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Source: Zoological Science, 18(6) : 757-770

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.18.757

ZOOLOGICAL SCIENCE **18**: 757–770 (2001)

[REVIEW]

Body Plan of Sea Urchin Embryo: An Ancestral Type Animal

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ABSTRACT—Sea urchin embryos are though to possess a body plan characteristic of early deuterostomes. Sea urchins contain homologs of *Otx, Lim, T-brain* and *Hox* gene cluster, which are involved in head and segment formation in vertebrate development, although the sea urchin has not evolved a head or segments. We described here that sea urchin *Otx* is involved in various aspects of early development and that the *Hox* genes do not obey spatial colinearity in sea urchin embryo. The *Otx* and *Hox* genes seems to be used subsequently for head formation and determining the anteroposterior axis respectively during chordate evolution. We propose that the Precambrian was a period where these regulatory genes were utilized in many different combinations during animal development, leading to the evolution of a wide range of body plans, many of which were successful. We also discuss the role of chromatin boundaries and the mechanism of cell specification along animal vegetal axis, especially differentiation of the large micromere progeny, which are the prospective primary mesenchyme cells and play a role as an organizer in sea urchin embryos.

INTRODUCTION

The animals alive today that are known to science are composed of approximately one million species displaying a tremendous diversity. All the basic types of multicellular animals burst on the scene in an evolutionary frenzy called the Cambrian explosion about 540 million years ago. Genes involved in morphogenesis are well conserved and the function of most of the genes are shared among all animal phyla, thus the divergent animals are thought to be derived from a common ancestor. It has been suggested that the early animals had already obtained almost all the genes responsible for the morphogenesis by the Precambrian period and that the evolution of genetic regulatory programs produced divergent animals. The molecular mechanisms involved in giving rise to the diverse animal phyla alive today needs to be elucidated, however the origin of animals has been elusive because of the dearth of animal fossils below the Precambrian-Cambrian boundary. In 1998, animals considerably older than the Cambrian finally began being found in the fossil record from the 570 million-year old Doushantuo phosphorites in southern China, and they were preserved in a way that reveals details down to the cellular level (Xiao *et al*., 1998; Li *et al*., 1998). The key to the exquisite preservation is calcium phosphate which is known for its faithful replication of delicate tissues. The Doushantuo phosphorite contained large populations of animal embryos in cleavage stages as well as algae and sponges. The manner of cleavage of the embryo suggested that the Doushantuo fossils are most probably bilaterian. Although gastrulae or later developmental stages have not yet been identified, these finding suggested that they could be broadly equivalent to blastaea or planuloids *sensu* Haeckel (1874), embryos of microscopic animals as envisioned by Davidson *et al.* (1995) and that the morphology of the Precambrian animals resemble larvae of marine invertebrate alive today.

We have been studying molecular mechanisms of early development using sea urchin embryos which are though to possess a body plan characteristic of early deuterostomes (Davidson *et al*., 1995). Sea urchins are simple deuterostomes with bilaterally symmetrical, enterocoelous larvae. *Otx* and *Hox* genes, which are well conserved in metazoans, are involved in the head formation and anteroposterior positional information of segments in chordate respectively. How were these transcription factors used in the ancestral animals which had not yet evolved head and segments? In this review, we focus on the function of *Otx* and *Hox* genes in sea urchin early development, and also we describe the factors involved in the

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primary mesenchyme cell differentiation and new findings using an advantage of sea urchin embryos as an animal model.

Development of sea urchin embryo

In sea urchin embryogenesis it has been suggested that the initial territories are specified by a combination of asymmetric distribution of cytoplasmic determinants and cell-cell interactions. During cleavage, a maternally regulated, reproducible pattern of cell divisions partitions the egg cytoplasm among blastomeres that consequently have defined sizes and orientations relative to each other. The geometric precision of cleavage restricts the range of cell-cell interactions that take place in the normal embryo, with the result that the fates of blastomeres at different positions along the animal-vegetal axis are reproducible and predictable, although most blastomeres have the potential to assume a wide variety of fates until early gastrula stage. At the 16-cell stage, animal-vegetal polarity is morphologically evident. From animal to vegetal are arrayed tiers of eight mesomeres, four macromeres, and four micromeres. At the 32-cell stage, the mesomeres have divided to give two tiers of 8 cells, while in the vegetal hemisphere the macromeres have divided to give one tier of 8 cells, and the micromeres have divided to give large and small micromeres (Fig. 1). At the 60-cell stage blastomeres clonally originated from founder cells divide the embryo into five distinct territories: small micromeres, large micromeres, vegetal plate, oral ectoderm, and aboral ectoderm (Fig. 2). The vegetal plate develops into archenteron and secondary mesenchyme cells which produce muscle and pigment cells in later stage. The territories are identified by the expression of specific marker genes and their cell lineages (Davidson, 1989, 1991) (Fig. 2).

