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Homeobox-containing gene transiently expressed in a spatially restricted pattern in the early sea urchin embryo

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ABSTRACT **In the sea urchin embryo, the lineage founder cells whose polyclonal progenies will give rise to five different territories are segregated at the sixth division. To investigate the mechanisms by which the fates of embryonic cells are first established, we looked for temporal and spatial expression of homeobox genes in the very early cleavage embryos. We report evidence that PlHbox12, a paired homeobox-containing gene, is expressed in the embryo from the 4-cell stage. The abundance of the transcripts reaches its maximum when the embryo has been divided into the five polyclonal territories—namely at the 64-cell stage—and it abruptly declines at later stages of development. Blastomere dissociation experiments indicate that maximal expression of PlHbox12 is dependent on intercellular interactions, thus suggesting that signal transduction mechanisms are responsible for its transcriptional activation in the early cleavage embryo. Spatial expression of PlHbox12 was determined by whole-mount *in situ* hybridization. PlHbox12 transcripts in embryos at the fourth, fifth, and sixth divisions seem to be restricted to the conditionally specified ectodermal lineages. These results suggest a possible role of the PlHbox12 gene in the early events of cell specification of the presumptive ectodermal territories.**

In metazoan organisms, commitment of cells to a particular fate or set of fates takes place by three known modes. Syncitial specification is the mechanism used by *Drosophila* and most insect embryos. Blastomere specification is largely conditional in most invertebrate embryos, and conditional specification is also the major mechanism operating after cellularization in *Drosophila*. Finally, in most invertebrate embryos the fates of some blastomeres are mostly determined by autonomous specification processes (1, 2). In the sea urchin embryo, specification of cell fates is both cell autonomous and conditional. Only the four micromeres that arise at the vegetal pole at the fourth division appear to be autonomously specified (3). If removed from the embryo and cultured, the micromeres will in fact differentiate in skeletogenic mesenchyme cells, form spicules, and express the cell-lineage marker genes (4–7).

Founder cells that are conditionally specified constitute a large fraction of the sea urchin embryo (3). Lithium and phorbol 12-myristate 13-acetate, which are known to affect the inositol phosphate and the protein kinase C second messenger pathways (8–10), respectively, alter cell fate during development. Therefore, signal transduction mechanisms, activated by ligand–receptor interactions, are most probably involved in the specification of adjacent blastomeres. Initial specification of founder cells ends at the sixth cleavage. After segregation of the lineages, the sea urchin embryo at the 64-cell stage can be divided into five polyclonal territories that will differentiate into various structures of the pluteus (11, 12).

The molecular details of blastomere specification in the sea urchin remain to be elucidated. To clarify the role of the zygotic genome in the expression of regulatory genes that might be involved in such a process, we searched for developmental controlling genes, focusing on the homeobox-containing genes. Several homeobox-containing genes have been isolated from different sea urchin species (13–17). However, the expression of all but the maternal SpOtx (18) occurs after the cell lineages have been specified and/or in adult tissues, suggesting a role in developmental formation of the adult body plan, rather than involvement in cell specification during early embryogenesis.

We have isolated several homeobox-containing genes from the sea urchin *Paracentrotus lividus*, and in this paper we present evidence for spatially restricted localization for the transcripts of a divergent homeobox gene, which is transiently transcribed during very early embryogenesis.*

MATERIALS AND METHODS

cDNA Library Construction and Screening. A 32- to 64-cell stage cDNA library was constructed in λ ZAP II vector (Stratagene) according to the instructions suggested by the manufacturer. Plaques of the unamplified library (1.5×10^5) were screened with the ^{32}P-end-labeled oligonucleotide (5′ to 3′) ATCTGGTTTCAGAACCGGAGGATGAA. Hybridization and washes were carried out at 37°C in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 6× SSC/0.5% SDS, respectively. Recombinant plaques were purified and BlueScript plasmids were excised from the λ arms according to the Stratagene protocol. Both DNA strands of the positive clones were sequenced.

Embryo Culture and Cell Dissociation. *P. lividus* embryos were cultured at 20°C until the desired stage. To prepare dissociated cells, eggs depleted of fertilization envelopes were washed twice in Ca^{2+}-free seawater and cultured in the medium described by Giudice and Mutolo (19). To prevent reaggregation, cells were regularly passed through a 28-μm Nitex screen. Both dissociated cells and control embryos were collected at different times and total RNA was extracted according to Chomczynski and Sacchi (20).

