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Altered expression of spatially regulated embryonic genes in the progeny of separated sea urchin blastomeres

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Summary

We have examined the importance of the extracellular environment on the ability of separated cells of sea urchin embryos (*Strongylocentrotus purpuratus*) to carry out patterns of mRNA accumulation and decay characteristic of intact embryos. Embryos were dissociated into individual blastomeres at 16-cell stage and maintained in calcium-free sea water so that daughter cells continuously separated. Levels of eleven different mRNAs in these cells were compared to those in control embryos when the latter reached mesenchyme blastula stage, by which time cells in major regions of the intact embryo have assumed distinctive patterns of message accumulation. Abrogation of interactions among cells resulted in marked differences in accumulation and/or turnover of the individual mRNAs, which are expressed with diverse temporal and spatial patterns of prevalence in intact embryos. In general, separated cells are competent to execute initial events of mRNA accumulation and decay that occur uniformly in most or all blastomeres of the intact embryo and are likely to be regulated by maternal molecules. The ability of separated cells to accumulate mRNAs that appear slightly later in development depends upon the presumptive tissue in which a given mRNA is found in the normal embryo. Messages that normally accumulate in cells at the vegetal pole also accumulate in dissociated cells either at nearly normal levels or at increased levels. In one such case, that of actin CyIIa, which is normally restricted to mesenchyme cells, *in situ* hybridization demonstrates that the fraction of dissociated cells expressing this message is 4- to 5-fold higher than in the normal embryo. In contrast, separated cells accumulate significant levels of a message expressed uniformly in the early ectoderm but are unable to execute accumulation and decay of different messages that distinguish oral and aboral ectodermal regions. These data are consistent with the idea that interactions among cells in the intact embryo are important for both positive and negative control of expression of different genes that are early indicators of the specification of cell fate.

Key words: sea urchin embryo, messenger RNA, cell dissociation, tissue-specific gene expression, differentiation.

Introduction

The sea urchin pluteus is a relatively simple larva that develops from six major groups of cells (reviewed by Hörstadius, 1973; Okazaki, 1975*a*,*b*). This embryo has been the subject of extensive and elegant experimental embryology since Driesch (1891, 1892) first began investigation of the developmental capacities of embryo fragments. This work defined the fate map of the embryo and demonstrated the importance of localized maternal information in early development. In addition, analysis of the developmental capacities of parts of embryos and of abnormal combinations of blastomeres clearly established a crucial role for cell–cell interactions in the differentiation of some tissues (reviewed by Hörstadius, 1973). The techniques of molecular biology made possible an equally extensive analysis of gene expression in this embryo, focusing mainly on genome structure and temporal patterns and levels of control of gene expression in the whole embryo (reviewed by Davidson, 1986). To a large extent these two bodies of knowledge remain unconnected: Consequences of experimental manipulations have been interpreted primarily by morphological criteria, while characterization of gene expression in whole embryos has revealed little about which individual gene products are involved in the determination and differentiation of diverse cell types, or when distinctions are first made among cells at the molecular level.

Three approaches have allowed us to begin to relate expression of specific gene products to specific developmental events. These are techniques for enrichment of

individual tissues (Okazaki, 1975*a*; McClay & Marchase, 1979; Harkey & Whiteley, 1980; reviewed by McClay, 1986), production of antibodies directed toward determinants restricted to specific regions or cell types of the embryo (reviewed in McClay & Wessel, 1985), and development of *in situ* hybridization techniques for elucidating spatial patterns of distribution of individual mRNAs (Cox *et al.* 1984; reviewed by Angerer *et al.* 1987). These approaches have identified individual proteins and RNAs that provide sensitive indicators of early commitments of cells to specific pathways of differentiation (reviewed by Angerer & Davidson, 1984). An important conclusion that emerged from use of molecular markers is that the major groups of blastomeres in sea urchin embryos have already established distinctive patterns of gene expression at the transition between cleavage and early blastula stage.

Analysis of specific gene products that signify early steps in the commitment of cells to different developmental fates provides new criteria for analyzing the effects of experimental manipulations of the embryo. These criteria have the advantages of greater independence because individual gene products may be expressed, although an entire morphological structure is not created, and of being much earlier indicators of developmental decisions than are the morphological consequences of those decisions (Angerer & Davidson, 1984; Sargent *et al.* 1986; Gurdon, 1987; Wilt, 1987). This kind of approach has been used recently to help unravel the interactions of genes involved in *Drosophila* segmentation, for example, by examining mutations in individual genes not for their morphological effects, but for the alterations they produce in the patterns of expression of mRNAs or proteins encoded by other genes (for recent reviews see Akam, 1987 and Scott & Carroll, 1987). In studies of inductive interactions in *Xenopus* embryos, several clones representing mRNAs expressed specifically in endoderm, mesenchyme or ectoderm have been used as indicators of commitment of cells to specific pathways of differentiation (Gurdon *et al.* 1984, 1985; Sargent *et al.* 1986). In the present study, we analyze the extent to which programs of expression of different genes in the sea urchin embryo can be executed by cells when they are deprived of cell–cell and cell–substratum interactions and positional cues normally provided by the intact embryo. To this end, we have examined the ability of separated cells derived from blastomeres continually dissociated beginning at 16-cell stage to regulate appropriately a variety of individual genes with diverse and well-characterized spatial and temporal patterns of expression.

Materials and methods

Embryo culture and dissociation techniques

Gametes from *Strongylocentrotus purpuratus* adults (Marinus Co., Westchester, CA) were collected after intracoelomic injection of 0·5 M-KCl. After washing, eggs were fertilized in filtered Harvey's artificial sea water, pH 8·1 (SW), containing 10 mM-para-aminobenzoic acid (Sigma) to prevent hardening of fertilization membranes (McCarthy & Spiegel, 1983). When the embryos reached 16-cell stage (approximately 6 h; Fig. 1A), fertilization membranes were removed by passing the embryo suspension through 54 μm Nitex cloth (Showman & Foerder, 1979). Embryos were dissociated by washing three times in calcium- and magnesium-free artificial sea water containing 1 mM-EDTA (CMFSW; Hynes & Gross, 1970) at 4°C unless otherwise noted. In later experiments (numbers 8 and 9, Table 2) embryos were dissociated by washing in 'cell dissociation medium' [CDM: 20% distilled H_2O, 40% CMFSW, 40% 1 M-dextrose; Harkey & Whiteley (1985)] rather than CMFSW. In this case, separated cells were also collected from CDM by centrifugation at 300 *g*, but onto a 1 M-sucrose cushion. Dissociated blastomeres were cultured at 15°C starting at $\sim 1 \times 10^5$ cells ml^{-1} in calcium-free sea water (CFSW; Giudice & Mutolo, 1970) containing 1 mM-[ethylene-bis(oxyethylenenitrilo)]-tetraacetic acid (EGTA) to separate daughter cells after each division. During the entire dis-

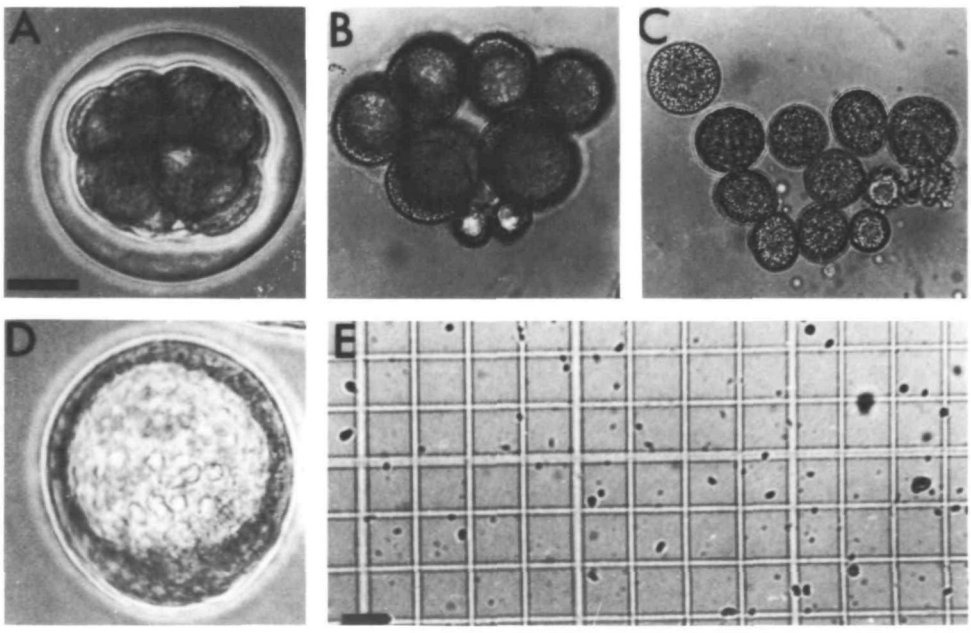

Fig. 1. Dissociation and culture of blastomeres. Intact embryos are shown with the animal pole at the top. (A) An intact 16-cell embryo, still within the fertilization membrane. (B) A 16-cell embryo after removal of the fertilization membrane and most of the hyaline layer by the first washes in CMFSW. (C) Dissociated blastomeres after 3 washes with CMFSW. (D) Mesenchyme blastula stage embryo from a control culture at 24 h. (E) Dissociated cells at 24 h, after culture in CFSW to ensure continued separation. Scale bar = 25 μm; A–D are at the same magnification.

sociation procedure, intact control embryos were held at 4°C; culture in normal SW at 15°C was resumed at the same time that dissociated cells were placed in CFSW at 15°C.

