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Expression of homeobox-containing genes in the sea urchin (*Parancentrotus lividus*) embryo

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Abstract

Two homeobox-containing genes that belong to different homeodomain classes have been isolated from a sea urchin genomic library. One, PlHbox11, is the sea urchin homologue of the human and mouse Hox B3 gene, the other, PlHbox12, shows about 55% identity with paired class genes. Expression profile analysis of the two sea urchin Hbox genes suggests that they play different roles during embryogenesis. In fact, PlHbox11 transcripts are rare and are detected only in the pluteus larva and in the Aristotle's lantern and intestine of the adult. The PlHbox12 gene is, on the contrary, transiently expressed in the very early embryo already at the four cell stage; it accumulates at the 64 cell stage and disappears at later stages of development. *In situ* hybridization experiments to 16 and 32 cell stage whole mount embryos showed localization of the PlHbox12 mRNA to part of the mesomere-macromere region of the early cleavage embryo. These observations suggest a possible role of this gene in early events of cell specification.

Introduction

The sea urchin embryo belongs to a group of invertebrates that are characterized by an invariant cell lineage (Cameron *et al.*, 1987), i.e. the cleavage planes relative to the animal-vegetal (A-V) axis, occur in the same positions in all embryos of the species. The unfertilized egg is polarized along the animal-vegetal axis that, as shown almost a century ago by Boveri (1901), is established during oogenesis. This represents the primordial embryo axis. The second axis, the oral-aboral (O-A) or dorso-ventral (D-V) axis, is specified after fertilization within the first cleavage (Cameron *et al.*, 1989), although it seems that it is not firmly established until later in development (see Jeffery, 1992). The first two cleavage planes are perpendicular and vertical, intersecting along the A-V axis. The third cleavage is equatorial and separates the vegetal from the animal pole cytoplasm (Davidson, 1986; Giudice, 1986). Evidence, obtained by a series of classical embryo manipulations (reviewed by Hörstadius, 1973), indicates that the unfertilized sea urchin egg provides the spatial information that will establish the pattern of cleavage and the fate of the blastomeres (reviewed in Davidson, 1989; Spinelli & Albanese, 1990). This positional information ought to be asymmetrically distributed along the A-V axis and is used by the blastomeres to perform a function appropriate to their position in the embryo. The invariant cleavage pattern predicts, in fact, that the fate of a definite cell lineage should be specified, at least in part, by certain maternal cues inherited by the region of the egg cytoplasm occupied by the founder cells. This type of specification is cell autonomous. In the sea urchin embryo only the four micromeres that arise at the vegetal pole at the fourth division appear to be autonomously specified (reviewed in Davidson, 1986; 1989). If removed from the embryo and cultured, the micromeres will differentiate in skeletogenic mesenchyme cells, form spicules (Okazaki, 1975) and express the spiculegenic gene SM

50 whose expression, in the undisturbed embryo, is restricted to the primary mesenchyme cells (Benson *et al.*, 1987; Sucov *et al.*, 1988; Livingston & Wilt, 1990). However, cell autonomous expression is not confined to the vegetal pole only. Two zygotic genes encoding for secreted metalloproteases, whose transcripts are spatially restricted to the animal emisphere (Lepage & Gache, 1990; Lepage, Ghiglione & Gache, 1992; Lepage, Sardet & Gache, 1992), show the correct temporal activation in dissociated blastomeres (Ghiglione *et al.*, 1993).

