage (H), or no micromeres were deleted (i.e., control; I). All embryos are oriented similarly, with the vegetal plate down and have been processed by whole-mount in situ hybridization for *Endo16* (blue/black stain). 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 (H) gives consistently strong staining in even more cells and dramatically rescues gastrulation (from Ransick and Davidson, 1995).

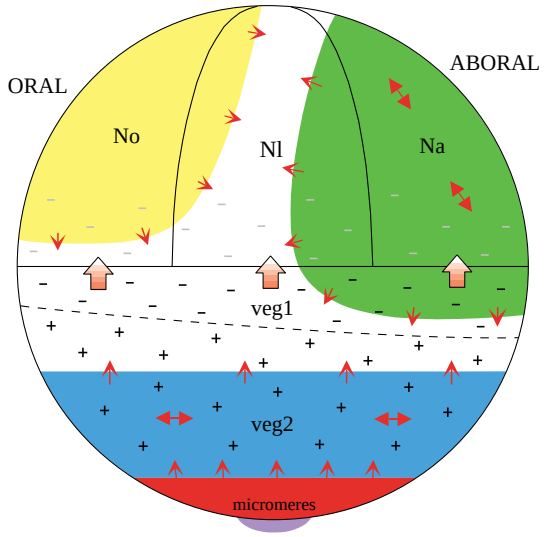

Fig. 4. Model for signaling interactions in the late cleavage-mid blastula-stage embryo. The color coding is as in Figs 1 and 2. Arrows indicate signaling interactions. Each presumed interaction is discussed in text. The – and + signs denote presence and absence, respectively, of negatively acting regulators for *Endo16* (and presumably other endoderm-specific genes) above the future endoderm-ectoderm boundary (dotted line).

transduction pathway: (1) the terminal transcription factor required for the function of the ectoderm repression module is of the CREB family (Montminy, 1997; Brindle et al., 1995), and (2) the spatial repression that it mediates is abrogated by introduction of a construct encoding an activated Ras protein (C.-H. Yuh and E. H. Davidson, unpublished data). It is not known whether the signal emanates from above, below or within the *veg*$_1$ ectodermal blastomeres [the view we favor and that is indicated in Fig. 4 by + and – signs].

Vegetal-to-ectoderm signal

This interaction is apparently required for differentiation of oral ectoderm, and possibly aboral ectoderm as well. The evidence derives from experiments summarized in Table 1 (isolates 2, 3 and 7). Thus cultured mesomere tiers fail to form stomodaeum or ciliated band, while addition of macromeres yields normal oral morphology with a polarized pattern of EctoV expression. Two items of evidence (see Table 1, isolate 2) indicate that a very early signal may indeed pass across the 3rd cleavage plane: (1) Henry et al. (1989) found (in *L. pictus*) that oral differentiation could occur in mesomere tiers removed from the embryo as late as 4th cleavage, but not if they are removed at 3rd cleavage and (2) Wikramanayake et al. (1995) found (in *L. pictus*) that the aboral ectoderm-specific genes (*LpS1*, *LpC2*) are not expressed in mesomeres separated at 3rd cleavage unless macromeres are recombined with them. However, this requirement was not observed in *S. purpuratus*, in which isolated 3rd cleavage mesomere tiers are competent to express these aboral ectoderm markers at normal levels. The difference may be one of timing of the signal, or of the appearance of a sufficient amount of it, relative to the cleavage clock. The signal is shown traversing the plane of 3rd cleavage in Fig. 4 because that is the interface tested in the experiments that suggest its existence, but it could continue to emanate from the endodermal domain below the prospective endodermal/ectodermal boundary.

Possible signals from the aboral and oral polar blastomeres to the lateral ectoderm

By the hatching blastula stage (18 hours), *CyIIIa* expression is detectable in about half the blastomeres on one side of the embryo (Cox et al., 1986), i.e., the prospective aboral side but, at mid-cleavage, individual blastomeres in the lateral regions still give rise to both oral and aboral clones (Cameron et al., 1993). This is not true of the polar blastomeres, which generate either all oral or all aboral clones (Cameron et al., 1987), as illustrated in Fig. 2A. We infer that specification across the O/A axis may be progressive. This raises the possibility that additional signals emanate from the polar oral and aboral blastomeres.

Interactions among ectoderm cells

These interactions are implied by the results of Hurley et al. (1989) and Stephens et al. (1989), which show that some aboral ectoderm-specific genes, i.e., *CyIIIa*, *Spec1* and *Spec2a*, are not expressed at normal levels in disaggregated cells. Similarly, *PlHbox12* expression (see above) is also extinguished in disaggregated cells (Di Bernardo et al., 1995). Interactions may occur within both the oral and aboral ectoderm territories but no oral ectoderm markers are available for this stage.

Many new interactions are set up later in development, but these lie beyond the scope of this paper, except for those noted en passant in the following discussion of endomesoderm specification. For example, there is excellent evidence for interactions required for skeletogenic patterning that occur between the skeletogenic mesenchyme and the ectodermal wall, and for other interactions between the skeletogenic mesenchyme and secondary mesenchyme cells at the gastrula stage (Ettensohn, 1990, 1992; Ettensohn and Ingersoll, 1992; McClay et al., 1992; Armstrong et al., 1993; Guss and Ettensohn, 1997; Ettensohn et al., 1997). There is also a complex set of interactions within the forming archenteron that is responsible for its differentiation into a tripartite gut (McClay and Logan, 1996). A feature of all of these later signaling events is that they involve some cells that are migratory or to some extent motile. In contrast, all the interactions presented in Fig. 4, and those that occur within the vegetal plate at the blastula stage, take place between stationary cells, in situ. Therefore they occur with respect to the initial spatial coordinates of the egg and, in some cases, with respect to specific cleavage planes. This is a characteristic of pregastrular conditional specification in sea urchin embryos as in most other invertebrate embryos. The signaling that mediates these specification processes most likely requires immediate cell-to-cell contact, implying tethered ligands or at least a very high degree of restriction in diffusion.

SPECIFICATION OF ENDOMESODERMAL COMPONENTS IN BLASTULA-EARLY GASTRULA STAGES

Following the cleavage and early blastula stage specification events summarized in Fig. 4, and the foregoing discussion, a major developmental process of the mid-late blastula stage is

the specification of endodermal versus mesodermal territories. The vegetal domain of the embryo includes the progenitors of all embryonic and adult mesoderm (see Fig. 1). This domain now consists of *veg$_2$* progeny, the skeletogenic mesenchyme lineages and the eight small micromere descendants. In euechinoids, the skeletogenic mesenchyme ingresses during the blastula stage and this is the initial mesodermal component of the embryo to delaminate. The remaining blastomeres form what is sometimes called the 'definitive vegetal plate' (mesenchyme blastula stage). Its mesodermal components arise from a disc-like field of cells in the central region of the definitive vegetal plate, as we discuss in detail below, while the peripheral region produces foregut and midgut endoderm (Fig. 2C). Only recently it has been found that *veg$_1$* components abutting the *veg$_2$* lineage boundary also contribute to the endoderm (Logan and McClay, 1997; Ransick and Davidson, 1998). Late in gastrular invagination the *veg$_1$* components move in to form the hindgut and portions of the midgut. But gastrulation is initiated entirely by the *veg$_2$* descendants, and indeed depends on their early conditional specification, as reviewed above (cf. Fig. 3E-I).

