Endoderm Differentiation *in Vitro* Identifies a Transitional Period for Endoderm Ontogeny

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The vegetal plate of the sea urchin embryo is specified during early cleavage divisions of the embryo as shown by the classical experiments of Horstadius (reviewed in ''Experimental Embryology of Echinoderms,'' 1973, Clarendon, Oxford). Not until gastrulation, though, do the cells within this territory differentiate into their characteristic cell types. Vegetal plate descendents comprise the coelomic epithelium, circumesophageal muscle, basal cells, pigment cells, and endodermal epithelium. We report here that cells of the endodermal lineage acquire the ability to differentiate autonomously several hours prior to gastrulation, between the late blastula and early mesenchyme blastula stages. Cells dissociated from whole embryos after the late blastula stage have the ability to differentiate *in vitro,* independent of cell contacts and of the embryonic environment. In contrast, preendoderm cells removed from the embryo prior to the late blastula stage show no ability to differentiate when cultured *in vitro* even though cells of other lineages, e.g., ectoderm and skeletogenic mesenchyme, show morphological and molecular differentiation in these same cultures. We have used the expression of the endoderm-specific gene products Endo 1 and LvN1.2, detected by RNase protection assays and by *in situ* immunolabeling, to quantify endoderm differentiation independent of embryonic or cellular morphology. These studies define a transitional period in the ontogeny of the endoderm, from cells reliant on interactions to promote fate specification and organization of territories to later events involved in morphogenesis that result from cell-type-specific gene expression. © 1996 Academic Press, Inc.

INTRODUCTION

Gastrulation is a period of dramatic cell rearrangements within an embryo that results in new cell interactions between the repositioned cells and tissues. These new cell interactions are crucial for the subsequent period of organogenesis. Gastrulation in the sea urchin embryo is highlighted by the ingression of mesenchymal cells and the invagination of the endodermal epithelium. Ingressing mesenchyme cells form the mesodermal derivatives of skeleton, coelom, muscle, and pigment, whereas the invaginating epithelium forms the endodermal digestive tract. The first morphological indication of endoderm differentiation occurs at primary invagination when the vegetal plate epithelium bends into the blastocoel. This inpocketing is followed by a process referred to as secondary invagination whereby

the tube elongates across the blastocoel and contacts a specific area on the ectoderm, the ''target site,'' to form the mouth opening (Hardin and McClay, 1990; reviewed in Ettensohn and Ingersoll, 1992).

Endoderm cells begin to express lineage-specific genes during gastrulation. In the sea urchin *Lytechinus variegatus* two gene products, LvN1.2 and Endo 1, that are expressed specifically by endoderm cells have been identified. Endo 1 is a 320-kDa glycoprotein of unknown function that was identified by a monoclonal antibody (Wessel and McClay, 1985). Translation of this protein begins at gastrulation and the mature glycoprotein concentrates at the apical cell surface. LvN1.2 is a 25-kDa protein that appears to be associated with the vesicularization of membrane in the apical region of the digestive epithelium (Wessel *et al.,* 1989). Although Endo 1 and LvN1.2 are encoded by different genes, their spatial and temporal accumulation profiles are identical, i.e., mRNA and protein accumulation begins during primary invagination and is restricted to the differentiating endoderm of the hindgut and midgut regions. Whether these

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gene products actually participate in morphogenesis is not known, but they have proven important as indicators of endoderm differentiation independent of the morphology of the cell or embryo (Livingston and Wilt, 1990; Malinda and Ettensohn, 1994).

Cells of the endodermal epithelium in the sea urchin are descendents of the vegetal plate, one of five territories of the early embryo (Davidson, 1989). Specification, or conditional assignment of fate within the vegetal plate, occurs early in cleavage, probably at or shortly after the fourth cell division (Ransick and Davidson, 1993). The mechanism of specification of the vegetal plate has not been elucidated but probably results from a combination of gene regulatory factors postulated to be localized in the egg (Davidson, 1989) and cell interactions (reviewed in Horstadius, 1973; Ransick and Davidson, 1993). Although determinants stored asymetrically in the egg have only recently been identified (Di Carlo *et al.,* 1994; K. Flytzanis, personal communication) and their role, if any, in vegetal plate specification has yet to be established, cell interactions are known to be crucial for the establishment of the vegetal plate territory. For example, when Horstadius transplanted the vegetal-most blastomeres, the micromeres, from one embryo to an ectopic position in the animal cap of a host embryo (Horstadius, 1973), he observed the formation of a second vegetal plate in the host embryo at the site of the transplantation, in an area that normally would form only ectoderm. By following the fates of each of the host and donor blastomeres in these chimeras, Horstadius showed that the micromeres do not contribute to the new vegetal plate (Horstadius, 1973). Instead the micromeres altered the fate of the adjacent, host blastomeres to develop an ectopic vegetal plate which then had the capacity to develop into all the normal vegetal plate descendants, including secondary mesenchyme derivatives and endoderm. Recently, it was shown that morphogenesis of the ectopic endoderm was indistinguishable from the endogenous vegetal plate descendents (Ransick and Davidson, 1993). Recent evidence demonstrates that micromeres also influence the specification of vegetal plate cells during normal development (Ransick and Davidson, 1995).