RNase Protection Assay. The RNase protection assay was performed by hybridizing 100 μg of total RNA, extracted from embryos at different developmental stages, with a [^{32}P]UTP-labeled antisense RNA transcribed from an intron-containing genomic subclone. Hybridization was carried out in 80% formamide/0.4 M NaCl/40 mM Pipes, pH 6.4/1 mM EDTA at 50°C. The hybrids were digested with RNase A and RNase T1 and the protected hybrids were fractionated onto a denaturing 6% acrylamide gel. Gels were dried and exposed to x-ray film at −80°C.

Whole-Mount *in Situ* Hybridization. Linearized plasmids containing cDNA inserts of 376 and 524 bp, respectively, corresponding to the 5′ and 3′ regions of the full-length cDNA, were transcribed *in vitro* in the presence of digoxigenin 11-

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*The sequence reported in this paper has been deposited in the GenBank data base (accession no. X83675).

UTP RNA labeling mixture. The two RNA probes were mixed in equimolar amounts. Whole-mount *in situ* hybridizations were performed according to Harkey *et al.* (21) and Lepage *et al.* (22) with slight modifications. Fixed embryos were incubated with proteinase K (20–30 μg/ml) and hybridized in sealed capillaries with the RNA probes (30 ng/ml) for 16 h at 45°C. After hybridization, to melt out nonspecific hybrids and to wash out excess probe, the embryos were incubated in several steps in 2× SSC/0.1% Tween 20 and 0.1× SSC/0.1% Tween 20, respectively, for 15 min at 60°C. Antibody reaction and staining were as described (22).

RESULTS

PlHbox12 Is a Divergent Homeobox-Containing Gene. To isolate homeobox-containing genes expressed during early embryogenesis of the sea urchin, we screened a cDNA library from 32- to 64-cell stage embryos with an oligonucleotide encoding the most conserved region of helix III of the homeodomain. We isolated four recombinants that, as revealed from the comparison of their nucleotide sequences, contained identical cDNA inserts. The nucleotide and the predicted amino acid sequences of one such clone, denoted PlHbox12, are shown in Fig. 1*A*. PlHbox12 cDNA is ≈900 bp long and contains the coding information for 227 amino acids. Its size corresponds to that of an RNA band detected by Northern blot hybridization (see ref. 17 and Fig. 3*C*). The homeodomain is close to the N terminus of the protein, and the C terminus is rich in threonine and serine residues, suggesting that the activity of this homeoprotein might be modulated by phosphorylation and dephosphorylation events. The amino acid sequence comparison of homeodomains shown in Fig. 1*B* indicates that PlHbox12 encodes a divergent homeodomain. The greatest similarity was found with the *Drosophila* gooseberry proximal, 58.3% identity (23), and with the sea urchin *otx* (18) and *otp* (24), 48.3% and 43.3% identity, respectively. The identity increases if we take into account that many substitutions are conservative. The paired box, a second sequence-specific DNA binding domain (25), is absent. Furthermore, unlike the paired class proteins that contain serine in position 50 of the homeodomain (see Fig. 1*B*), this residue, which gives sequence specificity of binding of the protein (26), is substituted by glutamine in PlHbox12, suggesting that the encoded homeodomain could specifically bind Antennapedia class target sequences. Sequence analysis of a genomic clone (data not shown) revealed that the coding sequence of the helix III of the homeodomain is interrupted by an intron. As shown in Fig. 1*B* (arrow), the intron–exon boundary lies at valine 47. The interruption of the coding sequence at this amino acid residue is conserved in *otp* (24), *otd* (27, 28), and many other homeogenes.