Specification of veg$_1$ endoderm

After the 7th cleavage, the positions of cleavage planes within the *veg1* domain become variable (Logan and McClay, 1997). Therefore the conditional specification of *veg$_1$* endoderm progenitors by signals from *veg$_2$* affects a variable population of cells and cannot depend on lineage. Furthermore, only some *veg$_1$* cells become endoderm; nor are endodermal *veg$_1$* descendants rigidly confined to hindgut and midgut. Occasionally in *S. purpuratus*, and frequently in *L. variegatus*, individual *veg$_1$* progeny are found even in foregut. *veg$_1$* descendants remain plastic during most of the blastula period and the only role that lineage plays in their specification is to separate them initially from their earlier-specified *veg$_2$* sister cells. In *S. purpuratus*, more of the *veg$_1$* progeny become endodermal on the oral than on the aboral side and, perhaps in consequence, the archenteron is biased in position toward the oral side of the late gastrula. In *L. variegatus*, there is no regularity in the *veg$_1$* endodermal contribution with respect to the O/A axis.

An interesting aspect of the late specification of *veg$_1$* cells is that it occurs well in advance of their invagination. This can be visualized in *S. purpuratus* by the activation of the *Endo16* gene just after *veg$_2$* invagination has occurred at the early gastrula stage. The *Endo16*-expressing *veg$_1$* cells now constitute an eccentric ring around the blastopore that is widest on the oral side, and this corresponds exactly to the *veg$_1$* components that will later invaginate to form the hindgut (Ransick and Davidson, 1998). *SpKrox-1*, a zinc-finger transcription factor (Wang et al., 1996) that is activated in macromeres late in cleavage and is then expressed throughout the definitive vegetal plate, begins to be transcribed in the future endodermal components of *veg$_1$* as well, and can be detected by in situ hybridization several hours earlier than is *Endo16* (unpublished observations of A. Ransick). The initial specification of the endodermal *veg$_1$* constituents thus probably occurs between the end of cleavage and the mesenchyme blastula stage (since the targets of this specification arise only in the 7th-9th cleavage divisions).

Comparative considerations: primitive and derived aspects of endoderm specification

Of living echinoderm classes, the echinoids (sea urchins) are most closely related to the holothuroids (sea cucumbers), while the asteroids (starfish) belong to a different evolutionary branch that also includes the ophiuroids (brittle stars) (Smith et al., 1993; Littlewood et al., 1997). It is therefore particularly interesting to compare some recent results on endoderm specification in starfish to those discussed in the foregoing; shared mechanisms are likely to represent basal aspects of the echinoderm way of doing business. No micromeres are produced in starfish embryos, nor are there any skeletal elements in the embryo. As noted earlier, the starfish egg is polarized in the A/V axis and the ability to produce an archenteron in starfish eggs is an autonomous, localized property of the vegetal blastomeres. Specification of the endodermal constituents has been examined experimentally, in *Asterina pectinifera*, by Kuraishi and Osanai (1992, 1994). A brief summary of results relevant to the present argument is as follows. (1) 'Maternal factors responsible for gut formation' are present at the vegetal pole of the egg, and if ≥7% of the cytoplasmic volume is cut off the vegetal pole of the oocyte, gastrulation does not occur, archenteron formation fails to take place, and a gut marker, alkaline phosphatase, is not expressed. (2) The macromere lineage in *Asterina* is like that of echinoids, in that the macromere granddaughters constitute similarly arranged *veg$_2$* and *veg$_1$* rings. (3) The fate of *veg$_2$* blastomeres is the same as in echinoids, in that their progeny form the vegetal plate. (4) The fate map of the vegetal plate is also similar, the peripheral regions giving rise to the foregut and midgut endoderm, and the central region of mesodermal constituents. (5) Following the initial phase of gastrulation, and after a pause of several hours, the lower *veg$_1$* progeny invaginate to form the hindgut and the lower part of the midgut, while the upper *veg$_1$* progeny produce the ectoderm surrounding the blastopore. (6) The *veg$_1$* endodermal constituents are specified as a consequence of signals from the *veg$_2$* cells that abut them, as demonstrated convincingly in a blastomere recombination experiment utilizing the alkaline phosphatase marker.

We may therefore conclude that all aspects of the endoderm specification process that we have described for sea urchins are primitive for echinoderms with one very illuminating exception. This is the inductive role of the micromeres in the *veg$_2$* specification process, as it occurs in euechinoids. In these sea urchins, it is the micromeres that are autonomously specified at the vegetal pole while, in the asteroids, there are no micromeres and instead the *veg$_2$* lineages are autonomously specified at the vegetal pole. The micromere induction system is a derived echinoid character correlated with, and possibly following from, the precocious cleavage-stage segregation of skeletogenic and those coelomic sac mesodermal lineages of which the micromeres are the founder cells. It would be interesting to know how recent this developmental mechanism is in origin. In *Eucidaris tribuloides*, a cidaroid sea urchin, the skeletogenic lineage founder cells are also segregated precociously, but micromeres per se form only irregularly (Wray and McClay, 1989): do they play the same inductive role? All modern sea urchins descend from a single cidaroid-like genus that survived the Permian extinction 230 mya (Smith, 1984) and the mode of *veg$_2$* specification in a living cidaroid would thus be revealing.