The large micromeres are thought to play a role as an organizer and initiate a cascade of signal transduction toward overlaying cells (Davidson, 1989). The large micromeres induce the overlying macromere progenies, specifying the vegetal plate (Ransick and Davidson, 1993, 1995). A complete respecification of cell fate occurs when 16-cell stage micromeres from the vegetal pole of a donor embryo are implanted into the animal pole of an intact recipient embryo (Fig. 1). Thus, the large micromeres, which are the prospective primary mesenchyme cells (PMCs) play a key role in the cell fate specification and axis determination during sea urchin embryogenesis.

The mesomeres at 16-cell stage display a character similar to the animal cap of amphibian embryo. The mesomeres are normally fated to form epithelial cells. When the mesomeres are combined with micromeres and/or large micromeres, the chimera embryoid develops three germ layers and forms an almost normal embryo (Livingston and Wilt, 1990; Khaner and Wilt, 1991) (Fig. 1).

Macromeres at 16-cell stage develop into ectoderm, endoderm and secondary mesenchyme cells (SMCs). Borders between ectoderm and endoderm and between endoderm and SMCs are negotiated by inductive interactions among macromere progeny during the late cleavage-to-mesenchyme

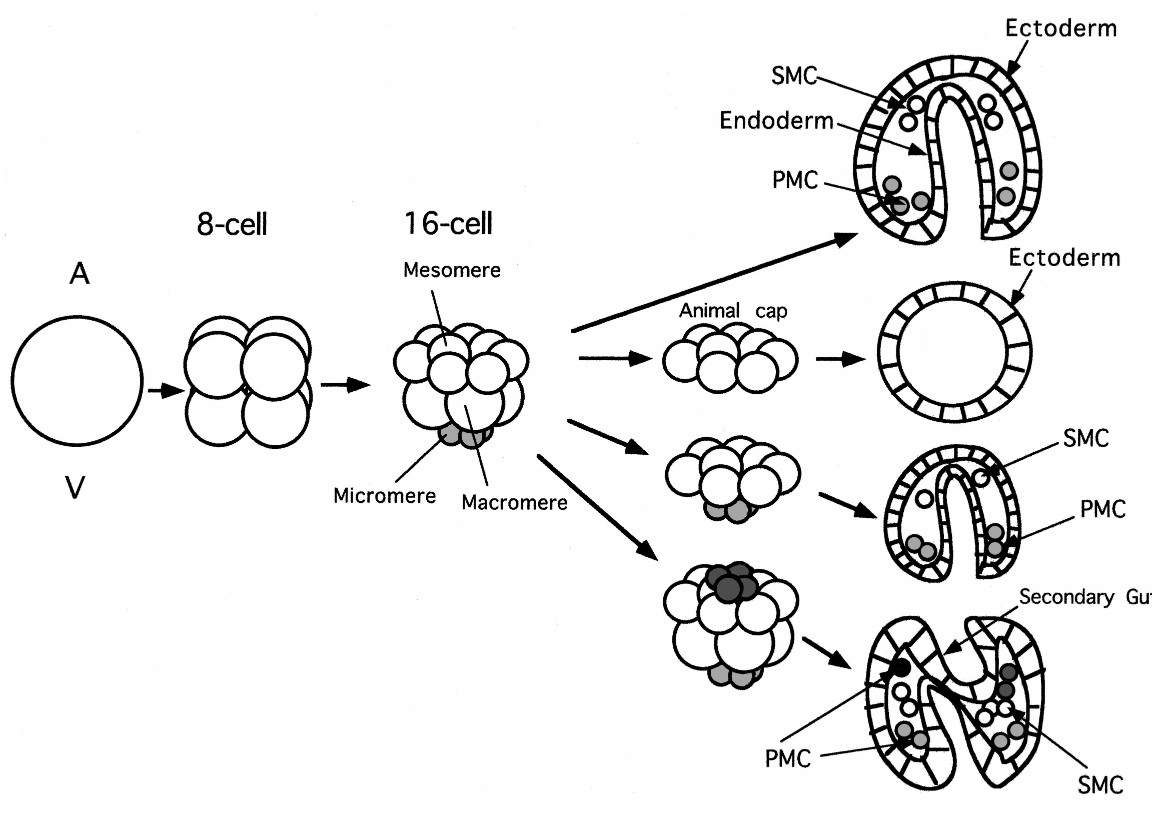
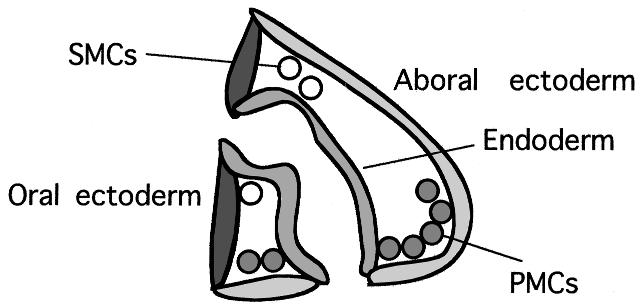


Fig. 1. Development of sea urchin embryo. Micromere descendant cells (gray) play a key role in sea urchin development. Micromeres (black) implanted into animal pole induce secondary gut.



