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A Normal Epithelial-Mesenchymal Transition as a Model for Studying Metastatic Onset

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Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. One goal of this research was to examine the relationship between Tbx2/3, which is implicated in breast cancer, and EMT. Tbx2/3, was cloned and characterized. A polyclonal antibody was generated and functional assays performed to determine the role of Tbx2/3 during development and to link these observations to the tumorigenesis observed in breast cancer patients with amplifications of chromosome region 17q23 containing the Tbx2 locus. FGF signaling was also further examined during this funding period but the results of these studies were incomplete. Functional characterization of Ets showed that it is activated by the ras pathway activates and that activated Ets is involved in the specification events that are necessary for mesoderm to undergo EMT in the sea urchin.
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Introduction:

Epithelial-mesenchymal transitions (EMTs) are indicative of cancer metastasis and correlate with poor patient survival. These transitions, however, are a normal part of animal development. The EMT that occurs during mesenchyme formation in the sea urchin is highly representative of the transition that occurs during cancer metastasis in that identical signaling pathways and effector molecules are involved. This transition has been well characterized in the sea urchin and thus provides an in vivo model system to further elucidate the changes that cells undergo during EMTs. This study sought to identify signaling pathways and molecular correlates to EMT. In particular studies include an examination of Notch and Fibroblast Growth Factor (FGF) signaling pathways as well as members of the T-box and Ets transcription factor families. The goal of these studies is two-fold: first to enhance the knowledge on endogenous EMTs in order to have a basis of comparison for those occurring during metastasis and second, to identify new genes involved in the process and therefore provide potential targets for new therapies aimed at blocking cancer progression.
During this annual period, progress was made on the research proposed. This progress is summarized below.

**Aim 1:** ‘What role do Ets factors play in epithelial-mesenchymal transitions’.

Ets proteins comprise a family of winged-helix transcription factors that bind to DNA monomerically at a consensus 10bp site. These factors are regulated by ras dependent MAP kinase phosphorylation of their N-terminal regulatory domains and cooperate with other ras responsive AP1 transcription factors to regulate gene expression (Yang et al., 1996; Wasylyk et al., 1997). Ras has been implicated in oncogenesis since a high number of human cancers exhibit permanently active ras molecules (Downward, 1997). Ets factors, being downstream of ras signaling, may therefore be involved in cancer progression by contributing to the transforming potential of activated ras. Additionally, Ets factors have been directly implicated in breast cancer progression and the invasiveness of certain cancers. Ets proteins overexpressed in mammary tumor cells include ESX/ESE-1, PEA3 and ERM (Dittmer and Nordheim, 1998). Invasive breast cancers produce Ets1 while non-invasive ones do not (Delannoy-Courdent et al, 1996).

As well, blockage of Ets dependent transcription by either antisense Ets1 or expression of the Ets DNA binding domain alone is sufficient to block invasiveness in vitro (Iwasaka et al., 1996; Chen et al., 1997, Foos et al., 1998). Ets factors have been identified in the sea urchin embryo (Wei et al., 1999; Qi et al., 1992; Chen et al., 1988) and one such factor is expressed prior to an epithelial-mesenchymal transition (Kurokawa et al., 1999). Overexpression of this factor results in embryos producing an excess of mesenchymal cells, while expression of a C-terminal DNA binding domain alone exhibits the opposite phenotype.

The goal of this aim was to perturb Ets function in a similar manner in order to characterize the effects on integrins, cadherins and known effectors of EMT. Another goal was to determine where Ets factors stand in the sea urchin endomesoderm gene network as well as how Ets might be regulated.

During this past period, a gene regulatory network that controls the specification of mesoderm and endoderm in the sea urchin embryo was worked out collaboratively with the laboratory of Eric Davidson at the California Institute of Technology, in conjunction with our lab. This network is based on large-scale perturbation analyses in combination with computational methodologies, genomic data, cis-regulatory analysis and molecular embryology (Davidson et al, 2002). According to the model, Ets is upstream of the EMT and appears necessary for the differentiation of the primary mesenchyme cell population.

QPCR analysis places Ets upstream of known terminal mesenchymal genes, including Msp 130, Sm 50, Ficolin, Sm27, and Msp L. Additionally, Ets appears to be upstream of two more general endomesoderm specific genes, Deadringer and Fox B (Figure 1). We asked how Ets might be regulated, either by specific transcriptional activation or a phosphorylation event. A paired box transcription factor is upstream of Ets transcription (Olivieri et al, 2002, 2003). It had been shown that Ets was a downstream target of phosphorylation by the Ras-Raf-MEK-ERK signaling pathway in cell culture (Rabault et al, 1996; Fafeur et al, 1997; Paumelle et al, 2002), so an inhibitor of ERK was utilized to
determine if the pathway might work similarly in the urchin. Indeed, the inhibitor of
ERK prevented the specification of mesenchymal cells and a failure of EMT. If ERK
acts through Ets in this sequence, the prediction that follows is that a constitutively
active form of Ets might rescue the drug-induced inhibition of EMT. This appears to be
the case since the injection of RNA encoding a constitutively active Ets causes the
specification of excess mesenchymal cells, even in the presence of the drug. Thus, the
ras pathway, through Ets, regulates the progression of specification, leading to ingestion.
When Ets functions, it specifies the apparatus required for EMT. A later unknown step
provides the proximal signal to initiate the EMT.

Two publications from this work were published during this period [Oliveri, P.,
Davidson, E.H. and McClay D.R. (2003). Activation of pmar1 controls specification of
micromeres in the sea urchin embryo. Dev Biol 258, 32-43; Peterson, R.E. and McClay,
D.R. (2003). Primary mesenchyme cell patterning during the early stages following
ingression. Dev Biol 254, 68-78.] and a reprint of Oliveri et al is attached in the appendix
for more detailed analysis of the results described above.

AIM 2: ‘To characterize the role of FGF and Notch signaling during epithelial-
mesenchymal transitions’

Months 1-18 in the ‘Statement of Work (SOW) were allocated to study Notch signaling,
T-box genes, and the relation between the two. Murine mammary tumor virus (MMTV)
induced tumorigenesis previously identified the INT3 gene as a locus for viral insertion
(Gallahan and Callahan 1987, 1997). The INT3 gene encodes a mammalian Notch
homologue, Notch4. Viral insertion generates a truncated protein consisting of only the
intracellular portion of the receptor, and thus, a constitutively active signal. In the sea
urchin, a similar form of the Notch protein (LvN\textsuperscript{\textasciitilde}) when expressed results in an increase
in the number of migratory mesenchyme cells (Sherwood and McClay 1999).

The first aspect of this aim was to characterize a possible downstream component of the
Notch pathway that may be involved in the EMT. One possible candidate, the T-box
gene Brachyury, was characterized, but was shown not to be a direct target of Notch
signaling. However, it was significant because the work showed that Brachyury
functioned in gastrulation movements of the endoderm. Brachyury may be important in
coordinating adhesive and cytoskeletal changes, since these are necessary for gastrulation
movements (Gross and McClay, 2001). During the course of this study, another sea
urchin homologue of a T-box gene, Tbx2, was cloned because it had recently been
implicated in breast cancer progression (Barlund et al., 2000; Jacobs et al, 2000). We
refer to this gene as Tbx2/3 due to the fact that the Tbx2/3 subfamily of T-box genes is
represented by only one gene, which is a predecessor of the duplication event that
generated individual Tbx2 and Tbx3 genes. As mentioned in the last report, Tbx2/3
expression was analyzed with polyclonal antisera generated against recombinant protein.
Nuclear expression of LvTbx2/3 initiated at the mesenchyme blastula stage embryo and
persisted throughout the larval stage of the embryo. Tbx2/3 was expressed
asymmetrically in the dorsal region of all three germ layers of the embryo throughout this
developmental time period. The molecular linkage of dorsal specific Tbx2/3 expression
within all three germ layers was demonstrated by perturbing events thought to pattern the dorsal ventral axis of the embryo. Based on these perturbations, it appears Tbx2/3 is downstream of the specification of endoderm, mesoderm and ectoderm, including the initial dorsal-ventral specification events. This data indicates that Tbx2/3 may be proximal to the morphogenic movements that shape dorsal structures.

During this past funding period, Tbx2/3 was better characterized. It appears as though early on during the initial ingression of mesenchyme cells that the expression of Tbx2/3 is dynamic. The entire population of primary mesenchyme cells expresses Tbx2/3 during their initial ingression into the blastocoeol of the embryo. Later, expression is restricted to only the dorsal mesenchymal cells. This is the time that skeletal patterning begins to shape these cells into the spicule skeleton of the embryo, providing further evidence that Tbx2/3 function lies downstream of the signals that set up the dorsal-ventral axis. To determine the function of Tbx2/3, ectopic mRNA expression studies were performed. Overexpression of Tbx2/3 mRNA produced abnormal morphological phenotypes and patterning deficiencies in the derivatives of all three germ layers. Yet, normal marker expression remained in each germ layer, indicating that ectopic Tbx2/3 does not perturb germ layer specification. Therefore, the phenotype may be due to genes directly downstream from Tbx2/3 that may be involved in patterning and morphogenesis. Thus, Tbx2/3 appears to inessential for mesenchyme formation or the EMT and therefore may not be a useful target for therapeutics aimed at the EMT. Yet, the work is significant because it shows that Tbx2/3 is necessary for important morphological movements of the skeletal mesenchyme cell population which require changes in adhesive molecules and the cytoskeleton in order to occur.


Months 18-36 of the SOW were allocated to the study of FGF signaling. Work on FGF signaling during this period has not progressed well due to the inability to isolate an endogenous sea urchin FGF receptor. However, during the past year an effort to sequence the entire genome of the sea urchins S. purpuratus and L. variegatus has progressed rapidly with support from the Human Genome Research Institute of the National Institute of Health. The sequencing is being done at the Baylor College of Medicine, Human Genome Sequencing Center, Houston, Texas. Since the inception of this effort in March of 2003, already 0.4 X of the genome has been covered. A search of this database was performed and several hits were discovered for FGF receptors. At the present time, an effort is being made to clone and characterize these receptors in order to determine if FGF signaling plays a role in the EMT of mesoderm in the sea urchin embryo.
Figure 1.

Model for Endomesoderem specification to 24 hours
View from the genome
May 21st, 2003
Figure 1: The endomesoderm gene regulatory network. Ets is downstream of the primary mesenchyme specific paired box transcription factor, pmar1. Ets is upstream of the endomeseoderm specific genes deadringer (Dri), and foxb as well as the terminal primary mesenchyme genes msp l, msp130, sm50, ficolin and sm27. See purple box labeled PMC.
Key Research Accomplishments:

- Characterization of the downstream targets of activated Ets.
- Characterization of the upstream phosphorylation events necessary for the activation of Ets.
- Determination that Ets functions before EMT and is necessary for the specification of mesenchymal mesoderm.
- Determination that Tbx2/3 functions downstream of dorsal-ventral specification signals, possibly as a proximal factor necessary for the expression of genes necessary for morphological changes in all three dorsal germ layers.
- Cloning of FGF receptor found in the sea urchin genome project database.
Reportable outcomes:


- Poster Presentation- July 2002, ‘Society For Developmental Biology Annual Meeting’ Madison, WI.
Conclusions:

The overall goal of this research is to provide an in vivo insight into the mechanism whereby adhesions are assembled and disassembled during EMT and metastasis and thereby identify potential targets for therapeutics aimed at preventing the spread of breast cancers. Genes and signaling pathways implicated in EMT and the invasiveness of breast cancers include FGF, Notch and T-box and Ets family transcription factors. During the past funding period, studies were performed relating to each of these pathways and factors. Ets factors were shown to increase the amount of mesenchyme in the embryo and Ets was placed with the framework of the sea urchin endomesoderm network. Several genes were shown to be downstream of Ets and one upstream transcription factor was shown to impinge upon Ets transcription. It was shown that the ras pathway, through Ets, regulates the progression of specification necessary the EMT to occur. A second goal of the study was to examine the role of the T-box family member, Tbx2/3, might play in EMT. Tbx2/3 is expressed in all dorsal regions of the embryo, downstream of β-catenin or β-catenin downstream genes, BMP2/4 and likely growth factor signaling. Tbx2/3 functions in these regions in pattern formation, and possibly in the morphological movements of the mesenchyme cell population. Lastly, FGF signaling was studied. Endogenous FGF receptors were identified from the sea urchin genome sequencing database and are in the process of being cloned.