Cell interactions leading to vegetal plate specification begin early in development and involve a G-protein/protein kinase C (PKC)-mediated pathway. Treatment of early embryos or animal caps with either Li$^+$ ions or the phorbol ester, 12-0-tetradecanoylphorbol 13-acetate, induces the formation or expansion of vegetal plate differentiation, including endoderm cells (Livingston and Wilt, 1990, 1992; Nocente-McGrath *et al.,* 1991). Since this vegetalizing effect is similar to the effect of transplanted micromeres, it is thought that micromere/macromere interactions are mediated, at least in part, by signal transduction pathways that utilize G-proteins and PKC activation. To effect a change in vegetal plate specification, however, embryos must be treated before the blastula stage. Treatment of embryos during the late blastula stage or later in their development has no effect on endodermal specification (Livingston and Wilt, 1992; Nocente-McGrath *et al.,* 1991). Thus, the window

for specification of vegetal plate cells, including endoderm specification, occurs early in embryogenesis.

In this study we have identified a transitional period in endodermal ontogeny between the periods of vegetal plate specification during early cleavage and endoderm differentiation at gastrulation. We refer to this transition as ''commitment,'' the developmental phenomena of cells acquiring the ability to differentiate autonomously (Gurdon, 1987; Slack, 1991; Kimmel *et al.,* 1991). If an isolated cell differentiates *in vitro,* we conclude that the cell was committed to its fate, or committed to autonomous differentiation, prior to the time of isolation. Cells cultured from embryos dissociated prior to or during the late blastula stage were unable to express endoderm markers, whereas cells isolated from embryos following the late blastula stage had acquired the ability to differentiate *in vitro* and express endoderm-specific genes. We conclude that this transition at the late blastula/early mesenchyme blastula stage is indicative of the cell's developmental commitment to morphogenesis.

METHODS

Embryos and reagents.   Adult *Lytechinus variegatus* were obtained from Tracy Andachat of Duke University Marine Laboratory (Beaufort, NC) and from Sue Decker and Scott's Services, both of Miami, FL. Gametes were obtained and embryos were cultured as previously described (McClay, 1986).

Cell cultures.   Embryos were dissociated as described in McClay (1986). Embryos were washed twice in 10 vol of calcium-magnesium free seawater (CMF; McClay, 1986) at 4°C, then incubated in 10 vol of hyaline extraction media (McClay, 1986) at 4°C for 10 min and dissociated in CMF. After dissociation, cells were filtered through 20 μm Nitex to remove any nondissociated cell clumps so that the cell suspension was greater than 98% single cells. Cells were then diluted in CMF to approximately 10^5 cells per milliliter and plated onto tissue culture plates that previously had been coated with 1% protamine sulfate or poly-L-lysine (15,000–50,000 kDa), washed with distilled water, and air-dried. Cells were allowed to settle onto the wells (10-ml cell suspension for 100-mm dishes; 1 ml for each well of a 24-well plate; 4.5 ml for each well of a 6-well plate) for 20–30 min, after which the CMF was replaced with ASW containing 2–4% heat-inactivated horse serum (BRL, Bethesda, MD). For suspension cultures, cells were incubated in an Erlenmeyer flask on a New Brunswick G-24 gyratory shaker at 200 rotations/min.

In vitro nuclear transcription assay.   Nuclei were isolated from different stage embryos of *Lytechinus variegatus* according to Morris and Marzluff (1983) and nuclear transcription reactions were performed as described by Marzluff and Huang (1984) with slight modifications. Briefly, the nuclear ''run-on'' reactions contained 100 μCi [α-^{32}P]UTP (New England Nuclear), 1.5 mM each of ATP, GTP, and CTP; 0.15 mM (unlabeled) UTP, 180 mM KCl, 10 mM MgCl$_2$, 0.02 mM S-adenosylmethionine, 1 mM spermidine, 80 units RNAsin (Promega, Madison, WI), and 100 μl of approximately 1×10^8 nuclei per milliliter in a total reaction volume of 200 μl. The run-on reactions were performed at 23°C for 30 min. The reactions were terminated by the addition of 200 μl of 1% SDS and 10 mM EDTA. Radiolabel incorporated into run-on transcripts was measured by TCA precipitation (Marzluff and Huang, 1984).

To assay the *in vitro* nuclear transcription reactions, the radiolabeled transcripts from above were hybridized to the target DNA immobilized on nylon filters (MSI, Westboro, MA). Target DNAs in excess of transcript abundance were spotted onto filters by slot blotting. The DNAs were then hybridized and washed as described (Bruskin *et al.,* 1981) except that hybridizations were performed at 37°C for 72 hr using equivalent incorporated radioactive counts for each sample.

