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Lithium evokes expression of vegetal-specific molecules in the animal blastomeres of sea urchin embryos

(blastomere separation/alkaline phosphatase/spicule matrix protein RNA)

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ABSTRACT **The mechanism of determination of early embryonic cells has been investigated using sea urchin embryos. An efficacious method of isolating blastomere pairs from the animal or vegetal half of sea urchin embryos was developed. The overt differentiation of separated animal and vegetal blastomere pairs resembles that of separated animal and vegetal hemispheres isolated by manual dissection. Treatment of animal blastomeres with LiCl caused them to display a morphology resembling that of isolated vegetal blastomeres. The effects of separation of animal and vegetal blastomeres and of treatment of animal blastomeres with LiCl were examined at the molecular level using gut alkaline phosphatase and a spicule matrix protein RNA as markers of differentiation. Histochemical staining and *in situ* hybridization studies showed that these markers are normally only expressed in vegetal blastomeres but that their expression can be evoked in animal blastomeres by treatment with LiCl.**

The mechanisms of determination of early embryonic cells are a problem of fundamental importance in biology. Classical studies have shown that the animal and vegetal halves of very early sea urchin embryos are already determined; when separated, the two halves follow widely divergent paths of differentiation. Separated animal halves form hollow epithelial spheres with exaggerated cilia and little overt differentiation. Vegetal halves are able to form more normal embryos with archenterons, skeletal spicules, and pigment cells, which will occasionally go on to form a rather normal pluteus larvae (for reviews, see refs. 1–3).

Treatment of embryos with a variety of agents, including LiCl, results in a pattern of differentiation in which archenterons are exaggerated (and/or exogastrulated), and the surface epithelium is reduced. Such embryos are said to be "vegetalized" (4). Von Ubisch (5) reported in 1929 that treatment of the separated animal hemisphere with LiCl resulted in the subsequent formation of guts and spicules by these blastomeres. This result suggests that the capability to form vegetal structures also resides in the animal blastomeres, but in a suppressed or inactive form, and that LiCl may somehow allow vegetal structures to form in blastomeres that have an otherwise quite different determination and fate.

LiCl has also been shown to affect cell fate and pattern formation in amphibians (6, 7), suggesting that it alters a cellular process important in determination of cell fate in developing systems. Although LiCl may affect a variety of cellular processes, recent studies showing that LiCl has specific effects on secondary-messenger pathways in several different cell types (8–10) have stimulated our interest in perturbations caused by lithium.

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We have devised a simple method to study isolated blastomeres in culture that allows one to study the mechanisms of cell determination. The old finding that LiCl may respecify blastomere fate has been confirmed. In addition, we have shown that LiCl not only elicits vegetal structures in animal blastomeres, but also causes the appearance of molecular markers that serve known differentiated functions specific to vegetal cell lineages.

The first marker used is alkaline phosphatase, a gut-specific enzyme expressed in cells derived from the macromere lineage in normal development (11). The second marker, spicule matrix protein (SM50) RNA, is a transcript encoding a major protein component of the skeletal spicule of larvae (12, 13), derived solely from micromeres. Use of these molecular markers and the new culture system allows quantitative study of determination and its respecification by LiCl in sea urchin blastomeres.

MATERIALS AND METHODS

Isolation and Culture of Blastomeres. Gametes of *Strongylocentrotus purpuratus* were obtained, and eggs were fertilized and cultured by conventional methods (14–16). At the second cleavage division, the embryos were settled two times, resuspended in Ca^{2+}-free sea water and cultured with stirring to the 16-cell stage. At this time the embryos were centrifuged and resuspended in Ca^{2+}- and Mg^{2+}-free sea water; this was repeated until the majority of the blastomeres were dissociated into pairs. The disaggregated embryos were then placed in ice-cold Ca^{2+}-free sea water in a Petri dish that contained a layer of 1% agarose in sea water. The blastomere pairs were isolated using a micropipette and cultured on agarose in Jamarin sea water (Jamarin Laboratory, Osaka, Japan) with or without 20 mM LiCl. Those blastomeres cultured with LiCl were transferred after 6–8 hr to Jamarin sea water without LiCl.