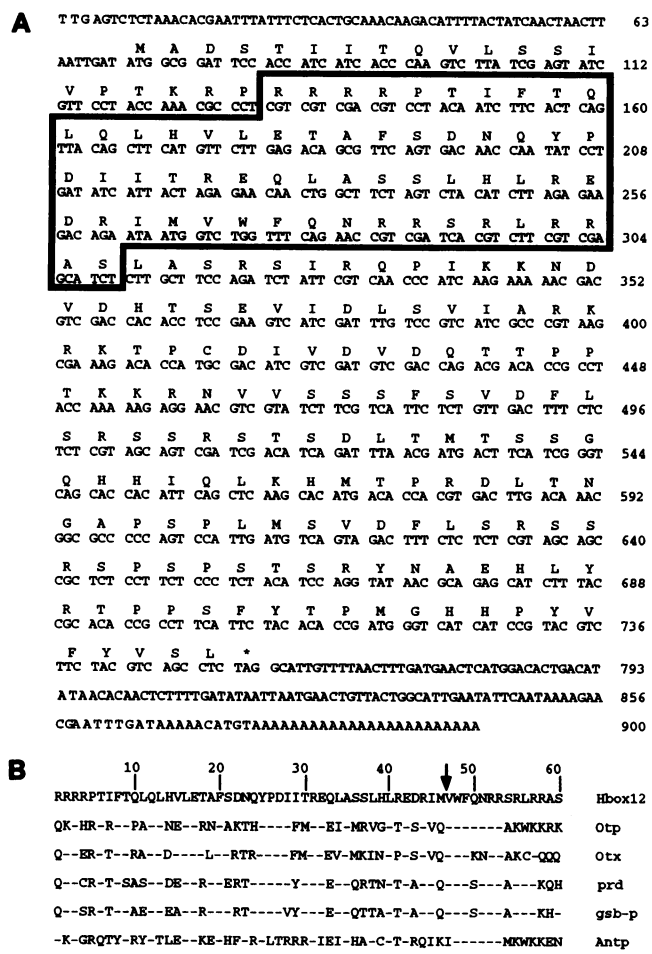

FIG. 1. (*A*) PlHbox12 cDNA and deduced amino acid sequences. The homeodomain is boxed. (*B*) PlHbox12 homeodomain comparison with sea urchin orthopedia (Otp) and orthodenticle (Otx) and *Drosophila* paired (prd), gooseberry proximal (gsb-p), and Antennapedia (Antp). Dashes indicate amino acid identity; arrow points to splice site.

PlHbox12 Gene Is Transiently Expressed During Early Cleavage. The temporal expression of PlHbox12 was determined by an RNase protection assay. For this experiment, an antisense RNA derived from a 591-bp genomic fragment containing the homeodomain coding region interrupted by a 300-bp intron was hybridized to total RNA from unfertilized eggs and from several different developmental stages. The result is shown in Fig. 2. RNase protected bands of the expected sizes of 159 and 135 bp (arrows) and several others of smaller sizes were detected from 4- to 8-cell stage embryos.

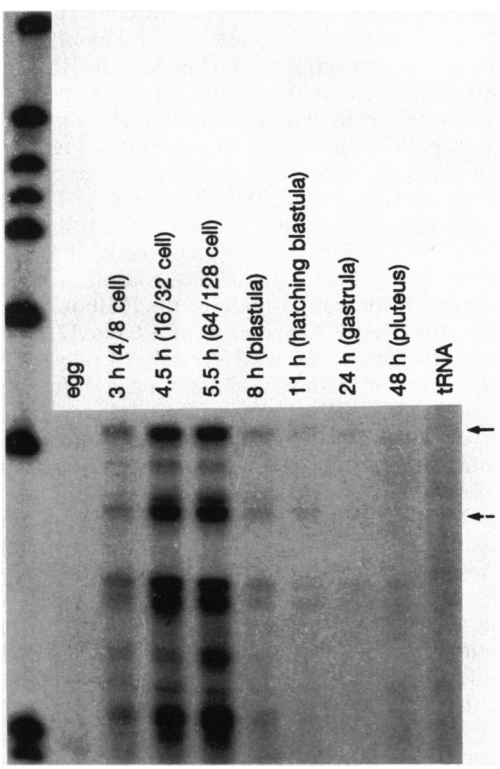

FIG. 2. Temporal expression of PlHbox12 gene. Total RNA was extracted from unfertilized eggs and from embryos. Hours of development and developmental stages (in parentheses) are indicated. RNase protection assays were carried out as described. RNase-resistant hybrids were run on denaturing polyacrylamide gel along with labeled *Hpa* II-digested pUC19 fragments. Arrows indicate expected RNase protected fragments.