Table 2 lists early vegetal plate-specific genes that are expressed in all *veg$_2$* cells during the blastula stage, e.g., *Endo16* and several genes encoding transcription factors including *HpHnf-3B* (Harada et al., 1996) and *SpKrox-1* (Wang et al., 1996). These genes are evidently activated, directly or indirectly, in consequence of two different signals, as portrayed in Fig. 4. The first is the micromere-to-*veg$_2$* signal, and second is the *veg$_2$*-to-*veg$_2$* signal, as discussed above. Since the same genes in the same cells are downstream of both signals, the micromere-to-*veg$_2$* signal and the *veg$_2$*-to-*veg$_2$* signal are likely to be the same, i.e., to utilize the same ligands and receptors. The same argument pertains to the later *veg$_2$*-to-*veg$_1$* signal, which again results in activation of the same genes, e.g., *Endo16* and *SpKrox-1* in *veg$_1$* progeny, when these cells are specified prior to invagination. The *veg$_2$*-to-*veg$_1$* signaling system also exists in *Asterina*, and possibly the *veg$_2$*-to-*veg$_2$* signaling system. We conclude that this signaling system and its linkages to the vegetal-plate-specific transcription apparatus are primary features of cells deriving from the vegetal cytoplasm of the egg in echinoderms. Thus we can imagine that, in the divergence of the echinoids, the sequestration of the skeletogenic micromeres at the vegetal pole included the sequestration of this same signaling capacity. Step by step as this lineage evolved, the initial activation of vegetal plate-specific genes would have become more dependent on this signal, but it is still not absolutely dependent on it (cf. Fig. 3A; Ransick and Davidson, 1995).

How endoderm specification might work: a parallel input model

Prior to specification measurable by marker gene expression, β-catenin concentrates in the nuclei in all cells whose progeny will ever produce endoderm or mesoderm. It is present in the nuclei of micromeres and *veg$_2$* cells as early as 7th cleavage, and is clearly visible in the lower *veg$_1$* cells by early blastula stage 32 (Miller and Moon, 1996; D. R. McClay, personal communication). To borrow a current cliché, nuclear β-catenin in the sea urchin embryo marks the 'identity' of endomesodermal progenitors. Given that the output of a specification process is spatially differential transcriptional activity, a simple way to think about 'identity' functions of this kind is that they represent one of several parallel inputs into the *cis*-regulatory systems primarily affected by a specification event. Here we would argue that nuclearization of β-catenin is upstream of transcription factors that interact with primary vegetal plate-specific *cis*-regulatory elements, but that to generate transcriptional activity these elements require other factors in addition. The advent of the additional factors would determine the spatial domains and the temporal occurrence of gene expression.

These arguments predict a specific organization of the primary *cis*-regulatory targets of vegetal-plate-specific genes, in particular those encoding zygotic endoderm-specific transcription factors normally activated by the micromere signal in *veg$_2$* progeny, and later by *veg$_2$*-to-*veg$_2$* and *veg$_2$*-to-*veg$_1$* signals. We imagine that these *cis*-regulatory elements exist in a state of balance in that their response depends on the levels of binding of both the endomesodermal 'identity' factor(s), and of other factors downstream of intercellular signal transduction pathways (a caution is that gross overexpression of either *cis*-regulatory input could easily obliterate this balance, revealing only the activity of the gene that is overexpressed). In starfish, the equivalent endogenous transcription factors are autonomously active; in sea urchins, they initially require the micromere signal for activity. Later, in both organisms, they act downstream of the signal, now of *veg$_2$* origin.

When embryos are treated with LiCl, the vegetal cap of nuclearized β-catenin intensifies and expands throughout the *veg$_1$* domain (D. R. McClay, personal communication), and LiCl causes all *veg$_1$* cells to become specified as endoderm (Cameron and Davidson, 1997). As has long been known, the period of sensitivity to LiCl treatment for alteration of vegetal fates is cleavage (Hörstadius, 1973). These facts suggest that the teratogenic 'vegetalization' effects of LiCl are in general the consequence of changes in the activity of an endogenous egg cytoplasmic pathway that conveys the endomesodermal 'identity' functions erected along the A/V axis during cleavage, rather than of changes that affect the activity of the interblastomere signaling systems that establish the detailed expression patterns. LiCl treatment also shifts the lower boundary of VEB gene expression towards the animal pole (Ghiglione et al., 1993). As we have seen, VEB gene expression is a reflection of the early autonomous A/V system, which is clearly not responsive to interblastomere signaling.

Fig. 5 summarizes the idea set forth in this section. Similar arguments may be made for both *veg$_2$* and *veg$_1$* specification, and also for specification of the mesodermal domain that arises during the blastula stage in the center of the vegetal plate (see below). The gist of this argument is that the persistent primordial A/V identity system is the mechanism by which the cytoarchitectural spatial coordinates of the egg ultimately effect transcriptional specificity through the blastula stage. Since the endomesodermal 'identity' system is LiCl-sensitive, this teratogen spatially perturbs all endomesodermal specification. HMG-box factors (LEF1, TCF1) are among those functioning downstream of the GSK3-β-catenin activity pathway, and these factors characteristically affect the architecture of *cis*-regulatory DNA-protein complexes (Falvo et al., 1995; Giese et al., 1991, 1992; Behrens et al., 1996). Thus they might be expected to set the concentration window within which the second, and signal-dependent, transcription factor(s) will be functional within the *veg$_2$* and *veg$_1$* domains. In the animal pole cells, which lie outside the primordial endomesodermal identity domain, we would argue that the threshold for response to the micromere signal is different. Thus in the induction of a second vegetal plate by micromeres transplanted to this region, β-catenin and the associated transcription factor(s) would be missing from the regulatory complex. In the absence of nuclear β-catenin, the lower threshold for the signal-dependent factor might no longer exist. Now the inductive signal alone suffices to activate specific genes, even without the endogenous endomesodermal gene-activating capacities at the vegetal pole. In fact, when micromeres are transplanted to the animal pole and a second vegetal plate forms from mesomere derivatives, nuclearization of β-catenin is not observed in the responding cells (D. R. McClay, personal communication). This confirms that the transcription factors downstream of the micromere signal and those downstream of nuclear β-catenin are in fact different factors. Fig. 5 provides the general argument that the interactions between the endogenous, originally maternal

spatial regulatory system, and the particular conditional (or other) embryonic specification systems, occurs by integration of their respective inputs at the *cis*-regulatory level (*cf.* Arnone and Davidson 1997).

Origin of embryonic and adult mesoderm

The initial mesodermal cell type to arise, the skeletogenic mesenchyme, is on comparative grounds to be regarded as a heterochronic, early segregation of what would otherwise be a secondary mesenchymal cell type since, in *Eucidaris*, the skeletogenic mesenchyme delaminates at gastrulation, along with other mesenchymal cell types. The secondary mesenchyme gives rise to two additional free wandering cell types, i.e. pigment cells, and fusiform blastocoelar cells. Muscle cells and coelomic sac cells evert from the sides of the archenteron tip late in gastrulation, with the formation of the bilateral coelomic sacs. It is not known whether any of the mesenchymal cell types of the embryo, i.e., skeletogenic, pigment and blastocoelar cells, are ancestral to equivalent mesodermal components of the adult rudiment, e.g., whether the pigment and skeletal elements of the latter develop anew from mesodermal stem cells during larval life. As detailed in the following section, however, the major mesodermal components of the adult body plan, i.e., body wall musculature, internal water vascular system, tube feet and coelomic linings, all clearly derive from the coelomic sacs.