Morphological Characteristics. After 3 days in culture at 15°C, embryoids were examined for the appearance of gut-like invaginations and spicules. Invaginations could be seen under a dissecting microscope and were scored as positive when an organized, hollow group of cells was present in the blastocoel cavity or had evaginated as a group. Spicules could be observed in embryoids flattened with a coverslip by use of the 10× objective of a differential interference contrast microscope.

Alkaline Phosphatase Activity. Embryoids cultured for 4 days were placed in a drop of sea water on a microscope slide. A drop of fast blue RR salt (4-benzoylamino-2,5-dimethoxybenzene-diazonium chloride hemi[zinc chloride] salt) in 2.5% (wt/vol) naphthol AS-MX phosphate (Sigma) was added, and the slides were incubated 15 min in a moist chamber at 37°C. When levamisole was used, it was added to the drop of sea water (final concentration = 10 mM) 5 min before addition of substrate.

Abbreviation: SM50, spicule matrix protein RNA.

Probe Preparation. The SM50 probe is derived from a 1.3-kb sequence that was isolated and characterized by Sucov *et al.* (12) and Benson *et al.* (13) and subcloned into the pSP65 transcription vector (17). Sp6 polymerase (Promega Biotec) and [^{3}H]UTP were used to synthesize ^{3}H-labeled single-stranded RNA probe according to Melton *et al.* (17).

***In Situ* Hybridization.** Embryoids were placed in a drop of Jamarin sea water on a poly(L-lysine)-coated slide and allowed to settle 10 min. A coverslip was then used to squash the embryoids, as described for intact embryos by Hough–Evans *et al.* (18). The slides were then fixed in glutaraldehyde by the method of Cox *et al.* (19). The protease treatment, hybridization steps, RNase A digestion, and autoradiography were all performed according to Cox *et al.* (19). NTB (Kodak) emulsion-coated slides were exposed at 4°C for 10–14 days before development.

RESULTS

Separation of Blastomeres. The near total removal of Ca^{2+} from embryo cultures during the second cell division, combined with the modest shear of stirring, results in the dissociation of these embryos into groups of two to eight blastomeres by the time of the fourth cell division. Washing such partially dissociated embryos once in Ca^{2+}- and Mg^{2+}-free sea water and then once in Ca^{2+}-free sea water will produce cell pairs of recently divided blastomeres—members of the pairs being held together by a mid-body connection. Pairs of equal-sized mesomeres can then be distinguished from macromere/micromere pairs, and the different types of pairs can be removed and cultured separately (Fig. 1).

The synchrony of the cell divisions and the location of the third cleavage plane in these cultures is crucial to the success of these techniques. Asynchronous cultures resulted in an inability to distinguish 16-cell-stage mesomere pairs from 32-cell macromere pairs. For this same reason embryos must be dissociated shortly after the fourth cell division, kept at 4°C, and isolated before a subsequent cell division can occur. The third cleavage plane delineates the boundary between the animal and vegetal halves of the embryo, thus separating two areas of widely divergent developmental potentials. Displacement of the third cleavage plane towards the vegetal pole occurs in some cultures and results in animal blastomeres with a greatly increased tendency to form vegetal structures, a result first observed by Driesch (20). In the experiments reported here, only synchronous cultures that exhibit an equatorial third cleavage plane were used for blastomere separations.

Overt Differentiation of Cultures. The development of mesomeres with and without LiCl treatment and of untreated macromere/micromere pairs was observed during 3 days of culture. After separation all of the blastomeres divide in apparently normal fashion, and after one-day culture all blastomere types form an embryoid resembling an early blastula, with a small blastocoel cavity (Fig. 1 *D–F*). In some cases the cavity failed to expand, and the embryoids remained small and thick-walled, although this was not the usual case. Untreated mesomeres formed thin-walled, hollow, ciliated embryoids without spicules or gut (Fig. 1*G*). The mesomere isolates often had some larger cells embedded in the wall and sometimes show a thickened area at one pole. The blastocoel in the macromere/micromere pairs did not expand to the extent seen in mesomere pairs. However, invagination occurred, and bipartite and tripartite guts were formed. Skeletal spicules were also often present (Fig. 1*I*). These embryoids from macromere/micromere pairs were ciliated, but the cilia were reduced in size compared with mesomeres, and the embryoids were less motile. Mesomeres treated with LiCl formed embryoids that ranged in appearance from that of untreated blastomeres to that of macromere/micromere pairs (Fig. 1*H*). Although some LiCl-treated mesomeres formed hollow ciliated balls, these mesomeres usually invaginated to form structures ranging from short internal cell masses to hollow bipartite and tripartite gut-like structures. Spicules were also formed in many of the embryoids from LiCl-treated mesomere cultures, and their appearance seemed independent of the appearance of invaginations. The length of cilia and motility of the embryoids varied, with the more vegetalized embryoids generally being less motile.