Activation of pmarl controls specification of micromeres in the sea urchin embryo

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Abstract

pmarl is a transcription factor in the paired class homeodomain family that was identified and found to be transcribed in micromeres beginning at the fourth cleavage of sea urchin development [Dev. Biol. 246 (2002), 209]. Based on in situ data, molecular perturbation studies, and QPCR data, the recently published gene regulatory network (GRN) model for endomesoderm specification [Science 295 (2002) 1669; Dev. Biol. 246 (2002), 162] places pmarl early in the micromere specification pathway, and upstream of two important micromere induction signals. The goal of this study was to test these three predictions of the network model. A series of embryo chimeras were produced in which pmarl activity was perturbed in one cell that was transplanted to control hosts. At the fourth cleavage, micromeres bearing altered pmarl activity were combined with a normal micromereless host embryo. If β-catenin signaling is blocked, the micromeres remain unspecified and are unable to signal to the host cells. When such β-catenin-blocked micromeres also express Pmarl, all observed micromere functions are rescued. The rescue includes expression of the primary mesenchyme cell (PMC) differentiation program, expression and execution of the Delta signal to induce secondary mesoderm cell (SMC) specification in macromere progeny, and expression of the early endomesoderm induction signal necessary for full specification of the endoderm. Additionally, Pmarl expressed mosaically from inserted DNA constructs causes induction of Endo 16 in adjacent cells, demonstrating further that Pmarl controls expression of the early endomesoderm induction signal. Based on these experiments, Pmarl is an important transcription factor necessary for initiating the micromere specification program and for the expression of two inductive signals produced by micromeres. Each of the tests we describe supports the placement and function of Pmarl in the endomesoderm GRN model. © 2003 Elsevier Science (USA). All rights reserved.

Keywords: Gene regulatory network; Sox; Delta; β-Catenin; Paired homeodomain

Introduction

The recently published gene regulatory network (GRN) for endomesoderm specification provides a new focus for our view of early development (Davidson et al., 2002a,b). That view of development is a progression of specification sequences with many inputs, beginning with cytoplasmically localized information, and later with coordinating inputs of short-range signals from other cells. Each level of specification results from a complex integration of transcription factor inputs (Davidson, 2001). The endomesoder-
molecular function and cis regulatory analyses that will test and challenge predictions of the network. The present set of experiments is among the first of the biological tests that will, along with the cis regulatory solutions, vastly reinforce confidence in the authenticity of the network. Other efforts are underway with many genes to establish whether the predicted connections between transcription factors are direct as indicated in the current model, or whether there is a more indirect relationship between genes in question.

The specification of micromeres is a component of the larger endomesoderm GRN. This cell lineage gives rise to the PMCs, the cells responsible for production of the embryonic skeleton. Micromeres have fascinated embryologists for more than 100 years because they are so easily identified at the fourth cleavage as a result of an unequal cell division at the vegetal pole. Bovari (1901a,b), established their lineage and fate. In the 1920s and '30s, Hörstadius carried out numerous blastomere manipulation experiments, including micromere transplantation (reviewed by Hörstadius, 1939). Micromeres transplanted to the animal pole of a host 16-cell-stage embryo induced an ectopic gut that invaginated from the site of transplantation. These results suggested that micromeres somehow induce endoderm and are important for placement of axial properties. In the absence of micromeres, the embryo lacks a gut (after a long delay, the embryo sometimes regulatively compensates and eventually replaces the gut). Ransick and Davidson (1993) later showed that micromeres begin endoderm induction very early, and that the induction of an ectopic gut at the animal pole exhibits expression of the same molecular markers as the endogenous gut. These experiments suggested that micromeres begin their specification sequence immediately after fourth cleavage and that micromeres are an important source of an early inductive signal (ES) for the endomesoderm lineage.

Embryological experiments demonstrated that micromeres themselves require no further input from the rest of the embryo beyond fourth cleavage since micromeres, isolated at the 16-cell stage, differentiate as PMCs and make skeletal spicules in culture (Okazaki, 1975). These cells require horse serum, so a caveat of the above experiment is the potential requirement for growth factors or other factors present in the horse serum. Nonetheless, numerous experiments have shown that micromeres isolated at fourth cleavage and transplanted to host embryos give rise exclusively to skeletogenic progeny, no matter where they are placed, so by fourth cleavage, in the context of the living embryo, they display autonomous specification. At the 16-cell stage, the nuclei of micromeres become positive for β-catenin, a transcriptional cofactor in the wnt signaling pathway (Logan et al., 1999; Peifer et al., 1991), and β-catenin is necessary for micromere specification (Emily-Fenouil et al., 1998; Logan et al., 1999; Wikramanayake et al., 1998). Though β-catenin is the earliest known input in micromere specification, other components of micromeres have been found to participate in steps that lead to PMCs. Several transcription factors are expressed exclusively in the micromeres, and perturbation of these has substantial effects on PMCs (Kurokawa, 1999; Davidson et al., 2002b; Amore et al., 2002). The endomesoderm GRN models a hierarchical pattern of gene activation and repression based on perturbation of one and observing the effect on expression of other transcription factors known to be in the micromeres. Based on logic, these studies reveal that maternal nuclear β-catenin and Otx activate transcription of pmarl at fourth cleavage, and Pmarl then initiates the micromere specification sequence. Pmarl is proposed to be a direct target for the β-catenin, and is further proposed to be upstream of the early endoderm induction signal (ES), the expression of Delta, and the transcriptional apparatus that specifies the differentiated PMCs. Several substantial pieces of information lead to this model. First, Pmarl is expressed specifically in micromeres beginning at the fourth cleavage, so it is expressed in the right place at the right time. Second, experimental activation of pmarl in other parts of the embryo causes an ectopic specification of mesenchyme-like cells. Third, upregulation of pmarl leads to upregulation of other micromere lineage transcription factors, but the reciprocal activation is not true (Oliveri et al., 2002).

A later functional property of micromeres is the expression of the Delta ligand to activate Notch in veg 2 cells, a necessary step in the induction of SMCs (Sherwood and McClay, 1999). Delta is released between the 8th and 10th cleavage and is expressed by micromeres at the right time for this signal to activate Notch on the adjacent veg 2 cells (Sweet et al., 2002). Thus, shortly after the micromeres initiate their own specification, they begin to signal to other lineages in the embryo. Ransick and Davidson (1993) showed that a different micromere signal induces endoderm beginning between the 4th and 6th cleavage.

Given this rich background, there are two sources of information necessary to validate the GRN model for the micromeres and extend it. First, since many of the perturbation studies utilize injections that alter gene regulation in the entire embryo, biological studies are necessary to authenticate the function of the GRN in the specific cells where specification events are thought to occur. Second, cis regulatory analysis of pmarl and other genes in the micromere GRN will strengthen confidence in the proposed transcriptional activation sequence. The purpose of this paper is to test experimentally the micromere portions of the GRN, especially as they relate to pmarl function, using blastomere transplantation approaches. Experiments show that all three major functions of the micromere progeny are dependent on pmarl function. The ES and the Delta signal are downstream and essentially independent of the regulatory apparatus controlling PMC differentiation. Thus, we confirm that pmarl activation is one of the most crucial steps in the activation and specification of the micromere lineage.
Fig. 1. Pmarl rescues embryos in which β-catenin nuclearization is prevented. All embryos at 36 h. (A) Control embryo. PMCs are stained with a PMC marker, and in the center of the PMC ring, the blastopore of the archenteron is visible (arrowhead). (B) An embryo as in (A) but with micromeres removed at the 16-cell stage. The embryo has no PMCs, and no archenteron (16/16 cases). (C) Micromeres from embryos injected with Δ-cadherin were returned to a micromereless embryo as in the inserted diagram, grown to 36 h, then stained with the PMC marker. There were no PMCs and no archenterons (35/36 cases). Control micromeres added to micromereless embryos resembled control embryos with PMC rings and archenterons (17/17 cases; data not shown). (D, E) Micromeres from embryos injected with both Δ-cadherin RNA and pmarl RNA were transplanted to the vegetal pole of micromereless control embryos. The embryos had PMC rings and archenterons (arrowhead in D) (29/30 cases). Green stain is a PMC marker; red stain (in D) or blue stain (in A–C and E) is directed against β-catenin in adherens junctions. In all experimental cases, one micromere was transplanted to the vegetal pole of a micromereless host.

Fig. 2. Pmarl expression is sufficient to specify any blastomere as a skeletogenic mesenchyme cell precursor. (A) Host micromereless embryos that do not receive micromeres at the vegetal plate have no PMCs and fail to gastrulate by 38 h (18/18 cases). (B) The experiment. Eggs were injected with Δ-cadherin RNA, pmarl RNA, and rhodamine dextran. At the 16-cell stage a single, double-injected mesomere (red cell in diagram) was transferred to the vegetal pole of a micromereless control embryo. (C) The Pmarl-expressing mesomeres ingress as PMCs, stain with a PMC marker (red), and the archenteron is normally completed by 38 h (17/18 cases). (D) Ingressed mesomeres (stained green with a PMC marker) form a normal-looking PMC ring. The red stain is β-catenin in adherens junctions. (E) When transplanted to the vegetal plate of embryos with endogenous micromeres, the double-injected mesomeres still make PMCs, (arrowheads pointing to red-stained mesomere-neo-PMC cells) and join into the vegetal plate ring along with the endogenous PMCs. (F) The skeleton produced by a Pmarl-expressing mesomere transformed into a PMC is entirely of mesomere origin (rhodamine-labeled mesomere cells are pseudocolored in the host embryo, and an additional spicule is out of focus in the background).
Micromanipulation and imaging

Eggs of *Strongylocentrotus purpuratus* were shed in SW following injection of animals with 0.5 M KCl. Eggs were washed in SW and fertilized with dilute sperm in SW containing para-aminobenzoic acid (15 mg/100 ml SW). Cultures were incubated at 16°C. Just prior to surgery, the embryos were passed through 102-micron Nitex mesh four times to remove fertilization envelopes. Alternatively, if embryos were previously injected with RNA, they were pipetted into a narrow bore pipet that manually shears off the fertilization envelope without damaging the embryo. Surgeries were adapted from McClay et al. (2000). Briefly, to transplant cells from *S. purpuratus*, it was necessary to pretreat the embryos by incubation for 30 s in HEM (McClay, 1986), containing 2 mg/ml BSA (HEM/BSA). The BSA eliminates problems with the very sticky hyaline layer surrounding the *S. purpuratus* embryo, and the HEM softens the hyaline layer so that cells can be transplanted.

Embryos to be manipulated were transferred from the HEM/BSA to modified Kiehart chambers (Kiehart, 1982). The chambers were modified so that two glass needles could be inserted into the chamber at 90° to each other. One of the glass needles served as a suction pipet, and the other needle served as a holding and manipulating pipet. These were driven by two joystick micromanipulators. The suction applied was by mouth. For cell transplantations, donor cells were pulled into the suction pipet one at a time. The bore of the pipet was preadapted to a slightly smaller I.D. than the diameter of the cell to be transplanted so that inside the pipet the cell took on a sausage shape. The cell was transferred to a host embryo and inserted into position. The sausage shape helped with the insertion between cells in that the donor cell overlapped initially both to the inside and the outside of the host cell layer. Within a minute, the donor cell rounded up and was retained in position by the host. Embryos were then transferred back to SW. For all operations, the embryos were in HEM for less than 5 min each, and sham control cell to control embryo operations, development was indistinguishable from unoperated control embryos both in timing of morphogenetic events and in final phenotype.

Several operations were performed. Many of the experiments required that host embryos were first made micromereless. Micromeres were removed early in the 16-cell stage and the embryos were inspected to assure that all 4 micromeres were removed. Donor cells were inserted in the cleft between the 4 macromeres. When these embryos were inspected at the 32-cell stage, the transplanted donor cells were in the same position as control micromeres relative to macromeres. Because the insertion cleft is small, generally only 1 or 2 of the 4 micromeres were transplanted. Nevertheless, previous work demonstrated that 1 micromere conveys an inductive signal that is virtually indistinguishable compared with the normal 4 micromeres (with only a reduction in the number of pigment cells indicating a reduced level of induction) (McClay et al., 2000).