RNA isolation

RNA was extracted essentially according to Nemer *et al.* (1984), and the concentration and purity were determined spectrophotometrically.

RNA blot analysis

RNA samples were denatured and 5 μg lane^{-1} fractionated by electrophoresis on 1% agarose gels containing formaldehyde as described by Maniatis *et al.* (1982). After transfer overnight in 20 × SSC, membranes (Gene Screen Plus; DuPont/NEN) were baked at 80°C *in vacuo* for 2 h. Individual comparisons of mRNA levels were made among samples analyzed on the same blot. Single-stranded RNA probes were transcribed from restriction endonuclease truncated DNA templates with SP6 or T7 RNA polymerase according to Lynn *et al.* (1983) to a final specific activity of 2×10^8 disints min^{-1} μg^{-1} using [α-^{32}P]UTP (>3000 Ci mmol^{-1}; Amersham). Template DNA was removed by digestion with DNase I (RNase-free; FPLC-pure grade; Pharmacia) and, after addition of ammonium acetate to 2 M, RNA was precipitated with isopropanol (Maniatis *et al.* 1982). Blots were prehybridized in a solution containing 50% deionized formamide, 2 × SET (1 × SET is 0·15 M-NaCl, 0·03 M-Tris–HCl, 2 mM-EDTA, pH 8·0), 1% SDS, 1 × Denhardt's, 1 mg ml^{-1} phenol:chloroform-extracted yeast RNA (type VI, Sigma), and 10% polyethylene glycol 8000 (Sigma) at 55°C for 1–2 h. The prehybridization solution was replaced with fresh solution containing approximately 1×10^6 cts min^{-1} of RNA probe ml^{-1}, and the filters were incubated overnight at 55°C in bottles on a tissue culture roller (Rollacell™, New Brunswick Scientific). Filters were washed at 68°C (approximately T_m – 15°C) according to the method of Church & Gilbert (1984), with two washes in wash buffer 1 (40 mM-sodium phosphate, pH 6·8, 0·5% bovine serum albumin [Sigma], 1 mM-EDTA, 5% SDS) for a total of 10 min, and three washes in wash buffer 2 (40 mM-sodium phosphate, pH 6·8, 1 mM-EDTA, 1% SDS) for a total of 30 min, then autoradiographed with Kodak XAR-5 film. Filters were rehybridized with different probes after removal of the previous probe with two 15 min washes in 0·1 × SET, 0·1% SDS at 95°C. Autoradiography was carried out to several extents of exposure for each assay and signals were quantified by laser densitometry to identify exposures in the linear response range of the film. Signals were corrected for minor differences in quantity of RNA on the blot by normalizing to signals obtained with a probe complementary to a 512 nt region of *S. purpuratus* mitochondrial 16S rRNA (Wells *et al.* 1982; Eldon *et al.* 1987).

In situ *hybridization to dissociated cells*

Cells from cultures or control embryos were adjusted to 10^6 cells ml^{-1} and 5 μl drops were pipetted onto microscope slides which had been treated with triethoxyaminopropyl silane and activated with paraformaldehyde according to the procedure of Gottlieb & Glaser (1976). For controls, intact embryos were collected by centrifugation, resuspended in 0·1 vol. of CMFSW at 0°C and gently agitated until the cells separated. The time needed to separate control cells was ≤30 min and all manipulations were performed on ice to minimize RNA degradation. Slides were dried by brief heating at 50°C and then placed on an ice-cold metal tray, and the cells were fixed with 0·3% glutaraldehyde in 2·5% (w/v) NaCl, 50 mM-sodium phosphate, pH 7·5, for 1 h. Slides were washed briefly in fixation buffer several times and passed through graded ethanols and xylene. Samples were then processed for *in situ* hybridization as described by Cox *et al.* (1984), beginning after the normal deparaffinization step in xylene and using ^{3}H-labeled RNA probes ($1{\cdot}4\times10^8$ disints min^{-1} μg^{-1}) at saturating concentrations. Slides were autoradiographed and developed as described by Angerer & Angerer (1981).

Probe templates

The DNA templates used to transcribe RNA probes are described in the following references: cytoplasmic actins CyI, CyIIa, CyIIb, and CyIIIa (Cox *et al.* 1986); Spec1 and Spec2a (Hardin *et al.* 1988); Spec3, the 1·74 kb *Eco*RI fragment used in Eldon *et al.* (1987); collagen (Venkatesan *et al.* 1986); SpHbox1 (Angerer *et al.* 1989); SpMW5 and SpMW9 (M. Winkler, Univ. of Texas, Austin, personal communication). All probes represent *S. purpuratus* sequences, and the spatial distributions during embryogenesis of the corresponding mRNAs are described in 'Results'.

Results

Rationale and probe specificities

In these experiments, we dissociated embryos of *S. purpuratus* at 16-cell stage (approximately 6 h), cultured the blastomeres under conditions in which daughter cells continue to separate, and compared levels of specific mRNAs in these cell cultures to those in control embryos when the latter reach mesenchyme blastula stage (24 h; see Fig. 1). We chose 16-cell stage for dissociation because it presents the first morphological distinction among blastomere types: 8 mesomeres, 4 macromeres and 4 micromeres arrayed along the animal–vegetal axis (Fig. 1A). Micromeres can execute a normal pattern of gene expression (Harkey & Whiteley, 1983) and, in the presence of serum, differentiate autonomously to form primary mesenchyme cells in culture (Okazaki, 1975*a*). The developmental capacities of macromeres and mesomeres have not been analyzed by molecular assays. However, interpretations based on morphology indicate that these regions of the embryo, especially the animal hemisphere, cannot differentiate normally in isolation (Hörstadius, 1973). These observations suggest that regulation of individual genes expressed with different tissue specificities might be differently affected in blastomeres separated at this stage. We chose 24 h as the time to measure accumulation of individual mRNAs for several reasons. (1) By this time most, if not all, different cells in the normal embryo have initiated distinctive patterns of gene expression (reviewed by Angerer & Davidson, 1984 and Davidson, 1986). (2) In most cases, it avoids ambiguity of interpretation that might result for those mRNAs whose tissue specificity changes significantly in intact embryos at later stages (see below). (3) By this time mRNAs accumulate to levels that can be measured accurately.

We used probes for 11 different mRNAs whose spatial patterns of accumulation throughout development have been well characterized by *in situ* hybridization. Distributions of these mRNAs in late cleavage (~12 h) and mesenchyme blastula stage embryos (24 h)

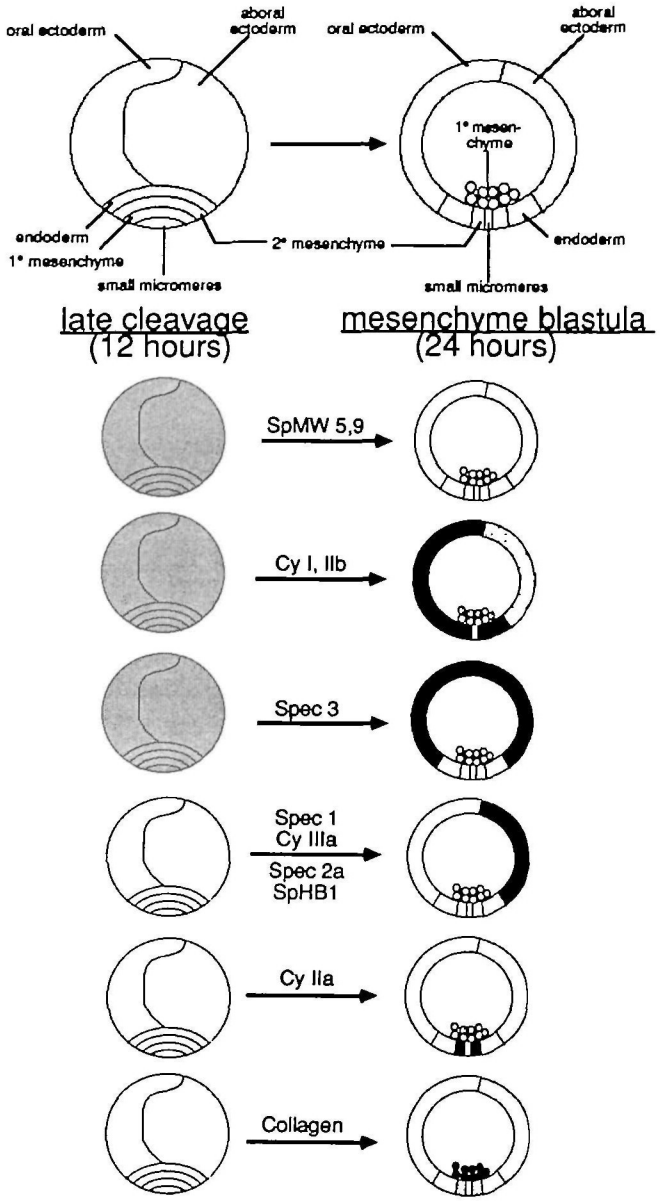

Fig. 2. Distribution of individual mRNAs during cleavage and at mesenchyme blastula stage. The diagrams at top depict fates of regions of the 12 h (140–200 cell) embryo (left), and 24 h mesenchyme blastula (right). Below each diagram are shown the spatial distributions of the mRNAs determined at either 12 (left) or 24 (right) h of development. Unfilled regions indicate the absence of detectable mRNA and increasing abundance is denoted by progressively darker shading. Additional details are given in the text.

are diagrammed in Fig. 2. These mRNAs encompass a variety of patterns of developmental prevalence and cell type specificity, of which we describe only those features most important for the work presented here.