The other mode of blastomere specification is conditional specification. Founder cells that are conditionally specified constitute a large fraction of the sea urchin embryo (Hörstadius, 1973; Davidson, 1989). Lithium and TPA, that are known to affect the inositol phosphate-protein kinase C second messenger pathways (Livingston & Wilt, 1989; 1992), alter cell fate during development. Therefore, signal transduction mechanisms, activated by ligand-receptor interactions, are most probably involved in the specification of adjacent blastomeres. Classical experiments carried out with fifth or sixth cleavage embryos showed that indeed the fate of blastomeres of isolated animal emisphere, that in the undisturbed embryo will give rise to oral and aboral ectoderm structures (Cameron *et al.*, 1987; Davidson, 1989; Livingston & Wilt, 1990), can be respecified if micromeres were transplanted at the vegetal side. In fact, from these chimeric recombinants a normal pluteus larva, with the endoderm respecified from the animal cap blastomeres, developed (Hörstadius, 1935). This and other blastomere recombination experiments led Hörstadius (1939) to believe that the vegetal and the animal poles create a double gradient of substances, as was proposed by Runnström (1929), that would regulate the reorganization of blastomeres along the A-V axis. This is to say that cell fate along the A-V axis is controlled by gradients of morphogens. In *Drosophila* maternal gene products that are asymetrically and graded distributed in the egg act as morphogens and are involved in the specification of the embryonic axes (Nüsslein-Volhard, 1991). In the specification of cell fate and organization of embryonic body plan along the anterior-posterior (A-P) axis, a key role is played by *bicoid* (Driever & Nüsslein-Volhard, 1989; St Johnston & Nüsslein-Volhard, 1992). This homeodomain-containing transcription factor, by activating the gap gene *hunchback*, will initiate a regulative cascade along the A-P axis in which, besides other regulatory genes, the HOM-C complex is involved (Gehring, 1987; Akam, 1987).

Morphogens of the *bicoid* type have not been characterized in sea urchin egg or very early developing sea urchin embryo so far. This could in part be explained by the different mode of cell fate specification between *Drosophila* and sea urchin (Davidson, 1990). In the latter, at early cleavage stages, inductive interactions between neighbouring cells are probably involved in such a mechanism. On the other hand, zygotic (Gache, Lepage & Ghiglione, 1992) and maternal (Romancino *et al.*, 1992; Di Carlo, personal communication) genes whose expression is spatially restricted to the animal region have been isolated. These genes encode for neither transcription factors nor growth factors but their protein products show also the same asymmetrical distribution in the embryo. However, their role if any in the mechanism of specification along the embryonic axes has yet to be determined.

Davidson (1989; 1990) proposed a model to reconcile the regulative behaviour of sea urchin embryo with the invariant cell lineage and the subsequent invariant patterning of gene expression. According to this model, conditional specification along the embryonic A-V axis occurs by a series of receptor-ligand interactions that would activate mechanisms of signal transductions. Recent transplantation experiments are in agreement with such a model. In fact, when 16 cell stage micromeres of a donor embryo were implanted into the animal pole of a recipient embryo at the 8 or 16 cell stage, the fates of adjacent cells were respecified from presumptive ectoderm into vegetal founder cells. The ectopic vegetal plate invaginated during gastrulation in a second archenteron. The gut-specific Endo-16 gene showed the typical spatial restricted pattern of expression in the induced archenteron (Ransick & Davidson, 1993). Hence, the fates of some lineages are specified by inductive intercellular interactions and are strictly dependent on the fixed geometry of cleavage planes. This would suggest that there is no requirement for zygotic genome activity to express developmental controlling genes before cell lineage specification. However, such genes could instead be transcriptionally activated by the intercellular signals that are generated by the interacting blastomeres during very early cleavage.

Despite such a wealth of information, the molecular details of blastomere specification in sea urchin are not well understood, nor has the involvement of regulatory genes been definitely ruled out in such a process.

We searched for developmental controlling genes in sea urchin and focused our attention on the

homeobox-containing genes. The HOM/HOX gene clusters have been conserved throughout evolution (Boncinelli *et al.*, 1988; Duboule & Dollé, 1989; Krumlauf, 1992) and ample evidence has been accumulated to show identical function for both genetic systems: they specify positional identities along the A-P axis (McGinnis & Krumlauf, 1992). In the sea urchin (*Tripneustes gratilla*) several homeobox-containing genes have been isolated (Dolecki *et al.*, 1986; Dolecki & Humphreys, 1988; Dolecki, Wang & Humphreys, 1988; Wang *et al.*, 1990). All seem to have a role in regional specification in late embryo, as in vertebrates, rather than being involved in axial specification during oogenesis or very early embryogenesis (Davidson, 1991). In fact, they are expressed in the embryo after the cell lineages have been specified and/or in adult tissues. The only available data on spatial expression of a Hbox gene (TgHbox-1) showed accumulation of the transcript in a restricted region of the aboral ectoderm in mid-late embryogenesis after the expression of the histospecific Spec gene had occurred (Angerer *et al.*, 1989).