At the blastula stage, all progenitors of the coelomic sacs and the secondary mesenchyme are located in the central region of the vegetal plate (including the small micromere constituents of the future coelomic sacs). Lineage labeling studies of Ruffins and Ettensohn (1993, 1996) have provided key evidence as to the disposition of the various sorts of mesodermal precursors within the vegetal plate of the late blastula, excepting the skeletogenic precursors, which have already ingressed. Specification of the mesodermal cell types remaining within the vegetal plate at this stage cannot have been completed prior to hatching blastula stage (the small micromeres could be an exception), since individual cells labeled with diI at this stage often give rise to progeny which appear in more than one mesodermal cell type when the embryos are examined later on (Ruffins and Ettensohn, 1993). However, specification probably takes place within the next several hours, since by mesenchyme blastula stage most vegetal blastomeres labeled by this method produce small clones of cells consisting of only a single cell type. Some of these cell types are not committed to given fates until much later, in that skeletogenic redifferentiation can be elicited by depletion of primary skeletogenic mesenchyme until mid-gastrula stage (Ettensohn, 1990). In *S. purpuratus*, some pigment cells delaminate and differentiate at the start of gastrulation, when eight pigment cell progenitors appear that express a specific cell surface marker (Gibson and Burke, 1985). By pluteus stage, there are about 30 pigment cells embedded in the aboral ectoderm in this species (Gibson and Burke, 1985; Miller et al., 1996). In *L. variegatus*, there may be as many as 26 pigment cell precursors in the vegetal plate and the ultimate number is about 60 (Ruffins and Ettensohn, 1996; Ettensohn and Ruffins, 1993). Similarly, there are in *L. variegatus* about 36 blastocoelar-type mesenchyme precursors in the vegetal plate, and about twice this number in the pluteus-stage embryo. The number of coelomic pouch cells (including the eight small micromere descendants), however, is about the same in the vegetal plate as in the coelomic pouches of the late embryo, in both *S. purpuratus* and *L. variegatus*, totaling about 20 in each species (10 in each pouch) (Cameron et al., 1993;

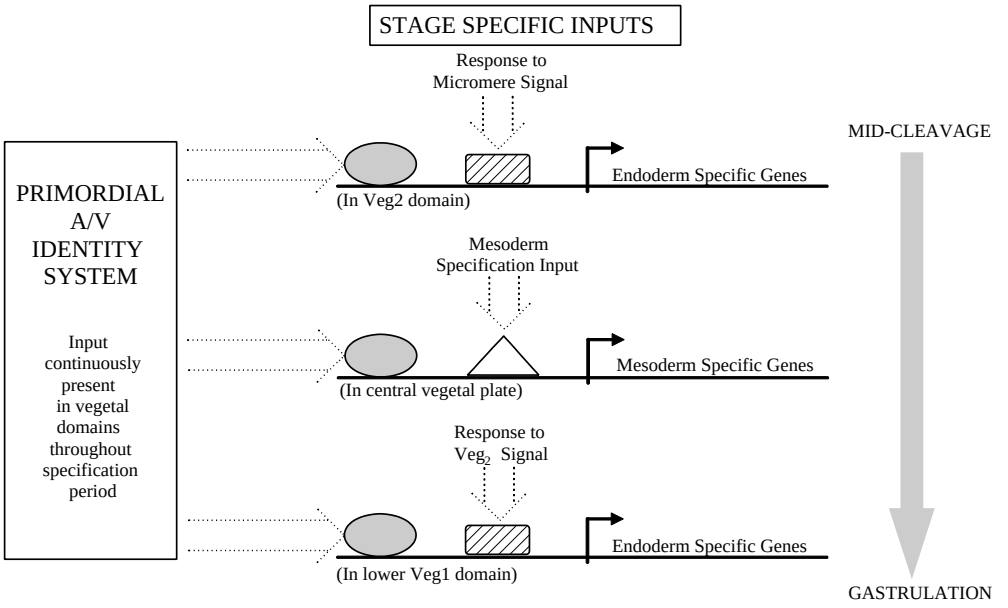

Fig. 5. Scheme of endomesodermal specification model discussed in text, in which the relevant *cis*-regulatory systems respond to parallel inputs. One of these inputs consists of maternal transcription factor(s) presented in the nuclei in consequence of a primordial A/V 'identity' system, indicated as solid symbols. This system responds to LiCl treatments during cleavage, which result in the expansion of the domains of endomesodermal specification. The second input in each type of *cis*-regulatory system consists of transcription factor(s) downstream of particular specification functions operating at given times and places, shown as open and hatched symbols. Under normal conditions, both inputs are required for gene expression. The earliest set of genes figured encode endoderm-specific products and are expressed in the veg_2 cells constituting the definitive vegetal plate, in response to signals from the macromeres. Mesoderm-specific genes are activated in the center of the vegetal plate during blastula stage, perhaps in part due to autonomous vegetal factor(s) as in starfish eggs (see text). Endodermal genes are later activated in a new set of cells of veg_1 origin, in response to signals from veg_2. The target genes, signals and transcription factors utilized in these veg_1 cells at least in part overlap those utilized earlier in the initial specification of the vegetal plate, while the timing is set by the $veg_2 \rightarrow veg_1$ signal. See text for discussion and references.

Ettensohn and Ruffins, 1993; Ruffins and Ettensohn, 1996). Although a few muscle cells extrude from the coelomic pouches to form the esophageal sphincter of the larval gut (Tamboline and Burke, 1992), most coelomic pouch cells are to be regarded as set-aside cells, anlage of the coelomic structures of the rudiment (Peterson et al., 1997).

The mechanism by which the cell types that arise from the vegetal plate are specified remains largely mysterious. The pattern of specification is biased along the O/A axis, so that more pigment cell precursors arise on the aboral side of the polar region of the vegetal plate and more blastocoelar cells on the oral side in *L. variegatus* (Ruffins and Ettensohn, 1996); and more coelomic pouch precursors arise on the oral side in *S. purpuratus* (Cameron et al., 1993). With this exception, the specification pattern has a cell-by-cell, salt-and-pepper quality with respect to cell type, suggesting intrinsic cellular decisions rather than position-dependent patches of cells of given states of specification, or the regularity that might be implied from a strict lineage-based formula.