RNA isolation and detection. Total RNA was isolated from cells and embryos of different stages of *Lytechinus variegatus* using a modification of Chomczynski and Sacchi (1987; Biotecx, Houston, TX). Accumulation of LvN1.2 RNA was measured by a RNase protection assay using antisense ^{32}P-riboprobes transcribed *in vitro* from recombinant Bluescript plasmids essentially as described (Kreig and Melton, 1987). The LvN1.2 cDNA template corresponded to the 5′ end of the LvN1.2 cDNA (nucleotides 1–245; Wessel *et al.,* 1989) subcloned into Bluescript. The template was linearized with *Eco*RI and transcribed to yield an antisense riboprobe using T7 RNA polymerase. An ubiquitin cDNA clone was isolated from an *L. variegatus* prism stage cDNA library (L. Berg and G. M. Wessel, unpublished results) and an *Xho*I fragment, which corresponded to the repeated polyubiquitin sequence (Gong *et al.,* 1991), was cloned into Bluescript. Following linearization with *Eco*RI, transcription with T7 RNA polymerase resulted in a 130-nt riboprobe with 95 bases protected following RNase treatment. Riboprobes were gel-purified and used in a RNase protection assay using the RPA II kit following the manufacturer's protocol (Ambion, Inc., Austin, TX) with 3 µg total RNA isolated from embryos or cell cultures. Densitometry was used to quantify expression of both LvN1.2 and ubiquitin mRNA signals.

Antibody labeling of in vitro cultures. Cells were washed once with ASW at room temperature and then fixed with 10% formaldehyde in CMF at 4°C for 30 min or with 100% methanol at −20°C. Fixed cells were then washed 3× with ASW and incubated with antibodies. For indirect immunolabeling with Endo 1, hybridoma supernatant was diluted four times in ASW and incubated with the fixed cultures at room temperature for 30 min. For immunolabeling with anti-LvN1.2, protein-A-purified immunoglobulins from rabbit sera were diluted in ASW and then incubated on the fixed cultures for 30 min at room temperature. Cultures were then washed with ASW 3× and the appropriate secondary antibody (rabbit anti-mouse or goat anti-rabbit; Organon Technica, Research Triangle, NC) conjugated to either fluorescein (diluted 30 times in ASW) or horseradish peroxidase (diluted 100 times in ASW) and incubated on cultures for 30 min at room temperature. Cultures were also labeled with Endo 1 directly conjugated to the fluorochrome Cy3 (Biological Detection Systems, Inc.) according to the manufacturer's specifications. Fluorescence visualization was accomplished on a Zeiss Axioplan microscope equipped with epifluorescence. Horseradish peroxidase activity was detected with 4-chloro-1-naphthol (Harlow and Lane, 1988) and visualized on a Zeiss IM35 microscope.

For labeling of cells *en masse,* cells were removed from the culture well with a stream of sea water through a Pasteur pipet and attached to a coverslip previously coated with 1% protamine sulfate. Cells were fixed with either 100% MeOH or 3.7% formaldehyde, washed with ASW, labeled with the appropriate antibodies, and visualized.

RESULTS

Endoderm cells undergo a distinct developmental transition at the early mesenchyme blastula stage that enables the preendoderm cells to differentiate autonomously. We refer to this transition as a developmental commitment (for example Slack, 1991; Kimmel *et al.,* 1991). To identify commitment in preendoderm cells, we dissociated cells from whole embryos at various stages of development, cultured the cells *in vitro,* independent of the embryonic environment, and then examined their ability to express Endo 1 and LvN1.2. We were unable to isolate preendoderm cells free of other cell types, so we have relied on cell-type-specific markers to indicate endoderm differentiation in a mixed population of cells. The cultures contain all cells of the embryo and it is possible that some interactions of cells occur randomly by soluble factors, by extracellular matrix components, or by direct cell–cell interactions. Each of these possible interactions, however, was minimized by dilution of the cells in culture and by replacing the culture media. The culture media used in these experiments consisted of filtered artificial sea water containing 2–4% heat-inactivated horse serum. Under aseptic conditions, this medium supported cell viability for at least 3 days. An important feature of these cultures was to plate cells onto protamine sulfate or small polymers, less than 50,000 kDa, of poly-L-lysine. This treatment enabled cell spreading and cell shape changes to occur, but minimized their aggregation.

To monitor endoderm-specific differentiation in cell cultures we used RNase protection assays to monitor the level of LvN1.2 mRNA accumulation and *in situ* immunolabeling with antibodies to both the Endo 1 and LvN1.2 proteins to quantify endoderm differentiation on an individual cell basis. A time course of embryo dissociation and cell culture is shown in Fig. 1A. Embryos from a sibling population cultured throughout the experiment were periodically dissociated for cell culture and for immediate RNA isolation as a zero time point. Dissociated cells were plated in tissue culture dishes and were incubated until intact embryos reached the pluteus stage. RNA was then isolated from the cells of each culture and compared to the LvN1.2 levels of the intact embryos sampled at the time of each dissociation. Ubiquitin mRNA accumulation was used as a standard in each of these RNase protection assays.