Territory	Markers
Aboral ectoderm	Ars, Spec2a, Cyllla
Oral ectoderm	EctoV, Hpoe
Endoderm	Endo 16
PMCs	SM30, SM50, msp130
SMCs	HpTa

Fig. 2. Territories and territory specific markers of the sea urchin embryo. Ars; arylsulfatase (Akasaka *et al*., 1990; Mitsunaga-Nakatsubo *et al*., 1998), Spec2a; calmodulin like protein (Cox *et al*., 1986), CyIIIa; cytoplasmic actin (Hardin *et al*., 1988), EctoV; hyaline layer glycoprotein protein (Coffman and McClay, 1990), Hpoe; hyaline layer glycoprotein (Yoshikawa, 1997), Endo16; cell adhesion protein (Nocente-McGrath *et al*., 1989), SM30; spicule matrix protein (George *et al*., 1991; Akasaka *et al*., 1994), SM50; spicule matrix protein (Benson *et al*., 1987; Katoh-Fukui *et al*., 1992), msp130; lipid-anchored glycoprotein (Parr *et al*., 1990), HpTa; Brachyury (Harada *et al*., 1995).

blastula period (Angerer and Angerer, 2000).

During the cleavage stage, the embryonic cells form an epithelial ball with monolayer-cells (blastula). After hatching, the cells around the vegetal pole migrate into blastocoel (mesenchyme blastula). These cells are PMCs which later form spicules. Then the cells around vegetal pole begin to invaginate to form the archenteron(gastrula). The invagination site represents the anus. At the late gastrula stage, SMCs appear at the tip of the archenteron. The SMCs develop into muscles and pigment cells later. The archenteron is bent and opens into a stomodeum. The oral side of the ectoderm is referred to as oral ectoderm, and the other side is referred to as aboral ectoderm (prism).

Hox7 and Hox11/13b are involved in pattern formation in sea urchin embryos

It is well known that *Hox* gene complexes have highly conserved function in determining the anteroposterior axis. Sea urchins also possess a single *Hox* gene complex containing 10 genes (Martinez *et al*., 1999)(Fig. 3a). However, in the embryo, only two Hox genes (*Hox7* and *Hox11/13b*) are expressed (Angerer *et al*., 1989; Dobias *et al*., 1996). The expression of *Hox7* protein is restricted to the aboral ectoderm, and *Hox11/13b* expression is restricted to oral ectoderm, endoderm and SMCs in sea urchin embryos after the gastrula stage (Fig. 3b).