A considerable number of genes are now known that are expressed during embryogenesis only in the endomesoderm, as summarized in Table 2. All genes encoding products other than the transcription factors in Table 2 are eventually expressed in either endodermal or mesodermal cell types in the late embryo but not both, though in the cases of *S9* and *Endo16* the initial phase of expression, in the whole of the vegetal plate, clearly includes both endodermal and mesodermal territories. Ultimately, however, *S9* is mesodermal and *Endo16* is endodermal. A particularly interesting expression pattern (so far known only in *L. variegatus*) is that of the Notch protein. At mesenchyme blastula stage, a pattern of apical cellular localization emerges that is initially confined to a broad ring of cells extending beyond the definitive vegetal plate of the mesenchyme blastula-stage embryo. This ring perfectly excludes the central mesodermal domain of the vegetal plate (Sherwood and McClay, 1997). The Notch protein continues to be localized apically in the archenteron during invagination, except at the tip where mesodermal cells are delaminating. This is the first early macromolecular localization pattern discovered that directly reflects the endomesodermal fate map of the vegetal plate as seen in Figs 1 and 2C, and it implies that transcription factors downstream of *Notch* contribute to the early specification of mesodermal as opposed to endodermal territories. Two genes encoding transcription factors listed in Table 2, i.e. the zinc-finger gene *SpKrox-1* (Wang et al., 1996), and the brachyury genes *SpT-1* and *HpT-1* (Harada et al., 1995; and unpublished data) are also expressed in the vegetal plate of the hatching blastula stage, but they are expressed throughout, irrespective of the future boundary between endoderm and mesoderm. The other transcription factors listed begin to be expressed only later. It is interesting that LiCl treatment expands the outer boundary of the *Notch* apical localization ring upward, so that 60% more cells express *Notch* apically (Nocente-McGrath et al., 1991; Ransick et al., 1993; Cameron and Davidson, 1997; Sherwood and McClay, 1997; D. R. McClay, personal communication), concomitantly with the expanded domain of nuclear β-catenin and of endodermal fate caused by LiCl treatment. *Notch* may thus serve to mediate an aspect of the 'identity' system built into the early embryo, which we believe may be the target of LiCl treatment, at least in respect to its action on vegetal cell specification, i.e., apical *Notch* could be downstream of the system evinced by β-catenin nuclearization. LiCl treatment also causes an expansion of 35% in the area of the inner *Notch*-free radial zone, and the production of excess mesodermal derivatives therefrom (Sherwood and McClay, 1997). One possibility is that there are autonomous vegetal factors promoting expression of mesoderm-specific genes since, in *Asterina*, the evidence is clear that if the vegetal-most cytoplasm is removed from the egg, secondary mesenchyme cell types fail to form (Kuranishi and Osanai, 1994). These mesoderm factors could function together with the same vegetal 'identity' factors downstream of β-catenin as we propose for endoderm-specific genes (Fig. 5). Thus it is possible that key endodermal and mesodermal *cis*-regulatory elements operate similarly: both might utilize the β-catenin system plus, in the case of mesoderm, autonomously activated factor(s) and, in the case of endoderm, signal-activated factors. The role of the pathway demarcated by apical expression of *Notch* could be to repress mesoderm-specific genes (Sherwood and McClay, 1997), as suggested by the exclusive relation between apical *Notch* and mesodermal fate, in both normal and LiCl-treated embryos. Thus the *Notch* pattern suggests that the transcriptional state of the endodermal and the mesodermal domains must be distinct within the vegetal plate, i.e., expression of a yet unidentified set of mesoderm-specific transcription factors might be installed in the central region of the vegetal plate during the mid to late blastula stage, but repressed in the endodermal domains. Irrespective of these speculations, we note that the subsequent process of cell-by-cell specification of different mesodermal cell types is different from any occurring elsewhere in the embryo.

REGULATORY REQUIREMENTS IN THE DEVELOPMENT OF THE EMBRYO AND OF THE ADULT RUDIMENT

Maternal transcription factors

It has been obvious for a long time that, in order to explain phenomena such as localization of developmental potential in egg cytoplasm and other aspects of embryogenesis, the egg must contain transcription factors and/or mRNAs that encode them (e.g., Davidson, 1968). The initial set of conditional specifications in the sea urchin embryo were proposed to work by means of regional covalent modification of maternal transcription factors, in response to signaling amongst blastomeres (Davidson, 1989). This probably remains the best way to think about conditional specification in the cleavage-stage embryo, but there is little direct biochemical evidence. In *C. elegans* most of the blastomeres are also specified by short-range signaling during cleavage, and components of several familiar signaling systems as well as several maternal transcription factors mediate these specification processes (Thorpe et al., 1997; Bowerman et al., 1992, 1993; Mello et al., 1994; Rocheleau et al., 1997; Moskowitz et al., 1994).

Many transcription factors have been identified by their functional interactions with well-characterized *cis*-regulatory systems active in the early sea urchin embryo, and it is a remarkable fact that, in every case so far investigated, mRNA encoding those factors, and/or the factor itself, exists in the cytoplasm of the unfertilized egg. Some genes encoding transcription factors that are only expressed zygotically in the

early embryo have also been found by homology cloning, e.g., *PlHbox12* (Di Bernardo et al., 1995) and *SpKrox-1* (Wang et al., 1996), both mentioned earlier. Nonetheless, the maternal representation of embryonic factors is impressive. For example, all of the nine factors required for control of the *CyIIIa* gene are probably present in unfertilized egg cytoplasm (Calzone et al., 1997) as is the Otx factor (Mao et al., 1996). The concentrations of these factors per egg is often about the same as later on, per embryo. This is true, for instance, for SpP3A2 (Zeller et al., 1995a), SpGCF1 (Zeller et al., 1995b), SpZ12-1 (Wang et al., 1995) and SpRunt-1 (Coffman et al., 1997), though genes encoding all these factors are transcribed zygotically as well (op cit; see also Li et al., 1997 and Cutting et al., 1990). During cleavage, the maternal factors move into the nuclear compartments, as shown by confocal imaging for SpOtx-1 (Chuang et al., 1996) and SpCoup-1 (C. Flytzanis, personal communication), or by direct measurements on nuclear and cytoplasmic fractions, e.g., SpP3A2 (Zeller et al., 1995a).

There is yet no direct evidence for territorially confined covalent modification of maternal transcription factors in sea urchin embryos, but covalent modification seems to be their general property. Every one of nine different embryo transcription factors chosen only because immunological probes were available were shown to exist in multiple forms, mainly due to diverse states of phosphorylation (Harrington et al., 1997). In three cases examined further, the distribution of the variants changes greatly between fertilization and blastula stage. Exploration of such modifications in given territories of the cleavage-stage embryo, particularly in respect to maternal transcription factors or their cofactors that respond as immediate-early targets during specification, seems more than ever an important research objective.