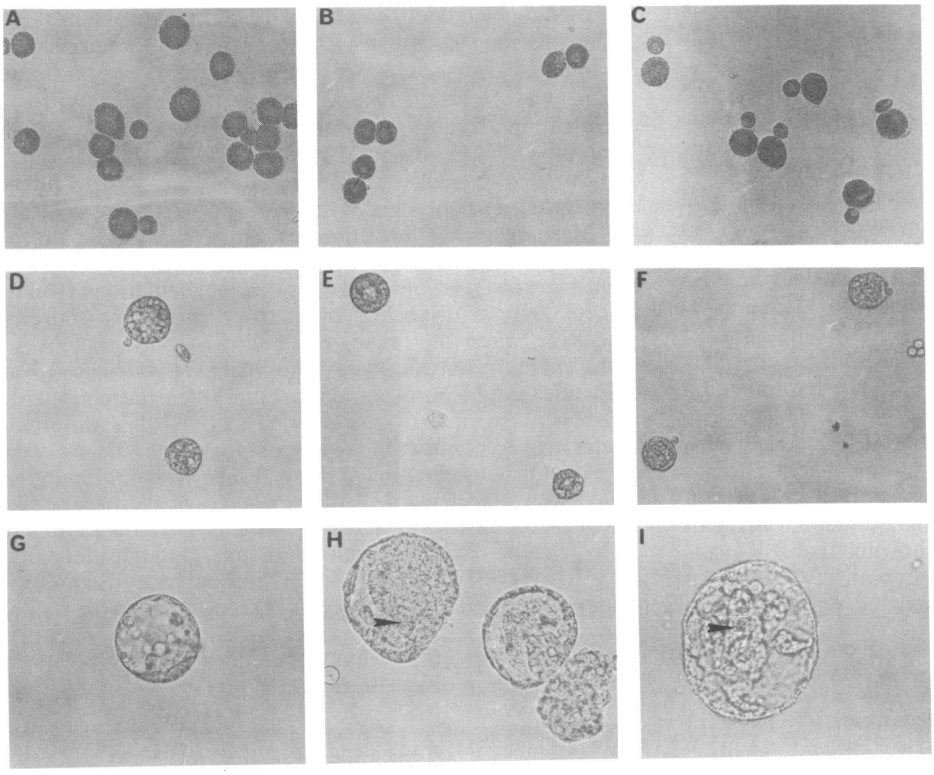

FIG. 1. Photographs showing overt differentiation of cultures. (*A*) Partially dissociated culture of 16-cell-stage embryos, (*B*) isolated mesomere pairs, (*C*) isolated macromere/micromere pairs. Embryoids derived from the following sources are shown: mesomeres cultured 1 day (*D*); mesomeres exposed to LiCl for 7 hr, then cultured 1 day (*E*); macromere/micromere pairs cultured 1 day (*F*); mesomeres cultured 3 days (*G*); mesomeres exposed to LiCl for 7 hr and then cultured for 3 days (*H*); and macromere/micromere pairs cultured 3 days (*I*). (*A–F*, ×75; *G–I*, ×150.) Arrowheads, spicules.

Nomarski differential interference microscopy was used to confirm the presence of calcified skeletal elements in these embryoids. Mesomeres treated with LiCl and macromere/micromere pairs formed birefringent skeletal rods, whereas untreated mesomeres did not (Fig. 2).

Quantitative Summary of Cultures. Twelve separate experiments using different females were scored for the appearance of gut-like invaginations and spicules (Fig. 3). Over 80% of the macromere/micromere pairs gave rise to invaginations and spicules. Untreated mesomeres gave rise to embryoids with <6% showing spicules or invaginations. Half of the experiments using untreated mesomeres gave rise to cultures showing no vegetal characteristics whatsoever, whereas five of the remaining six experiments gave rise to cultures showing between 3% and 12%. One experiment using untreated mesomeres, with only 12 embryoids scored, gave rise to a culture showing 30% vegetal characteristics with gut-like invaginations and spicules. LiCl treatment resulted in >50% of the mesomeres producing invaginations and spicules. In all individual cultures, treatment with LiCl substantially increased the amount of vegetal differentiation as compared to untreated controls. Recent experiments with another species, *Lytechinus pictus*, exhibit an even higher percentage of embryoids with vegetal characteristics from higher doses of LiCl (data not shown).