We have isolated several homeobox-containing genes from a *Parancentrotus lividus* genomic library and in this paper we present some data on the characterization and expression for two of them. We also show some evidence for spatial restricted localization in embryos at 16–32 cell stage for the transcripts of a Hbox gene that is transiently transcribed during very early embryogenesis.

Material and methods

Embryo culture, RNA extraction and blot hybridization

Paracentrotus lividus adults were purchased from a commercial supplier. Gametes were collected and eggs fertilized (Giudice, 1973). Embryos were cultured at 18°C until the desired stage. The extraction of total RNA, from different stages of development and from adult tissues, and the RNA blot hybridization were carried out as previously described (Gianguzza *et al.*, 1989).

Reverse transcriptase coupled—polymerase chain reaction

Conditions for RT/PCR were essentially as described in Palla *et al.* (1993). Briefly, 1 μg of DNase I-digested total RNA was incubated with reverse transcriptase in the presence of gene specific downstream oligonucleotide at 42° for 30 min. The resulting cDNA was amplified with Taq polymerase and the upstream primer for 30 cycles.

Whole mount in situ *hybridization*

Fixed embryos (Angerer *et al.*, 1987) at 16 and 32 cell stages were rehydrated, treated with 30 μg/ml Proteinase K for 15 min, and refixed with 4% paraformaldehyde. Hybridization was carried out overnight at 45°C with sense and antisense DIG-11UTP labelled RNA probes, transcribed from a linearized pGEM4Z containing the full length copy of PlHbox12 cDNA. RNA hybrids were detected by adding the blocking reagent (Boehringer Mannheim) to a final concentration of 1% and incubated for 30 min at room temperature. This step was followed by an incubation with the alkaline-phosphatase-conjugated antibody (Lepage, Sardet & Gache, 1992). Staining was carried out for 1–3 h, in the dark, in the presence of the chromogenic substrates NBT and BCIP (Boehringer). The reaction was stopped by several washes in PBS containing 0.1% Tween 20–1 mM EDTA. Embryos were finally dehydrated, resuspended in Terpineol (Sigma) and photographed under bright field illumination on MC 80 Axioskop microscope (Zeiss).

Results

Cloning and sequencing homeobox-containing genes

To investigate on the role played by the homeodomains in early embryogenesis of sea urchin we isolated homeobox-containing genes and determined their expression during development. A genomic library was screened with an oligonucleotide encoding for the most conserved region of the helix III of the homeodomain. The identification of the homeobox in the restriction enzyme fragments of several positive clones was carried out by Southern blot hybridization. These DNA fragments were subcloned in plasmid vectors and sequenced. The alignment of the predicted amino acid sequences of the homeobox of two genomic clones indicates that PlHbox11 and PlHbox12 do not belong to the *antennapedia* class homeodomain (Fig. 1A). According to Laughon (1991) PlHbox11 belongs to