Regulatory requirements in early sea urchin embryogenesis

As defined earlier, the sea urchin develops by a typical 'type 1' embryonic process such as is widespread among invertebrates (Davidson, 1991). Several properties of this form of embryogenesis are relevant to the present topic. In type 1 embryogenesis, the embryo genomes are activated at once (there is no 'mid-blastula transition'). Specification of many blastomeres occurs in situ during cleavage, in advance of any cell migration, either by autonomous processes or by short-range signaling across the cleavage planes. Even before cleavage ends, specification results in differential expression of genes encoding not only zygotic transcription factors but also terminal differentiation products, though morphologically differentiated structures will not arise until much later. Examples of genes encoding differentiation products discussed in this review, all of which begin to be expressed during mid-late cleavage, include the *SM50* gene activated in the skeletogenic lineage, the *CyIIIa* cytoskeletal actin gene and the *Spec* Ca^{2+} binding genes activated in the aboral ectoderm, and the *Endo16* gene, activated initially in blastomeres of the veg_2 lineage component. Evidence so far suggests that the regulatory organization controlling the activation of the initial sets of downstream genes exemplified by these may be relatively simple, as indicated in Fig. 6. The salient feature of this diagram is its shallowness: it lacks stacked cascades of genes encoding transcription factors which then activate other genes controlling other more downstream transcription factors, and then finally the differentiation genes. There is only one level of zygotically expressed genes encoding transcription factors in Fig. 6, those that initially respond to maternal transcription factors upon their regional presentation (though not shown, this network would also include 'horizontal' transcriptional interactions, direct or indirect). Fig. 6 may well be an oversimplification. However, this is the organization implied by the observations noted above that all of the transcription factors binding in the *cis*-regulatory system of the terminal *CyIIIa* differentiation gene (Kirchhamer et al., 1996; Calzone et al., 1997) are initially present maternally. The maternal factors may simply turn on their own genes zygotically, or territory-specific gene batteries may be set up by them, or both, but there is so far no hint of a deep regulatory cascade overlying any of the downstream genes here considered.

Powerful and detailed structure/function analyses of *cis*-regulatory systems that function in early development are now appearing, mainly from sea urchin and *Drosophila* embryos (reviewed by Arnone and Davidson, 1997). A well-supported set of properties is emerging from these studies that indicate the general character of the *cis*-regulatory elements that 'interpret' embryonic specification functions. We now know that the basic rules of functional *cis*-regulatory organization for systems that integrate spatial regulation information are similar, whether these are meant to operate in the syncytial *Drosophila* embryo or the cellular sea urchin embryo. *Cis*-regulatory elements that execute spatial specification functions must process multiple inputs. They must integrate positive and negative interactions, respond to signals and lineage-specific and temporal inputs, perform switch functions, and so forth. Further discussion lies beyond the scope of this paper (see Arnone and Davidson, 1997; Yuh et al., 1998), except to stress that these kinds of *cis*-regulatory specification systems are

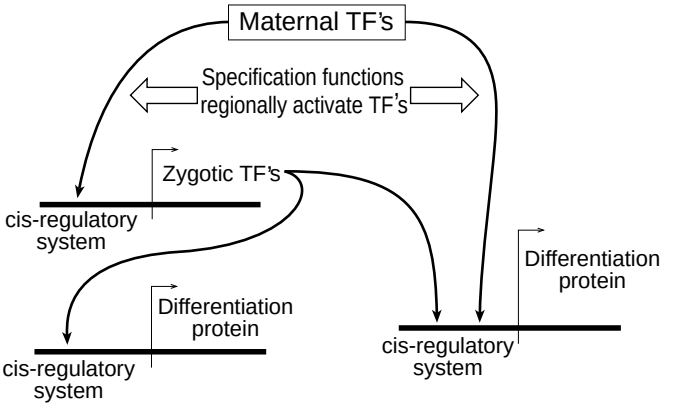

Fig. 6. A simplified view of the shallow depth of the gene regulatory network controlling transcriptional activity in the early sea urchin embryo. It is unlikely that there are many 'layers' (or internal nodes) in the network since even genes at the termini of the network, such as the aboral ectoderm-specific *CyIIIa* cytoskeletal actin gene, initially utilize factors of maternal origin. Thus, the diagram shows only one level of genes encoding transcription factors that are expressed zygotically. This diagram is of course intended to be relevant only to the early stages of specification and the initial tiers of differential gene expression; later on, as new cell types and structures arise under the influence of new signaling interactions the regulatory network in use must expand.

likely pan-metazoan (or at least pan-bilaterial metazoan). Their structural design provides the genetic conditions for embryonic specification mechanisms. Since they operate in the basic processes of type 1 embryogenesis, the evolutionary advent of such *cis*-regulatory systems may have corresponded with the advent of this relatively simple form of embryogenesis itself.

Temporal interrelations

In order to place the many different regulatory and developmental processes discussed in this essay in temporal context, we have mapped them against developmental time (i.e., starting with fertilization) in Fig. 7. At the bottom of this figure are all the specification events by which the fate map of Fig. 1 is realized. This phase has mainly occurred by gastrulation and it is followed by morphogenetic events not discussed in this essay, and later by postembryonic development, as briefly considered in the following. A key feature of the diagram is the long persistence of the primordial A/V 'identity' system, i.e., of the continuing influence on transcriptional readout that we have inferred. In Fig. 7, this refers only to endomesodermal specification, and there may be other aspects of this primordial spatial control system of which we have yet to learn.

POSTEMBRYONIC DEVELOPMENT OF THE RUDIMENT

An entirely different set of processes underlies the development of the adult body plan in the imaginal rudiment. Fig. 8 traces the diversification and expression of the coelomic mesoderm, as the construction of the adult body plan begins within the imaginal rudiment. At the end of embryogenesis, the coelomic anlagen consist of about ten cells each. The embryo as a whole had yet to undergo any growth, since feeding can be initiated only at the end of embryogenesis with the development of the ciliary band, the innervated oral apparatus and the muscular sphincters of the gut. From this point of view can be seen the advantage of