This complex protection pattern was reproducible, observed in a number of experiments (see, for instance, Fig. 3*B*). Since by RNA blot hybridization we detected a single RNA band (see Fig. 3*C*), the multiple RNase-resistant fragments obtained in the RNase protection assay might be due to allelic polymorphism of the PlHbox12 transcripts or to overdigestion with RNase. The expression profile of Fig. 2 indicates an absence of PlHbox12 mRNA in the unfertilized egg and a sharp increase in abundance of the PlHbox12 transcripts in embryos until the 64- to 128-cell stage of development. The transcripts abruptly decline in abundance thereafter and return to an undetectable level by the gastrula stage. From these experiments, we conclude that PlHbox12 mRNA derives from transcription of the zygotic genome and that expression is transient and occurs during early/mid-cleavage.

Maximal Expression of PlHbox12 Depends on Intercellular Interactions. Expression of PlHbox12 was also determined in dissociated blastomeres. Fertilized eggs were cultured for several hours in Ca^{2+}-free seawater. This simple method prevented cells from reassociating without interfering with cell division (19, 29). Division of blastomeres and the absence of intercellular interactions were monitored by microscopic observations, counting cells at regular time intervals and comparing their increasing number to that of control embryos (data not shown). Samples from control and dissociated embryos were collected at different time intervals and total RNA was extracted. Cell viability was also checked by detecting the hatching enzyme transcripts in control and dissociated embryos. The hatching enzyme gene is expressed autonomously (30, 31) from the zygotic genome. In *P. lividus* embryos, the abundance of transcripts reaches a peak at the prehatching blastula stage (32), so we expected comparable levels of expression in dissociated and control embryos at 4 and 6 h of development. The results of RNA blot hybridization presented in Fig. 3*A* confirmed this prediction. In contrast, when the same RNA samples were probed with PlHbox12 antisense RNA in an RNase protection assay (Fig. 3*B*), the RNA samples from dissociated blastomeres hybridized to a very low extent with the antisense probe, whereas the control embryos showed a protection pattern similar to that of Fig. 2. Fig. 3*C* shows RNA blot analysis carried out with RNA samples from a different experiment. Again, the expression of PlHbox12 is severely affected in dissociated embryos. From these results, we conclude that maximal expression of the PlHbox12 gene is dependent on intercellular interactions. Alternatively, calcium ions are required for transcription of the PlHbox12 gene.

Spatially Restricted Expression of PlHbox12. We used digoxigenin-labeled antisense RNA probes to determine the spatial expression of PlHbox12 in embryos at different stages of development. RNA·RNA hybrids were detected by staining the embryos with an alkaline phosphatase-conjugated anti-digoxigenin antibody. The results are shown in Fig. 4. The most striking observations were the prevalent localization of the PlHbox12 transcripts in some but not all of the animal blastomeres and in a fraction of the macromere and vegetal tiers and the absence of staining in the micromeres at the vegetal pole. This spatially restricted pattern of expression is already evident in embryos at the 16-cell stage (Fig. 4*A*). Preferential hybridization seemed also to occur on the blastomeres located on one side of the embryo. In fact, as shown in Fig. 4*B*, two animal 2 tier blastomeres and two macromeres, respectively, belonging to the animal 2 and macromere tiers of a 32-cell stage embryo, are clearly stained on one side of the embryo and unstained on the opposite side. Fig. 4 *C* and *D* shows two different focal planes of another fifth-cleavage embryo. Again, we may observe that the stained macromeres are located on one side (Fig. 4*C*) and that not all animal blastomeres reacted with the antisense probe (Fig. 4*D*). The distribution of PlHbox12 transcripts in embryos at the 64-cell stage agrees with the spatial expression pattern seen in embryos at the fourth and fifth divisions. Indeed, no expression of the PlHbox12 gene seems to occur in the autonomously specified micromeres at the vegetal pole (Fig. 4 *E* and *F*), whereas its transcripts are spatially restricted toward blastomeres of the animal cap and of the vegetal 1 tier, located on one side of the embryo. The specificity of hybridization is shown in Fig. 4 *G* and *H*. As expected, the embryos at the gastrula stage, in which expression of PlHbox12 has been turned off (Fig. 2), did not hybridize with the antisense PlHbox12 RNA (Fig. 4*G*), nor did the 64-cell stage embryos with the sense probe (Fig. 4 *H*).