(1) SpMW5 and SpMW9 are abundant maternal mRNAs that probably represent the set of genes encoding proteins required for cell division (Grainger *et al.* 1986). Both messages are uniformly distributed in eggs and embryos (L. Angerer, unpublished observations) and decrease in abundance gradually during cleavage so that their concentrations are much reduced by 24 h. Variations in abundance of these messages during cleavage suggest that these genes are transcribed for a short period after fertilization (L. Kelso & M. Winkler, personal communication).

(2) The temporal and spatial patterns of expression of the linked cytoplasmic actin genes CyI and CyIIb are indistinguishable, although the abundance of CyIIb mRNA is several-fold lower in all cases examined (Shott *et al.* 1984; Lee *et al.* 1986; Cox *et al.* 1986). CyI mRNA, which has been examined in greater detail, is present at very low concentration in maternal RNA and accumulates rapidly and uniformly in the embryo towards the end of cleavage (Lee *et al.* 1986; Cox *et al.* 1986) as the result of transcriptional activation between 9 and 12 h (Hickey *et al.* 1987). At mesenchyme blastula stage (20–23 h), this message begins to disappear rapidly from aboral ectoderm and, to a lesser extent, from presumptive oral ectoderm and primary mesenchyme cells, while continuing to accumulate in presumptive secondary mesenchyme and endoderm (Cox *et al.* 1986).

(3) The Spec3 gene product is associated with ciliogenesis and synthesis of this message is coordinated with that of a β-tubulin mRNA (Eldon *et al.* 1987; Harlow & Nemer, 1987). This mRNA initially accumulates uniformly in the embryo at late cleavage stage and then, shortly before hatching blastula stage, disappears from all cells at the vegetal pole while continuing to accumulate uniformly in ectoderm during the next phase of development (Eldon *et al.* 1987).

(4) Cytoplasmic actin CyIIIa and Spec1 mRNAs are present at relatively low concentration in maternal RNA, only about 3–4 % of the levels found at gastrula stage (Lee *et al.* 1986; Bruskin *et al.* 1981; Hardin *et al.* 1988). The maternal copies of CyIIIa mRNA are not detectably localized in the egg (Cox *et al.* 1986). Both messages begin to accumulate at about 9 h of development as the result of transcriptional activation (Spec1, Cabrera *et al.* 1984; CyIIIa, Hickey *et al.* 1987) and through the remainder of development they are confined to aboral ectoderm and its precursors (Lynn *et al.* 1983; Cox *et al.* 1986; Hardin *et al.* 1988). The Spec1 (and Spec2a, see below) message encodes a protein belonging to the calmodulin/troponin C/myosin light chain group of calcium-binding proteins (reviewed in Hardin *et al.* 1988).

Two other probes detect mRNAs that are also restricted to aboral ectoderm, but which accumulate later during the differentiation of this tissue. Spec2a mRNA, encoded by a small gene family whose members are structurally related to Spec1 (Hardin *et al.* 1988), does not accumulate until after hatching blastula stage. SpHbox1, a homeobox-containing mRNA, accumulates in aboral ectoderm beginning at mesenchyme blastula stage (Dolecki *et al.* 1986) and becomes progressively restricted to the aboral end of the pluteus larva (Angerer *et al.* 1989).

(5) The collagen probe detects an mRNA that accumulates mainly in primary and, to a lesser extent,

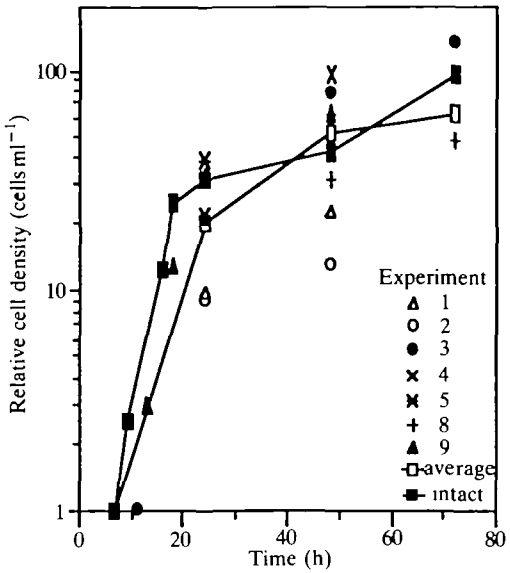

Fig. 3. Dissociated cells continue to divide at nearly normal rates. Cultured dissociated cells were counted with a hemocytometer at the indicated times. To facilitate comparison, data from individual experiments are shown normalized so that the initial cell density at the completion of the dissociation procedure is equal to 1. Experiments are numbered as in Table 2. The average value for dissociated cells at each time is shown by □, and cell numbers for normal *S. purpuratus* embryos, ■ (Hinegardner, 1967) are shown after normalization of the number at 6 h (16-cell stage) to 1.

in presumptive secondary mesenchyme cells beginning just before hatching blastula stage. This message is one of the first detectable zygotic gene products in primary mesenchyme cells (Angerer *et al.* 1988).

(6) Actin CyIIa mRNA is first detectable just after hatching (around 20 h; Lee *et al.* 1986). At mesenchyme blastula stage, *in situ* hybridization shows that it is found primarily in presumptive secondary mesenchyme cells and, briefly, in some primary mesenchyme cells. This mRNA is restricted to secondary mesenchyme cells through late gastrula stage, but subsequently disappears from these cells and accumulates to similar high levels in part of the endoderm (Cox *et al.* 1986).

Separated cells continue to divide at nearly normal rates, but do not show morphological signs of differentiation

Dissociated blastomeres were cultured in stirrer flasks at 15 °C in calcium-free sea water containing EGTA (CFSW). Under these conditions there was little, if any, aggregation of separated cells. Cell viability at 24 h, monitored by trypan blue dye exclusion, was always greater than 95 %. Viable cells were counted at 24, 48 and 72 h and, as shown in Fig. 3, the kinetics of increase in cell number were similar to those of intact embryos: There is a rapid increase between 6 and 24 h followed by a slower increase between 24 and 72 h. Although viable cell counts are probably less accurate at later stages, due to the small size of the cells, the average number of separated cells at 72 h was at least within a factor of 2 of that in normal embryos. At all times we observed some variation in cell size. At 24 h, the largest cells (3 % of the population), had volumes about one-half that of micromeres, but the volumes of the large majority of cells were two- to fivefold less than those of these large cells. Thus virtually all cells make at least some divisions after initial separation, and the population on average makes almost the normal number of divisions.

Dissociated cells showed no morphological differentiation characteristic of cells in the mesenchyme blastula. They remained spherical, with centrally located nuclei, and were not visibly ciliated. At longer culture times (48 or 72 h), there was no evidence of pigment granules characteristic of some secondary mesenchyme cells or of spicule material normally synthesized by primary mesenchyme cells.

Levels of individual mRNAs are affected differently in dissociated cells

To compare concentrations of individual mRNAs in separated cells and control embryos, total RNA was purified at 24 h as described in Materials and methods and analyzed on blots hybridized with ^{32}P-labeled RNA probes. All blots were rehybridized with a probe for mitochondrial 16S rRNA that is present at constant concentration throughout development (Wells *et al.* 1982; Eldon *et al.* 1987), and these signals were used to normalize for slight differences in loading. This correction was usually 5–10 % and never more than 20 %. Examples of these data, shown in Fig. 4, demonstrate that the levels of different mRNAs are affected by cell dissociation in dramatically different ways. SpMW5 and SpMW9, abundant maternal mRNAs, are the only messages examined that decrease in abundance in normal embryos between 6 and 24 h and these continue to decay, although to reduced extents in dissociated cells. (The blot shown in Fig. 4 has been overexposed to show the signal for RNA extracted from intact cells. Quantification of the time course of this decay is illustrated in Fig. 5.) In the particular experiment shown in Fig. 4, effects of dissociation on other mRNAs range from almost complete failure to accumulate (e.g. Spec1), through levels similar to those in control embryos (e.g. actin CyI) to significant overaccumulation (e.g. actin CyIIa).