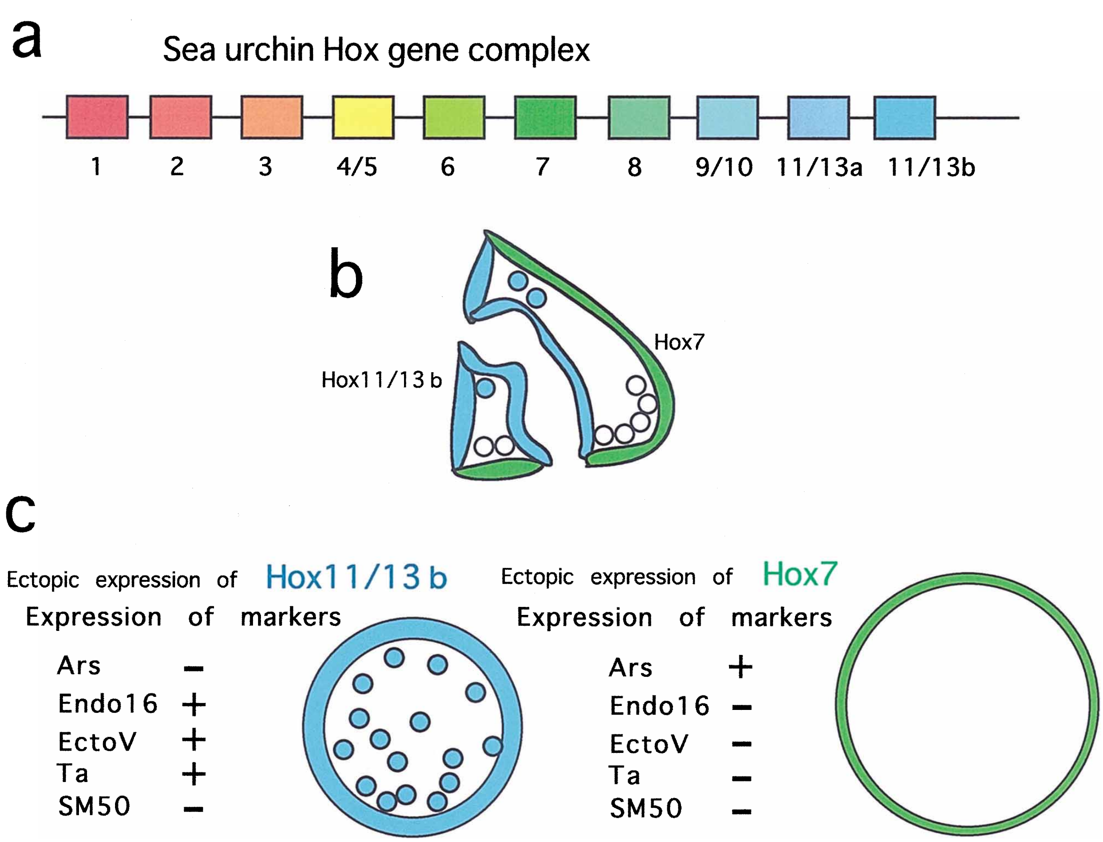


Fig. 3. Sea Urchin Hox gene complex. (a) Structure of sea urchin Hox gene complex. (b) Expression territories of Hox7 and Hox11/13b in sea urchin embryo. (c) Ectopic expression changes the morphology and expression pattern of marker genes.

The nuclear localization of Hox7 and Hox11/13b was demonstrated by immunostaining (Dobias *et al*., 1996; Ishii *et al*., 1999), supporting the idea that both Hox proteins are involved in transcriptional control. With the aim of gaining insight into the role of *Hox7* and *Hox11/13b* in the sea urchin development, we performed *Hox7* and *Hox11/13b* over-expression experiments by injecting mRNAs of *Hox7* and *Hox11/13b*. The overexpression of *Hox7* represses the development of oral ectoderm, endoderm and mesenchyme cells. On the contrary, overexpression of *Hox11/13b* represses the development of aboral ectoderm and PMCs. The data suggests that *Hox7* and *Hox11/13b* are expressed in distinct non overlapping territories, and over expression of either one inhibits territory specific gene expression in the domain of the other (Fig. 3c).

Since the Hox7 overexpressing embryos develop into epithelial balls consisting of aboral ectoderm cells alone, it is likely that Hox7 is part of the circuit responsible for the differentiation of aboral ectoderm cells. However, it has been shown that the aboral ectoderm cells have initiated a tissue-specific program of gene expression before the *Hox7* message achieves a significant fraction of its peak abundance (Angerer L. M. *et al*., 1989). Thus, Hox7 can not be involved in the initial specification of aboral ectoderm. Quantitative RT-PCR reveals that the overexpression of Hox7 does not activate aboral-ectoderm specific Ars gene expression. Thus, it does not seem likely that Hox7 is involved in aboral ectoderm differentiation by activating aboral ectoderm-specific genes.

Nickel (Sunderman, 1989) ions have been implicated as chemical reagents which disturb the signal transduction. It has been shown that the intracellular signaling mediated by calcium ions is involved in the differentiation of oral-aboral ectoderms (Akasaka *et al*., 1997). It is likely that cell to cell signals regulate aspects of the ectodermal patterning during early development. The Hox7 overexpressing embryo is resistant to oral ectoderm induction by $NiCl_2$. Thus, Hox7 seems to be involved in the last crucial part of the cascade which results in the oral-aboral differentiation. In *Hox11/13b* overexpressing embryos, both oral ectoderm (EctoV) and endoderm specific (*Endo16*) genes are expressed in the epithelial cells, however the morphological differentiation of archenteron and oral ectoderm was not observed. It seems likely that factors other than *Hox11/13b* are necessary for the complete development of endoderm and oral ectoderm.

We propose that an important function of both of *Hox7* and *Hox11/13b* genes in sea urchin embryo is to maintain specific territorial gene expression by each one, and their function does not depend on cell position along the axis of the embryo (Ishii *et al*., 1999). Deschamps and Wijgerde (1993) reported that there are two phase of *Hox* gene expression in mouse development. One phase of *Hox* gene expression, which is well known, takes place at the early somite stage. Another phase of *Hox* gene expression takes place at the gastrula stage, much earlier than the somite stage. They showed that *Hox-2.3* and *Hox-2.4* start to express at the late streak stage at the allantois and the most posterior part of the streak. In *C. elegans* also, the *Hox* genes are reported to be responsible for the specification of cell fate in a position independent manner (Cowing and Kenyon, 1996; Wittmann *et al*., 1997).