When mesomeres were transplanted to the vegetal or animal poles, they were taken from the donor embryo at the 32-cell stage and 1 mesomere daughter was transplanted into the same position as micromeres. When mesomeres (or micromeres) were transplanted to the animal pole, they were inserted between the 4 blastomeres closest to the animal pole, again inserting the sausage-shaped cell into the cleft. The transplanted donor cells were prelabeled with rhodamine dextran (10,000 mw; 1:5 dilution of a 50-mg/ml stock into the injection medium). We avoided the lysinated form of rhodamine dextran, which prevented the dye from being fixed with the tissue; in preliminary experiments, the high lysine content of the fixable dye tended to reduce the effectiveness of injected molecules.

Prior to fixation, the embryos were examined by fluorescent microscopy and scored for phenotype. They were then fixed for 20 min with 2% paraformaldehyde in SW, postfixed 5 min in MeOH (on ice), and washed into SW containing 4% normal goat serum (SW-NGS). Throughout antibody-staining reactions, the embryos remained in SW-NGS and all antibodies were diluted in this solution. Embryos were stained with antibodies to PMCs [1D5, a monoclonal recognizing MSP-130 (Wessel and McClay, 1985)], β-catenin (Miller and McClay, 1997a), cadherin (Miller and McClay, 1997b), SoxB1 (Kenny et al., 1999), pigment cells (Gibson and Burke, 1985), and Notch (Sherwood and McClay, 1997). Embryos were examined by confocal microscopy using either a Zeiss 410 or 510 scanning confocal microscope.

DNA and RNA constructs and injection of into eggs

A Hatching Enzyme promoter--pmarl DNA construct was engineered by replacing the GFP coding region already present in a HE-GFP clone described by Bogarad et al. (1998). The GFP was removed by using the *NheI* and *Xhol* unique sites present in the construct, and the coding region of *pmarl*, obtained by digestion with *SpeI* and *Xhol*, was inserted (Oliveri et al 2002). The subclone was checked by restriction digestion analysis of known sites and by sequencing. The DNA clone obtained was linearized by using *KpnI* and injected as described by McMahon et al. (1985), with the exception of the glycerol in the injection solution. RNAs for injection were synthesized as described by Oliveri et al. (2002). For micromere rescue and blastomere micromanipulation experiments, RNA was injected along with rhodamine dextran (10 pg/pl; Sigma). The injection concentration of the probes was: Δ-cadherin RNA (60 ng/μl), *pmarl* RNA (5.6 ng/μl) for both the single injection and the coinjection experiments. The injection volume for each egg was approximately 2 pl. In experiments to determine the minimum amount of *pmarl* sufficient to rescue the
\(\Delta\)-cadherin phenotype, \(\Delta\)-cadherin mRNA at 60 ng/\(\mu\)l was coinjected with pmarl RNA at 5.6, 2.8, 1.1, and 0.56 ng/\(\mu\)l.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization was performed as described by Ransick and Davidson (1995), with the following modifications. The endo16 probe was transcribed in presence of 10× DIG RNA labeling mix (Roche) and the GFP probe in presence of 10× Fluorescein RNA labeling mix (Roche). The two probes were hybridized to fixed embryos at the same time under the conditions described by Oliveri et al. (2002). The detection of the two different probes was obtained following the conditions described by Hauptmann (2001) and using the NBT/BCIP ready-to-use tablet (Roche) for the DIG-labeled probe and the Fast Red (Roche) for the Fluorescein-labeled probe.

**Results**

Pmarl expression rescues cadherin-inhibited specification of micromeres

\(\beta\)-Catenin enters the nuclei of micromeres shortly after their birth at the fourth cleavage (Logan et al., 1999). Experimentally, the nuclear entry of \(\beta\)-catenin and its subsequent association with TCF-LeF (Vonica et al., 2000) is required for micromere specification. If nuclear entry of \(\beta\)-catenin is blocked by expression of the cytoplasmic tail of cadherin (Wikramanayake et al., 1998), or by blocking GSK-3 (Emily-Fenouil et al., 1998), micromeres fail to be specified. Oliveri et al. (2002) found that one of the first markers to be expressed in micromeres following nuclear entry of \(\beta\)-catenin is pmarl, a transcription factor of the paired homeodomain family. pmarl expression is greatly diminished if \(\beta\)-catenin fails to enter the micromere nuclei. Manipulation of pmarl in whole embryos demonstrated that it acts as a repressor. The most direct evidence for this is that introduction of mRNA encoding an Engrailed (en) repressor domain with pmarl homeodomain fusion forces the pmarl-en fusion to act as an obligate repressor for target genes. This construct produces identical effects to introduction of mRNA encoding the natural Pmarl protein. Both cause all downstream micromere genes to be globally derepressed. Therefore, the micromere-specific expression of the pmarl gene normally has the effect of repressing a gene encoding a repressor of these downstream genes. It thereby allows them to become active exclusively in micromeres and their descendants. In addition, the Pmarl protein includes a sequence motif that shows similarities to several well-known transcriptional repressors (Oliveri et al., 2002).

To ask how pmarl works in the micromeres, we began a series of studies in which expression of pmarl was modified only in the micromeres. Three functions were examined: (1) expression of the ES (Ransick and Davidson, 1995), a signal necessary for correct specification of the endomesoderm and for on-time invagination of the archenteron, (2) expression of the Delta signal, necessary to activate Notch in the veg 2 cells to specify SMCs (Sherwood and McClay, 1999; Sweet et al., 2002); and (3) differentiation of micromere progeny into skeletogenic mesenchyme cells. Earlier transplantation studies showed that, if normal micromeres are transplanted from a donor 16-cell-stage embryo to a host micromereless embryo, the micromeres behave just as they do in an unmanipulated embryo (McClay et al., 2000). If a micromereless host received transplanted micromeres, the micromeres ingress as PMCs, express surface molecular markers, and produce a skeleton. The embryo gastrulates at the correct time (32-36 h for \textit{S. purpuratus} at 16°C). SMCs are specified normally, based on the appearance of pigment cells by 48 h, then coelomic pouches, a muscular pharynx, and blastocoelar cells in pluteus larvae at 72 h. If, in the same operation, micromeres are not replaced, as in Fig. 1B, most embryos fail to gastrulate. In a large number of trials, many fewer than 20% of the micromereless embryos eventually gastrulate. Those few embryos that eventually recover and gastrulate become albino pluteus larvae, their archenteron invagination is delayed to beginning at 55–72 h, and PMCs plus nonpigmented SMCs become replaced only in those embryos that eventually gastrulate. Thus, gastrulation and specification of SMCs are severely compromised by the absence of micromeres. In embryo recombinations, when \(\beta\)-catenin-induced input was eliminated from micromeres by transplantation of \(\Delta\)-cadherin-expressing micromeres to replace normal micromeres on uninjected host embryos, the embryos developed as if they were micromereless (Fig. 1C). The chimeras lacked PMCs, invagination of the archenteron was greatly delayed if it occurred at all, and all such embryos were albino, presumably as a consequence of a failure of the micromeres to express Delta.

When pmarl RNA was injected along with the same \(\beta\)-catenin-inhibiting concentration of \(\Delta\)-cadherin, and micromeres transplanted at the 16-cell stage, normal-appearing development returned (Fig. 1D and E). The double-injected micromeres became PMCs, a skeleton formed, and archenterons invaginated at the correct time (32-36 h), in almost all of the transplant combinations (greater than 95% of 136 transplant combinations in 19 separate experiments) (Fig. 1D and E). When embryos were cultured longer, they displayed the full range of SMCs, including pigment cells (see Fig. 3 below), a muscular pharynx, coelomic pouches, and blastocoelar cells. Thus, expression of pmarl rescued all the known functions of micromeres, including production of both induction signals and the production of a normal skeleton. Whole embryos that expressed pmarl alone or \(\Delta\)-cadherin plus pmarl formed a mass of mesenchymal cells.
The Pmarl rescue of micromeres is effected by fewer copies of RNA than normally are synthesized by micromeres

A general concern of the RNA injection experiments, like the rescue experiment described above, is that the phenotype observed could be caused by a vast overexpression of pmarl that forces an artificial conversion of cells to the mesenchyme phenotype. Although the Δ-cadherin: pmarl ratio of injected mRNA was 10:1, the described rescue of the β-catenin-devoid phenotype was obtained with the injection of roughly 20- to 40-fold excess amount of pmarl mRNA into whole eggs, relative to calculated amounts of endogenous pmarl RNA in micromeres (see Materials and methods for injection conditions). If one conservatively considers no degradation of the injected RNA and assumes equal partitioning of the injected RNA throughout the cytoplasm, the micromeres at the moment they are born (4th division) will inherit just 1.6- to 3.2-fold excess pmarl mRNA relative to untreated embryos [the micromeres segregate 8% of egg cytoplasm (Davidson, 1988)]. Thus, the observed rescue is unlikely to result from vast overexpression of pmarl.

To experimentally establish the minimum amount of pmarl necessary to rescue micromere specification, we analyzed effects of a dilution series of RNA injections. One might predict that the dominant repressor function of Pmarl, to extinguish a ubiquitous repressor and thereby activate the micromere-PMC cell program, can be reached with a relatively low number of transcripts. To test that hypothesis, we determined the smallest amount of pmarl necessary to overcome the Δ-cadherin-altered phenotype. pmarl RNA at increasing dilutions was injected into eggs along with the minimum number of molecules of Δ-cadherin RNA known to eliminate β-catenin signaling (Δ-cadherin: pmarl ratio of transcripts 20:1, 40:1, and 100:1). Embryos were scored at 24 h of development for the resulting phenotype. Using this approach, we find the minimum number of pmarl copies/cell necessary to rescue the micromere specification, as well as transfect the other blastomeres, is 0.4- to 0.8-fold the normal amount of pmarl RNA present in micromeres at 5th cleavage. This corresponds to about 66–130 copies per micromere relative to 163 copies/cell maximally present in normal embryos between the 4th and 8th cleavage (Oliveri et al., 2002). Thus, pmarl rescues micromere specification even when present at a fraction of levels expressed in control embryos.

Ectopic Pmarl expression specifies the micromere program in all cells of the embryo

In micromeres, the endomesoderm GRN predicts Pmarl to repress a ubiquitous repressor expressed everywhere in the early embryo. If this ubiquitous repressor normally blocks micromere specification elsewhere in the embryo as well, the model predicts that ectopic expression of pmarl should force nonmicromeres into the micromere specification pathway. The mesenchymal phenotype of pmarl mRNA-injected whole embryos tends to support this hypothesis. But, are those Pmarl-expressing cells converted to functional micromeres? To address this question, pmarl RNA was either injected with Δ-cadherin RNA or alone (with an identical phenotypic outcome). At the 16-cell stage, a single mesomere (or macromere in other cases) was removed from the animal pole of injected embryos and transplanted to the vegetal pole in place of four removed micromeres of an un.injected control embryo. Mesomeres normally produce only oral and aboral ectoderm. Fig. 2 shows the remarkable result that the progeny of the transplanted Pmarl-expressing mesomeres behave in almost every way like micromeres. The Neo-PMCs of the chimera ingress, the archenteron invaginates at the correct time demonstrating that the transplanted mesomere conveys the ES pathway. The mesenchymal phenotype of pmarl shows the remarkable result that the progeny of the transplanted Pmarl-expressing mesomeres are pigmented (Fig. 2C). The transformed mesomeres make a normal-appearing PMC ring (Fig. 2D and E), and they make a skeleton (Fig. 2F) (18/18 cases). When examined at 48 h., embryos with transformed mesomeres are pigmented (Fig. 3C), suggesting that the Delta ligand was produced correctly (4/6 cases), while control micromereless embryos (18/18), micromereless embryos that received uninjected mesomeres (9/9), or micromereless embryos that received mesomeres with Δ-cadherin only (6/6), failed to gastrulate, failed to make PMCs, and failed to make pigment cells (data not shown). Pluteus larvae with Pmarl-transformed mesomeres were observed to have a muscular pharynx, coelomic pouches, and blastocoelar cells, indicating that the full range of SMCs was specified (data not shown). The only abnormal aspect of the mesomere-PMC conversion observed was somewhat abnormal pattern of the skeleton. With this exception, early ectopic pmarl expression is able to convert a mesomere from its normal ectodermal fate into cells of the micromere-PMC lineage, and the conversion is almost complete. A similar outcome is observed if pmarl-injected macromeres are transplanted to the vegetal plate (data not shown). We conclude that expression of pmarl is sufficient to launch the micromere specification program. Since any cell expressing Pmarl is specified as a micromere/PMC, we suggest that no other maternal components are necessary in micromeres other than those that activate pmarl.