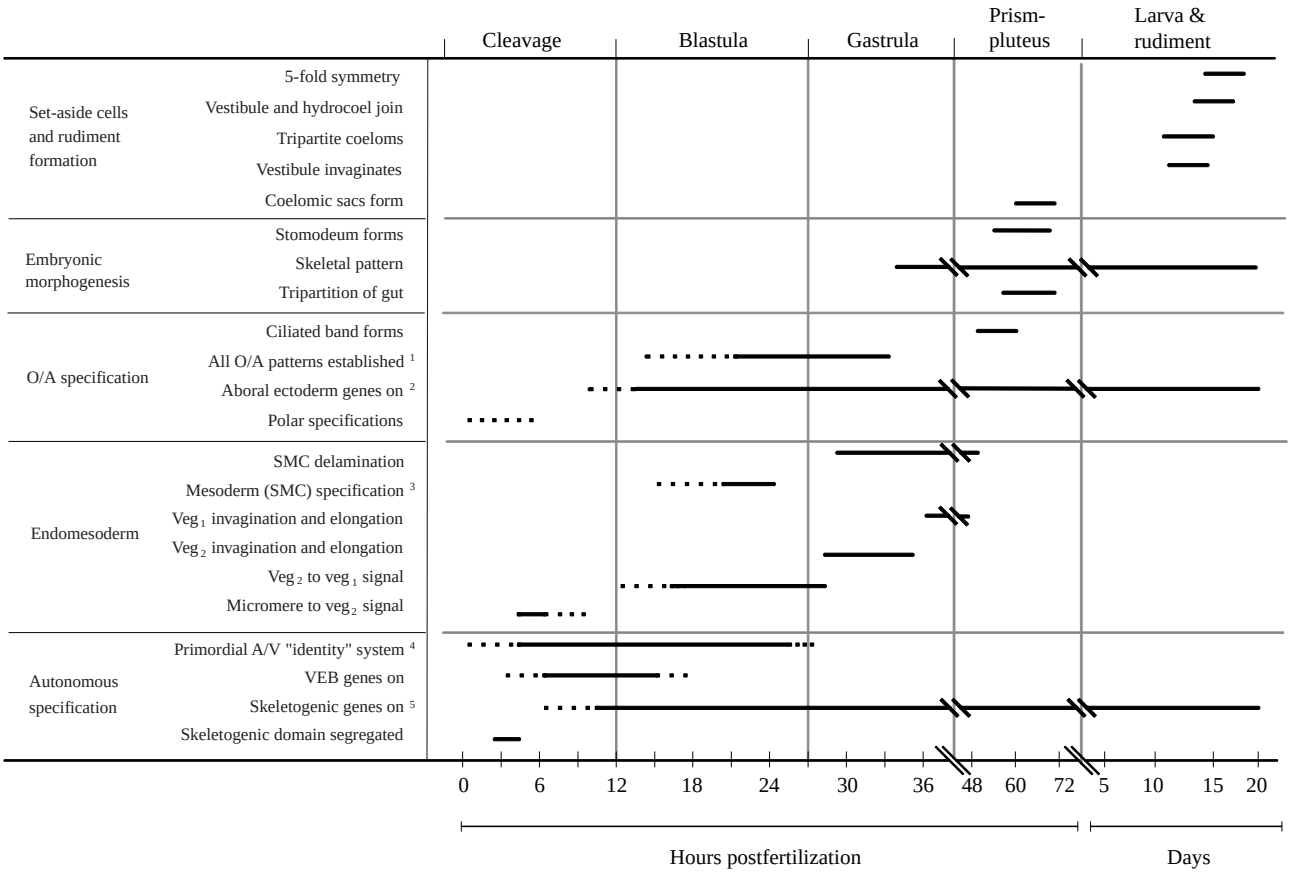

Fig. 7. Temporal interrelations of developmental processes discussed in text. The time scale is for *S. purpuratus* at 15°C. Dots represent uncertainty or heterogeneity when more than one marker is being considered.
[1]This refers to definitive oral ectoderm expression of EctoV in presumptive oral blastomeres and of Spec1, Spec2a and arylsulfatase in presumptive aboral ectoderm; *CyIIIa* expression is aboral ab initio (see text for details and references).
[2]Initial timing from Hickey et al. (1987) and Lee et al. (1992); run-on data for *CyIIIa*.
[3]The initial SMCs to delaminate are pigment cells, followed during gastrulation by blastocoelar cells, coelomic pouch cells and presumptive muscle cells (see text). Estimated for *S. purpuratus* from *L. variegatus* data of Ruffins and Ettensohn (1993, 1996) and Sherwood and McClay (1997).
[4]As discussed in text; see Fig. 5.
[5]Measurements of Killian and Wilt (1989) on *SM50* transcripts.

confining set-aside cell populations such as those of the coelomic sacs to the smallest possible number of cells. At the end of embryogenesis in *S. purpuratus* there are about 1500 cells, and just before metamorphosis when the juvenile sea urchin is ready to emerge, about 150,000 (Cameron et al., 1989b). Over 90% of these are in structures that will be included in the juvenile. Thus the specifically larval tissue is the product of only two to three further rounds of division, on the average, after the end of embryogenesis. In contrast, the cell populations that form the rudiment expand dramatically. This can be seen most clearly in the fate of the mesodermal components descendant from the coelomic sacs. As shown in Fig. 8A, these give rise to bilateral tripartite coeloms, which grow up along the esophagus, down over the sides of the midgut and transversely. The vestibular invagination (Fig. 8A) expands greatly and, as it meets the middle coelom on the left side, it forms a bilayered disc composed of ectoderm and mesoderm. A complex process of morphogenesis ensues, the course of which need not concern us here (Hyman, 1955).

Most of the major phylotypic echinoderm features of the ventral portion of the adult body plan form from derivatives of the vestibular floor, the middle and posterior coeloms on the left side and numerous mesenchyme cells of unknown origin (Fig. 8B). These features include the radially symmetric water vascular system, the circumoral and the five radial ganglia of the echinoid CNS, the five initial podia, the five large tooth sacs, the central mouth, the five initial sets of spines and the ventral portions of the endoskeletal test. The dorsal portion of the body plan, including the genital plates, arises from elements of the right coeloms and test-forming constituents. The larval midgut is retained but the esophagus and hindgut are resorbed and rebuilt after metamorphosis. The gonads appear much later (Houk and Hinegardner, 1980); the germ cells are among the coelomic sac derivatives, but it has been excluded that they derive uniquely from the descendants of the small micromeres since sea urchins raised from embryos lacking micromeres are normal with respect to fertility (Ransick et al., 1996). At least some of the cells of coelomic sacs thus have remained entirely unspecified as embryogenesis terminates.

Even from an external point of view, it is obvious that developmental mechanisms are required for adult body plan formation that differ from those underlying embryogenesis. The spatial coordinates of territorial specification in the embryo are determined by the cytoarchitectural coordinates of the egg, as we have seen in some detail, and some of the prominent early specifications occur within and with reference to given components of the canonical cell lineages. None of these mechanisms are available in rudiment development. The product of embryogenesis is in fact very different from that of postembryonic development in much more than scale. Thus the completed embryo is composed entirely of single cell layers plus some mesenchymal cells, as illustrated in Fig. 2D. For example, the oral and aboral ectoderm and the gut are all only one cell thick while, as Fig. 8 shows, the rudiment develops as a series of apposed tissue layers, beginning with the vestibule (green)/hydrocoel (blue) apposition, which then generates additional layers by infolding and growth, and is further underlain by extensions of the somatocoel (orange). In addition, the embryo/larva has only a small number of thinly spaced nerve cells, while the adult rudiment develops the dense ganglia of its radial CNS (purple). The mesoderm of the embryo proper consists exclusively of three mesenchymal cell types plus a few sphincter muscle cells, while that of the rudiment gives rise to a much greater number of differentiated cell types and, most importantly, forms large three-dimensional structures such as the water vascular system, the tube feet and the tooth sacs, as well as coelomic linings.