Recently Arenas-Mena *et al*. (2000) have shown a spatially sequential and colinear arrangement of expression domains of five posterior genes of the Hox cluster in the somatocoel during larval stage. The sequence of Hox gene expression patterns within the somatocoel was cross orientated to the adult anterior-posterior axis. They also reported that a remarkable number of apparent co-options of Hox gene use in developing structure. The findings in sea urchin development, and those from work in *C. elegans* and mouse early embryo suggest that *Hox* gene cluster may originally have had functions in the establishment of the territories or cell-fate, and do not necessarily obey spatial colinearity. In some embryos, the genes are used subsequently for determining the anteroposterior axis at the somite stage.

Otx is involved in various aspects in sea urchin development

The *Otx* gene family is a member of the bicoid class homeobox genes. The product contains a well conserved homeodomain which has a lysine at position 51 that confers DNA-binding specificity for the sequence motif TAATCC/T (Hanes and Brent, 1989, 1991; Treisman *et al*., 1989). Orthodenticle (*Otd*) cDNA was first isolated form *Drosophila* as a homeobox protein related to head formation (Finkelstein *et al*., 1990; Cohen and Jurgens, 1900; Finkelstein and Perrimon, 1990). Subsequently, cDNA homolog (*Otx*) of *Otd* were isolated from various vertebrates. These Otx proteins also have been shown to be essential for pattering the anteriormost aspects of the brain in vertebrates. The mouse has two types of Otx which are expressed in restricted regions of developing brain and are responsible for head formation (Simeone *et al*., 1993; Matsuo *et al*., 1995). In zebrafish three types of Otx are present in the developing diencephalon and midbrain (Mori *et al*., 1994). In sea urchin development, two isoforms of Otx are expressed (Mao *et al*., 1994; Sakamoto *et al*., 1997). The first type of Otx protein, referred to as an

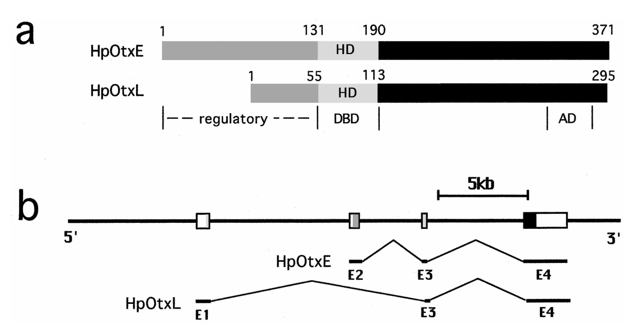


Fig. 4. Structure of Otx gene and protein. (a) Structure of Otx protein. HD: homeodomain, DBD; DNA binding domain, AD; activation domain. (b) Structure of Otx gene. Boxes represent exons. Gray regions in the box represent coding regions. Lower panel indicates splicing pattern of Otx gene products.

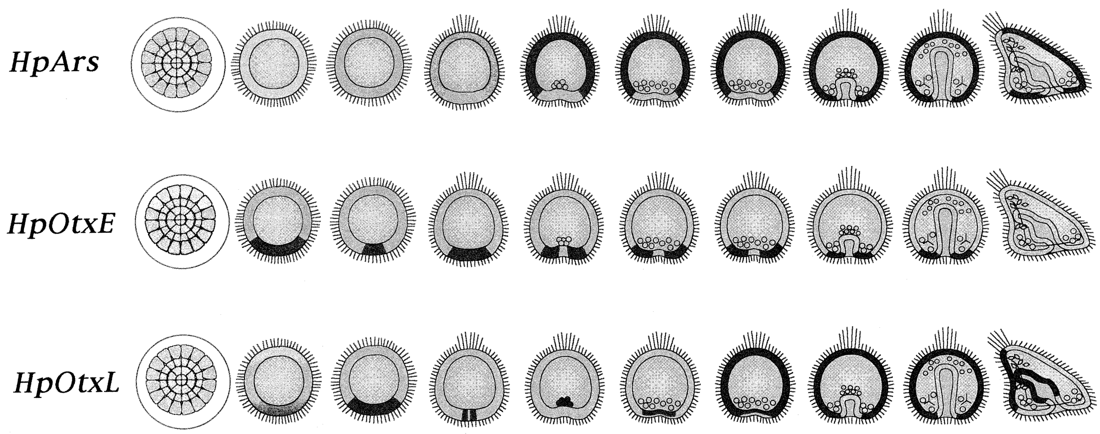


Fig. 5. Spatial expression pattern of HpArs, HpOtxE and HpOtxL. Regions of the embryo where genes are expressed are indicated by the dark gray.