Pmarl expression in micromeres is upstream of the two signals that induce endoderm and secondary mesoderm

The SMC induction signal, Delta, is expressed by micromeres between the 8th and 10th cleavage (McClay et al., 2000; Sherwood and McClay, 1999; Sweet et al., 1999 2002). Previous work showed that ectopic pmarl expression activates ectopic delta RNA expression (Oliveri et al., 2002). To confirm that Pmarl is upstream of Delta, and importantly, that the Delta produced in response to pmarl activation has SMC inductive activity, we manipulated pmarl in micromeres and mesomeres as above and then
Fig. 3. Pmar1 expression is necessary for production of the SMC induction signal. (A, B) cad/pmari double-injected micromeres were transplanted to the vegetal pole of control micromereless host embryos. (A) At 48 h, the cad/pmari-injected cells were PMCs (Portion of ring of fluorescent cells in A). (B) At the same plane of focus, that embryo also had nonfluorescent (i.e., induced) pigment cells (arrowheads) (5/6 cases examined). (A) Epifluorescent image of the same embryo showed in (B) (bright field). (C) pmar1-expressing mesomeres transplanted to the vegetal pole of micromereless embryos also induce the host to produce pigmented cells (arrowheads) (4/6 cases examined). Control micromereless embryos were albino (6/6 cases examined in this experiment; data not shown).

Fig. 4. Pmar1 is upstream of the early endoderm induction signal. An assay for a consequence of the ES signal examines nuclear SoxB1 immunocytoologically (green nuclei). In each case, PMCs were removed as micromeres or ingressed, leaving only endomesoderm at the vegetal plate (with the exception of C).
examined the ability of those micromeres, when transplanted to the vegetal plates of unmanipulated micromereless embryos, to induce pigment cells. Fig. 3 shows that when Pmarl is present in the transplanted micromeres, a pigmented embryo results by 48 h. (23/30 cases). Embryos are albino in the absence of pmarl-expressing micromeres (6/6 analyzed cases). Manipulation of pmarl in micromeres has the predictable effect on that induction sequence (no pmarl expression, no induction). Embryos with pmarl-expressing mesomeres also produce pigment cells by 48 h (Fig. 3C). If grown to the pluteus stage, embryos with transplanted Pmarl-expressing micromeres or mesomeres also have a muscular pharynx, coelomic pouches, and blastocoelar cells (data not shown). We conclude that pmarl expression is sufficient for the production of the Delta induction signal, a signal that is necessary for SMC specification.

The early endomesoderm induction signal (ES) is first detected between the fourth and sixth cleavage (Ransick and Davidson, 1995), i.e., if micromeres are present during that time endo 16 message is later detected normally in the vegetal plate. If micromeres are removed, endo 16 expression is reduced progressively depending on whether micromeres are removed at the 6th, 5th, or 4th cleavage. The provisional endomesoderm GRN proposes pmarl expression to be required for rapid activation of the ES in the micromeres (Davidson et al., 2002a,b). The next tests examined this prediction that place pmarl immediately upstream of the production of the ES.

To examine the ES, two assays were developed that require this signal. The first assay measured disappearance of nuclear SoxBl (Fig. 4). SoxBl is a transcription factor present maternally in all cells (Kenny et al., 1999). Early in development, maternal SoxBl protein concentration is greatly reduced in micromere nuclei, and after hatching is cleared from the nuclei of veg 2 cells and ultimately to the entire endomesoderm region. Fig. 4 shows that SoxBl clearance requires the presence of micromeres and that requirement fits parameters of the ES. An assay was designed, using immunohistochemical disappearance of nuclear SoxBl from the veg 2 cells as a measure, to examine the proximal consequences of the ES (Fig. 4). The ES also is a prerequisite for on-time gastrulation and for later expression of endo 16 in the vegetal territory, providing two additional another assays to assess ES activity (Ransick and Davidson, 1995) (Fig. 5). We used both of these assays to ask if pmarl expression in cells is necessary for the ES response in neighboring cells.

If micromeres are absent, there is no SoxBl clearance from the vegetal plate (Fig. 4B) at 20 h (early mesenchyme blastula stage), and the archenteron fails to invaginate until at least 24–36 h beyond normal invagination time in a small percentage of embryos. Control embryos with micromeres eliminate SoxBl from a large region of the vegetal plate that is about 13 cells wide, as counted from confocal sections (Fig. 4A), and gastrulate beginning at 30–32 h. If Δ-cadherin-expressing micromeres are transplanted to micromereless control embryos, SoxBl clearance from endomesoderm fails to occur (Fig. 4C) and the archenteron fails to invaginate. If the transplanted micromere expresses a rescuing level of Pmarl, SoxBl is cleared from the veg 2 cells, indicating that the ES is produced and functional (Fig. 4D), and the archenteron begins invagination at 30–32 h.

Fig. 5 demonstrates that pmarl is upstream of the early signal using a different experimental approach. The entire coding region of pmarl was placed under the control of the hatching enzyme (HE) cis regulatory element. The HE promoter is a well-characterized regulatory region that allows expression of a downstream gene everywhere in the embryo except in the most vegetal territories (Wei et al., 1997). Groups of 200–300 eggs were injected with constructs: hatching enzyme promoter-driven pmarl (HE-pmarl) and/or the hatching enzyme promoter-driven gfp (HE-gfp) (Bogarad et al., 1998). Double injected embryos concatenate the two DNA constructs and express them mosaically, in the same cell(s) (Arnone et al., 1997). Thus, if double injected, a gfp-expressing clone of cells will also express pmarl. The easily detected GFP therefore is used as an ectopic pmarl expression marker. As shown already by the whole embryo mRNA injections, all cells of the embryo have the capability of acquiring a micromere fate if they express pmarl (Oliveri et al., 2002). Therefore, ectopic cell

(A) Normal SoxBl clearance from the endomesodermal cells of the vegetal plate by 20 h (316/316 controls examined in 19 experiments). (B) If micromeres are removed during 4th cleavage, SoxBl fails to clear from the endomesodermal vegetal plate (34/36 cases examined). (C) If micromeres lacking β-catenin input are present at the vegetal plate, those micromeres fail to convey the ES, and no SoxBl is cleared from vegetal plate nuclei (3/3 cases examined) (D) Pmarl expression by transplanted micromeres rescues the ES as seen by the vegetal clearance of SoxBl from endomesoderm. The Pmarl-expressing PMCs ingressed (as they did in controls) (11/12 cases examined). All embryos shown were fixed at 20 h of development. Micromeres were labeled with rhodamine dextran to track location of micromere progeny. Prior to fixation, the location of PMCs was noted; then, with fixation, the red dye was lost from the cells. Fig. 5. Mosaic expression of pmarl in ectopic positions leads to endoderm induction in adjacent cells. (A) Schematic map of the two constructs used to induce mosaic ectopic pmarl and gfp expression via DNA injection. Both the pmarl coding sequence (red) and the gfp sequence (green) were placed under the control of the hatching enzyme promoter (HE). The hatching enzyme regulatory sequence normally drives expression ubiquitously, except in micromeres. (B, C) Embryos injected only with HE-gfp construct. As expected, GFP was expressed mosaically in patches of cells, except by PMCs as indicated by the green false color, in 95% of the cases. (E, F) Eggs received HE-gfp and HE-pmarl DNA. Again, GFP was expressed in a mosaic pattern (green cell clones), but in these embryos, GFP was expressed abundantly in PMCs, indicating that, wherever pmarl is expressed, the cells were transformed into PMCs. Note that the embryos show extra PMCs and a reduced blastocoel. In (D), eggs were injected with the HE-gfp construct and were examined by double in situ analysis at mesenchyme blastula stage with probes to gfp (brown-red) and an endoderm marker, endo 16 (blue-purple). The gfp is expressed only in non-PMCs tissues, and endo 16, as expected, is expressed in the vegetal plate only. By contrast, in (G), eggs that had been double injected with the gfp and pmarl constructs express endo 16 (blue-purple) in an extra patch of cells (purple arrowhead) adjacent to gfp (and pmarl)-expressing cells ingressed in the blastocoel.
clones expressing pmarl should exhibit micromere functions, including release of induction signals. Fig. 5B and C shows that, when the GFP reporter is expressed alone, it is expressed mosaically in nonmicromeric regions in all of the analyzed cases, as expected. The HE promoter drives the expression of the GFP either in single clones (Fig. 5C) or in multiple different clones (Fig. 5B) in 85–99% of the injected embryos, depending on the embryo batch. By contrast, at 24 h of development, GFP-expressing cells in the double injected embryos ingress into the blastocoel reflecting the behavior of PMCs (Fig. 5E and F). These embryos, in 86% of the cases, show reduced blastocoelar cavities with extra mesenchyme cells in them. Thus, the HE promoter activates pmarl expression ectopically. This activates the micromere-PMC regulatory program, and as a consequence, GFP now is expressed in mesenchyme cells.

The next test asked if those double-injected, transformed cells convey the early induction signal. Ectopic expression of pmarl not only should convert cells into the micromere lineage as described above, but also should induce adjacent cells to be specified as endomesoderm. This prediction was tested by double in situ detection of endol6, an early endomesoderm marker, and gfp at 24 h of development after coinjection of HE-gfp and HE-pmarl (on 66 embryos) or HE-gfp alone as control (on 58 embryos). In Fig. 5G, the gfp/pmarl expression (red-brown) is shown to be in cells that have ingressed into the blastocoel from an ectopic position relative to the endogenous vegetal plate (endol6 stain at the bottom of Fig. 5G). This phenotype is observed in 88% of the injected embryos. Embryo detection of RNA by in situ hybridization was about the same as direct observation of the GFP (88 vs 86% of embryos expressing extra mesenchyme cells). Adjacent to the ectopic gfp/pmarl cells, ectopic endol6 expression (dark blue-purple) is seen in an ectopic patch of cells (arrowhead in Fig. 5G), with the endogenous patch of endol6 seen at the bottom (75% of the scored double injected embryos show adjacent patches of cells positive for endol6 expression). Embryos injected with HE-gfp alone show the normal vegetal plate expression of endol6 and nonmesenchyme patches of gfp-expressing cells in 98% of the cases (Fig. 5F). We conclude from these two experiments that pmarl expression is upstream of the ES.

**Discussion**

**Use of blastomere transplantation to test gene regulatory network logic**

The GRN for micromere specification and function published by Oliveri et al. (2002) was based entirely on whole embryo data. Several kinds of experimental information underlie the provisional GRN. The most basic is the time and place of expression of all the genes that participate in the network. Causal relationships within the GRN emerged from extensive QPCR observations on the specific consequences to other genes after perturbing regulatory genes specifically in micromeres and their descendants. WMISH experiments were carried out to reveal the effects on other genes of the spatial misexpression of pmarl. The pmarl gene encodes a transcriptional repressor normally active only in micromeres. Its function is proposed to inactivate an otherwise globally active repressor(s), thereby allowing micromere-specific genes to be expressed only in that lineage. To those data, the experiments in this manuscript add direct biological tests of predictions of the GRN. The correct placement of three major functions of pmarl in micromeres is supported in micromere transplantation experiments. Further, ectopic activation of pmarl transfects other cells of the embryo to carry out the micromere program showing that expression pmarl is sufficient for those cells to become PMCs.

Fig. 7 is a current version of the micromere specification GRN, modified somewhat from that shown in Oliveri et al. (2002), and simplified to focus on the results of tests in the present set of experiments. None of the other modifications, which are based on more recent data (http://www.its.caltech.edu/~mirsky/qper.htm), affect any aspects of the GRN directly relevant to the present work. The GRN is essentially a logic map, intended to display the regulatory
activators that account for the specific functions executed by the micromere lineage up to the point of ingestion (20 h in *S. purpuratus*). There are three such functions: the expression of the “early signal” (ES) required for veg2 endomesoderm specification; the subsequent expression of the Delta signal required for specification of pigment and other mesoderm cell types from veg2 blastomeres lying adjacent to the micromeres; and the installation of a skeletogenic regulatory state in the micromeres.