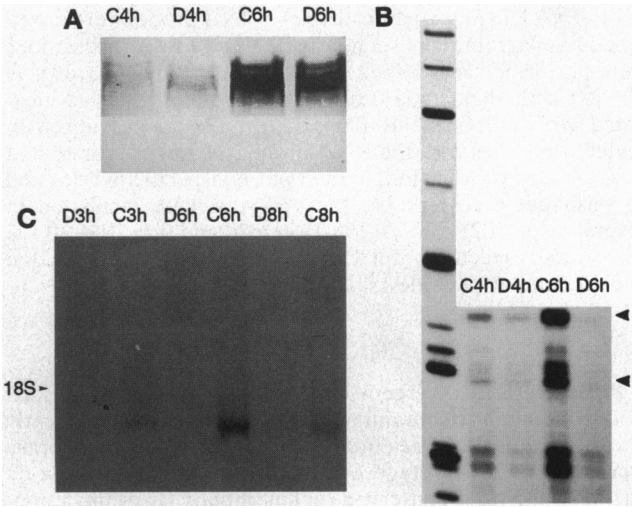

FIG. 3. Pattern of expression of hatching enzyme and PlHbox12 genes in dissociated and control embryos. Total RNAs were extracted from control (C) and dissociated (D) embryos. Control cultures in *A* and *B* at 4 h of development were a mixed population of 8-cell, 16-cell, and 32-cell stage; at 6 h, the embryos were at 64-cell and 128-cell stages. Control cultures in *C* were 8-cell and 16-cell stages at 3 h; 64-cell and 128-cell stages at 6 h; and prehatching blastulae at 8 h of development. (*A*) RNA blot hybridization. Total RNAs were fractionated onto a formaldehyde-agarose gel, transferred to a nylon membrane, and hybridized with a digoxigenin UTP-labeled antisense transcript from the hatching enzyme cDNA clone. Stringent washes were performed in 0.1× SSC at 65°C. Hybrids were detected by incubation with an anti-digoxigenin alkaline phosphatase-conjugated antibody and stained as described by the manufacturer (Boehringer Mannheim). (*B*) RNase protection assay. Conditions were as described in Fig. 2. Expected RNase protected bands are indicated by arrowheads. Marker is an *Hpa* II-digested pUC19 plasmid labeled at the 3′ ends. (*C*) Northern blot analysis of RNA samples from an embryo dissociation experiment, different from that of *A* and *B*, hybridized with a ^{32}P-labeled antisense PlHbox12 RNA. Hybridization conditions and washing were as in *A*. Position of the 18S rRNA is indicated by an arrowhead.

DISCUSSION

Embryonic cell specification requires that developmental asymmetries need to be established first. Several pieces of evidence indicate that maternal acting genes, encoding signaling molecules and transcriptional and translational regulators, set up the axes in embryos of different phylogenetic groups (33). In the sea urchin, one axis, the animal–vegetal axis, is established during oogenesis, while the specification of the second embryonic axis, the oral–aboral axis, might be initiated at the 2-cell stage (34). The molecular mechanisms of cell specification along the embryonic axes in the sea urchin have not been sorted out. Genes that potentially could be involved in such a process have been isolated. For instance, orthodenticle-related transcripts that are evenly distributed in the unfertilized eggs and in the early cleavage embryos have recently been identified in *Strongylocentrotus purpuratus* (18). Zygotic (22) and maternal (35) transcripts that are spatially