early-type Otx (HpOtxE), appears in early development and gradually decreases in amount by the gastrula stage. The second type of Otx protein, referred to as a late-type of Otx (HpOtxL), appears at the blastula stage and remains until gastrula stage. The nucleotide sequence reveals that the homeobox and downstream regions through to the C-terminus are identical in the two types of HpOtx proteins, while the N-terminal region has different polypeptides (Sakamoto *et al.*, 1997)(Fig. 4a). These distinct Otx proteins are generated from a single gene by altering the transcription start site and by an alternative splicing (Kiyama *et al.*, 1998) (Fig. 4b). Whole-mount in situ hybridization using isoform specific probes reveals a complex and dynamic change of expression patterns in the three germ layers (Li *et al.*, 1997; Mitsunaga *et al.*, 1998) (Fig. 5), suggesting that the Otx is not merely required for the differentiation of specific territories. *Otx* genes seem to be involved in the various aspect of sea urchin early development.

The overexpression of Otx isoforms alters the fate of entire embryonic cells to ectoderm cells (Mao *et al.*, 1996; Mitsunaga *et al.*, 1998). Furthermore, disrupting Otx function by dominant repression causes a specific inhibition of aboral ectoderm- and endoderm-specific gene expression and blocks the formation of aboral ectoderm and endoderm cell types (Li *et al.*, 1999). These dramatic changes of morphogenesis produced by overexpression and reduced function indicate that Otx plays an important role in the sea urchin embryos. We suggest that *Otx* may originally have had functions in various aspects in early development, and the gene was used subsequently for head formation during chordate evolution.

Otx target gene

Despite important role of Otx in embryogenesis, the direct target genes have not been demonstrated in any other species other than sea urchins. *HpArs* (Sakamoto *et al.*, 1997; Kiyama *et al.*, 1998), *Spec2a* (Gan *et al.*, 1995) and *Endo16* (Yuh *et al.*, 1998) have been reported as direct target genes of Otx in sea urchin embryo. *HpArs* is transcriptionally activated late in blastula, and after the gastrula stage it is expressed exclusively in the aboral ectoderm throughout embryonic development (Sasaki *et al.*, 1988; Akasaka *et al.*, 1990a, 1990b). In order to detect cis-regulatory elements responsible for the transcriptional activity of genes, we introduce reporter(*Luc*)-fusion constructs into fertilized eggs. To analyze the function of transcription factors *in vivo*, we introduce effector(transcription factor)-constructs together with reporter-constructs (Kiyama *et al.*, 1998). Microinjection is a very useful technique to introduce DNA into fertilized egg; however, it does have some disadvantages: a certain amount of skill is required to perform the microinjection, and the number of embryos that can be injected at one session is limited to approximately 1000. We applied the particle gun method to sea urchin embryos and obtained excellent expression of the introduced DNA (Akasaka *et al.*, 1995). We introduce DNA into approximately 100,000 eggs per one shot. The expression of the construct of interest can be normalized by concomitantly-introducing a reference construct; CMV *Renilla* luciferase, which allows highly quantitative experiment. When the linearized plasmid DNAs are introduced into fertilized egg, they form random concatenates, and during the early development of the embryos replicate repeatedly (McMahon *et al.*, 1985). The stable incorporation of exogenous DNA into the chromosomal DNA of embryos occurs, and the exogenous DNAs persist until postmetamorphosis juvenile stage (Flytzanis *et al.*, 1985).

In gene transfer experiments, a 229-bp fragment, referred to as C15, in the first intron of *HpArs* was found to have enhancer elements (Iuchi *et al.*, 1995). This region contains a tandem repeat of core consensus sequences of orthodenticle-related protein (Otx) binding sites, which serve as the major source of positive control for *HpArs* gene (Sakamoto *et al.*, 1997) (Fig. 6a). The time course of expression of HpOtxL is similar to that of *Ars* gene. Thus, HpOtxL is very likely to be involved in the activation of the *Ars* gene. In order to eliminate the contribution of the endogenous HpOtx to the expression of the reporter, we used a Gal4 DNA binding domain-HpOtx fusion protein together with a reporter containing Gal4 binding sites and lacking HpOtx sites. This should provide a test of the ability of HpOtx to transactivate the *Ars* promoter-enhancer without the background contribution by the endogenous HpOtx. The reporter construct was generated by ligating Gal4