In initial experiments, we tested two variations in culture conditions that might affect accumulation of these mRNAs. To determine the effect of absence of calcium in the medium, we cultured dissociated cells at 10-fold lower concentration in normal sea water with faster stirring in order to maintain separation (Arceci & Gross, 1980). This control is especially important in the case of Spec1 (and probably Spec2a) mRNA, which encodes a calcium-binding protein (Muesing *et al.* 1984). We also cultured cells in the presence of 2 % horse serum, which has been reported to improve viability of dissociated cells from embryos of *Lytechinus* (Arceci, 1980) and *Strongylocentrotus* (McCarthy & Spiegel, 1983) and to be required for differentiation of primary mesenchyme cells (Okazaki, 1975*a*). No effect on mRNA accumulation was observed when Ca^{2+} or

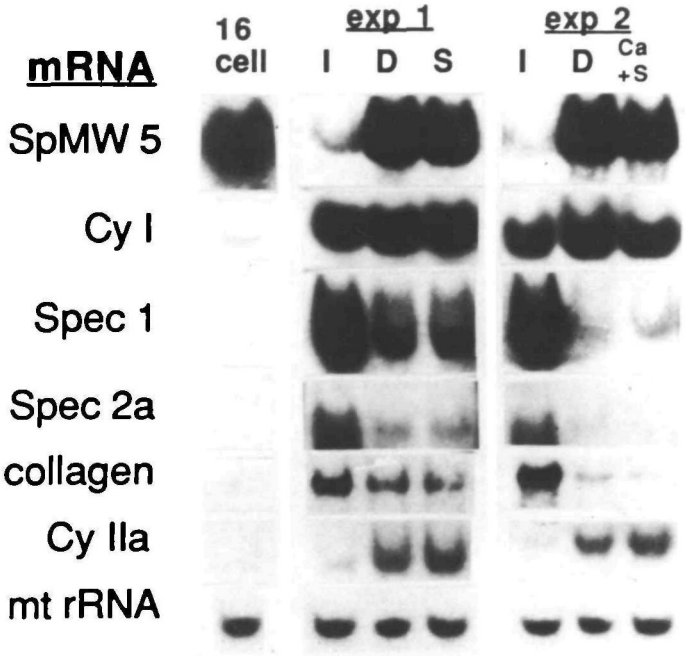

Fig. 4. Representative RNA blot hybridizations with specific RNA probes. (16-cell), 16-cell embryos before dissociation at 6 h; (I), intact 24 h mesenchyme blastulae; (D), 24 h dissociated cells cultured in CFSW; (S), 24 h dissociated cells cultured in CFSW + 2 % horse serum; and (Ca + S), 24 h dissociated cells cultured in normal sea water + 2 % horse serum. Quantification of signals for the complete set of probes is shown in Table 1. The bottom lanes (mt rRNA) show an example of rehybridization with a mitochondrial rRNA probe to compare relative amounts of total RNA on the blots.

Table 1. *Lack of effect of culturing dissociated cells in serum and/or calcium**

	Experiment 1		Experiment 2	
	Addition to CFSW		Addition to CFSW	
Probe	None	Serum	None	Ca + serum
CyI	120	120	80	94
CyIIa	200	150	210	400†
CyIIb	310	350	100	110
CyIIIa	50	54	7	8
Spec1	40	36	4	3
Spec2a	16	19	3	5
Spec3	66	65	13	12
Collagen	27	43	13	20
SpHbox1	0	0	0	0
SpMW5	680	630	700	550
SpMW9	660	710	690	490

* Values are the abundance of mRNA in dissociated cells at 24 h as a percentage of the amount in intact embryos.
† We consider this 2-fold difference to lie within the variability observed among dissociated cell experiments for CyIIa mRNA; see Table 2 and text.

serum was added separately (data not shown) or when they were added simultaneously (Fig. 4 and Table 1). Under these different conditions we detected no differences in division rate or morphology of the separated cells compared to those of cells cultured by our standard method (data not shown).

Results from 9 separate experiments using the standard culturing protocol are summarized in Table 2. The values given are mRNA abundances in dissociated cells at 24 ± 1 h expressed as percentages of the values for corresponding control mesenchyme blastulae. Included are data from one experiment (5) in which cells were dissociated at 15 °C, the normal culture temperature, rather than at 4 °C as in all other experiments. This variable also did not have a significant effect. The levels of some mRNAs in dissociated cells relative to those in normal control embryos varied considerably among different cultures. In order to determine what portion of this variation could be attributed to differences in levels of individual mRNAs among control cultures, we

Table 2. *mRNA levels in dissociated cells*

	Experiment number										
Probe	1	2	3	4	5†	6	7	8‡,§	9‡,‖	Mean	±S.E.M.*
CyI	120	80	150	110	130	73	78	110	78	100	90–110
CyIIa	190	210	370	1400	100	260	68	340	120	230	170–300
CyIIb	310	100	340	60	280	360	99	69	88	150	120–190
CyIIIa	50	7	8	4	54	5	2	13	25	11	7–16
Spec1	40	4	28	0	32	17	20	21	20	14	9–21
Spec2a	19	4	15	19	28	23	–	–	31	17	10–31
Spec3	66	13	35	23	100	18	20	32	57	33	26–41
Collagen	27	13	61	74	20	15	33	56	48	33	26–40
SpHbox1	0	0	0	0	11	5	16	–	9	3	2·0–4·5
SpMW5	680	700	390	340	430	360	1150	720	420	530	460–610
SpMW9	660	690	340	790	390	140	480	230	410	400	330–490

Values represent the amount of mRNA detected in dissociated cells at 24 h in each experiment as a percentage of the amount in intact embryos for that experiment.
–, not determined.
* Because the values given are ratios, means and standard errors of the mean were calculated using logarithmically transformed data.
† Dissociation performed at 15 °C rather than 4 °C.
‡ Dissociation performed in CDM rather than CMFSW (see Materials and methods).
§ Experiment from which cells were analyzed by *in situ* hybridization (see Fig. 5).
‖ Experiment from which time course data were obtained (see Fig. 4).

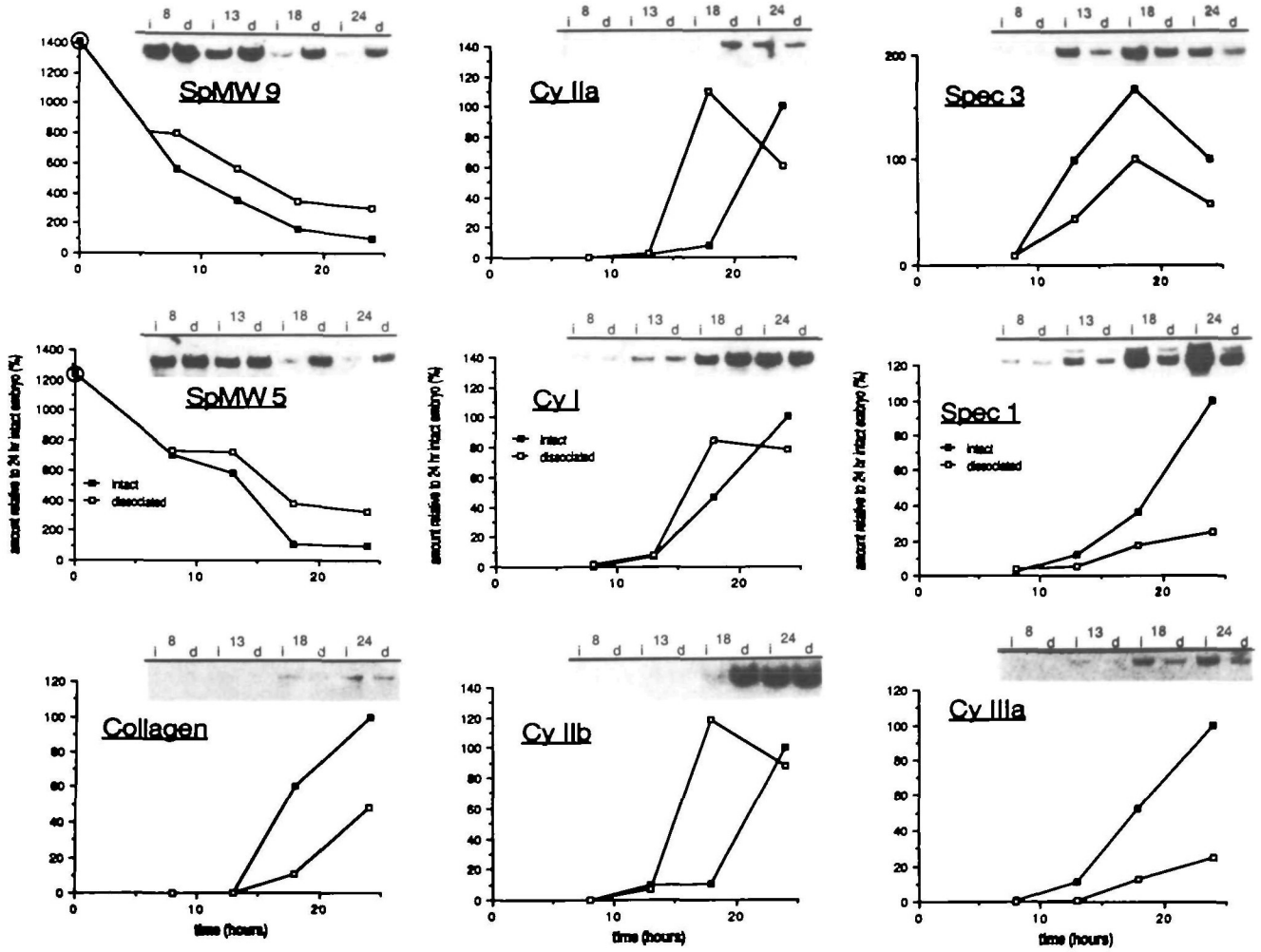

Fig. 5. Temporal patterns of abundance of individual mRNAs in intact embryos and dissociated cells. For each mRNA the amount in intact embryos (■) and in cultures of cells dissociated beginning at 16-cell stage, 6 h (□), is shown as a percentage of the amount in control cultures at 24 h. Representative RNA blot signals are shown for each mRNA in the insets, which are labeled with sampling time in h for either intact embryos (i) or dissociated cells (d). Relative amounts of SpMW5 and SpMW9 mRNAs in eggs, shown circled, were calculated from analysis of a separate batch of embryos (data not shown).