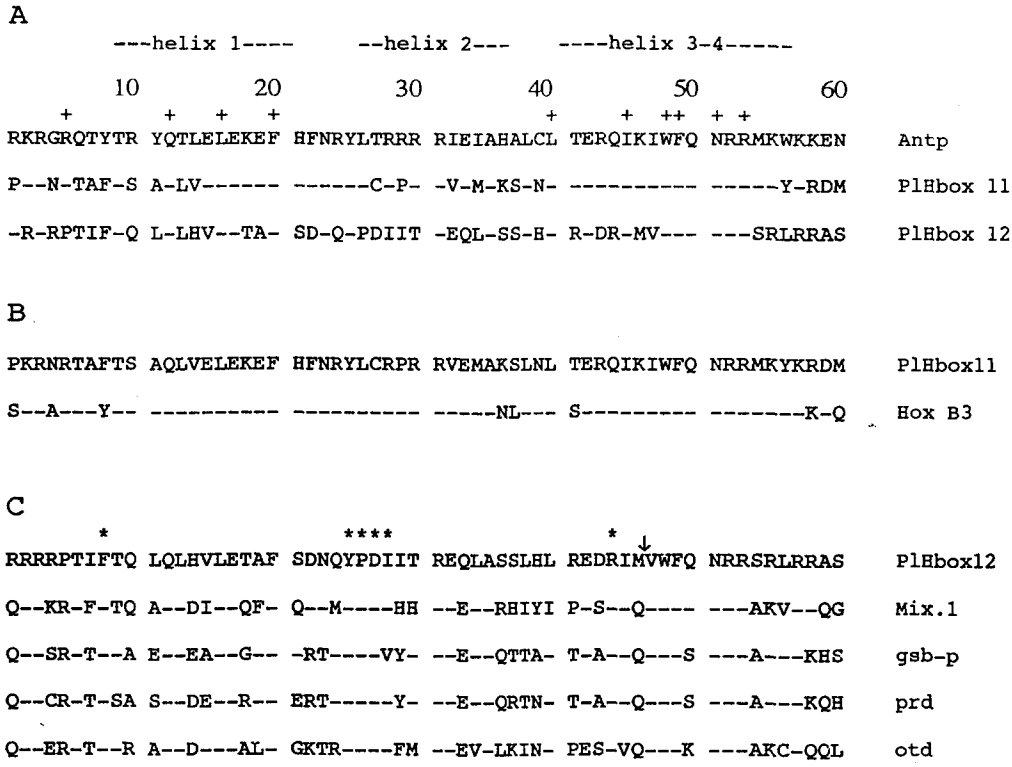

Fig. 1. Homeodomain amino acid sequence comparison. A: PlHbox11 and PlHbox12 homeodomains are aligned with the Antp gene homeodomain. The invariant amino acids are indicated by +. B and C: amino acid sequence comparison among: B PlHbox11 and human and mouse Hox B3. C: PlHbox12 and paired class genes. Asterisks denote the amino acids shared between PlHbox12 and paired class genes and not generally present in genes of different classes. Both PlHbox genes are respectively aligned with genes that belong to the same group. Identities are marked by dashes, while different amino acids are shown as one-letter code translation.

the group 11 homeodomain. The alignment of Fig. 1B shows, in fact, 87% amino acid identity between PlHbox11 and Hox B3 (Scott, 1993). Hox B3 is localized at the most 3′ region of the human and mouse clusters and its expression domain borders span into the anterior region of the mouse embryo (reviewed in Boncinelli *et al.*, 1991; Hunt & Krumlauf, 1991). PlHbox12 encodes for a divergent homeodomain. The highest similarity was found with some homeodomains of the paired class (Fig. 1C). PlHbox 12 is 53.3% identical to the *Xenopus laevis Mix.* 1 gene which begins to be expressed in the vegetal region of blastula stage embryos (Rosa, 1989); it shares 58.3% identity with the *Drosophila* gooseberry proximal protein, 52% with *gooseberry* distal and *orthodenticle* and 57% with the *paired* gene product (Burri *et al.*, 1989). The identity increases if we take into account the fact that many substitutions are conservative. All but *Mix.* 1 paired genes contain the paired box, a second sequence-specific DNA binding domain (Triesman, Harris & Desplan, 1991; Zannini *et al.*, 1992). We recently cloned and sequenced a full length PlHbox12 cDNA and observed that such a domain is absent. Furthermore, unlike paired class proteins that contain ser in position 50 of the homeodomain (see Fig. 1C), this residue which gives binding specificity to the protein (Hanes & Brent, 1989) is substituted by gln in the PlHbox12 gene suggesting that this homeodomain could specifically bind *Antp* class target sequences. All but one of the invariant amino acids that specifically interact with the bases and the phosphates of the recognition sequence of the DNA target are conserved in PlHbox11 and PlHbox12 homeodomains. However, the Arg-5, the most invariant residue of the amino terminus of the homeodomain (Laughon, 1991), that specifically contacts and hydrogen bonds with the O2 of the thymidine residue in the minor groove (Kissinger *et al.*, 1990), in PlHbox12 is substituted by Pro. Interestingly, the Thr-6 that in the engrailed homeodomain contacts the phosphate backbone of the DNA (Kissinger *et al.*, 1990) is also conserved in PlHbox11 and PlHbox12. Sequence analysis of genomic and cDNA PlHbox12 clones revealed that the coding sequence of the helix III of the homeodomain is interrupted by an intron. As shown in Fig.