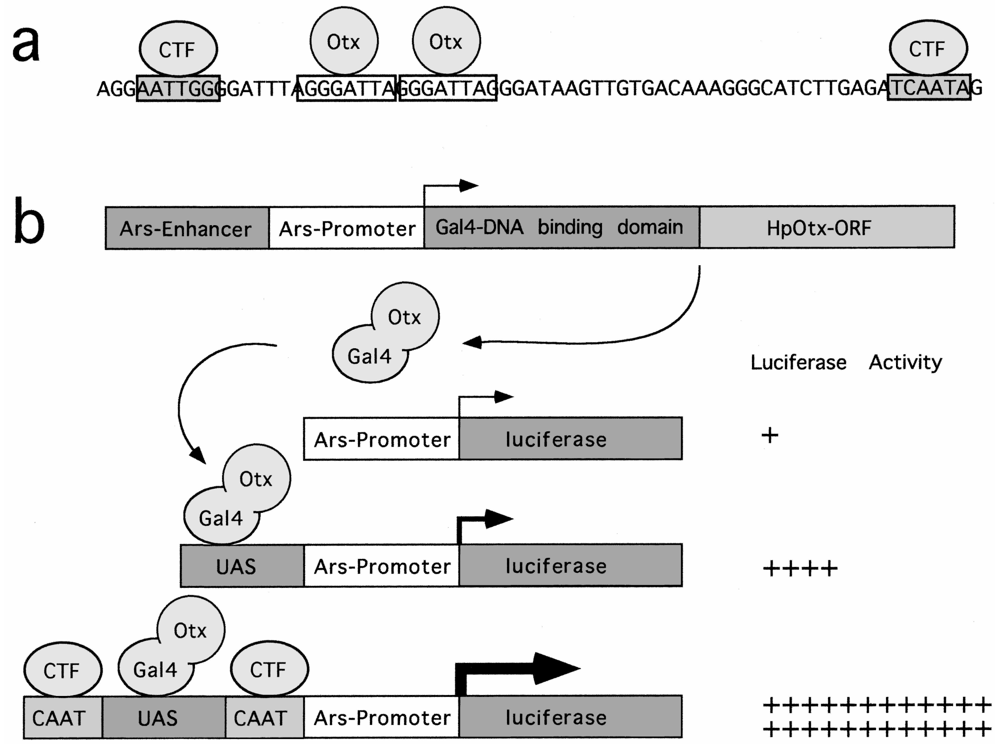


Fig. 6. Structure of HpArs enhancer and transactivation assay with the Gal4-Otx fusion protein. (a) Structure of HpArs enhancer. Sequences in open boxes indicate Otx binding sites. Sequences in gray boxes indicate CTF binding sites. (b) Upper panel indicates the structure of the expression construct of the Gal4-Otx fusion protein. Lower panel indicates luciferase reporter gene driven by different combination of cis-elements, and the expression levels.

binding site (UAS), (in stead of *Ars* enhancer C15 which contains Otx sites) and an *Ars* promoter, (*Ars194*) in which the Otx binding site has been deleted, conjoined to the luciferase reporter gene (Fig. 6b). The different construct combinations were introduced into fertilized eggs, which were cultured to the gastrula stage. HpOtxL successfully activates the *Ars* promoter by 4 fold, but that HpOtxE does not under the same conditions. The Gal4-HpOtxL fusion protein does not activate the expression of the luciferase reporter construct in which the Gal4 binding sites are deleted. The results indicate that Gal4-HpOtxL fusion protein activates *Ars* promoter-directed gene activity at the UAS site.

Otx activates ars gene with CAAT binding protein

We thought the 4 fold expression activated by Gal4-HpOtxL fusion protein was much less than endogenous *Ars* enhancer C15 activity. We wished to know if regions of the C15 enhancer other than Otx sites are needed for maximum enhancer activity. Therefore, we generated an enhancer in which UAS was substituted for the Otx sites, and in which 5' and/or 3' deletions (Δ5' and/or Δ3') were made in the modified C15 enhancer. When both of the 5' and 3' regions of the C15 enhancer were fused to UAS (C15(UAS)), the fusion enhancer C15(UAS) shows approximately 3-fold greater activity than UAS alone without the expression of Gal4-HpOtxL fusion protein. Furthermore, the activity of the *Ars* promoter(*Ars194*) bearing C15(UAS) was enhanced 5 fold by the expression of Gal4-HpOtxL fusion protein (Fig. 6b). The results indicate that both the 5' region and 3' region of the C15, in addition to Otx sites, are required for a maximum response to HpOtxL.