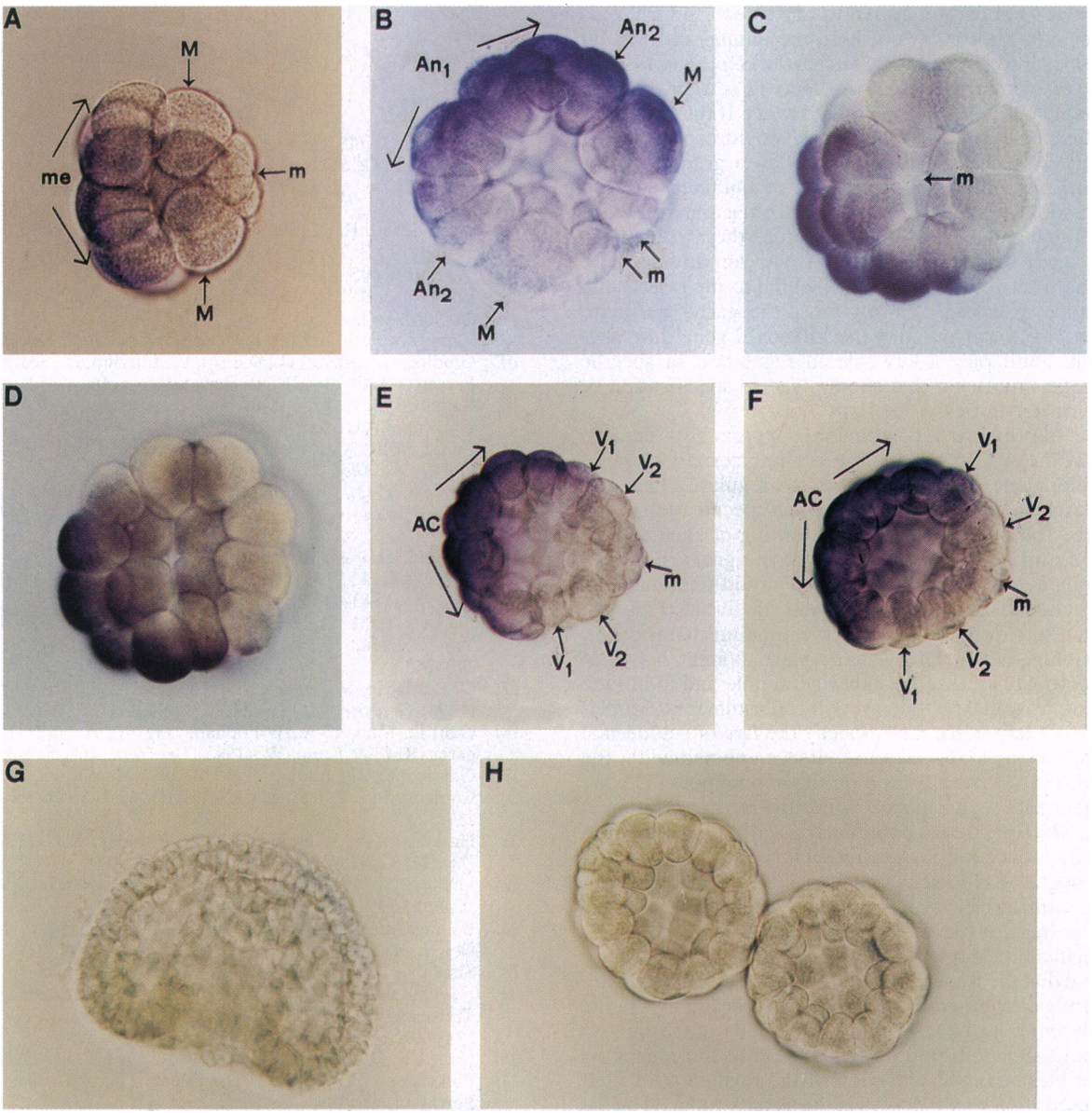

FIG. 4. Spatial expression pattern of PlHbox12 in *P. lividus* embryos. Whole-mount *in situ* hybridization was performed with digoxigenin UTP-labeled antisense. (*A–G*) and sense (*H*) PlHbox12 RNA. (*A*) Optical cross section of a 16-cell stage embryo oriented with the micromeres (m) to the right. The mesomeres (me) and the macromeres (M) are also indicated. (*B*) Optical cross section of a 32-cell stage embryo. The animal 1 (An_1) and animal 2 (An_2) tiers, macromeres (M), and large and small micromeres (m) are indicated. (*C*) Vegetal pole view of a 28-cell stage embryo with the four micromeres (m) surrounded by the eight macromeres. (*D*) Animal view of the same 28-cell stage embryo. (*E* and *F*) Optical cross sections (side views) of two different sixth-cleavage embryos, oriented with the vegetal pole to the right. AC, animal cap; V_1 and V_2, vegetal 1 and vegetal 2 tiers, respectively; m, micromere. (*G*) Gastrula stage embryo hybridized with antisense PlHbox12 transcript. (*H*) Sixty-four-cell stage embryos hybridized with sense probe.

restricted to the animal region have also been described. Finally, PlHbox12, for the reasons outlined below, might also be involved in the mechanism of initial specification of the embryonic cells.