The several specific features of the GRN shown in Fig. 7 that were directly challenged by the experiments in this manuscript are: (1) the prediction that expression of the *pmarl* gene is all that is required to transduce the essential 

 activates of the *pmarl* gene is all that is required to cause any cell in the embryo to execute all three of the functions normally carried out by the micromere lineage; (3) the corollary prediction that no prelocalized micromere “factors” or other features are needed downstream of *pmarl* gene for it to execute its role in micromere specification, nor are there any signals from the veg2 cells adjacent to the micromeres required; and (4) the prediction implicit in the GRN of Fig. 7 that, once *pmarl* is expressed, micromere specification carries on autonomously.

### Tests of the predictions

The experiments of Fig. 1 show that expression of *pmarl* in micromeres alone suffices to rescue skeletogenic specification dependent on the β-catenin input. The experiment in Fig. 2 generalizes this same result to blastomeres normally fated to become ectoderm (mesomeres). Therefore, *pmarl* expression suffices to produce the skeletogenic regulatory state, and its downstream effector functions, in cells of any lineage, position, and (normally) fate. This means that there can be no requirement for any other maternal micromere-specific factors downstream of *pmarl* itself.

In the experiments of Figs. 1–3, pigment cells are formed in the host embryos from host embryo precursors. This induction depends on expression of *pmarl* (i.e., presence of *pmarl* mRNA) in transplanted micromeres also bearing Δ-cadherin (Figs. 1 and 3). No pigment cells are found if the transplanted micromeres express Δ-cadherin mRNA alone or if the micromeres are simply removed from an otherwise normal embryo. Mesomeres expressing *pmarl* also suffice to induce pigment cells if transplanted to a micromereless host. Therefore, as Fig. 7 predicts, *pmarl* expression suffices to permit inductively functional Delta expression, even in mesomeres. The dependence of *delta* expression in micromeres on β-catenin nuclearization is therefore due entirely to the fact that the β-catenin input is needed for *pmarl* expression (Oliveri et al., 2002).

There is extensive evidence that *delta* expression lies downstream of *pmarl*. It cannot lie upstream because the *delta* gene is expressed much later than is *pmarl*. Nor is the *delta* gene wired in parallel with *pmarl* with independent inputs because perturbation of *pmarl* expression specifically affects *delta* expression in the embryo and also at the cis-regulatory level (Oliveri et al., 2002; unpublished data from our laboratory). Nor can *delta* be upstream of the ES because this signal is passed at a stage prior to the time when the *delta* gene is activated (Ransick et al., 1995). A recent experiment of Sweet et al. (2002) shows that *delta* expression by mesomeres endows these cells with the ability to generate endoderm and skeleton, as well as mesoderm. However, blockade of *delta* translation with a Morpholino antisense oligo shows that, in a normal context, both skeleton formation and endoderm specification proceed normally (Sweet et al., 2002). The animal cap experiment thus indicates a train of regulative respecifications, and does not in fact conflict with the evidence underlying the present experiment, which places *pmarl* upstream of *delta*, and *delta* specifically responsible for the induction of pigment cells (Oliveri et al., 2002; Davidson et al., 2002; Sweet et al., 2002).

Figs. 4–6 concern the dependence of the ES on *pmarl* expression, also indicated in Fig. 7. Clearance of SoxB1 from endomesoderm nuclei is used as an early index of the effect of the ES on the veg2 endomesodermal precursors (unpublished data). Fig. 4 shows that this clearance fails in micromereless embryos and in embryos to which micromeres expressing Δ-cadherin mRNA are transplanted. But again, transplanted micromeres expressing both Δ-cadherin mRNA and *pmarl* mRNA lead to SoxB1 clearance in the adjacent cells (Fig. 4), and a normal archenteron is also induced to form. Very dramatically, SoxB1 clearance can even be induced ectopically by transplanting mesomeres expressing *pmarl* mRNA to the animal pole (Fig. 6). In Fig. 5, it is similarly demonstrated that *endo16*, an endomesodermal marker gene, is activated in mesomeres adjacent to clones of cells producing Pmarl from a genetic expression construct. The ES mRNA is therefore also likely to be an early transcriptional product of the micromeres. Combined, these experiments show that expression of the ES in any cell requires *pmarl* expression; that the need for the β-catenin input for ES expression is again by way of the *pmarl* gene; and that no micromere-specific features downstream of *pmarl* expression are required for ES emission.

### The micromere GRN

From these experiments, it can be concluded that no additional inputs downstream of *pmarl* gene expression are exclusive to micromeres. This means that the cis-regulatory element of *pmarl* is almost certainly the furthest upstream zygotic transcriptional apparatus in the micromere specification GRN. This gene is activated immediately upon the birth of the micromeres (Oliveri et al., 2002), so temporal considerations make it extremely unlikely that there is any additional micromere-specific zygotic transcriptional function upstream of it. The *pmarl* cis-regulatory element is already in hand, and its inputs will soon be known. The
they induce ectopic nuclear clearance of SoxBl (6/6 embryos examined). (A) The vegetal pole of control embryos clears SoxBl due to the presence of micromeres that induce a secondary axis if transplanted to the animal pole. (B) If control micromeres are placed at the animal pole of a control embryo, SoxBl clearance is present at the animal pole if control micromeres were present (4/6 embryos examined). Green is immunostaining for SoxBl, and red is a specific PMC antibody. The hyaline layer outside the embryo is also stained nonspecifically with the red antibody (see Materials and methods for details).

Additional evidence that lies outside the scope of this paper explicitly places all the known skeletogenic subnetwork regulators, i.e., tbr, ets, aoxl, dri, and foxb, downstream of the pmarl derepression system (QPCR perturbation experiments, op cit.; Amore et al., 2003; and much unpublished additional data of P.O. and E.H.D.). Thus, overexpression or knockout of these regulators affects skeletogenic functions (for ets, Kurokawa et al., 1999; for dri, Amore et al., op. Cit.; for tbr, Davidson et al., 2002a and Croce et al., 2001; plus other unpublished data). But these perturbations do not affect expression of the ES or the Delta signal, for example, as has been shown clearly in each case by morpholino knockouts followed by measurement of delta transcript level (data on QPCR web site, op cit.). As illustrated in Fig. 7, this is because the delta gene and the skeletogenic regulatory apparatus are hooked up in parallel to the pmarl derepression system.

A final point that emerges into the foreground of this work is that the GRN affords a direct, two-way transit between the evidential world of experimental embryology and the sequence-based logic relationships wired into the genomic regulatory system. This is of great interest for the design of future research. On the one hand, it breathes conceptual import into what has been a largely empirical branch of classical embryology. On the other hand, it provides a vivid means of testing the completeness of the logic predictions of the GRN, where the readout can be perceived directly, at the level of cell fate and function.

Fig. 6. Pmarl-expressing micromeres or mesomeres have the capacity to induce a secondary axis if transplanted to the animal pole. (A) The vegetal pole of control embryos clears SoxBl due to the presence of micromeres (now visible as PMCs (red) in the blastocoel (6/6 embryos examined)). (B) If control micromeres are placed at the animal pole of a control embryo, they induce ectopic nuclear clearance of SoxBl (6/6 embryos examined). (C) That same ectopic SoxBl clearance is present at the animal pole if micromeres expressing pmarl are transplanted to the animal pole (4/6 embryos examined). Green is immunostaining for SoxBl, and red is a specific PMC antibody. The hyaline layer outside the embryo is also stained nonspecifically with the red antibody (see Materials and methods for details).

Fig. 7. Micromere specification gene regulatory network. The network shown is modified from Oliveri et al. (2002). The diagram represents the "view from the genome": all the known interactions at gene level relevant for the micromeres-PMC specification. The colored lines indicate the interactions analyzed in this paper; the gray lines are not considered here. More details are available in the Web site: http://www.its.caltech.edu/~mirsky/endomes.htm. Arrows indicate activating positive functions and bars repressive interactions. The pmarl gene (red) is depicted in the middle of the GRN and is activated by maternal β-catenin (orange) and maternal Otx. Pmarl is modeled to repress an unknown ubiquitous repressor(s) (blue). That repressor(s) prevents the micromere specification pathway from moving forward to activate specific early micromere-PMC transcription factors (tbr, ets, aoxl, dri, and foxb). These, acting in different combinations, are responsible for the expression of the skeletogenic differentiation genes. The repressor also blocks the expression of delta (yellow), schematically represented as a membrane protein. The Delta ligand is responsible, through an interaction with Notch (yellow arrow), for the specification of the secondary mesenchyme cells (lower box on the right; Sherwood and McClay 1999; Sweet et al., 2002). When Pmarl silences the repressor(s), delta is expressed and the SMC specification pathway is activated. Pmarl also activates the production of the ES, illustrated as embedded in the schematic membrane (green). The double arrow from pmarl to the unknown gene coding for the ES indicates a possible indirect interaction. So far, Pmarl has been shown to work as a repressor (Oliveri et al., 2002). The ES function is indirectly connected (green dashed line) to SoxBl (black) clearance from the endomesoderm territory (upper right box). Thus, all three known early functions of the micromeres are enabled by Pmarl expression.
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References


LvTbx2/3: a T-box family transcription factor involved in formation of the oral/aboral axis of the sea urchin embryo

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SUMMARY

T-box family transcription factors have been identified in many organisms and are frequently associated with patterning events during embryonic development. With an interest in the molecular basis of patterning in the sea urchin embryo, we identified several members of the T-box family in 

Lytechinus variegatus. Here, we report the cloning and characterization of an ortholog of the Tbx2/3 subfamily, LvTbx2/3. To characterize the spatial distribution of LvTbx2/3 protein throughout sea urchin embryogenesis, a polyclonal antiserum was generated. Nuclear localization of LvTbx2/3 initiated at the mesenchyme blastula stage and protein was present into the pluteus stage. Localization was asymmetric throughout this period and costaining with marker genes indicated that asymmetry was about the oral/aboral (O/A) axis. Asymmetric distribution of LvTbx2/3 was observed in the aboral territories of all three germ layers. In the skeletogenic mesoderm lineage, LvTbx2/3 expression was dynamic because expression appeared initially in all skeletogenic mesenchyme cells (PMCs) but, subsequently, became refined solely to the aboral ones during skeletogenesis. To determine if the aboral expression of LvTbx2/3 is linked between germ layers, and to place LvTbx2/3 in the sequence of events that specifies the O/A axis, the effects of a series of perturbations to O/A polarity were examined. Preventing the nuclear localization of β-catenin, pharmacological disruption of the O/A axis with NiCl2, overexpression of BMP2/4 and disruption of the extracellular matrix all blocked LvTbx2/3 expression in all germ layers. This indicates that expression of LvTbx2/3 in the aboral territories of each germ layer is a common aspect of O/A specification, downstream of the molecular events that specify the axis. Furthermore, blocking the nuclear localization of β-catenin, overexpression of BMP2/4 and disruption of the extracellular matrix also prevented the oral (stomodaeal) expression of LvBrachyury (LvBrac) protein, indicating that the O/A axis is established by a complex series of events. Last, the function of LvTbx2/3 in the formation of the O/A axis was characterized by examining the phenotypic consequences of ectopic expression of LvTbx2/3 mRNA on embryonic development and the expression of marker genes that identify specific germ layers and tissues. Ectopic expression of LvTbx2/3 produced profound morphogenetic defects in derivatives of each germ layer with no apparent loss in specification events in those tissues. This indicates that LvTbx2/3 functions as a regulator of morphogenetic movements in the aboral compartments of the ectoderm, endoderm and mesoderm.

Key words: Sea urchin, T-box, Tbx2/3, Oral/aboral, Morphogenesis

INTRODUCTION

Cell-fate specification in the sea urchin embryo is achieved through an initial polarization of the unfertilized egg along the animal/vegetal (A/V) axis and, after fertilization, through cell-cell interactions within and between blastomere layers during cleavage and blastula stages. Such polarizations, signaling events and cell-cell interactions activate region-specific genes and segregate the 60-cell stage embryo into five embryonic territories: the small micromeres; the skeletogenic mesenchyme; the vegetal plate endomesoderm; the oral ectoderm; and the aboral ectoderm (Davidson, 1998). Although there is much data about the development of the primary, A/V axis of the embryo (reviewed in Angerer and Angerer, 2000), less is known about the specification of the secondary, O/A axis in the sea urchin. In some species of sea urchins, the O/A axis can be predicted by the plane of first cleavage, whereas in others, the future axis cannot be related to the position of cleavage planes until the third cleavage or later (Cameron et al., 1989; Henry et al., 1992). Signaling events seem also to be involved (Wikramanayake et al., 1995; Wikramanayake and Klein, 1997).