Table 3. *Uniformity of abundance of different mRNAs in different cultures of control embryos**

Probe	±S.E.M.†
CyI	81–130
CyIIa	84–120
CyIIIa	89–110
Spec1	89–110
Spec3	87–120
Collagen	86–120

* Levels of the listed mRNAs were determined in 7 cultures (Table 2, experiments 1–7) of control embryos at 24 h by analysis of samples on the same RNA blot. The average of values for each different mRNA was taken as 100 %, and standard errors were calculated.

† Because the values given are ratios, standard errors were calculated using logarithmically transformed data.

hybridized six of the probes to blots containing RNAs from seven batches (experiments 1–7) of control 24 h mesenchyme blastulae. Table 3 shows the standard errors for these measurements, with the average of values among different batches for each mRNA taken as 100 %. The small variation in mRNA levels among batches of control embryos derived from different females at different times spanning two breeding seasons indicates that absolute levels of these mRNAs are rather tightly regulated in normal embryos. We conclude that differences observed for the ratios presented in Table 2 reflect primarily differences in mRNA abundance among batches of dissociated cells. The data for dissociated cells do not reveal any consistent correlation of values that could be attributed to differences in cell viability among experiments. Levels of different messages expressed primarily or exclusively in ectoderm (CyIIIa, Spec1, Spec3 and Spec2a) show some correlation which we consider further in the Discussion.

Temporal pattern of mRNA accumulation in dissociated cells

To determine whether dissociation of cells leads to

alterations in timing of accumulation of individual messages, we examined intermediate time points covering the period during which, in normal embryos, levels of the abundant maternal mRNAs (SpMW5 and SpMW9) decrease while those of the other mRNAs increase: 8 h, allowing only time for any transient effects of the dissociation to pass; 13 h, when intact embryos (~200-cell stage) first detectably accumulate some of these mRNAs; 18 h, about 300-cell blastula stage, the usual time of hatching and before primary mesenchyme cells have ingressed; and 24 h, about 400-cell mesenchyme blastula stage. To minimize variation, all samples were derived from the same single-pair fertilization. Results for individual mRNAs are shown in Fig. 5, in which values for separated cells are shown as percentages of control levels at 24 h; insets in each panel show the RNA blot signals.

Based on differences in the kinetics of accumulation of individual mRNAs in dissociated cells, we consider them in four classes. (1) Both maternal RNAs, SpMW5 and SpMW9, decay in control embryos and in dissociated cells, although the latter maintain consistently higher levels. (2) Three mRNAs, those encoding actins CyIIa, CyIIb, and CyI, surprisingly show initial accumulation to higher levels in separated cells and then level off or decline while they continue to accumulate in control embryos. (3) Spec3 mRNA accumulates and decays with kinetics similar to those in control embryos, although at all stages levels are reduced compared to controls. (4) All other messages accumulate at lower (collagen) or much lower (Spec1, actin CyIIIa) rates in dissociated cells than in controls, but beginning at about the same time. Analyses at 48 and 72 h show that the levels of all of these mRNAs decline after 24 h (data not shown).

These data provide additional insight to the sources of variation in the standard 24 h assays shown in Table 2. First, the kinetics of accumulation of actin CyIIa and CyIIb and, to a lesser extent, CyI mRNAs, differ significantly in dissociated cells and embryos. Clearly small differences of timing in different cultures would have large effects on the ratios presented in Table 2 and, as expected, these transcripts show the greatest variability of the newly accumulating mRNAs at 24 h. Second, although the abundances of SpMW5 and SpMW9 mRNAs are about 5-fold greater, on average, in dissociated cells at 24 h, this difference appears to result from a slight pause in decay after dissociation, followed by resumption at a rate within 2-fold of that in control cells. For Spec1 and actin CyIIIa, these data show that at no time are dissociated cells capable of accumulating levels of mRNA similar to those in control embryos.

Number of dissociated cells expressing actin CyIIa and Spec1 mRNAs

RNA blot assays do not distinguish whether changes in abundance of individual mRNAs in dissociated cells are due to corresponding changes in the number of cells expressing those mRNAs, the abundance of the mRNA per cell, or both. We used *in situ* hybridization to address this question for two of the messages. Because separated cells cannot be identified by morphology, this approach is useful only when changes in mRNA expression are relatively large. Furthermore, levels of hybridization that appear high on sections of intact embryos correspond to relatively few autoradiographic grains per single cell. We analyzed Spec1 mRNA because it is the most abundant representative of the set of messages that is restricted to presumptive aboral ectoderm of the normal embryo (Lynn *et al.* 1983) and because its expression is greatly reduced in dissociated cells. We examined actin CyIIa message because, in the normal 24 h blastula, it is confined to a small subset of cells, the secondary mesenchyme, but it is markedly overexpressed in dissociated cells.

For this analysis, dissociated cells were allowed to settle onto slides and processed for *in situ* hybridization. For a control population, 24 h embryos from the same experiment were dissociated into individual cells in CMFSW, and these separated cells were immediately processed in the same manner. Histograms of the percentage of cells with different numbers of autoradiographic grains are presented in Fig. 6. For Spec1 mRNA, these data show a class of heavily labeled cells in control embryos (Fig. 6A) that is absent from the dissociated cell population (Fig. 6B). Fig. 6C shows a difference histogram constructed by subtracting the values for each grain density class of Fig. 6A from the corresponding value of Fig. 6B. This difference shows that dissociated cells lack the class of labeled cells present in control embryos, denoted by the shaded bars in Fig. 6C, corresponding to about 40 % of the total cells. *In situ* hybridization to sections of intact embryos in the same experiment shows a single labeled region including ~35 % of cells that constitute aboral ectoderm (Lynn *et al.* 1983; Hardin *et al.* 1988; data not shown). It is clear that few, if any, dissociated cells accumulate quantities of Spec1 mRNA as high as those found in presumptive aboral ectoderm of intact embryos. This result excludes an interpretation in which a subset of presumptive aboral ectoderm cells expresses Spec1 mRNA at normal levels while other cells do not.

Hybridization with the actin CyIIa probe revealed that many more cells accumulate this mRNA in dissociated populations than do in control embryos. Comparison of the histogram for control cells (Fig. 6D) to that for dissociated cells (Fig. 6E) clearly demonstrates a large increase in the percentage of cells with higher grain densities (mode of 6–7/arbitrary unit area). The difference histogram shown in Fig. 6F shows that ~35 % of the dissociated cell population (shaded bars) accumulate CyIIa mRNA whereas only about 8 % of cells, the presumptive secondary mesenchyme, express CyIIa message in normal blastulae (Cox *et al.* 1986). We conclude that the large increase in abundance of CyIIa mRNA in dissociated cell populations reflects primarily a similar increase in number of cells expressing this message.