A difference in the viability of the different cultures can also be seen (data not shown). Approximately 65% of untreated mesomere pairs survived 3 days in culture. Treatment with LiCl reduced this number to ≈50%, indicating a slight toxic effect of LiCl. A much lower percentage (20%) of isolated macromere/micromere pairs survived. This reduced viability of isolated vegetal blastomeres was noted by Driesch (20), but the reasons for it are unknown. We have observed it routinely with blastomeres of both *S. purpuratus* and *L. pictus*.

Alkaline Phosphatase Staining. Alkaline phosphatase activity can first be detected in the archenteron of the gastrula, and it continues to accumulate and be expressed in the pluteus larvae (21–24) (Fig. 4*E*). This enzyme is unusual in that the inhibitor levamisole, used as an inhibitor of nonintestinal alkaline phosphatase in some other organisms (25), is a potent specific inhibitor of the activity in sea urchins (Steve Benson, personal communication) (Fig. 4*F*). The treated and untreated embryoids that developed from isolated blastomeres were tested for alkaline phosphatase activity to determine whether any correlation exists between morphological indi-

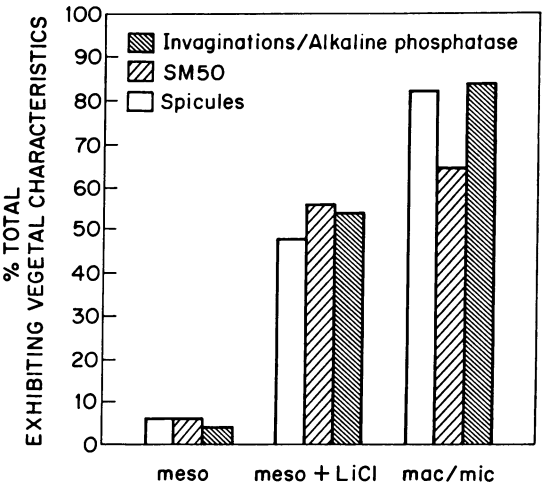

FIG. 3. Quantitative summary of blastomere cultures. Bar graphs show the proportion of embryoids from untreated mesomeres (meso), mesomeres treated with LiCl (meso + LiCl), and macromere/micromere pairs (mac/mic) that exhibited invaginations or alkaline phosphatase activity, spicules, and SM50 expression. The number of embryoids examined for invaginations and spicule is as follows: meso, 240; meso plus LiCl, 140; and mac/mic, 90. All embryoids with an invagination stained positively for alkaline phosphatase activity. Separate experiments were used to determine levels of SM50 expression. The number of embryoids used was as follows: meso, 27; meso plus LiCl, 28; and mac/mic, 10.

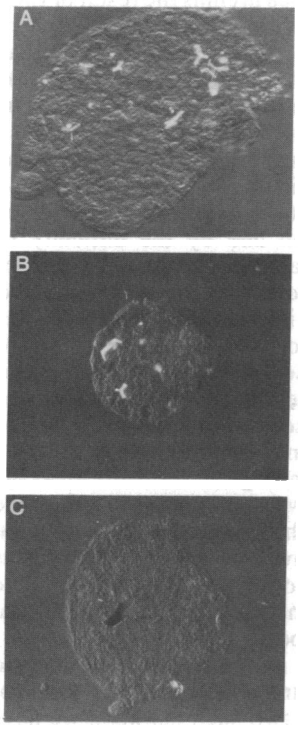

FIG. 2. Embryoids viewed with Nomarski optics. Photographs taken with Nomarski differential interference microscopy. Embryoids 3 days of age derived from macromere/micromere pairs (*A*), mesomeres treated with LiCl (*B*), and untreated mesomeres (*C*). (×125.)