Several genes are expressed asymmetrically about the O/A axis in the endoderm, mesoderm and ectoderm, but no genes have yet been identified that reflect the oral/aboral polarity extending between all three. In the endoderm, asymmetrically
localized gene products include apical LtvNotch protein, which is enriched on the aboral side (Sherwood and McClay, 1997). In the nonselective mesoderm, CyIIa is distributed orally, and OrCT, CAPK and Pi103 aborally (Miller et al., 1996; Rast et al., 2002). O/A patterning has been studied most in the ectoderm and, thus, many gene products with asymmetric distribution about the axis have been identified. Aboral genes include Spec1 and Spec2 (Lynn et al., 1983), CyIIa actin (Cox et al., 1986), arylsulfatase (Sasaki et al., 1995), Hox3/Hbox1 (Angerer et al., 1989), SpMta (Nemer et al., 1995) and P342 (Calzone et al., 1991); whereas oral genes include EctoV (Coffman and McClay, 1990) Otx (Li et al., 1997a; Yuh et al., 2002), SpCoup-TF (Vlahou et al., 1996), BMP2/4 (Angerer et al., 2000), PitOtp (Di Bernardo et al., 1999), Brachury (Gross and McClay, 2001) and goosecoid (Angerer et al., 2001).

Based on the expression patterns and likely function of members of the family of T-box genes in regionalization of body plans in other organisms, we hypothesized that they might play a similar role in the sea urchin embryo. Members of the T-box family of transcription factors have been identified in all metazoan organisms in which they have been sought (reviewed by Papaioannou and Silver, 1998; Smith, 1999). T-box genes are characterized by homology to the DNA-binding domain of Brachury, the founding member of the T-box gene family. The T-box encompasses ~180 amino acids and can be located anywhere in the protein (Kispert and Hermann, 1993). T-box proteins share little homology outside this region and it is in the T-box that the specificity for target promoters resides (Conlon et al., 2001). The T-box family includes transcriptional activators such as Brachury, Tbx5, VegT and Eomesoderm (Kispert et al., 1995; Horb and Thomsen 1997; Horb and Thomsen, 1999; Ryan et al., 1996) as well as transcriptional repressors such as Tbx2 and Tbx3 (Carreira et al., 1998; He et al., 1999). The importance of T-box genes in development is underscored by their involvement in a variety of human pathologies, including that of Holt-Oram syndrome (Jerome and Papaioarmou, 2001; Merscher et al., 1999), in DiGeorge syndrome (Jerome and Papaioannou, 2001; Merscher et al., 2001), Tbx3 in ulnar-mammary syndrome (Bamshad et al., 1997) and, possibly, Tbx2 in breast cancer (Jacobs et al., 2000).

Here, we report the identification and characterization of LtvTbx2/3, a member of the Tbx2/3 subfamily of T-box genes, during development of the sea urchin embryo. LtvTbx2/3 protein is concurrently expressed in the aboral territories of the endoderm, mesoderm and ectoderm. A series of perturbations to the molecular components that are thought to be involved in specifying the O/A axis revealed that the aboral distribution of LtvTbx2/3 appears to be a common aspect of O/A specification in each of these tissues. Specifically, LtvTbx2/3 expression is dependent on either beta-catenin or genes downstream of beta-catenin, and is prevented by ventralization with NCI2, overexpression of LtvBMP2/4 and disruption of the extracellular matrix (ECM). Thus, LtvTbx2/3 is expressed downstream of, or relatively late in, the sequence of events that serve to specify this axis. That LtvTbx2/3 expression can not be separated between the different tissues after perturbation indicates that O/A axis specification is linked in all three germ layers of the sea urchin embryo at the level of LtvTbx2/3 and may occur in parallel to the distinct specification events that give rise to the ectoderm, endoderm and mesoderm of the embryo. Ectopic expression of LtvTbx2/3 supports this conclusion that universal expression of LtvTbx2/3 profoundly affects the morphogenesis of ectoderm, endoderm and mesoderm without altering specification events of embryonic territories. Combined with the loss of expression of LtvTbx2/3 after perturbation of O/A specification, these results indicate that LtvTbx2/3 may be a downstream component of the O/A axis program that is involved specifically in morphogenesis of aboral territories in the embryo.

MATERIALS AND METHODS

Animals

Sea urchins (L. variegatus) were obtained from either Susan Decker (Hollywood, FL) or Jennifer Keller (Duke University Marine Laboratory). Gametes were harvested and cultured at 23°C as described (Hardin et al., 1992).

Cloning an LtvTbx2/3 fragment

Degenerate primers were designed that corresponded to the amino acids YTPEDDSP (forward)ATVAYQN (reverse) and used in a PCR reaction with cDNA template prepared from mid gastrula poly(A)+ mRNA. PCR conditions were 45 cycles of 96°C for 60 seconds, 40°C for 60 seconds, 72°C for 2 minutes 45 seconds. The amplified, 234 bp products were gel purified, cloned into the pGEMT vector by BLAST search.

cDNA library screens

Screens were performed essentially as described (Gross and McClay, 2001) with hybridizations performed at 55°C in 0.5 M Na2PO4 pH 7.2, 1 mM EDTA, 7% SDS, after Church and Gilbert (1984). After rescreens, nine clones were excised, sequenced and identified as LtvTbx2/3 fragments. A full-length open-reading frame was defined by overlapping individual fragments.

Northern analysis

Northern blotting (RNA gel blot hybridization) for LtvTbx2/3 was performed as described (Gross and McClay, 2001). Blots were given two 5 minute washes with 6x SSPE, 0.5% SDS at room temperature, one 45 minute wash with 1x SSPE, 0.1% SDS at 37°C and, one 45 minute wash with 1x SSPE, 0.1% SDS at 50°C. After washing, the blot was wrapped in plastic wrap and placed on film for 72 hours at ~70°C with an intensifying screen. It was then stripped in 20% formamide, 6x SSPE for 30 minutes at 65°C and reprobed as above with an L. pictus ubiquitin fragment as a loading control.

Antibody production

LtvTbx2/3 fusion protein was expressed following PCR amplification of a BamHI-Xhol fragment of LtvTbx2/3 (encoding amino acids 11-339) and subcloning into the pGEX4T-1 glutathione S-transferase (GST) expression system (AmershamPharmacia Biotech). Expressed, affinity-purified protein (80 μg) was mixed 1:1 with Freund's complete adjuvant and injected into each of three guinea pigs (Charles River, Raleigh, NC). Animals were boosted with 80 μg protein mixed 1:1 with incomplete Freund's adjuvant after 21, 42 and 70 days. Bleeds were performed 31, 53 and 80 days after the last injection and serum isolated as described (Harlow and Lane, 1988).

Western analysis

1500 late-gastrula embryos were homogenized in the presence of protease inhibitors, boiled and run on a 10% SDS-PAGE gel. Protein was blotted onto nitrocellulose, blocked overnight at 4°C.
in 2% milk, 1% bovine serum albumin (BSA) TBS and probed for 1.5 hours at room temperature with a 1:1000 dilution of either α-Tbx2/3 or preimmune serum in 2% milk, 1% BSA TBS. The blot was washed three times with TBS before applying goat α-guinea pig HRP-tagged secondary antibody (Jackson Immunoresearch Laboratories) at 1:5000 for 1 hour at room temperature. Labeled proteins were visualized by ECL (AmershamPharmacia Biotech).

Immunolocalization and image analysis
Embryos were fixed in 2% paraformaldehyde, 60% artificial sea water (ASW) for 10-12 minutes at room temperature, before being permeabilized for 60 seconds with ice cold, 100% methanol. They were then washed three times with PBS, blocked 10-20 minutes in PBS, 4% normal goat serum (NGS; GibcoBRL) and incubated overnight at 4°C in primary antibody, 4% NGS. After washing four times in PBS, they were blocked as above and incubated for 60 minutes at room temperature in secondary antibody, 4% NGS (either Cy3 or Cy5-conjugated; Jackson Immunoresearch Laboratories). Embryos were then washed four times in PBS and mounted in 70% glycerol. LvTbx2/3 and LvBrac sera were diluted 1:500 for all images. Undiluted supernatants of monoclonal antibodies (mAbs) 5a7 (EctoV), 5c7 (Endo1) and 295 were used with the above fixation and incubation conditions. All images were obtained using a 40× Plan-Neofluor oil-immersion objective (NA=1.3) on a Zeiss laser-scanning confocal microscope (Carl Zeiss, Thornwood, NY) mounted on a Zeiss Axiovert inverted microscope. Where necessary, 1 μm sections from single label images were rendered into 3D projections using Zeiss confocal software. Double labeled images were taken sequentially using appropriate filters and subsequently overlayed using Adobe Photoshop 5.0.

Chemical treatments
Treatment of embryos with either NiCl2 or β-aminopropionitrile (βAPN) were performed as described (Hardin et al., 1992; Wessel and McClay, 1987).

Generation of LvTbx2/3 constructs
Full-length LvTbx2/3 was generated by subcloning fragments from individual excised cDNA clones obtained in library screening (details available on request). For ectopic overexpression studies, an SpOtx 5′ UTR plus the first five amino acids of SpOtx was cloned in frame, 5′ to the LvTbx2/3 translation-start site. This leader sequence has been demonstrated to provide an excellent translation start site for mRNA constructs in the sea urchin (Sherwood and McClay, 1999). All clones were sequenced bidirectionally to verify fidelity.

mRNA preparation and injection
ΔLvG-cadherin and LvBMP2/4 were linearized and injected as described (Logan et al., 1999; Angerer et al., 2000). LvTbx2/3 was linearized with XhoI and used as a template to generate in vitro-transcribed 5′ capped mRNA using the T3 mMessage mMachine kit (Ambion). Concentrations of mRNA were determined by spectrophotometry, and by comparison to known amounts of RNA using both gel electrophoresis and dotting onto a 0.6% agarose gel.

Quantitative PCR (QPCR)
RNA was isolated using Trizol (Invitrogen). Reverse transcription reactions were performed using oligo dT priming and MMLV-reverse transcriptase (Gibco). Reactions were purified using a PCR-purification kit (Qiagen). QPCRs were performed using Roche LightCycler Fast Start SYBR as manufacturers instructions. Primers used were ubiquitin (Rast et al., 2000) and LvTbx2/3. A Tbx2/3 plasmid was used to generate a standard curve for quantification, and ubiquitin was used to normalize the cDNA samples. Each time point was determined from two independent batches, and each reaction was confirmed by gel electrophoresis.

RESULTS
Identification of a Tbx2/3 subfamily member in the sea urchin
LvTbx2/3 was PCR amplified from a mid-gastrula stage cDNA pool using degenerate oligonucleotides that correspond to evolutionarily conserved regions of the DNA-binding domain of other T-box proteins. Cloning and sequencing of the amplified fragment identified it as a L. varieglatus Tbx2/3 ortholog (LvTbx2/3). A mid-gastrula cDNA library was then screened and nine LvTbx2/3 cDNA clones recovered. Alignment of the sequences of these clones defined the full coding region of the gene. LvTbx2/3 encodes a 637 amino acid protein, based on the predicted open reading frame from the primary sequence data (Fig. 1A; GenBank accession number AY120889). Supporting LvTbx2/3 as a member of this T-box subfamily, LvTbx2/3 aligns in a phylogenetic tree of Tbx2/3 subfamily proteins (Fig. 1B).