Cells dissociated from embryos *prior* to early mesenchyme blastula stage *did not* accumulate detectable LvN1.2 mRNA (Fig. 1B). The stages tested included early blastula, before the embryos hatched from the fertilization envelope (BH; 6 hr); blastula immediately after hatching (AH; 8.5 hr); and late blastula (LB; 9.5 hr), when the vegetal plate of the embryo was flattened, but prior to mesenchyme cell ingression. In contrast, cells isolated from embryos following late blastula accumulated increasing amounts of LvN1.2, even though at the time of dissociation the embryos had no detectable *LvN1.2* transcriptional activity (Fig. 2) nor LvN1.2 mRNA accumulation (Fig. 1B). These stages included early mesenchyme blastula (EMB; 11 hr) during mesenchyme cell ingression; mid-mesenchyme blastula (MMB; 12 hr), when all mesenchyme cells have ingressed but prior to migration; and late mesenchyme blastula (LMB; 13 hr),

A

IN VITRO EXAMINATION OF ENDODERM DETERMINATION

FE - Fertilized egg
BH - Before hatching
LB - Late blastula
EMB - Early mesenchyme blastula
MMB - Mid-mesenchyme blastula

LMB - Late mesenchyme blastula
EG - Early gastrula
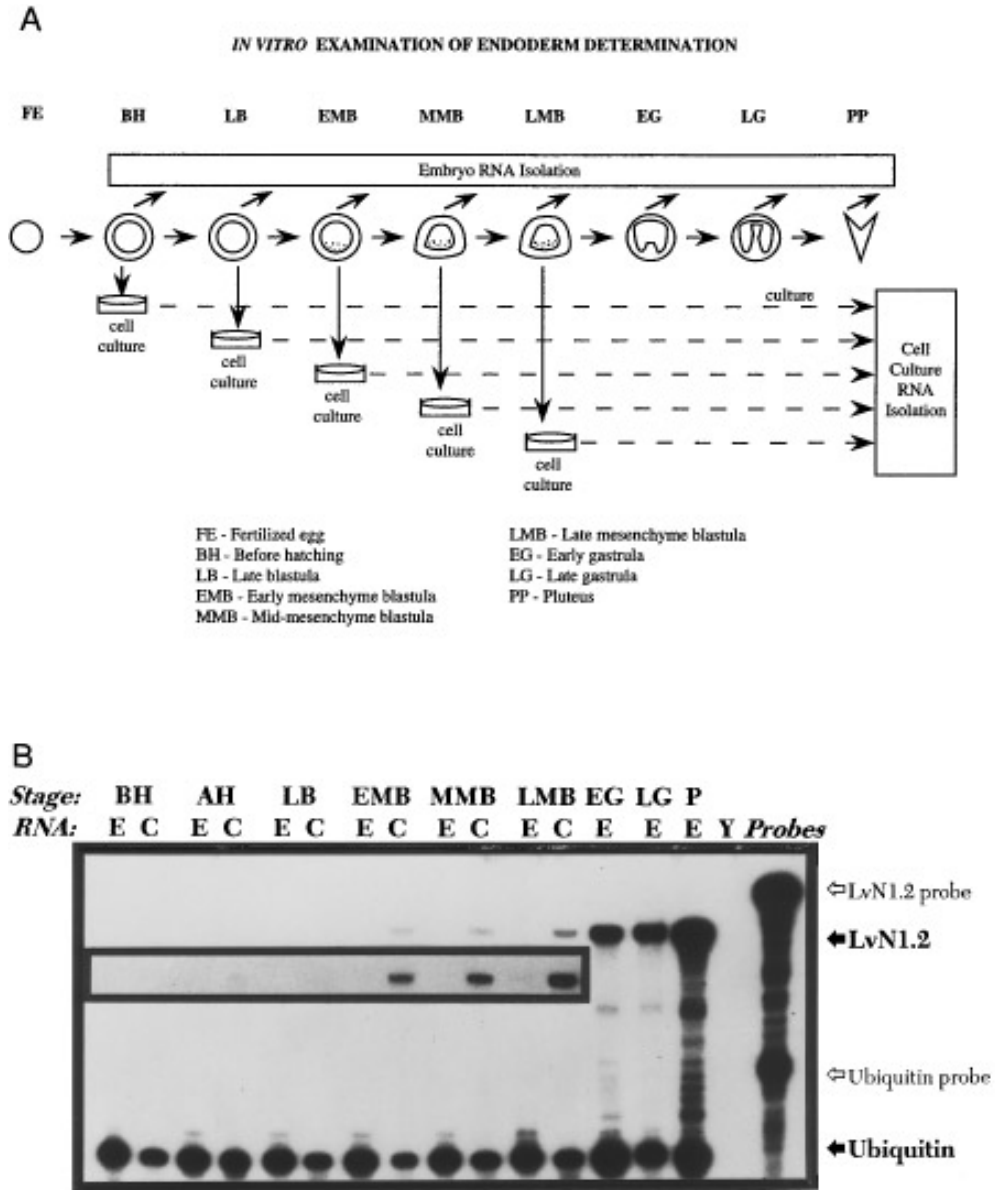LG - Late gastrula
PP - Pluteus