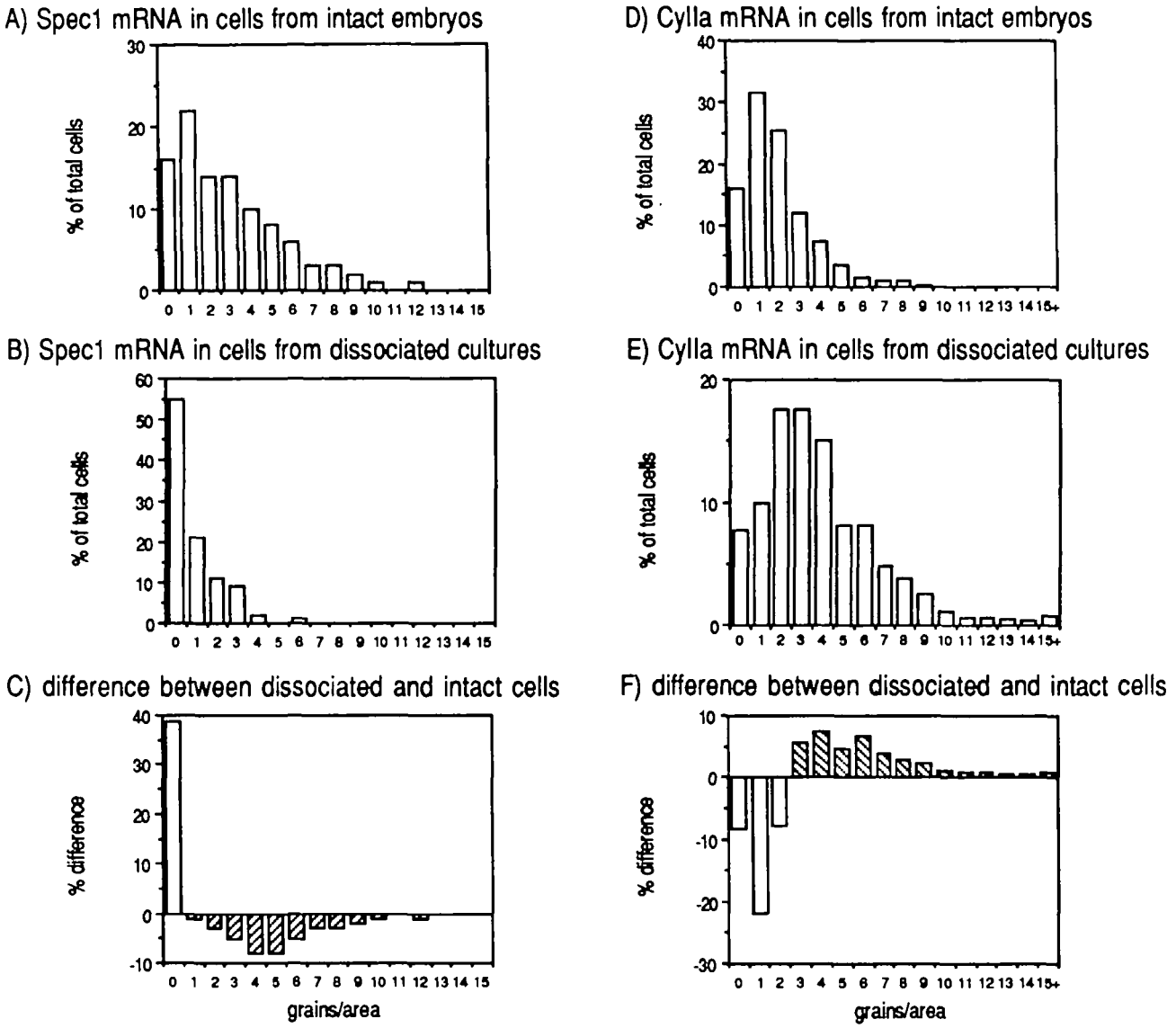

Fig. 6. Determination by *in situ* hybridization of the number of cells expressing Spec1 and actin CyIIa messages. Cells dissociated from intact embryos at 24 h (panels A,D) or cells separated at 16-cell stage and cultured until 24 h (B,E) were hybridized with either Spec1 (A,B) or actin CyIIa (D,E) probes. To avoid bias in the histograms due to the presence of a few percent of large cells, values for grains/cell were converted to grains/arbitrary area using approximate cell areas determined with an ocular micrometer. The % of the total number of cells counted is shown on the y-axis for each of the grain density classes on the x axis. The total number of cells for each panel was: (A) 370, (B) 501, (D) 522, (E) 754. Panels C and F are the difference histograms obtained by subtracting values for cells from intact embryos from those for dissociated cells. This presentation illustrates the distribution of labeling intensities for dissociated cells (shaded bars) that replace a similar percentage of cells in samples from control embryos (open bars). Shaded distributions above the line represent the population of cells expressing an mRNA in separated cells but not in the intact embryo; distributions below the line represent the reciprocal case in which cells expressing an mRNA in the intact embryo fail to do so in separated cells.

Discussion

Analyses of spatial and temporal patterns of gene expression in the early sea urchin embryo have shown that the major regions that give rise to different tissues establish distinctive patterns of mRNA accumulation by blastula stage (reviewed by Angerer & Davidson, 1984; Davidson, 1986). Many of the messages that have been examined in this study are essentially absent from the maternal RNA population and accumulate in only single or restricted sets of tissues as a result of new embryonic transcription. Several others exhibit initial uniform accumulation in the embryo followed by tissue-specific decay. Synthesis of high complexity nuclear RNA is thought to be initiated around 16-cell stage (Ernst *et al.* 1980) and accumulation of new zygotic tissue-specific mRNAs is first detectable around the 100- to 150-cell stage (Lee *et al.* 1986; Eldon *et al.* 1987; Hickey *et al.* 1987; Hardin *et al.* 1988). In the intact embryo, initiation of tissue-specific accumulation and

decay of different messages presumably signal early steps in commitment of blastomeres to restricted fates. The experiments presented here examine the extent to which separated cells can execute these early events of regulation of gene expression.

Evidence for the viability of dissociated cells

A concern in this experimental approach is that failure of cells to accumulate some mRNAs results from damage to the cells. We do not believe that the cell separation procedure, *per se*, is damaging because an extensive literature shows that separated blastomeres, including those separated in calcium-free sea water, can contribute differentiated cells in chimeras (reviewed by Hörstadius, 1973). Furthermore, experiments in which 16-cell blastomeres are immediately reaggregated show that these cells can reform embryo-like structures with differentiated tissues (reviewed by Spiegel & Spiegel, 1985) and can carry out patterns of RNA accumulation much more similar to those of control embryos (Hurley *et al.* in preparation). To avoid questions of long-term cell viability, we used a minimal separation time that is sufficient for the appearance and accumulation of a number of specific mRNAs. Several observations attest to the healthiness of separated cells during this period. (1) Between 6 and 24 h separated blastomeres continue to divide at rates close to normal and are still capable of dividing several more times, on average, during the next 48 h. Cells continue to exclude trypan blue dye during this period. (2) Separated cells accumulate significant quantities of several mRNAs, including actin CyI and CyIIb messages, that accumulate uniformly in all cells of normal embryos during this period. They also turn over several abundant maternal mRNAs (SpMW5 and SpMW9) at rates similar to those in normal embryos. Arceci & Gross (1980) have previously shown that micromeres, mesomeres and macromeres cultured separately are also able to execute the switch in synthesis from early (α) to late (β, γ, etc.) chromosomal histone variants. (3) Addition of 2% horse serum, reported to improve viability of separated cells (Arceci, 1980; McCarthy & Spiegel, 1983), and/or culturing in the presence of calcium, also had no detectable effect on message accumulation (Table 1, Fig. 3). (4) We found that cells dissociated at 16-cell stage and cultured to 24 h do not carry out unsolicited synthesis of the major 70×10^3 M_r heat shock protein (data not shown), although Roccheri *et al.* (1981) have shown that normal blastulae as well as cells dissociated and cultured as in our experiments develop the capacity to mount a heat-shock response. (5) Cells cultured up to 24 h in CFSW and then reaggregated incorporate [^{35}S]methionine at rates equivalent to those of intact embryos (our unpublished observations).

Cell-autonomous events vs. *those dependent on the integrity of the embryo*

Results from studies of classical experimental embryology, which examined the developmental capacities of parts of embryos and of abnormal combinations of early blastomeres, have been interpreted as providing evidence that the fates of most regions of the embryo are determined by reciprocal gradients of animalizing and vegetalizing tendency emanating from the corresponding poles and mediated by localized maternal factors (Rünnstrom, 1928; reviewed by Hörstadius, 1973, and Rünnstrom, 1975). Micromeres have clearly demonstrated inductive powers and the ability of their progeny to differentiate cytologically and biochemically as primary mesenchyme cells when cultured in isolation from other blastomeres (Okazaki, 1975*a*) strongly suggests that localized maternal factors are sufficient for their determination. In contrast, it is not clear whether the large mass of presumptive ectoderm contains a region of animal pole cytoplasm with analogous but reciprocal function to that of the vegetal pole (reviewed by Angerer & Davidson, 1984; Davidson, 1989). Isolated animal hemispheres cannot carry out much of the cytological differentiation observed in the ectoderm of intact embryos. Regardless of the exact mechanism envisioned, the classic experiments indicate that fates of macromeres and mesomeres are not irreversibly determined by 16-cell stage. The daughter cells of these 16-cell blastomeres retain considerable plasticity of fate at least through blastula stage, which can be revealed by altering the relative numbers and positions of different blastomeres (reviewed by Hörstadius, 1973). A straightforward, although probably simplified framework for discussing our results is that cell separation distinguishes between events that can be initiated within lineages of cells entirely by maternal molecules and those that are affected by the organization of the embryo. This 'organization' may encompass interactions among cells mediated by cell–cell contacts, by contacts with extracellular materials deposited by individual cells or their neighbors, and by short-range diffusion of molecules. Our results are consistent with greater maternally derived independence of events of gene expression for cells derived from the vegetal pole, than for those derived from the presumptive ectoderm.

As illustrated in Fig. 2, five of the mRNAs examined initially are expressed uniformly in the intact embryo. In general, early uniform expression and decay of messages appear to be maternally programmed. (1) The abundant maternal messages SpMW5 and 9 decay rapidly and uniformly in normal embryos. They also disappear from dissociated cells, although to a slightly lesser extent, perhaps related to the fact that a small percentage of these cells divides more slowly. (2) The mRNA encoding the cytoplasmic actin CyI accumulates early and uniformly in the embryo as does, presumably, the coordinately regulated actin CyIIb message. Subsequently, the abundance of these messages is modulated differently in different regions of the embryo. This includes down-regulation between 12 and 20 h in a portion of the ectoderm, which is one of the earliest manifestations of the differentiation of oral and aboral regions. The simplest explanation of the overaccumulation of both of these mRNAs is that it results from the inability of some separated cells to execute this tissue-specific down-regulation. The later decline in abundance of each message in separated cells presumably

results from the inability of these cells to carry out the subsequent accumulation that normally occurs in other tissues. (3) Spec3 mRNA, encoding a cilia-associated protein, also accumulates early and uniformly in the normal embryo. It continues to be expressed in all presumptive ectoderm (oral and aboral) but by blastula stage is undetectable in the smaller fraction of cells that are the precursors of mesenchyme and endoderm. Without the knowledge that the Spec3 protein is associated with cilia, we would predict that Spec3 mRNA would accumulate to nearly normal levels. In fact, while its kinetics of appearance and decay mimic those in normal embryos, its levels are somewhat lower at all stages, which is undoubtedly related to the fact that separated cells do not generate cilia. Suppression of Spec3 mRNA accumulation in dissociated cells may result from some feedback control, perhaps dependent on products of other genes. For example, it has been shown that Spec3 mRNA abundance is coordinately regulated with that of a message encoding β-tubulin in normal embryos and throughout cycles of deciliation and reciliation (Eldon *et al.* 1987; Harlow & Nemer, 1987).