Northern-blot analysis of LvTbx2/3 mRNA revealed that a 5.37 kb message appears first at the mesenchyme blastula stage and that it is present throughout the phuteus stage (Fig. 2). The highest concentrations of mRNA were observed during gastrula stages. Quantitative PCR analysis of the LvTbx2/3 RNA corresponds well with the Northern-blot data. No LvTbx2/3 mRNA is present in the egg but low concentrations start to be detected at the hatched blastula stage. In the mesenchyme blastula there are ~200 copies of LvTbx2/3 mRNA per aboral cell and this level is retained until the early prism stage when the number of copies per aboral cell drops. To characterize the temporal and spatial distribution of LvTbx2/3 protein, a polyclonal antiserum was generated in guinea pigs against recombinant LvTbx2/3 (amino acids 11-339 fused to GST). This serum was tested for immunoreactivity by protein analysis on SDS-PAGE gels and whole-mount immunofluorescent staining of fixed embryos. Western blots of protein extracts from late gastrula were probed with LvTbx2/3 polyclonal and preimmune sera to ascertain specificity (Fig. 3). Two immunoreactive bands were observed when blots were probed with LvTbx2/3 serum, one of ~70 kDa and one of 35 kDa. The 70 kDa band corresponds with the predicted size of LvTbx2/3 from primary sequence data (637 amino acids). The 35 kDa band was also recognized when blots were probed with preimmune serum, indicating that it is a nonspecific antigen. Whole-mount immunofluorescent analysis of fixed embryos stained with preimmune serum did not result in any distinct staining pattern at any stage examined (Fig. 4C). Additionally, embryos stained with LvTbx2/3 serum after preincubation with recombinant LvTbx2/3 protein did not stain positively at any stage examined (Fig. 4D).

Throughout development, the spatial distribution of LvTbx2/3 is asymmetric about the O/A axis in the endoderm, mesoderm and ectoderm
Fig. 4A,B shows two different orientations of prism-stage embryos costained with anti-LvTbx2/3 antiserum (red) and 5a7 mAB (green). 5a7 recognizes EctoV, a protein that is localized to the foregut and oral ectoderm (Coffman and McClay, 1990). LvTbx2/3 was localized to the nucleus, as expected given its role as a transcription factor. A striking asymmetry of LvTbx2/3 distribution was observed in the ectoderm, endoderm and skeletogenic mesoderm at this stage of
development. EctoV and LvTbx2/3 were present in complementary patterns, indicating that this asymmetry is about the O/A axis of the ectoderm and that LvTbx2/3 is restricted solely to the aboral territories of the embryo. The aboral distribution of LvTbx2/3 in the endoderm and mesoderm is apparent in Fig. 4B, where the protein is clearly localized to the aboral regions of the archenteron and skeletogenic mesoderm. As such, LvTbx2/3 is the first marker of O/A polarity expressed in the derivatives of all three germ layers of the sea urchin embryo. Additionally, the LvTbx2/3 characterization reported here is, to our knowledge, the first report of protein expression for a non-Brachyury T-box gene in any organism.

The ectoderm, endoderm and mesoderm are all specified prior to LvTbx2/3 expression. Because LvTbx2/3 was distributed in a subset of cells in each of these tissues, we next characterized the temporal details of LvTbx2/3 protein expression (Fig. 5). LvTbx2/3 was localized to the nucleus at all stages examined. At mesenchyme blastula stage, LvTbx2/3 protein was observed in cells of the presumptive endoderm and ectoderm but not the mesoderm, as neither the ingressed endoderm and ectoderm nor the presumptive skeletogenic mesenchyme blastula stage. Asymmetric distribution in the endoderm and ectoderm continued through mid-gastrula stage (Fig. 5E,F). Between mid-gastrula and late-gastrula stages, LvTbx2/3 started to be expressed in cells of the skeletogenic mesenchyme lineage and the asymmetric localization in the invaginated endoderm became more apparent (Figs 5, 6). LvTbx2/3 protein in early and late-plutei embryos is shown in Fig. 5I-K. From an animal view of an early pluteus embryo that has been optically sectioned and
projected so that the animal-most ectoderm is removed, asymmetric distribution of LvTbx2/3 was observed in the ectoderm of the embryo and in the archenteron (Fig. 5I). A vegetal projection of a similarly staged pluteus embryo revealed that LvTbx2/3 is present in the ectoderm that surrounds the anus and was very strong in the distal-most portions of the extending embryonic arms (Fig. 5I). Although the concentration of LvTbx2/3 began to decline at the late pluteus stage, it was still observed asymmetrically in the ectoderm, endoderm and skeletogenic mesenchyme (Fig. 5K).

When LvTbx2/3 first appeared in the skeletogenic mesoderm, it was present in all of the PMCs (Fig. 5G,H and data not shown). However, as development proceeded to the prism and pluteus stages, LvTbx2/3 became restricted to the aboral PMCs (Figs 4, 5). The spatial and temporal aspects of LvTbx2/3 expression in the skeletogenic mesenchyme were further characterized to determine precisely when this restriction occurs (Fig. 6). Fig. 6A shows the initial, panskeletogenic mesoderm distribution of LvTbx2/3. Two different levels of confocal projections from the same prism-stage embryo are shown (Fig. 6B,C). The asymmetric distribution of LvTbx2/3 protein is clearly confined to the aboral PMCs and not present in the ventrolateral clusters. An oblique view of another late-prism stage embryo stained for LvTbx2/3 (red) and 5a7 (green) reinforced this observation, because LvTbx2/3 persists in the aboral ectoderm and endoderm of the embryo whereas no expression is observed in the ventrolateral clusters of PMCs (Fig. 6D). In early-pluteus stage embryos, asymmetric distribution of LvTbx2/3 persists in the PMCs, endoderm and ectoderm (Fig. 6E,F). Thus, in the skeletogenic mesoderm LvTbx2/3 is restricted to the aboral PMCs between late gastrula and early prism stages at the time when skeletal patterning begins to shape the spicule skeleton.

LvTbx2/3 in the sequence of O/A axis specification and patterning

The striking asymmetry of the distribution of LvTbx2/3 about the O/A axis in the endoderm, ectoderm and mesoderm raises the possibility that O/A polarity might be either established or maintained by the same molecular component(s) in all three germ layers of the embryo. To place LvTbx2/3 in the framework of specification pathways and patterning events that impinge on the formation of the O/A axis, and to gain further insights into the mechanisms of O/A axis specification, the distribution of LvTbx2/3, 5a7 (Fig. 7A) and LvBrac (Fig. 7B) were examined under a variety of perturbations to this axis.

β-catenin/vegetal signaling in O/A patterning

The influence of β-catenin and β-catenin-dependent signaling on LvTbx2/3 expression was assayed first. Injection of a construct that encodes the cytoplasmic tail of the sea urchin ortholog of E-cadherin, LvG-cadherin (ALvG-cadherin) (Logan et al., 1999), serves as a 'sink' for cytoplasmic β-catenin by binding to it and preventing nuclear translocation and gene activation. Such embryos develop without endoderm

Fig. 2. Developmental northern blot of LvTbx2/3 expression. Poly(A)^+ RNA (3 μg per lane) was loaded (calculated by OD_260). Loading was verified by probing the blot with a ubiquitin fragment from L. pictus (data not shown). Egg: 60 cell stage; MB, mesenchyme blastula; EG, early gastrula; LG, late gastrula; PR, prism; PL, pluteus larva.

Fig. 3. Tbx2/3 polyclonal and preimmune sera controls. Western-blot analysis of protein extracts from late gastrula (1500 embryos) using polyclonal LvTbx2/3 and preimmune serum. In blots probed with LvTbx2/3, two immunoreactive bands appear, one of ~70 kDa and one of 35 kDa. A 35 kDa band was also recognized by preimmune serum, indicating the presence of a nonspecific antigen.
or mesoderm, and β-catenin depletion eliminates the O/A axis in the ectoderm (Wikramanayake et al., 1998; Logan et al., 1999). Expression of this construct resulted in uniform expression of EctoV (Fig. 7C) and prevented the expression of LvTbx2/3 (Fig. 7D). LvBrac and SpGsc (Goosecoid), two gene products that normally localize to the stomodaeum, are also not expressed when the nuclear translocation of β-catenin is prevented (Gross and McClay, 2001; Angerer et al., 2001). This indicates that the blockage of β-catenin nuclear localization prevents both aboral and oral (stomodaeal) specification.

Fig. 4. Aboral distribution of LvTbx2/3 protein (red) in prism-stage embryos, demonstrated by co-staining with 5a7 (EctoV; green). The EctoV antigen is expressed from late gastrula stages onward, solely in the oral ectoderm and foregut. Prism-stage embryos viewed aborally (A) and in a vegetal cross-section (B). Complementary expression is observed, indicating that LvTbx2/3 is distributed asymmetrically about the oral/aboral axis, and localized in aboral territories of the endoderm, ectoderm and mesoderm. In many prism and pluteus-stage embryos, a patch of cilia on the oral surface crossreacts with Cy3 secondary antibodies nonspecifically, as observed in the LvTbx2/3 (red) images. (C) Whole-mount, immunofluorescent analysis of fixed embryos probed with preimmune serum. No nuclear staining is observed at any stage (early prism stage shown). (D) Whole-mount, immunofluorescent analysis of fixed embryos probed with with polyclonal LvTbx2/3 serum that had been preincubated with recombinant fusion protein. No nuclear staining is observed at any stage examined (early pluteus stage shown).

Fig. 5. LvTbx2/3 protein is asymmetric throughout embryonic development. Cross-section images (A,C,E,G,I,K) and surface projections (B,D,F,H,J). LvTbx2/3 expression first appears at the mesenchyme blastula stage and is distributed asymmetrically in the presumptive endoderm and ectoderm, as viewed in cross-section (A) and in a vegetal-surface view (B). Early gastrula stage embryos in cross-section (C) and a vegetal view (D) maintain asymmetric distribution of LvTbx2/3 in the presumptive endoderm and ectoderm, whereas the endoderm and mesoderm that have invaginated into the blastocoel do not express protein. (E,F) Mid-gastrula stage embryo (cross-section and surface projection of the same embryo). LvTbx2/3 expression is maintained asymmetrically in the presumptive endoderm and ectoderm and is not present in invaginated endoderm or mesoderm. (G,H) Late gastrula distribution of LvTbx2/3 protein (cross-section and surface projection of the same embryo). Asymmetric expression is observed in the invaginated endoderm, the ectoderm and in all of the skeletogenic mesenchyme cells at this stage (also see Fig. 6). (I) Animal view of early pluteus embryo optically sectioned to remove the most superficial layers of ectoderm and expose the archenteron and stomodaeum. LvTbx2/3 is distributed asymmetrically in the surface ectoderm and the length of the archenteron. (J) Vegetal surface view. Distribution of LvTbx2/3 protein is asymmetric in the aboral ectoderm nuclei. High concentrations of LvTbx2/3 are also observed in the distal most nuclei of the extending pluteus arms. (K) Vegetal cross-section of a late pluteus embryo. Asymmetric distribution is maintained in cells of the ectoderm, endoderm and skeletogenic mesoderm.
Fig. 6. Dynamic expression of LvTbx2/3 in the skeletogenic mesenchyme cells (PMCs). (A) Expression in PMCs begins at mid-late gastrula stage in all PMCs. (B,C) Two views of the same early-platula stage embryo. (B) Superficial view of the PMCs under the ectoderm. At this stage, asymmetric distribution of LvTbx2/3 is observed in the PMC lineage. Consistent with the observation that 0/A polarity in the ectoderm, mesoderm and endoderm is maintained in all tissues (Fig. 7F). BMP2/4 in O/A specification
Recent evidence indicates that an animally derived BMP2/4 ortholog affects O/A specification (Angerer et al., 2000). In situ analysis localizes BMP2/4 mRNA to presumptive oral ectoderm at the hatching blastula stage. Ubiquitous overexpression of BMP2/4 mRNA animilizes the embryo, causing it to form a ball of squamous epithelium whereas lower concentrations radialize the ectoderm of the embryo, as indicated by the formation of multiple clusters of spicules. At concentrations of BMP2/4 mRNA that radialize the spicules, oral expression of LvBrac was prevented but vegetal LvBrac expression was normal (Fig. 7G). Aboral expression of LvTbx2/3 was not observed in the ectoderm, endoderm or skeletogenic mesoderm under such conditions (Fig. 7H). This indicates that ectopic expression of BMP2/4 prevented the normal expression of LvTbx2/3 in all three germ layers, and that O/A polarity in the ectoderm, mesoderm and endoderm is linked by some common genetic or molecular mechanism that is likely to be sensitive to changes in BMP2/4 levels. The failure to observe stomodeal LvBrac protein after ectopic expression of BMP2/4 indicates that BMP2/4 signals prevent the expression of a subset of genes in the oral ectoderm and do not uniformly oralize the embryo. Expression of LvBrac is normal in the vegetal blastopore region, indicating that, unlike in the stomodeum, LvBrac regulation in this region is refractory to ectopic BMP2/4 injected at this level.