B

FIG. 1.   Endoderm commitment begins at early mesenchyme blastula stage. RNase protection assay (B) of total RNA isolated from intact embryos and from cultured cells as shown in (A). LvN1.2 accumulated in cells cultured to 24 hr if the cells were obtained from embryos following late blastula stage. No signal was detectable in cultures of cells obtained from late blastula or younger embryos (inserted region in (B) shows 5× the exposure of the full figure). The probe lane shows the starting probe lengths (open arrow) prior to the RNase protection assay, and the solid arrow indicates protected probe lengths. Stages: FE, Fertilized egg; BH, before hatching (6 hr); AH, after hatching (8.5 hr); LB, late blastula (9.5 hr); EMB, early mesenchyme blastula (11 hr); MMB, mid-mesenchyme blastula (12 hr); LMB, late mesenchyme blastula (13 hr); EG, early gastrula (14 hr); LG, late gastrula (19 hr); PP, prism-early pluteus (24 hr); RNA: E, total RNA from embryos; C, total RNA from cultured cells; Y, yeast tRNA.

when mesenchyme cells have begun to migrate from their point of ingression but before any morphological signs of invagination. Early gastrulae (EG; 14 hr) have a noticeable invagination of endoderm and significant LvN1.2 mRNA (lane EG - E in Fig. 1B); mid gastrula (MG; 14 hr) is when

the invagination reached halfway across the blastocoel; late gastrula (LG; 19 hr) is when the invagination reached the ectodermal epithelia; and late prism-pluteus (PP; 24 hr) is when the embryos were triangulated and arm buds were detectable. Gastrula and postgastrula embryos have signifi-
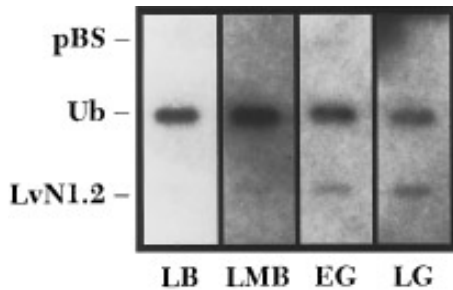
FIG. 2. LvN1.2 transcription begins at gastrulation. Nuclei were isolated from *Lytechinus variegatus* embryos at different stages and transcription of the endoderm-specific gene, LvN1.2, was measured by an *in vitro* run-on assay. Nuclei were isolated from whole embryos at the following stages: late blastula, LB; late mesenchyme blastula, LMB; early gastrula, EG; and late gastrula, LG. Target DNA sequences tested were: pBS, Bluescript plasmid; Ub, ubiquitin from *L. variegatus;* and LvN1.2.

cant LvN1.2 transcription (Fig. 2) and mRNA accumulation so their cells were not tested by *in vitro* culturing in this experiment. Figure 1 is one representative experiment of endoderm differentiation *in vitro* that was replicated in several independent trials.

Although we were unable to directly measure the transcriptional activity of LvN1.2 by nuclear run-on assays from cells cultured *in vitro,* we did determine that *in vivo,* LvN1.2 transcription begins just prior to gastrulation (Fig. 2). LvN1.2 mRNA synthesis was undetectable in nuclei isolated from the late blastula and faint signals were detectable at late mesenchyme blastula, though at levels significantly below that of early gastrula. Transcription of the ubiquitin gene, previously shown to be transcribed at equivalent levels in all cell types of all the developmental stages examined here (Tomlinson and Klein, 1990; Gong *et al.,* 1991), was used in these experiments to normalize the activity of the nuclear run-on assays between different stages. Thus, transcriptional activation appears to be an essential feature of the LvN1.2 mRNA accumulation that we measure *in vitro* and not a change in the stability of mRNA resulting from earlier, or constitutive, transcription.