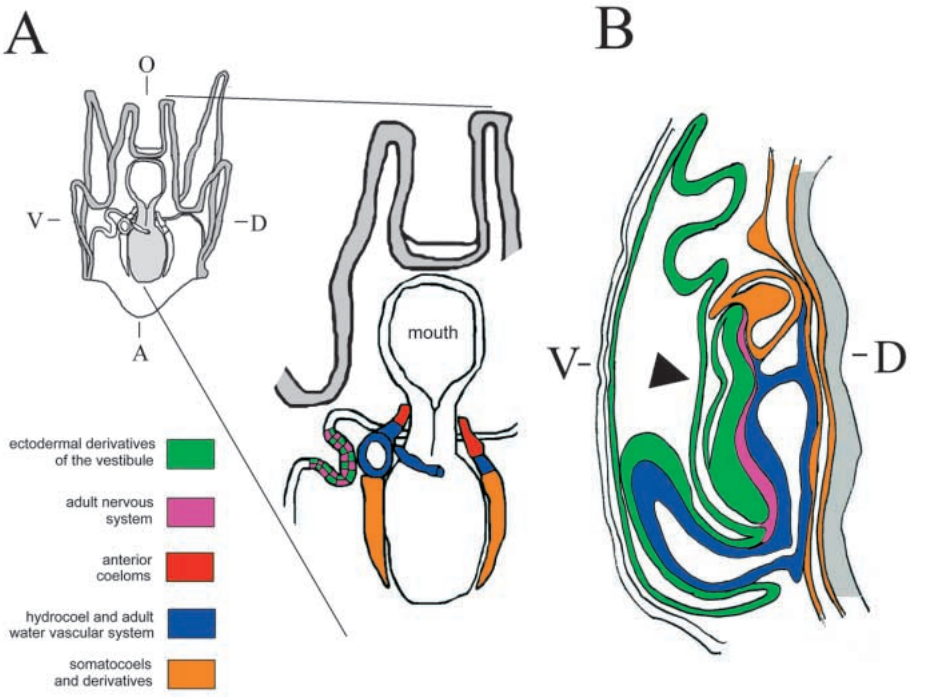

Fig. 8. Schematic representations of sea urchin larval and rudiment anatomy. The drawings emphasize the primordia of adult body plan structures color coded as indicated. (A) 2-week-old larval structures depicted in outline form. Inset, top left, shows the whole larva. The gut and ciliated band are shaded gray. The adult axes (D, dorsal and V, ventral) are shown together with the larval axes (O, oral, A, aboral). The enlarged portion of the figure shows only the larval mouth and stomach; the structures from which the rudiment will derive are the vestibule and coelom, as indicated. The coelom has divided into three parts: the anterior coelom (pink), middle coelom or hydrocoel (blue) and the posterior or somatocoel (orange). The green and purple checks indicates that the vestibular floor will give rise to both ectodermal and CNS derivatives. (B) A side view of the rudiment at about 4 weeks of development. The adult axes are indicated. The floor of the vestibule has proliferated, folded and fused to form several layers on the adult ventral surface. One of the five podia, which are elements of the water vascular system, is shown in the section. The podia develop from hydrocoelar (blue) and vestibular (green) precursors. The somatocoelar structures are a tooth sac and coelomic linings (orange). The CNS (purple) will consist of a circumoral nerve ring and five radial nerves (redrawn from von Ubisch, 1913; MacBride, 1903; Hyman, 1955).

Furthermore, excluding the set-aside cells, the embryo/larva is essentially eutelic; its cells divide only a set number of times and only a few times after cleavage has ended. For example, the whole archenteron is formed at gastrulation by invagination without further cell division (Hardin and Cheng, 1986; Hardin, 1989). In contrast the major phenomenon of rudiment development is the enormous expansion of undifferentiated cell layers such as those of the coeloms and the vestibule, and their specification, ab initio, during growth. This is the process that the mechanisms of type 1 embryogenesis do not encompass, i.e., specification of fields of growing, undifferentiated cells, without reference to lineage or primordial position in respect to the axes of the egg.

As yet we know little of the additional genetic elements called into play during rudiment development. In general, cell-type-specific genes activated during embryogenesis continue to function in the equivalent cells of the larva, e.g., *Spec1* and *CyIIIa* in both the embryo and larval aboral ectoderm, *SM30* in the embryonic and larval skeletogenic cells, and *Endo16* and *CyIIa* in the embryo and larval midgut (Arnone et al., 1998; A. Cameron, unpublished observations). It is interesting that *CyIIIa* is expressed exclusively in the embryo/larval phase of existence and, following collapse of the aboral ectoderm at metamorphosis, this gene is not utilized again (Shott et al., 1984; Cameron et al., 1989b). The embryo/larva is an independent developmental entity that is capable of complete development and, if fed, of a prolonged, free-living existence in the total absence of any rudiment (Davidson et al., 1995). The key issue is of course the nature of the regulatory apparatus utilized in rudiment development. *Distalless*, a transcription factor involved in many pattern formation processes in other organisms, is expressed in the vestibule during its invagination (Lowe and Wray, 1997), and another such gene, *Brachyury*, is also expressed in the rudiment (K. Peterson and R. A. Cameron, unpublished data). A very interesting recent observation highlights the qualitatively different modes of gene regulatory processes in the rudiment compared to those controlling embryogenesis. This concerns utilization of the Hox gene cluster of *S. purpuratus* (C. Arenas, P. Martinez, R. A. Cameron and E. H. Davidson, unpublished data). All of the genes of the single *Hox* gene cluster (Martinez et al., 1997; P. Martinez and E. H. Davidson, unpublished data) are transcribed once the rudiment begins to form, but only two of them, a *Hox4* and a *Hox6* gene, are expressed significantly in the whole process of embryonic development, up to and including formation of a free-living, feeding larva. The general role of genes such as these in the development of metazoan body plans is the specification of regional identity in growing cell populations with respect to the future parts of the organism. As predicted (Davidson et al., 1995), experimental observation now shows that this regulatory function is evidently required for the process of development shown in Fig. 8 of this paper, but not for the process shown in Fig. 2.

Sea urchin embryogenesis is driven by an elegant and evolutionarily ancient set of genetic regulatory mechanisms, but in themselves these have a relatively limited outcome, which does not approach what is needed for generation of any modern adult body plan. The comparative simplicity of these regulatory mechanisms is of course a powerful attractant, for they appear directly accessible to causal experimental analysis.

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