The ECM in O/A patterning
Disruption of the ECM with βAPN, a drug that prevents collagen crosslinking implicates the ECM in O/A specification or maintenance. Embryos treated with βAPN do not gastrulate and do not express the aboral-ectoderm-specific Spec1 gene (Wessel et al., 1989). The effects of ECM disruption on LvBrac and LvTbx2/3 expression was assayed (Fig. 7I). Neither stomodeal LvBrac (Fig. 7I) nor aboral LvTbx2/3 (Fig. 7J) were expressed following treatment with βAPN. This indicates that an intact ECM is necessary for specification and/or maintenance of gene expression in both the oral and aboral territories of the ectoderm, not solely in the aboral territory as previously thought. Normal expression of LvBrac in the vegetal blastopore region indicates that this perturbation did not affect LvBrac regulation in this region.

Functional characterization of LvTbx2/3
The results of the perturbation studies detailed above place aboral LvTbx2/3 expression downstream of several signals and specification events that are known to be involved in the formation of the O/A axis. To determine the role of LvTbx2/3 in the formation of this axis, ectopic mRNA expression studies were performed. Ectopic LvTbx2/3 expression produced drastic morphological defects in derivatives of all germ layers, suggesting that LvTbx2/3 functions in each germ layer (Fig. 8). Between 60-75% of embryos that ubiquitously express LvTbx2/3 mRNA displayed severe morphological abnormalities 24-48 hours post-fertilization (three- to fivefold overexpression obtained following injection of 0.75-1 pg/pl of mRNA amounting to 600-1000 copies of LvTbx2/3 mRNA per cell).

By 24 hours, control embryos injected with glycerol had formed a tripartite gut, characteristic skeletal structures and the embryonic shape appropriate for these stages of development (Fig. 8A,B). Embryos that expressed LvTbx2/3 ectopically were often delayed in gastrulation but did invaginate endoderm and gastrulate normally several hours after controls (data not shown). At 24 hours post-injection, embryos injected with LvTbx2/3 mRNA lacked normal skeletal rods and had a grossly mispatterned skeleton with several spicule clusters forming around the circumference of the embryo (Fig. 8C,D). Consistent with the observation that LvTbx2/3 is downstream of both germ-layer specification and O/A patterning events, embryos that ectopically expressed LvTbx2/3 had endoderm, ectoderm, skeletogenic mesoderm, pigment and blastocoelar cells. This indicates that germ-layer specification was not perturbed noticeably, rather, it is likely that aspects of patterning and later morphogenesis were affected. Because ectopic expression of T-box-family members might affect the function of other T-box proteins, these results must be considered cautiously. However, the interpretation that the
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Fig. 7. Perturbation of the oral/aboral axis and the consequences for \( L\nu Tbx2/3 \) expression. (A, B) Control embryos depicting normal expression of EctoV and \( L\nu Brac \). (A) Cross-sectional view of EctoV oral ectoderm distribution in the late gastrula. (B) Mid-gastrula surface view of normal blastopore and stomadai \( L\nu Brac \) expression. (C, D) Injection of \( L\nu G-cadherin \) mRNA animalizes the embryo by binding to endogenous \( \beta\)-catenin and preventing its nuclear localization. These embryos lack endoderm and mesoderm and, as previously reported, express the EctoV antigen uniformly (C). They do not express aboral \( L\nu Tbx2/3 \) (D). (E, F) \( NiCl_2 \) ventralization. (E) \( L\nu Brac \) expression expands to all ectoderm cells after ventralization with \( NiCl_2 \). (F) \( L\nu Tbx2/3 \) is not expressed in any germ layer of these embryos. (G, H) Ectopic expression of \( BMP2/4 \) radializes the ectoderm of the embryo, as indicated by the formation of multiple triradiate spicules (Angerer et al., 2000). Such embryos express oral levels of vegetal \( L\nu Brac \) around the blastopore but do not express oral \( L\nu Brac \) (G) and aboral \( L\nu Tbx2/3 \) in the tissue of any germ layer (H), indicating that ectopic expression of \( BMP2/4 \) antagonizes normal specification events along the A/V axis and in the O/A axis in all germ layers. (I, J) Disruption of the extracellular matrix with \( \beta\)APN, a drug that prevents collagen crosslinking, results in the failure to express oral \( L\nu Brac \), but vegetal expression of \( L\nu Brac \) is, apparently, unaffected (I). \( \beta\)APN also prevents \( L\nu Tbx2/3 \) expression.

**DISCUSSION**

**\( L\nu Tbx2/3 \): A T-box family transcription factor distributed asymmetrically in derivatives of all three embryonic germ layers**

Here, we report the identification and characterization of a novel sea urchin T-box gene, an ortholog belonging to the \( Tbx2/3 \) subfamily. The spatial restriction of \( L\nu Tbx2/3 \) protein to the aboral regions of each germ layer demonstrates that there is polarized gene expression about the O/A axis in the ectoderm, endoderm and mesoderm of the sea urchin embryo. Because all three tissues share this molecular component, O/A specification in each does not involve totally unique sets of proteins. Perturbations of either molecules or pathways involved in O/A axis formation indicate that \( L\nu Tbx2/3 \) acts
Fig. 8. Ectopic expression of LvTbx2/3 mRNA causes profound morphological defects in embryonic development but does not prevent expression of markers of ectoderm, mesoderm and endoderm specification. Nomarski (A) and polarized light (B) images of 24 hour control, glycerol-injected embryos. These embryos exhibit the morphology, skeletal pattern and tripartite gut characteristic of the pluteus stage of development. Embryos that ectopically express LvTbx2/3 mRNA (0.75-1.0 pg/pt; three to five times the endogenous copy number per nucleus, but in all nuclei) and imaged under Nomarski (C,E,G) or polarized light (D,F,H) optics are positioned to show a vegetal view. Twenty-four hours after injection, ectopic LvTbx2/3-expressing embryos appear radialized with multiple spicule clusters forming around the circumference of the embryo (D). They are, in many cases, delayed in gastrulation compared with control embryos as the archenteron has not yet reached the animal pole. The embryos contain derivatives of all germ layers, including pigment and blastocoelar cells derived from the nonskeletogenic mesenchyme, indicating that early specification events have not been eliminated. (E-H) embryos ectopically expressing LvTbx2/3 for 48 hours. These embryos exhibit severe morphological defects in tissues derived from all three germ layers. They lack the typical pluteus form, have drastically mispatterned skeletons and have archenterons composed of multiple chambers rather than the normal three. Two embryos (F,H) display the variability in the skeletal phenotypes. No two embryos that ectopically express LvTbx2/3 display identical defects in their skeletons, although all are severely mispatterned. When stained for terminal markers of pattern formation in these tissues, these embryos express markers for each known cell lineage. In the endoderm, the mid/hindgut marker, Endo1, is expressed and, in many cases, is localized to several of the additional chambers that have formed (I). LvBrac is normally expressed in two domains, a blastopore/hindgut domain and an oral/stomodaeal domain. Within the endoderm of injected embryos, LvBrac expression remains around the blastopore (J). EctoV is normally expressed in a refined domain corresponding to the oral ectoderm. In injected embryos, EctoV expression is still refined, indicating an oral axis has formed (K). (L) LvBrac is also expressed normally in the stomodeal domain, indicating that substructures have been specified in the oral ectoderm and that domain is not 'aboralized'. (M) mAb 295, which recognizes the ciliated band, a structure at the boundary between oral and aboral cells, is also expressed in these embryos. However, instead of being a tight band, in many cases the ciliary band is broadly dispersed, indicating a loss of a refined O/A boundary.

downstream of these events and may be proximal to the morphogenetic events that shape aboral structures. To our knowledge, no member of the T-box gene family, or of any transcription factor family, has been described that is distributed in such a strikingly polarized manner in the three germ layers of any organism.

O/A polarity in the sea urchin embryo

That LvTbx2/3 is expressed in the aboral territories of the endoderm, ectoderm and mesoderm provides a point of entry to examine the regulation of gene expression along the O/A axis in each of these tissues. To this end, we perturbed several events that are thought to be involved in patterning this axis and examined the effects on the expression of LvTbx2/3 and LvBrac, markers of aboral and oral gene expression, respectively. Results of these experiments suggest that either β-catenin or the expression of genes downstream of β-catenin is necessary for the expression of both proteins and, thus, gene expression along both the A/V and the O/A axes of the sea urchin embryo. Pharmacologically blocking formation of the aboral axis with NiCl₂ prevented LvTbx2/3 expression in all tissues, suggesting that gene expression along this axis is uniformly sensitive to this perturbation. In addition, it is possible that gene expression of axial information is controlled by mechanisms common to each germ layer rather than through different pathways in each.
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The results on protein distribution after ectopic expression of BMP2/4 are particularly interesting because previous experiments in Strongylocentrotus purpuratus embryos demonstrated that oral expression of EctoV was blocked by ectopic expression of BMP2/4 but the aboral domain of Spec1 expression increased (Angerer et al., 2000). In our study, ectopic expression of BMP2/4 in *L. variegatus* prevented the expression of both aboral *LvTbx2/3* and oral *LvBrac* (Fig. 7C,H). Thus, two aboral genes, Spec1 and *LvTbx2/3* differ in their response to ectopic expression of BMP2/4. The ectoderm of these embryos may be a locked in some sort of pre-aboral ectoderm state in which some aboral proteins are expressed but others are not because the signals necessary for their expression are inhibited by increased concentrations of BMP2/4. The most obvious explanation is that BMP2/4 might be a component of aboral specification, but, given the different Spec1 and *LvTbx2/3* responses to BMP2/4 perturbation, other, aboral-specification mechanisms must also exist. Evidence for a veg1-derived signal to overlying animal tissues has been observed recently (D.R.M. and J.M.G., unpublished observations), and this signal is sensitive to ectopic BMP2/4 expression. Thus, the defects in O/A gene expression described here might result from a perturbation to this signal. Further characterization of the BMP2/4 pathway, and the identification of more markers for O/A-axis formation in the ectoderm will likely clarify this issue. It is also possible that species-specific differences in specification of the oral and aboral axes might explain this discrepancy. *S. purpuratus* embryos at least partially differentiate aboral ectoderm autonomously, whereas *L. pictus* embryos require vegetal signaling to do so (Wikramanayake et al., 1995). Therefore, the loss of *LvTbx2/3* expression in embryos of *L. variegatus* following overexpression of BMP2/4 could reflect a slightly different role of this pathway in specifying structures along the O/A axis in *Lytechinus* species of urchins than that in *S. purpuratus*.

**LvTbx2/3 patterning and morphogenesis in the sea urchin embryo**

Based on perturbation studies, *LvTbx2/3* expression is downstream of the specification of endoderm, mesoderm and ectoderm, including initial O/A specification events in these tissues. When ectopically expressed, *LvTbx2/3* consistently produces abnormal morphological phenotypes and patterning deficiencies in derivatives of each tissue. Nevertheless, markers for specific germ layers and axial regions are expressed (Fig. 8). Thus, what is the role of *LvTbx2/3* in the aboral territories? Genes downstream of *LvTbx2/3* may be involved directly in patterning and morphogenesis, as suggested by the skeletal and endodermal phenotypes that result from the ectopic *LvTbx2/3* expression studies presented here. Several other T-box genes have also been noted to have distinct functions during morphogenesis. These include *Brachyury* in gastrulation movements (Kimmel et al., 1989; Conlon et al., 1996; Wilson and Beddington 1997; Gross and McCay, 2001), *Eomesodermin* in the formation of bottle cells and initiation of gastrulation (Ryan et al., 1996; Russ et al., 2000), *spadetail* in paraxial mesoderm migration (Griffin et al., 1998; Yamamoto et al., 1998), and *Tbx24* in somite segmentation (Nikaido et al., 1999). It will be of great interest to refine the position of *LvTbx2/3* in a network of O/A specification when more genes are identified in this gene-regulatory network. Also, using the differential screening technologies that are available currently, it should be possible to identify downstream targets of *LvTbx2/3* and determine their roles in patterning and morphogenesis along this axis.

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