The culture conditions used in these experiments supported the viability of all cell types of the embryo examined using the criteria of cellular phenotype, of cell-type-specific antibodies, and of trypan blue exclusion (>96%). We routinely observed the differentiation of primary mesenchyme cells, secondary mesenchyme cells (including pigment cells and muscle cells), and ectoderm (data not shown). In addition, using incorporation of bromodeoxyuridine into nuclei, we have determined that the cultured cells divide at a rate similar to that seen in intact embryos during the same period of development (Nislow and Morrill, 1988). Furthermore, since ubiquitin mRNA signals in the RNase protection assays remained relatively constant per unit of total RNA, we believe that transcription was maintained in most

cells under our culture condition. We also found that endoderm cells differentiate best in the absence of serum and that LvN1.2 expression is inversely proportional to serum concentration: a threefold difference was observed between no serum and 8% serum (data not shown). Heat-inactivated serum was routinely used in the cultures at 2–4% since, for example, primary mesenchyme cells differentiate best in the presence of these low levels of serum and we wanted to increase the viability of all cells in the culture to minimize the possible adverse effects of cell death on total culture viability. We conclude from these results that the culture conditions used here were permissive to support the survival, cell division, and differentiation of cells from several lineages of this embryo.

Examining LvN1.2 mRNA accumulation over time in cells derived from early mesenchyme blastula (EMB) revealed a reduced rate of accumulation of LvN1.2 mRNA over the 24-hr period of culture compared to that of intact embryos (Fig. 3A). However, in cells dissociated from early
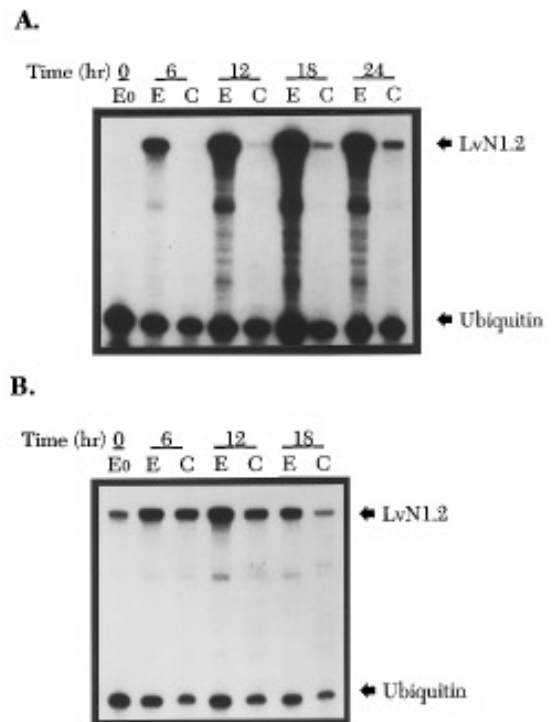

FIG. 3. Time course and stability of differentiation *in vitro.* Embryos were cultured to (A) the early mesenchyme blastula stage or (B) the early gastrula stage, and then divided into two groups. One population of embryos was cultured as intact embryos and RNA was isolated after 6, 12, 18, and 24 hr of additional culture. The other population of embryos was dissociated to single cells and cultured *in vitro.* RNA was isolated from these cell cultures at the same time as the intact embryos, i.e., following 6, 12, 18, and 24 hr of additional culture. Each set of RNA samples was then tested for accumulation of LvN1.2 mRNA by RNase protection assays using ubiquitin as a measure of RNA loading in each assay.

gastrulae, following initiation of endoderm differentiation *in vivo,* LvN1.2 mRNA accumulation was normal, i.e., comparable to levels seen *in vivo* (Fig. 3B). Culture of cells prior to EMB resulted in no detectable accumulation of LvN1.2, regardless of the time of culture (data not shown). The reason for the low mRNA accumulation in cultures from EMB is not known. Perhaps dissociation has removed signals present in the embryo that are required for differentiation, or perhaps commitment of preendoderm cells occurs sequentially throughout the vegetal plate. By dissociating embryos and isolating cells during this sequential event, we may have effectively excluded some cells in the population that are not yet committed. Until we can identify commitment directly, instead of by the functional bioassay of differentiation *in vitro,* we will not be able to distinguish between these possibilities. However, it is clear that *commitment* is dependent upon cell interactions that occur in the embryo and do not occur when the cells are removed from their neighbors and their normal environment.

Further evidence for a transitional period in endoderm differentiation relied on immunolabeling cell cultures and quantifying differentiated cells using endoderm-specific antibodies. Following dissociation and culturing similar to that described above, cells were processed for immunolabeling of LvN1.2 and Endo 1 and then quantified. Immunolabeling was performed either *en masse* by removal of cells from cultures and fixation on coverslips or *in situ* on the culture wells. We used an indirect immunoassay with either horseradish peroxidase-conjugated (Fig. 4; processed *en masse*) or fluorochrome-conjugated secondary antibodies to detect Endo 1-positive or LvN1.2-positive cells processed *in situ* (data not shown). Endo 1- or LvN1.2-positive cells were expressed as a percentage of the total cell population, which was then compared to endoderm cell counts derived from intact, sibling embryos. These data are displayed as a ''relative endoderm differentiation,'' the percentage of Endo 1- or LvN1.2-positive cells that differentiated *in vitro* relative to the percentage of Endo 1- or LvN1.2-positive cells that differentiated in intact embryos cultured for the same length of time (Fig. 4). Background values for this assay (treated the same as experimental wells but assayed without primary antibody) were 1–3%, which was in part probably the result of a trapping of antibody in cells that had lysed. Assays of Endo 1- or LvN1.2-positive cells were also performed immediately following embryo dissociation and resulted only in background values in the three stages shown here (data not shown).