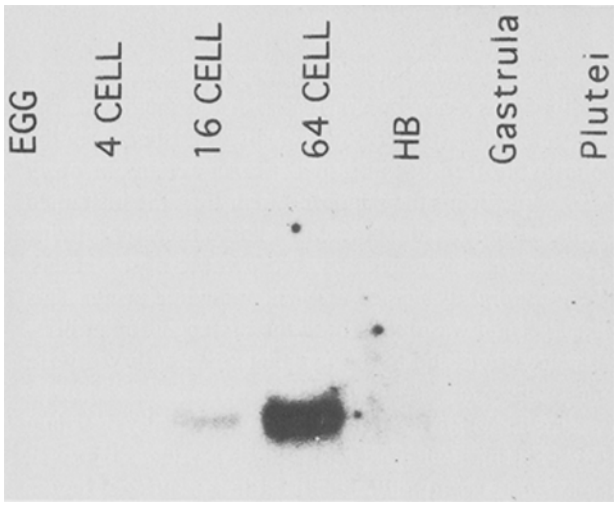

Fig. 2. Expression profile of PlHbox12 gene during development. 20 μg of total RNA of the indicated developmental stages were loaded on a denaturing agarose gel, transferred onto a nylon filter and hybridized with a ^{32}P labelled antisense transcript from the PlHbox12 cDNA. Washes were carried out under stringent conditions (0.1 × SSC at 65°C).

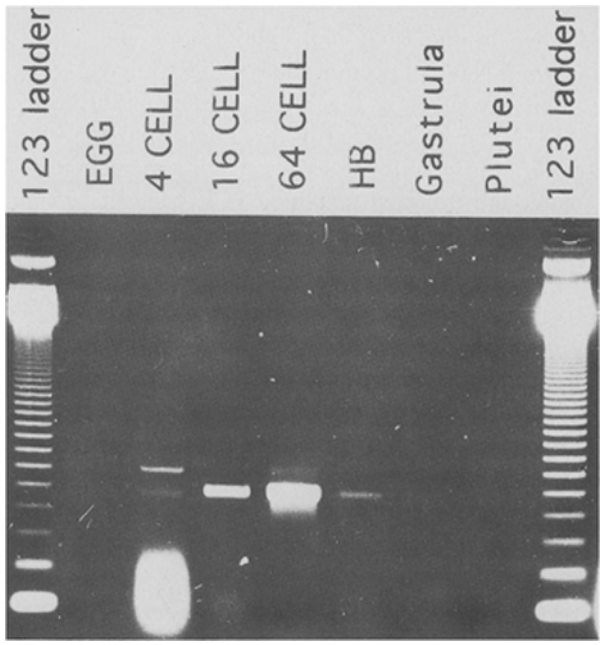

Fig. 3. Pattern of expression of the PlHbox12 gene during development detected by Reverse Transcriptase coupled-Polymerase Chain Reaction (RT-PCR). 1 μg of total RNA from eggs and embryos was reverse transcribed and then amplified by the use of specific oligonucleotides. RT-PCR products were loaded on a 1.5% agarose gel together with a 123 bp DNA ladder.

1C by an arrow, the intron-exon boundary lies at val 47. The interruption of the coding sequence at this amino acid residue is conserved in many other homeogenes (Allen *et al.*, 1991; Simeone *et al.*, 1993).

Expression of sea urchin Hbox genes during development

To study the role played by Hbox genes during sea urchin embryogenesis we determined their temporal expression by RNA blot hybridization. Antisense labelled RNA probes were hybridized to total RNA blots from several different developmental stages (from unfertilized eggs to pluteus larvae) and from two adult tissues (Aristotle's lantern and intestine). As shown in Fig. 2 the PlHbox12 probe hybridized with a RNA band of 0.9 kb in length. This size roughly corresponds to the full length PlHbox12 cDNA we recently cloned. PlHbox12 transcripts can be detected at the 4 cell stage and accumulate at 64 blastomeres. At gastrula stage, they become undetectable, as they were in the intestine and in the Aristotle's lantern (not shown). By applying the more quantitative RNase protection, the same embryonic expression profile of the PlHbox12 gene with a peak of protected RNA band at the 64 cell stage was obtained (not shown). From these experiments we conclude that PlHbox12 mRNA derives from the transcription of the zygotic genome and that expression occurs transiently during very early cleavage.