Zygotic transcripts of four of the genes examined, Spec1, actin CyIIIa, Spec2a and SpHbox1, accumulate specifically in precursors of aboral ectoderm during normal development. Expression of these messages in separated cells is reduced, on average, at least 7-fold (Table 2). This is a large reduction, especially in view of the tight control of mRNA abundance in different cultures of control embryos (Table 3). Two observations indicate that this reduction does not represent a simple retardation of development of ectoderm. First, the number of cell divisions by 24 h is not significantly reduced in separated cells. Second, assays at 48 and 72 h showed no further increase in the levels of these mRNAs (our unpublished observations).

If expression of Spec1 and actin CyIIIa mRNAs reflects differentiation of aboral ectoderm, a process that we suggest depends on cell–cell interactions, then why should these mRNAs accumulate at all in separated cells? Our *in situ* hybridization results show that Spec1 mRNA does not accumulate preferentially in a small subset of separated cells that might be derived from, for example, the extreme animal pole, or the blastomeres closest to the inductive influence of the cells at the vegetal pole. We cannot determine whether the population of cells expressing at low levels is restricted to presumptive aboral ectoderm. We suspect that continuous contact throughout cleavage is required for accumulation of normal levels of aboral ectoderm-specific messages, since experiments in which cells were separated at each cleavage stage from 4-cell to 128-cell have not identified any discrete stage at which expression of these messages becomes autonomous; nor does separation at stages earlier than 16-cell result in further reductions of the levels of these messages (Hurley *et al.* in preparation). Thus, it seems more likely that initial accumulation of Spec1 and CyIIIa mRNAs might be sponsored by maternal molecules. The fact that low levels of these mRNAs are found in eggs (Bruskin *et al.* 1981; Lee *et al.* 1986; Angerer *et al.* 1989) supports the idea that early zygotic accumulation of these mRNAs is a continuation of an oogenetic program.

Although accumulation of collagen and actin CyIIa messages is not a very early event in the normal embryo, the fact that their expression is confined to vegetal pole derivatives, primary and secondary mesenchyme cells (Cox *et al.* 1986; Angerer *et al.* 1988), predicts that they should be expressed at levels similar to those in normal embryos. Collagen mRNA, which in normal embryos accumulates mainly in primary mesenchyme cells, reaches a level that is, on average, about one-third that in control embryos. Autonomous differentiation of primary mesenchyme cells has been demonstrated only when they are cultured on a surface in serum and allowed to form a syncytium. Maintenance of normal levels of collagen gene activity may depend on homotypic cell contacts and/or deposition on an appropriate substratum of extracellular matrix material, of which collagen itself is an integral part (Wessel & McClay, 1985). In experiments similar to ours, Stephens, Kitajima and Witt (personal communication) have found that the primary mesenchyme cell-specific message Sm50, which encodes the major spicule matrix protein, is expressed in dissociated cells at levels about 50 % of those in control embryos.

Accumulation of CyIIa message to abnormally high levels in separated cells is a surprising result. Our *in situ* hybridization measurements demonstrate that the number of dissociated cells expressing this mRNA is 4- to 5-fold higher than in normal embryos. An interesting possibility is that expression of this message is normally repressed in cells adjacent to micromeres and that separation of cells prevents this repression. Both older experimental embryology (reviewed in Hörstadius, 1973) and more recent experiments indicate that the fates of cells derived from the vegetal portion of macromeres are not determined autonomously. For example, the ability of secondary mesenchyme cells to 'count' and replace primary mesenchyme cells removed from the blastocoel (Fukushi, 1962; Ettensohn & McClay, 1988), indicates that some form of communication among vegetal pole cells is involved in specifying different cell fates. It is interesting that the only other set of cells that normally expresses the CyIIa message is the differentiating endoderm of the prism and pluteus larva, which also derives from the vegetal portion of the macromeres.

Determination of the ectoderm

Our molecular data relate to morphological descriptions of the fate of animal half embryos when separated at progressively later stages up to mesenchyme blastula. These show increasing ability to differentiate oral and aboral regions (Hörstadius, 1935, 1936*a*,*b*). Animal halves from 16- to 32-cell-stage embryos produce mostly permanent blastulae with elongated cilia, and little or no definition of oral and aboral regions. Animal half embryos also progressively lose their competence for induction by implanted micromeres (Hörstadius,

1936*a*). These observations are part of the classical evidence that normal differentiation of the ectoderm requires continued communication with more vegetal cells during cleavage and early blastula stages. During this period, we now know that different aboral ectoderm-specific messages accumulate in sequence (for example, see Hardin *et al.* 1988). Separation of blastomeres from 16-cell stage onwards does not suppress early regulation of mRNA abundance characteristic of all ectoderm, but does interrupt the animal–vegetal interaction required for normal accumulation of aboral ectoderm-specific messages and, probably also, the tissue-specific down-regulation of mRNAs initially expressed uniformly in the ectoderm. The variation in ability of embryos in different cultures to express aboral ectoderm-specific mRNAs is molecular data that parallels morphological descriptions in the literature showing that animal regions from different batches of eggs vary considerably in the extent to which they can differentiate. Such differences may ultimately relate to variations in the size of maternal stockpiles of regulatory molecules.

Further investigation of the exact cellular interactions necessary for specification of oral and aboral ectoderm will require additional markers for messages expressed at different times, especially ones expressed specifically in oral ectoderm, and investigation of the corresponding gene activities in separated blastomere types alone and in different combinations.

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References

AKAM, M. (1987). The molecular basis for metameric pattern in the *Drosophila* embryo. *Development* **101**, 1–22.

ANGERER, L. M. & ANGERER, R. C. (1981). Detection of poly A^+ RNA in sea urchin eggs and embryos by quantitative in situ hybridization. *Nuc. Acids Res.* **9**, 2819–2840.

ANGERER, L. M., CHAMBERS, S. A., YANG, Q., VENKATESAN, M., ANGERER, R. C. & SIMPSON, R. T. (1988). Expression of a collagen gene in mesenchymal lineages of the *Strongylocentrotus purpuratus* embryo. *Genes Dev.* **2**, 239–246.

ANGERER, L. M., DOLECKI, G. J., GAGNON, M. L., LUM, R., WANG, G., YANG, Q., HUMPHREYS, T. & ANGERER, R. C. (1989). Progressively restricted expression of a homeo box gene within the aboral ectoderm of developing sea urchin embryos. *Genes Dev.* **3**, 370–383.

ANGERER, L. M., STOLER, M. H. & ANGERER, R. C. (1987). *In situ* hybridization with RNA probes – An annotated recipe. In In situ *hybridization: Application to Neurobiology,* (ed., K. L. Valentino, J. H. Eberwine, & J. D. Barchas), pp. 42–70. Oxford University Press, New York.

ANGERER, R. C. & DAVIDSON, E. H. (1984). Molecular indices of cell lineage specification in the sea urchin embryo. *Science* **226**, 1153–1160.

ARCECI, R. (1980). Ph.D. Dissertation,'University of Rochester, Rochester NY.

ARCECI, R. J. & GROSS, P. R. (1980). Histone gene expression: Progeny of isolated early blastomeres in culture make the same change as in the embryo. *Science* **209**, 607–609.

BRUSKIN, A. M., TYNER, A. L., WELLS, D. E., SHOWMAN, R. M. & KLEIN, W. H. (1981). Accumulation in embryogenesis of five mRNAs enriched in the ectoderm of the sea urchin pluteus. *Devl Biol.* **87**, 308–318.

CABRERA, C. V., LEE, J. J., ELLISON, J. W., BRITTEN, R. J. & DAVIDSON, E. H. (1984). Regulation of cytoplasmic mRNA prevalence in sea urchin embryos. Rates of appearance and turnover for specific sequences. *J. molec. Biol.* **174**, 85–111.

CHURCH, G. M. & GILBERT, W. (1984). Genomic sequencing. *Proc. natn Acad., Sci. U.S.A.* **81**, 1991–1995.

COX, K. H., ANGERER, L. M., LEE, J. J., DAVIDSON, E. H. & ANGERER, R. C. (1986). Cell lineage-specific programs of expression of multiple actin genes during sea urchin embryogenesis. *J. molec. Biol.* **188**, 159–172.

COX, K. H., DELEON, D. V., ANGERER, L. M. & ANGERER, R. C. (1984). Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Devl Biol.* **101**, 485–502.

DAVIDSON, E. H. (1986). *Gene Activity in Early Development.* Academic Press, Orlando, Florida.

DAVIDSON, E. H. (1989). Lineage specific gene expression and the regulative capacities of the sea urchin embryo: A proposed mechanism. *Development* **105**, 421–445.

DOLECKI, G. J., WANNAKRAIROJ, S., LUM, R., WANG, G., RILEY, H., CARLOS, R., WANG, A. & HUMPHREYS, T. (1986). Stage-specific expression of a homeo box-containing gene in the non-segmented sea urchin embryo. *EMBO J.* **5**, 925–930.

DRIESCH, H. (1891). Entwicklungsmechanische Studien. I. Der Werth der beiden ersten Furchungszellen in der Echinodermentwicklung. Experimentelle Erzeugung von Theil- und Doppelbildungen. II. Über die Beziehungen des Lichtes zur ersten Etappe der thierischen Formbildung. *Z. wiss. Zool.* **53**, 160–184.