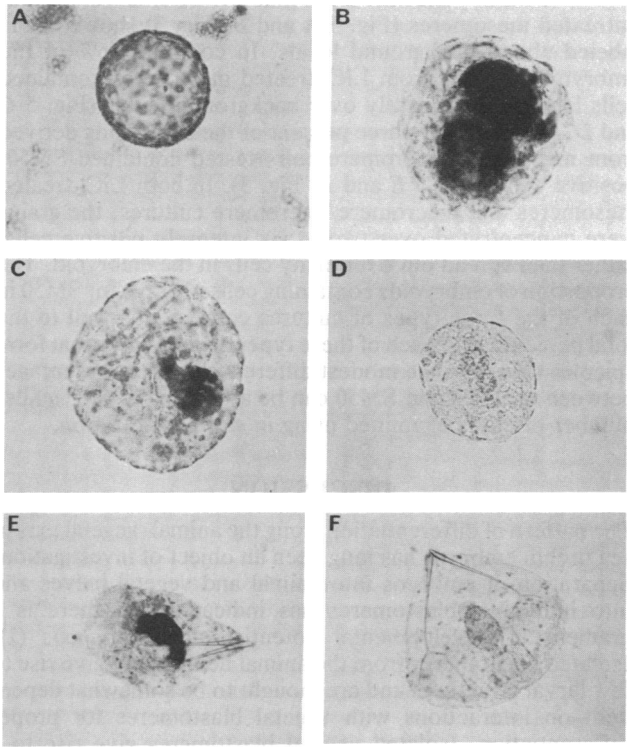

FIG. 4. Embryoids stained for alkaline phosphatase activity. Photographs of 4-day-old embryoids stained for alkaline phosphatase activity from untreated mesomeres (*A*), macromere/micromere pairs (*B*), mesomeres treated with LiCl (*C*), and control pluteus larva (*E*). The effects of preincubation with levamisole are shown for mesomeres treated with LiCl (*D*) and for control embryos (*F*). (*A–D*, ×150; *E–F*, ×75.)

cations of differentiation (invagination) and appearance of a gut-specific enzyme. After 4-day culture, staining for alkaline phosphatase activity was detected in the invaginated cells of LiCl-treated embryoids (Fig. 4*C*) and embryoids from macromere/micromere pairs (Fig. 4*B*), but not in embryoids from untreated mesomeres (Fig. 4*A*). The appearance of enzyme activity was not dependent on the extent of morphological differentiation of the archenteron. Small invaginations stained for activity, as did bipartite and tripartite guts; however, the larger, more developed guts stained much darker than did smaller invaginations.

The inhibitor levamisole was used to determine the specificity of the staining observed. Levamisole inhibited all alkaline phosphatase activity in LiCl-treated embryos (Fig. 4*D*) and macromere/micromere pairs (data not shown), as well as in normal control pluteus larva (Fig. 4*F*).

Expression of a Primary Mesenchyme-Specific Gene. We have examined the ability of LiCl to evoke the expression in animal blastomeres of genes that are only expressed during normal development in the descendants of the well-studied micromere lineage of the vegetal hemisphere. The SM50 gene, specific for primary mesenchyme, was chosen for study. SM50 encodes a 50-kDa protein that is a matrix glycoprotein of the endoskeletal spicule of the sea urchin larvae. It is expressed only in primary mesenchyme cells (which arise from micromeres), the four small cells formed at the vegetal pole at the fourth cleavage division, and is, therefore, an ideal marker for vegetal lineage-specific gene expression (12, 13).

SM50 antisense RNA transcripts were hybridized *in situ* to embryoids squashed onto poly(L-lysine)-coated slides. The use of squashed cells produces somewhat higher nonspecific background than sectioned material, but the background with antisense probe was identical to that found with control sense probes (data not shown). Less than 6% of embryoids from untreated mesomeres (Fig. 5 *A* and *B*, Fig. 3) showed cells labeled above background levels. In contrast, 56% of the embryoids formed from LiCl-treated mesomeres contained cells labeled significantly over background levels (Fig. 5 *C* and *D*, Fig. 3). Sixty-three percent of the embryoids derived from macromere/micromere pairs tested contained SM50-positive cells (Fig. 5 *E* and *F*, Fig. 3). In both LiCl-treated mesomeres and macromere/micromere cultures, the grains were concentrated over two to six intensely positive cells, rather than spread out over many cells in the embryoid. The proportion of embryoids containing cells positive for SM50 in each of the three types of cultures corresponds well to the total percentage of each of these types of embryoids that form spicules (Fig. 3). The modest differences in the percentages between spicules and SM50 can be attributed to the smaller number of cases examined using *in situ* hybridization.