Similar RNA blot analysis carried out with the antisense RNA of PlHbox11 did not produce, on the contrary, hybridization bands. No positive indication of transcripts from this gene, either in the embryo or in the adult, was obtained by performing RNase protection experiments. These negative results were interpreted as an indication that PlHbox11 mRNA belongs to the very low abundant mRNA class, expressed at very low level and perhaps in a few cell types. To test this possibility we exploited the most sensitive reverse transcriptase coupled-polymerase chain reaction (RT/PCR) to detect, by amplification, transcripts present in very few copies per embryo. Specific upstream and downstream oligonucleotide primers were synthesized for the two PlHbox clones. Total RNA from different developmental stages and adult tissues were reverse transcribed with the specific downstream primers and the resulting cDNAs amplified by PCR as described in material and methods.

To be certain that the amplified PCR products reflected the relative transcriptional level (mRNA abundance) in the embryo, we set up conditions of RT/PCR to detect the PlHbox12 transcripts, whose temporal expression profile during embryogenesis was

known by the RNA blot hybridization and quantitative RNase protection analysis (see above). The results are shown in Fig. 3. Once again, no PlHbox12 mRNA can be detected in the unfertilized egg, thus confirming zygotic transcription of this gene. Furthermore, the expected amplified PCR band of 574 bp begins to be detected in embryos at the 4 cell stage and it accumulates at the 64 cell stage. The RT/PCR assays performed on the RNA samples from embryos at later stages of development (plutei) and from adult tissues (Fig. 3 and results not shown) did not give rise to amplified products. Therefore, the RT/PCR analysis showed a temporal pattern of expression of the PlHbox12 gene and an accumulation profile of its transcripts during embryogenesis similar to those determined by the more conventional methods.

The expression of the PlHbox11 gene was also determined by RT/PCR. To be sure that the PCR products derived from amplification of cDNA are not due to DNA contamination, a control PCR, in which the same RNA samples were amplified by omitting the reverse transcriptase step, was also carried out. This control experiment was not needed for the PlHbox12 clone, due to the presence of an intron in the helix III region (see Fig. 1) that would have allowed the discrimination between genomic DNA and cDNA amplified PCR products. Nonetheless, as shown by ethidium bromide staining and Southern blot of the agarose gel (Fig. 4), omitting the reverse transcriptase step did not produce any specific PCR band in all RNA samples tested. On the other hand, if the RNA were reverse transcribed first and the resulting cDNA amplified by PCR, a specific band of the expected size of 374 bp has been obtained in the RNA samples from embryos at the pluteus stage and from the Aristotle's lantern and the intestine see Figure 4. No such amplified DNA band was detected in the RNA samples from unfertilized eggs and from embryos until gastrula stage.

Spatial distribution of PlHbox12 transcripts

To determine whether the expression of the more abundant PlHbox12 gene was spatially restricted in the embryo and whether the transcripts were asymmetrically distributed along the embryonic axes, we performed *in situ* hybridization to whole-mount embryos. A preliminary result is presented in Fig. 5. The figure shows the optical view of embryos at the 16 cell stage (A) and 28 cell stage (B) that were hybridized with single-stranded antisense RNA probe, labelled *in vitro* with digoxygenin-11-UTP, and stained with an alkaline-phosphatase-conjugated anti-digoxygenin antibody as described in material and methods. The hybridized blastomeres can be distinguished from the non-hybridized ones by their bluish colour. In black and white prints they appear darker than the unstained cells. However, in some cases some non-hybridizing blastomeres, as judged by microscopic observations, seemed slightly stained in black and white prints. This background was due to both the juxtaposition of blastomere layers and to the fact that some embryos were more pigmented than others.

As shown in Fig. 5, the label is not equally distributed in all blastomeres. On the contrary, the PlHbox12 transcripts seem to be localized in a restricted region of the embryo. In particular, at the 16 cell stage at least four mesomeres and two macromeres preferentially hybridized with the antisense probe, whereas staining seemed not to occur in the vegetal micromeres and in the other two macromeres (Fig. 5A). Figure 5B shows the hybridization result obtained with an embryo at the fifth division. At this developmental stage the embryo consists of two animal rings, *an*1 and *an*2 segregated from the equatorial division of the mesomeres, a ring of eight equally sized macromeres, and four large and four small micromeres. In the example shown in Fig. 5 the micromeres have not divided yet, due to their delayed cleavage (for a review see Hörstadius, 1973), so the embryo consists of 28 cells only. The staining is mostly localized in four out of eight macromeres and at least in four animal blastomeres. The *an*1 blastomeres in this particular optical view are probably covered by the other blastomeres, so from this experiment it can not be inferred whether they contained PlHbox12 transcripts. The four micromeres and four macromeres, as expected, showed very little, if any, hybridization. In conclusion, these results suggest that the PlHbox12 transcripts are spatially localized. They do not, however, show a graded distribution along the A-V axis. Rather, it appears from these and other experiments (not shown) that the distribution of PlHbox12 mRNA is restricted to the embryonic macro-mesomere regions only.