PlHbox12 is one of the earliest transcribed zygotic homeogenes identified in lower deuterostomes. It is transiently expressed during the early cleavages, from the 4-cell stage to the blastula stage. The PlHbox12 transcripts abruptly decline in abundance after the 64- to 128-cell stage of development. The mechanism responsible for the low stability of the PlHbox12 mRNA is not understood. The consensus A+U-rich motif, commonly found in multiple copies in the 3′ untranslated regions of lymphokine and protooncogene mRNAs and often considered to be mRNA instability determinants (36), is not found in the 3′ untranslated region of the PlHbox12 mRNA. Interestingly, PlHbox12 expression immediately precedes the specification of the first lineage founder cell (the Na blastomere) of the aboral ectoderm lineage that occurs at the 8-cell stage of development (11). In addition, maximal expression of PlHbox12 was observed concomitantly with segregation of the lineage founder cells of the five embryonic territories at the 64-cell stage of development (12). The results of *in situ* hybridization in whole-mount embryos also seem consistent with the possible involvement of PlHbox12 in the initial specification of cell fate. The staining pattern we observed in embryos at the fourth, fifth, and sixth divisions suggests that PlHbox12 transcripts are asymmetrically distributed along the embryonic animal–vegetal axis. In fact, as could have been predicted from the observed reduced expression of PlHbox12 in dissociated embryos, the autonomously specified micromeres at the vegetal pole did not hybridize with the antisense PlHbox12 RNA. Furthermore, in a number of experiments we

observed that staining was shifted to one side of the embryos and occurred in blastomeres of both the animal and vegetal tiers. According to the cell lineage chart of *S. purpuratus*, the fate of the blastomeres expressing PlHbox12 is to give rise to the presumptive ectoderm territories (11, 12). If the pattern of PlHbox12 expression is ectodermal, we would expect, at the 64-cell stage, absence of staining in the lower granddaughter cells of the macromeres. The results we obtained suggest, indeed, very low expression in the vegetal2 blastomeres. Since the oral–aboral axis is not apparent before the mesenchyme blastula stage, from the present results we cannot determine whether the positively reacting cells belong to the oral or the aboral ectoderm lineage. In any case, the early, transient, spatially localized activation of the PlHbox12 gene does suggest that it could play a key role in regulation of specific regional gene expression that should lead to specification of the ectoderm territories.

Classical blastomere recombination experiments demonstrated that the sea urchin embryo is characterized by regulative development (37). To reconcile the regulative behavior of the sea urchin embryo with the invariant specification of cell fate and the subsequent invariant patterning of gene expression, Davidson (3) proposed that inductive signals generated by short-range intercellular interactions would regionally activate, by posttranslational modification, maternal transcription factors and initiate the specification mechanism (3). Active skeletogenic regulatory factors were thought, however, to be primordially localized at the vegetal pole and to initiate an inductive cascade on the overlying blastomeres. Studies showing that micromeres can respecify the fate of ectodermal blastomeres to endodermal ones, if recombined with the animal half (38) or transplanted on the animal pole (39), are in agreement with such a model. Interestingly, transcription of PlHbox12, which encodes a potential transcriptional regulator, is drastically reduced in dissociated embryos, suggesting that this gene might be transcriptionally activated by intercellular interactions during cleavage. How can we reconcile the spatial restricted pattern of expression of PlHbox12 with the conditional specification model (3)? As a possible hypothesis, we may assume that the transcriptional regulators of PlHbox12 are not uniformly distributed along the animal–vegetal axis in the egg cytoplasm. In such a way, the fixed geometry of the division planes would allow differential segregation of the regulators among the blastomeres. Activation of the regulators of PlHbox12 transcription would occur via the signal produced by the interacting cells. According to this hypothesis, we expect that transcription of the PlHbox12 gene, at the 4- to 8-cell stage of development, occurs in a spatially restricted manner. Preliminary data (not shown) indeed suggest asymmetrical distribution of PlHbox12 in embryos at the 8-cell stage of development. Alternatively, activation or inactivation of globally present transcription factors at one end of the animal–vegetal axis might be responsible for the asymmetry of PlHbox12 expression along this axis. Functional analysis of the PlHbox12 promoter to identify the regulatory elements of both temporal and spatial expression should clarify this issue.

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