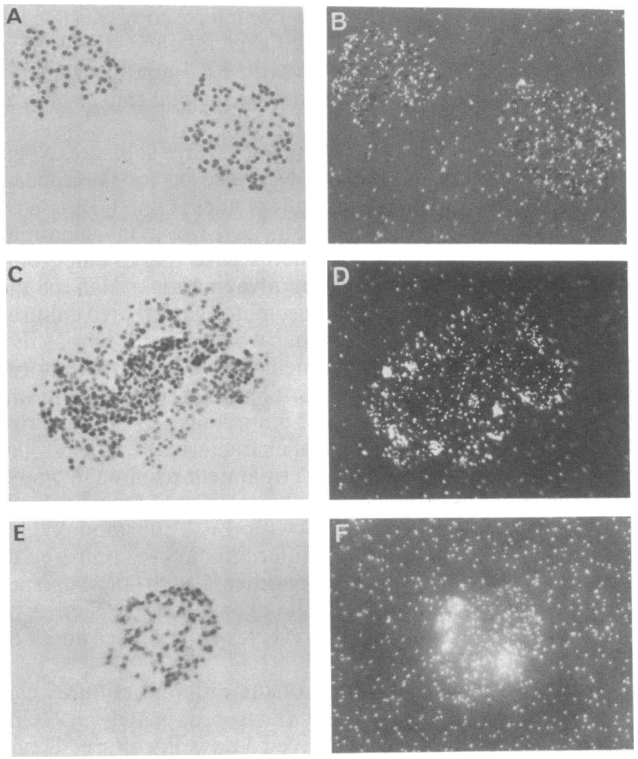

FIG. 5. Expression of a gene encoding a spicule matrix protein. *In situ* hybridization to 3-day-old embryoids using ^{3}H-labeled SM50 probe shown in bright-field (*A*, *C*, and *E*) and dark-field (*B*, *D*, and *F*) micrographs. (*A* and *B*) Untreated mesomeres; (*C* and *D*) mesomeres treated with LiCl; and (*E* and *F*) macromere/micromere pairs. (×150.) Background levels were determined with a sense-strand probe and gave results equal to levels from untreated mesomeres (*A* and *B*).

DISCUSSION

The pattern of differentiation along the animal–vegetal axis in sea urchin embryos has long been an object of investigation. Separation of embryos into animal and vegetal halves and into individual blastomeres has indicated that there is a gradient of developmental potential along this axis (1). Isolated blastomeres from the animal hemisphere give rise to few larval structures and are thought to be somewhat dependent on interactions with vegetal blastomeres for proper differentiation. Isolated vegetal blastomeres give rise to a number of larval structures and are more autonomous in their ability to differentiate. It is this proposed gradient of developmental potential that formed much of the basis for current ideas about determination.

The normal animal–vegetal pattern of differentiation is not irreversibly fixed, however, and can be altered by a variety of agents. One of the most potent of the "vegetalizing" agents is LiCl. Although LiCl may have sites of action yet to be determined, in recent years LiCl has been shown to block inositol phosphate metabolism (8) and to interact with G proteins in membranes (10). One action of LiCl, then, seems to be to interfere with signaling at the cell membrane via second-messenger pathways. In sea urchins the result of LiCl treatment is to cause an exaggeration of vegetal characteristics in developing embryos. It was also reported in 1929 that LiCl induced vegetal structures in isolated animal halves (5); this observation was also made in 1936 by Horstadius (26) but has not been studied quantitatively using modern methods.

Previous studies using separation of blastomeres and treatment with LiCl have been limited by their sole reliance on qualitative morphological criteria to assess the degree of animal or vegetal differentiation and by the difficulty in obtaining large amounts of isolated blastomeres by dissection. We have developed a method of obtaining isolated animal and vegetal blastomeres in relatively large quantities and have shown that the development of these blastomere pairs closely resembles that of separated animal and vegetal halves. The 4–6% level of vegetal differentiation found in embryoids derived from untreated mesomeres could be due to several factors: contamination of cultures during isolation with macromeres that have undergone the fifth cleavage division, the presence of a few large mesomeres in some cultures due to an unequal third cleavage division, the possibility of a few cultures derived from "vegetal eggs," as described by Horstadius (1), or even loss of a putative regulative influence exerted by homotypic cells in the animal cap when blastomeres are dissociated.