Results of the immunolabeling assays show that preendoderm cells acquire an ability to differentiate autonomously during the late blastula stage. When cultures were started from early mesenchyme blastula, Endo 1-positive cells *in vitro* reached 84% of the level of Endo 1-positive cells in intact embryos. In contrast, when cultures were started from early blastula or late blastula embryos, only 5 and 21% relative differentiation, respectively, was detected. The data were similar for LvN1.2 immunolabeling (early blastula: 4% (±4); late blastula: 24% (±17); early mesenchyme blas-

tula: 72% (±23)) except that, because the signals were less intense with this antibody, the deviation between experiments was greater than with Endo 1. In these immunolabeling assays, Endo 1- or LvN1.2-positive cells are weighed equally so that cells with strong signals count the same as cells with low, but positive, signals. From these results we conclude that the low level of LvN1.2 mRNA expression in cells cultured from EMB (Fig. 3A) is a result of lower LvN1.2 accumulation per cell and not a high level of expression in a minor population of cells. These data suggest that endoderm differentiation *in vitro* is similar to that in embryos, but the extent of expression of the marker genes during the transition period is sensitive either to the limitations of the *in vitro* culture system or to continued cell interactions.

DISCUSSION

The endoderm lineage of the sea urchin embryo was shown here to undergo a developmental transition between the late blastula and early mesenchyme blastula stages, enabling the cells to differentiate autonomously. We refer to this event as ''commitment,'' relying on the operational definition of others in several developmental systems (Gurdon, 1987; Slack, 1991; Kimmel *et al.,* 1991). Commitment, in contrast to ''determination,'' is used to describe the phenomenon shown here, since historically determination also refers to an ability of cells or tissues to differentiate in an ectopic location of the embryo (Slack, 1991). For example, the four micromeres of the sea urchin embryo, which are formed at the fourth cleavage division, are determined at the fourth cell division and differentiate into skeletogenic mesenchyme either when transplanted to an ectopic host location or when cultured *in vitro.* Although preendoderm cells may also have been determined at the early mesenchyme blastula stage, our assay system does not test such properties. Commitment is also used here to distinguish the observed phenomenon from specification, the process of conditional fate restriction, which in the vegetal plate of the sea urchin embryo appears to occur during early cleavage divisions and to result from a combination of cell interactions and stored maternal factors (Davidson, 1989; Ransick and Davidson, 1993).

Endoderm commitment is a relatively late event in the development of the sea urchin embryo and thus probably not the result of maternally stored information. Instead, endoderm commitment is probably a consequence of interactions between cells of the embryo prior to early mesenchyme blastula. Commitment in preendoderm cells occurs at the same time as cell positions within the vegetal plate shift as a result of the ingression of primary mesenchyme cells. Primary mesenchyme cells are located at the center of the vegetal pole and their ingression requires a shift of the epithelial cells to accommodate the loss of this population (Galileo and Morrill, 1985; Ettensohn and Ingersoll, 1992). One possible signal for commitment in endoderm is the
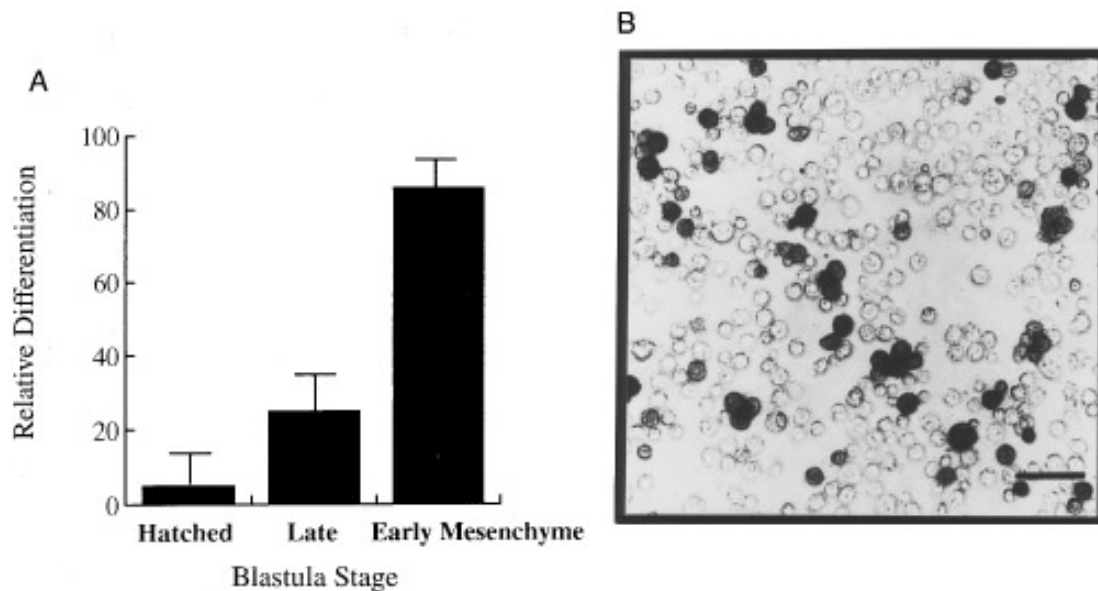
FIG. 4.   Quantification of differentiation in endoderm cells *in vitro.* Embryos were cultured to hatched blastula (8 hr), late blastula (10 hr), or early mesenchyme blastula (12 hr) when they were dissociated to single cells and plated *in vitro* in the presence of 4% horse sera. The cells were cultured for 24 hr and then fixed and immunolabeled with monoclonal antibody Endo 1. (A) Endo 1-positive cells are represented as the percentage of Endo 1-positive cells *in vitro* relative to the number of Endo 1-positive cells in sibling embryos cultured intact for the same time (which have reached early plutei). The data represent four separate experiments and the error bars indicate 1 SD. Statistical analyses show that late blastula and early mesenchyme blastula are significantly different ($P < 0.05$) from background, from hatched blastula, and from each other. Hatched blastula is not significantly different from background. (B) Representative field of cells cultured from early mesenchyme blastulae, cultured for 24 hr and then removed from the dish and processed in batch. Dark cells are Endo 1-positive cells. Bar, 50 μm.