DRIESCH, H. (1892). Entwickelungsmechanisches. *Anat. Anz.* **7**, 584–586.

ELDON, E. D., ANGERER, L. M., ANGERER, R. C. & KLEIN, W. H. (1987). Spec3: embryonic expression of a sea urchin gene whose product is involved in ectodermal ciliogenesis. *Genes Dev.* **1**, 1280–1292.

ERNST, S. G., HOUGH-EVANS, B. R., BRITTEN, R. J. & DAVIDSON, E. H. (1980). Limited complexity of the RNA in micromeres of sixteen-cell sea urchin embryos. *Devl Biol.* **79**, 119–127.

ETTENSOHN, C. & MCCLAY, D. R. (1988). Cell lineage conversion in the sea urchin embryo. *Devl Biol.* **125**, 396–409.

FUKUSHI, T. (1962). The fates of isolated blastoderm cells of sea urchin blastulae and gastrulae inserted into the blastocoel. *Bull. Marine Biol. Stat. Asamushi* **11**, 21–30.

GIUDICE, G. & MUTOLO, V. (1970). Reaggregation of dissociated cells of sea urchin embryos. *Adv. Morphog.* **8**, 115–158.

GOTTLIEB, D. I. & GLASER, L. (1976). A novel assay of neuronal cell adhesion. *Biochem. biophys. Res. Commun.* **63**, 815–821.

GRAINGER, J. L., VON BRUNN, A. & WINKLER, M. M. (1986). Transient synthesis of a specific set of proteins during the rapid cleavage phase of sea urchin development. *Devl Biol.* **114**, 403–415.

GURDON, J. B. (1987). Embryonic induction – molecular prospects. *Development* **99**, 285–306.

GURDON, J. B., BRENNAN, S., FAIRMAN, S. & MOHUN, T. J. (1984). Transcription of muscle-specific actin genes in early Xenopus development: Nuclear transplantation and cell dissociation. *Cell* **38**, 691–700.

GURDON, J. B., FAIRMAN, S., MOHUN, T. J. & BRENNAN, S. (1985). Activation of muscle-specific actin genes in Xenopus development by an induction between animal and vegetal cells of a blastula. *Cell* **41**, 913–922.

HARDIN, P. E., ANGERER, L. M., HARDIN, S. H., ANGERER, R. C. & KLEIN, W. H. (1988). The Spec2 genes of *Strongylocentrotus*

purpuratus: Structure and expression in embryonic aboral ectoderm cells. *J. molec. Biol.* **202**, 417–431.

HARKEY, M. A. & WHITELEY, A. H. (1980). Isolation, culture, and differentiation of echinoid primary mesenchyme cells. *Wilhelm Roux' Arch. devl Biol.* **194**, 377–384.

HARKEY, M. A. & WHITELEY, A. H. (1983). The program of protein synthesis during the development of the micromere-primary mesenchyme cell line in the sea urchin embryo. *Devl Biol.* **100**, 12–28.

HARKEY, M. A. & WHITELEY, A. H. (1985). Mass isolation and culture of sea urchin micromeres. In vitro *Cellular and Developmental Biology* **21**, 108–113.

HARLOW, P. & NEMER, M. (1987). Developmental and tissue-specific regulation of β-tubulin gene expression in the embryo of the sea urchin *Strongylocentrotus purpuratus*. *Genes Dev.* **1**, 147–160.

HICKEY, R. J., BOSHAR, M. F. & CRAIN, JR, W. R. (1987). Transcription of three actin genes and a repeated sequence in isolated nuclei of sea urchin embryos. *Devl Biol.* **124**, 215–227.

HINEGARDER, R. (1967). Echinoderms. In *Methods in Developmental Biology* (F. H. Wilt & N. K. Wessels, eds.), p. 139. Crowell-Collier, New York.

HÖRSTADIUS, S. (1935). Über die determination im verlaufe der eiaschse bei seeigeln. *Publ. Stat. Zool. Napoli.* **14**, 251–479.

HÖRSTADIUS, S. (1936*a*). Determination in the early development of the sea-urchin. *Collecting Net (Woods Hole).* **11**, 236–267.

HÖRSTADIUS, S. (1936*b*). Über die zeitliche determination im keim von *Paracentrotus lividus*. *Arch. Entwicklungsmech. Organ.* **135**, 1–39.

HÖRSTADIUS, S. (1973). *Experimental Embryology of Echinoderms*. Oxford Univ. Press (Clarendon), London and New York.

HYNES, R. O. & GROSS, P. R. (1970). A method for separating cells from early sea urchin embryos. *Devl Biol.* **21**, 383–402.

LEE, J. J., CALZONE, F. J., BRITTEN, R. J., ANGERER, R. C. & DAVIDSON, E. H. (1986). Activation of sea urchin actin genes during embryogenesis: Measurement of transcript accumulation from five different genes in *Strongylocentrotus purpuratus*. *J. molec. Biol.* **188**, 173–183.

LYNN, D. A., ANGERER, L. M., BRUSKIN, A. M., KLEIN, W. H. & ANGERER, R. C. (1983). Localization of a family of mRNAs in a single cell type and its precursors in sea urchin embryos. *Proc. natn. Acad. Sci. U.S.A.* **80**, 2656–2660.

MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

MCCARTHY, R. A. & SPIEGEL, M. (1983). Serum effects on the in vitro differentiation of sea urchin micromeres. *Expl Cell Res.* **149**, 433–441.

MCCLAY, D. R. (1986). Embryo dissociation, cell isolation, and cell reassociation. *Meth. Cell Biol.* **27**, 309–323.

MCCLAY, D. R. & MARCHASE, R. B. (1979). Separation of ectoderm and endoderm from sea urchin pluteus larvae and demonstration of germ-layer specific antigens. *Devl Biol.* **71**, 289–296.

MCCLAY, D. R. & WESSEL, G. M. (1985). The surface of the sea urchin embryo at gastrulation: A molecular mosaic. *TIGS* **1**, 12–16.

MUESING, M., CARPENTER, C. D., KLEIN, W. H. & POLISKY, B. (1984). High-level expression in *Escherichia coli* of calcium-binding domains of an embryonic sea urchin protein. *Gene* **31**, 155–164.

NEMER, M., TRAVAGLINI, E. C., RONDINELLI, E. & D'ALONZO, J. (1984). Developmental regulation, induction, and embryonic tissue specificity of sea urchin metallothionein gene expression. *Devl Biol.* **102**, 471–482.

OKAZAKI, K. (1975*a*). Spicule formation by isolated micromeres of the Sea Urchin Embryo. *Am. Zool.* **15**, 567–581.

OKAZAKI, K. (1975*b*). Normal development to metamorphosis. In *The Sea Urchin Embryo: Biochemistry and Morphogenesis*. (ed., G. Czihak), pp. 177–232. Springer-Verlag, New York.

ROCCHERI, M. C., DIBERNARDO, M. G. & GIUDICE, G. (1981). Synthesis of heat-shock proteins in developing sea urchins. *Devl Biol.* **83**, 173–177.

RÜNNSTROM, J. (1928). Plasmabau und determination bei dem Ei von Paracentrotus lividus Lk. *Arch. Entwicklungsmech. Organ.* **113**, 556–581.

RÜNNSTROM, J. (1975). Integrating factors. In *The Sea Urchin Embryo: Biochemistry and Morphogenesis*. (ed. G. Czihak), pp. 646–670. Springer-Verlag, New York.

SARGENT, T. D., JAMRICH, M. & DAWID, I. B. (1986). Cell interactions and the control of gene activity during early development of *Xenopus laevis*. *Devl Biol.* **114**, 238–246.

SCOTT, M. P. & CARROLL, S. B. (1987). The segmentation and homeotic network in early Drosophila development. *Cell* **51**, 689–698.

SHOTT, R. J., LEE, J. J., BRITTEN, R. J. & DAVIDSON, E. H. (1984). Differential expression of the actin gene family of *Strongylocentrotus purpuratus*. *Devl Biol.* **101**, 295–306.

SHOWMAN, R. M. & FOERDER, C. A. (1979). Removal of the fertilization membrane of sea urchin embryos employing aminotriazole. *Expl Cell Res.* **120**, 253–255.

SPIEGEL, E. & SPIEGEL, M. (1985). Cell-cell interactions during sea urchin morphogenesis. In *Developmental Biology, a Comprehensive Synthesis, vol. 2, The Cellular Basis of Morphogenesis* (L. Browder, ed.). Plenum Press, N.Y.

VENKATESAN, M., DE PABLO, F., BOGELI, G. & SIMPSON, R. T. (1986). Structure and developmentally regulated expression of a *Strongylocentrotus purpuratus* collagen gene. *Proc. natn. Acad. Sci. U.S.A.* **83**, 3351–3355.

WELLS, D. E., BRUSKIN, A. M., O'BROCHTA, D. A. & RAFF, R. A. (1982). Prevalent RNA sequences of mitochondrial origin in sea urchin embryos. *Devl Biol.* **92**, 557–562.

WESSEL, G. M. & MCCLAY, D. R. (1985). Sequential expression of germ-layer specific molecules in the sea urchin embryo. *Devl Biol.* **111**, 451–463.

WILT, F. H. (1987). Determination and morphogenesis in the sea urchin embryo. *Development* **100**, 559–575.

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