The recent development of an array of tissue-specific molecular markers now allows unambiguous identification of differentiated cell types. Using two such markers, we have

shown that LiCl treatment of isolated animal blastomeres can induce the appearance of enzyme activity and the accumulation of a transcript normally found only in cells of vegetal lineage.

A low level of alkaline phosphatase activity can be found in homogenates of unfertilized eggs and cleavage-stage embryos (21, 23). An increase in the activity of embryo homogenates is seen at mesenchyme blastula, and this increase continues through late pluteus stage. The increase has been shown to be due to the appearance of a new species of active enzyme at mesenchyme blastula (23). Histochemical studies (24) have shown that the enzyme activity is localized in the cells lining the lumen of the archenteron, which is derived from the macromere lineage (11), and is first detectable at early gastrula stage. The intensity of the staining increases during development before reaching a maximum at late pluteus stage. Treatment of whole embryos with LiCl has been shown to affect neither the time of onset of alkaline phosphatase activity nor the levels of activity reached (21).

Our results show that alkaline phosphatase activity is found only at low levels in cultured mesomeres, but this activity can be induced by treatment of mesomeres with LiCl. Only those LiCl-treated mesomeres that exhibit invaginated cells stain positively, and the activity is always localized in the invaginated cells. The staining of archenterons in LiCl-treated mesomeres can be inhibited by levamisole. The finding that a specific but idiosyncratic inhibition of gut alkaline phosphatase activity occurs in the archenterons of both normal embryos and embryoids derived from LiCl-treated mesomeres suggests that the same enzyme(s) is involved. Thus, the induction of archenteron-like structures by LiCl in animal blastomeres is accompanied by a corresponding induction of gut-specific enzyme activity. This is evidence that the effects of LiCl are manifested in both the morphological signs of morphogenesis and appearance of tissue-specific enzyme activity.

The SM50 transcript is specific to primary mesenchyme cells, which construct the embryonic skeleton. These cells are derived from micromeres, the four small blastomeres formed at the vegetal pole during the fourth cleavage division. The expression of this gene is remarkably autonomous; once the micromeres are formed, contact with either heterotypic (27) or homotypic (L. Stephens and F.H.W., unpublished work) blastomeres is not required for accumulation of the transcript to occur. This result implies that the information necessary for proper activation and expression of this gene is present at the vegetal pole of the egg. However, we have shown that treatment of animal blastomeres with LiCl can evoke the expression of this gene in blastomeres completely lacking cytoplasm of the vegetal hemisphere. The percentage of embryoids derived from LiCl-treated mesomeres that contain cells expressing SM50 corresponds approximately to the percentage of similar embryoids that contain spicules, suggesting that SM50 only accumulates in embryoids that go on to make spicules. This finding is molecular-level evidence that accumulation of a vegetal-specific gene transcript can be induced in animal blastomeres by LiCl.

Studies of embryonic development in several systems have indicated that differences in developmental potential along an embryonic axis are due to the asymmetric distribution of a molecule or molecules in the cytoplasm of the egg (28–32). Partitioning of these molecules into cells during cleavage gives rise to the various cell lineages. Such a system of animal and vegetal gradients has been proposed as an explanation for the gradient of developmental potential in sea urchin embryos (33, 34). The development of isolated animal and vegetal halves, the autonomy of micromere differentiation, and the observation that a vegetal displacement of the third cleavage plane can cause animal blastomeres to form vegetal structures all seem to support this theory. However, the fact that LiCl can induce vegetal differentiation in isolated animal blastomeres indicates that this simple model is not sufficient to describe the mechanisms involved in differentiation along the animal–vegetal axis (35).

We have shown that the induction of morphological indications of vegetal differentiation in animal blastomeres by LiCl is accompanied by the expression of lineage-specific molecular markers within these vegetal structures. This result suggests that the effector molecules needed to activate expression of these markers are present not only in vegetal blastomeres, but also in animal blastomeres as well. Because LiCl has been shown to interrupt signaling via secondary messengers, one possible explanation is that a signal at the cell surface is necessary to prevent expression of vegetal markers in animal blastomeres (35). The system described here should allow future experiments to determine the site of action of LiCl, which will, in turn, shed light on the mechanisms involved in cell determination in sea urchin embryos.