Discussion

This paper describes the characterization and expression of two homeobox-containing genes of the sea urchin *Paracentrotus lividus*. These two genes ought to play distinct regulatory roles in development, since their temporal expression is quite different. The activ-

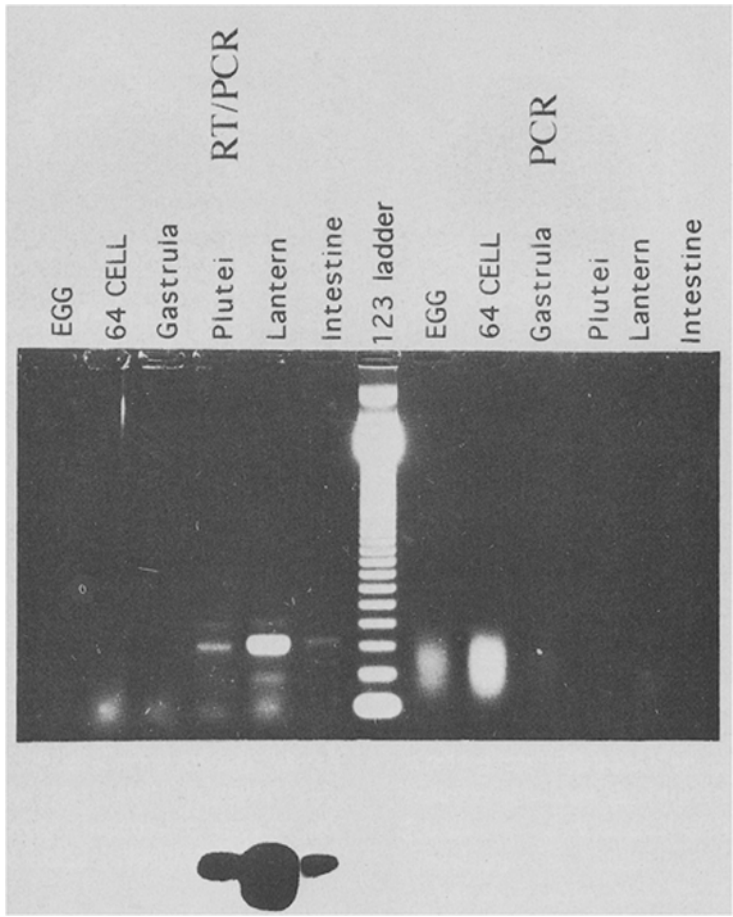

Fig. 4. Identification of PlHbox11 gene transcripts by RT-PCR. 1 μg of total RNA extracted from unfertilized eggs, embryonic stages and adult tissues were reverse transcribed with a specific downstream oligonucleotide and the resulting cDNAs were amplified by taq polymerase and a specific upstream oligonucleotide primer (RT/PCR). The same RNA samples underwent the cycle amplification reactions without the reverse transcriptase step. Aliquots of the amplified prodcuts were electrophoresed on an ethidium bromide stained 1.5% agarose gel (upper part of the figure). Samples were transferred onto a nitrocellulose membrane and hybridized with a specific PlHbox11 labelled probe (lower part of the figure). A final stringent wash was performed in 0.1 × SSC at 65°.

ity of the PlHbox11 gene product is most probably required for the postembryonic morphogenetic processes. Expression of this gene is in fact initiated in late embryo, after the embryonic territories have been specified, and in adult tissues. In this respect the function of PlHbox11 is similar to that of the majority of the HOX genes in vertebrate embryos where the initial tissue specification and differentiation occur before most homeobox-containing genes are activated (discussed in Davidson, 1991; Boncinelli *et al.*, 1991).