changing cell environment of preendoderm cells, stimulated either by a relative shift between secondary mesenchyme cells and endoderm cells in the vegetal pole vacated by primary mesenchyme cells or by interaction with the differentiating primary mesenchyme cells across the nascent basal lamina. Neither explanation, however, would be universal, since in embryos of the species *Eucidaris tribuloides,* endoderm differentiation and invagination *precede* the ingression of primary mesenchyme cells.

Differentiation of the endoderm is also known to involve changes in cell interactions. These include changes in cell–cell interactions (McClay and Chambers, 1978); cell–extracellular matrix interactions (Karp and Solursh, 1974; Wessel and McClay, 1986; Ingersoll and Ettensohn, 1994), and possibly also cell interactions with growth factors (Ramachandran *et al.,* 1993; Govindarajan *et al.,* 1995). Each of these interactions has a specific and profound effect on the manifestation of both the morphology and the molecular expression of endoderm cells. The requirement for such interactions during normal development may be a consequence of the vegetal plate being committed much later than other territories of the embryo. For example, the precursors of the skeletogenic mesenchyme, the micromeres, are committed (and determined) at the 16-cell stage (Okazaki, 1975). Commitment of ectoderm appears to be the earliest of the primary tissue types (Nemer *et al.,* 1985; Hurley *et al.,* 1989; Stephens *et al.,* 1989), where it was shown that at least partial differentiation of ectoderm occurs in cells dissociated from the embryo beginning at the 2-cell stage. Cells of the vegetal plate, however, are committed late in development and some populations remain pluripotent even until late in gastrulation (Ettensohn and McClay, 1988).

The phenomenon of commitment suggests that the activation of gene regulatory elements will occur in a cell-autonomous fashion *before* the cell begins to manifest a differentiated fate. It is interesting that the mRNA for Endo 16 and Msx accumulates in the vegetal plate territory of *Strongylocentrotus purpuratus* embryos at the early mesenchyme blastula stage (Nocente-McGrath *et al.,* 1989; Ransick and Davidson, 1993; S. Dobias and R. Maxson, personal communication) and may represent an early molecular indication of endoderm commitment that is important for subsequent endoderm autonomous gene expression. Endo 16 is a large protein that accumulates at the cell surface and is thought to mediate cell–extracellular matrix interactions (Soltysik-Espanola *et al.,* 1994). Msx is a protein of the homeodomain-containing class of gene regulatory factors that appears to have a significant influence on the expression of other genes in the embryo. Dominant negative expression of Msx in sea urchins inhibits endoderm differentiation

(R. Maxson, personal communication), making this regulatory factor a strong candidate to participate in the commitment phenomenon. In addition, a homologue of the forkhead family of HNF3 transcription factors is present in sea urchins transiently during the period when endodermal commitment occurs (Luke *et al.,* 1995). Forkhead proteins have been shown in other embryos to be involved in cell fate decisions, particularly of mesoderm and endoderm. Although we do not know the downstream targets of forkhead, and Msx in the vegetal plate, their expression in the embryo coincides with a transition in endoderm ontogeny, which is shown here to impart an ability on the cells to differentiate autonomously.

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