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1. Horstadius, S. (1973) *Experimental Embryology of Echinoderms* (Clarendon Press, Oxford, U.K.).
2. Davidson, E. H. (1986) *Gene Activity in Early Development* (Academic, New York), 3rd Ed.
3. Wilt, F. H. (1987) *Development* **100,** 559–575.
4. Lallier, R. (1964) *Adv. Morphogen.* **3,** 147–196.
5. Von Ubisch, L. (1929) *Wilhelm Roux' Arch. Entwicklungsmech. Org.* **117,** 80–122.
6. Kao, K. R. & Elinson, R. P. (1988) *Dev. Biol.* **127,** 69–77.
7. Regen, C. M. & Steinhardt, R. A. (1988) *Development* **102,** 677–686.
8. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312,** 315–321.
9. Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. S. & Wilson, D. B. (1986) *Science* **234,** 1519–1526.
10. Avissar, S., Schreiber, G., Danon, A. & Belmaker, H. (1988) *Nature (London)* **331,** 440–442.
11. Cameron, R. A., Hough-Evans, B. R., Britten, R. J. & Davidson, E. H. (1987) *Genes Dev.* **1,** 75–84.
12. Sucov, H. M., Benson, S., Robinson, J. J., Britten, R. J., Wilt, F. H. & Davidson, E. H. (1987) *Dev. Biol.* **120,** 507–519.
13. Benson, S., Sucov, H., Stephens, L., Davidson, E. H. & Wilt, F. H. (1987) *Dev. Biol.* **120,** 499–506.
14. Hinegardner, R. (1967) in *Methods in Developmental Biology,* eds. Wilt, F. H. & Wessels, N. K. (Crowell, New York), pp. 139–155.
15. Lutz, D. A. & Inoué, S. (1986) *Methods Cell Biol.* **27,** 90–109.
16. Hall, H. G. (1978) *Cell* **15,** 343–355.
17. Melton, D., Krieg, P. A., Rebagliati, M. R., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12,** 7035–7056.
18. Hough-Evans, S. B., Britten, R. J. & Davidson, E. H. (1988) *Dev. Biol.* **129** (1), 198–208.
19. Cox, K. H., DeLeon, D. V., Angerer, L. M. & Angerer, R. C. (1984) *Dev. Biol.* **101,** 485–502.
20. Driesch, H. (1900) *Arch. Entwicklungsmech. Org.* **10,** 361–410.
21. Gustafson, T. & Hasselberg, I. (1950) *Exp. Cell Res.* **1,** 371–375.
22. Pfohl, R. J. & Giudice, G. (1967) *Biochim. Biophys. Acta* **142,** 263–266.
23. Pfohl, R. J. (1975) *Dev. Biol.* **44,** 333–345.
24. Evola-Maltese, C. (1957) *Acta Embryol. Morphol. Exp.* **1,** 99–104.
25. Van Belle, H. (1972) *Biochim. Biophys. Acta* **289,** 158–168.
26. Horstadius, S. (1936) *Wilhelm Roux' Arch. Entwicklungsmech. Org.* **135,** 1–39.
27. Okazaki, K. (1975) *Am. Zool.* **15,** 567–581.
28. Dalcq, A. & Pasteel, J. (1938) *Bull. Acad. R. Med. Belg.* **6,** 261–308.
29. Sander, K. (1959) *Wilhelm Roux' Arch. Entwicklungsmech. Org.* **151,** 430–497.
30. Sander, K. (1976) *Adv. Insect Physiol.* **12,** 125–238.
31. Driever, W. & Nusslein-Volhard (1988) *Cell* **54,** 83–93.
32. Driever, W. & Nusslein-Volhard (1988) *Cell* **54,** 95–104.
33. Runnstrom, J. (1929) *Wilhelm Roux' Arch. Entwicklungsmech. Org.* **117,** 123–145.
34. Horstadius, S. (1939) *Biol. Rev.* **14,** 132–179.
35. Davidson, E. H. (1989) *Development* **105,** 421–445.