PlHbox12, on the contrary, is the earliest transcribed homeodomain coding gene identified in sea urchin so far. In fact, by three different methods we demonstrated that the abundance of PlHbox12 mRNA changes dramatically during early development, from the unfertilized egg to the pluteus stage. PlHbox12 transcripts are not maternal and they are barely detectable at the 4 cell stage (the earliest developmental stage tested). Their abundance increases sharply at the 64 cell stage; it abruptly declines thereafter and returns to an undetectable level at the gastrula stage. PlHbox12 is probably involved in regulative mechanisms of founder cell specification in the early sea urchin embryo. The spatially restricted expression of the PlHbox12 gene is consistent for such a role. The lack of expression in the vegetal micromeres, suggested by the result presented in Fig. 5, indicates that this homeodomain-containing transcription factor is not involved in the specification of the primary mesenchyme and small micromere founder cells. These territories are autonomously specified by inherited maternal regulatory factors (Cameron & Davidson, 1991).

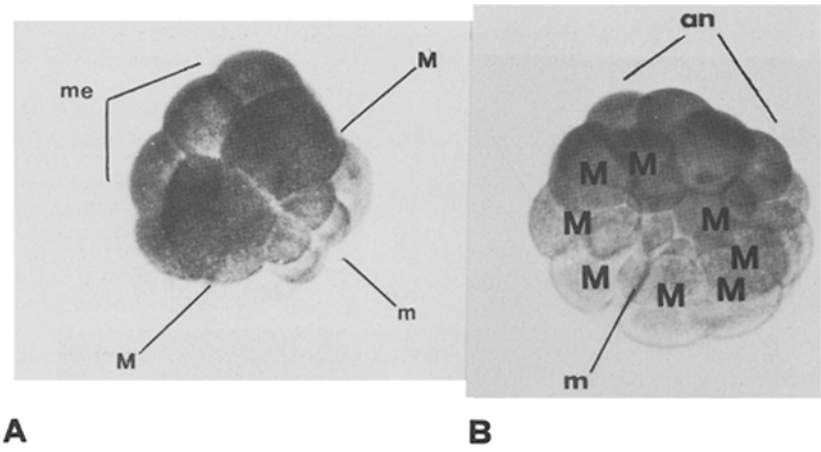

Fig. 5. Spatial pattern of expression of PlHbox12 in *P. lividus* embryos at 16 and 28 cell stage. *In situ* hybridization to whole mount embryos was performed with DIG-UTP labelled antisense RNA. A. Optical view of a 16 cell stage embryo. PlHbox12 transcripts are localized in two out of four macromeres (M) and in all four visible mesomeres (me). Micromeres (m) at the bottom are unstained although they appear darker than the underlying macromeres (see text). B. 28 cell stage embryo. The vegetal pole with the non-hybridizing micromeres (m), the ring of macromeres, half of which being clearly stained, and four hybridized animal blastomeres (an) are indicated.

Transcription of the PlHbox12 gene seemed to occur in half of the macromeres and in half or more animal blastomeres of the embryo at the fourth and fifth division. This result needed to be confirmed by a more detailed analysis aimed to the identification of the hybridizing blastomeres. However, in a number of experiments we observed that in a 16 cell stage embryo, preferential hybridization occurred in two adjacent macromeres and in at least four adjacent mesomeres of the animal cap only. Hence, according to the cell lineage chart of *Strongylocentrotus purpuratus* (Cameron *et al.*, 1987), the stained macromeres and mesomeres should respectively correspond to: i. either the couple VLM (right or left)/VAM or VLM (right or left)/VOM; ii. either NL1/No2/No1/NL2 or NL1/Na2/Na1/NL2. The fate of most of these blastomeres is to segregate founder cells of the ectoderm oral and aboral territories of the blastula. Both territories are specified by founder cells that are positioned in a fixed geometry by the invariant cleavage planes and are definitively segregated at the 64 cell stage. Experiments are in progress to obtain more information on the localization of PlHbox12 transcripts in earlier and later stages of development and to identify all blastomere descendants expressing the PlHbox12 gene. In any case, the early, transient, localized activation of this gene does suggest that it could play a key role in the regulation of specific-regional gene expression that should lead to specification of the ectoderm territories.

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