Besides the Otx sites, the enhancer C15 contains two CAAT sequences (Fig. 6a). We sought to determine the functional properties of these two sites. In order to analyze the role of Otx sites and CAAT sequences precisely, site-directed mutagenesis was employed to mutate these sequences. When one of the CAAT sequences was changed but the Otx sites were unaltered, a 15–34% reduction in the activity was observed. A significant (60%) decrease in the activity was observed when both CAAT sequences were mutated but the Otx sites were kept intact. When both Otx sites and CAAT sequences were mutated, the activity was reduced to background, indicating that together, both CAAT sequences and Otx sites are essential for the maximal enhancer activity. Furthermore, we have demonstrated that nuclear proteins prepared from embryos at mesenchyme blastula stage bind to the CAAT sequences in the enhancer C15 (Kiyama *et al*., 2000).

Functional domains in Otx

Because the fusion enhancer C15(UAS) responds appropriately to the expression of Gal4-HpOtxL fusion protein, we used C15(UAS) as a target sequence to address the question of which domains of HpOtxL are responsible for the activation of the *Ars* promoter. We fused portions of HpOtxL with the Gal4-DNA-binding domain and analyzed the ability of these fusion proteins to activate a luciferase reporter gene driven by *C15(UAS)-Ars194* in sea urchin embryos. The deletion analysis revealed that C-terminal region between amino acid 218–238 of HpOtxL are required for the transactivation, and that the N-terminal region of HpOtxL is responsible for the enhancement of transactivation of the *Ars* promoter. The N-terminal region of the other isoform, HpOtxE, represses the activity of its C-terminal region (Fig. 4a). These data suggest that Otx regulates the transcription of a target *Ars* gene by working cooperatively with a CAAT sequence binding factor(s), and that the transactivation domain and the regulation domain exists in the C terminal region and the N terminal region respectively.

Specificity of Otx target genes

The enhancer region of *Spec2a*, which is also specifically expressed in the aboral ectoderm, contains two Otx sites and two CAAT sequences (Gan and Klein, 1993). Considered together with the findings in the present study, it is possible that both Otx and CAAT sites are responsible for the specific expression of genes in the aboral ectoderm (Fig. 6a). On the other hand, the combination of a synthetic Otx site and the basal promoter of *Endo16* suffices to generate endoderm-specific expression (Yuh *et al*., 1998). Thus Otx regulates different genes in different territories in the embryo, and it is unlikely Otx is responsible for the territory-specific expression of the target genes. A previous report suggested that different transcription factors are involved in the expression of one gene and the factors form a regulatory network to achieve a complex gene expression in development (Yuh *et al*., 1998). We have suggested that HpOtxL activates *Ars* expression co-operatively with CAAT binding protein. It is likely that co-factor(s) interacting with Otx direct the specificity of the target genes.

Genes involved in animal-vegetal axis patterning

The cell fates of cells in the early embryo are progressively specified along the animal-vegetal axis. Embryos may inherit positional information in the form of asymmetrically distributed maternal determinants that are positioned during oogenesis. When unfertilized eggs are bisected through the equator and the two halves are fertilized, the animal half gives rise to an incompletely differentiated epithelial ball, while the vegetal half can produce a normal pluteus larva with derivatives of endoderm, mesoderm, and ectoderm (Horstadius, 1939; Maruyama *et al*., 1985). This experiment suggests the presence of determinants of fate in the vegetal pole and the requirement for cell-cell interactions to complete the fate specification of animal blastomeres. Recently, members of Wnt signaling system, such as GSK-3β, β-catenin and Tcf/Lef, have been reported to be factors which are involved in pattern formation of sea urchins along the animal-vegetal axis. Tcf/Lef is a partner of β-catenin in transcriptional regulation (Clevers and van de Wetering, 1997). Introduction into sea urchin embryos of a dominant negative form of Tcf/Lef that lacks the β-catenin binding domain produces animalized phenotypes (Huang *et al*., 2000; Vonica *et al*., 1999). The converse is also true: a constitutively active Tcf consisting of the strong transcription activation domain of VP16, vegetalized embryos (Vonica *et al*, 1999). Activation of GSK-3β promotes the degradation of β-catenin through a phosphorylation, ubiquitination and proteosome pathway, resulting in low level of free cytoplasmic β-catenin. Overexpression of GSK-3β causes severe animalization. Again the converse is true: expression of dominant negative (kinase-dead) GSK-3β blocks the turnover, leading to over accumulation of β-catenin in nuclei and vegetalization (Emily-Fenouil *et al*., 1998) (Fig. 7a). Treatment of embryos with LiCl, an inhibitor of GSK 3β, expands the domain of nuclear β-catenin into the presumptive ectoderm territory (Logan *et al*., 1999) and the embryos are vegetalized. The dominant negative Tcf/Lef can reverse the vegetalizing effect of LiCl (Vonica *et al*., 1999). An animal-vegetal asymmetry in the levels of nuclear β-catenin is seen in the embryos after 16 or 32-cell stage (Logan *et al*., 1999) (Fig. 7b). The

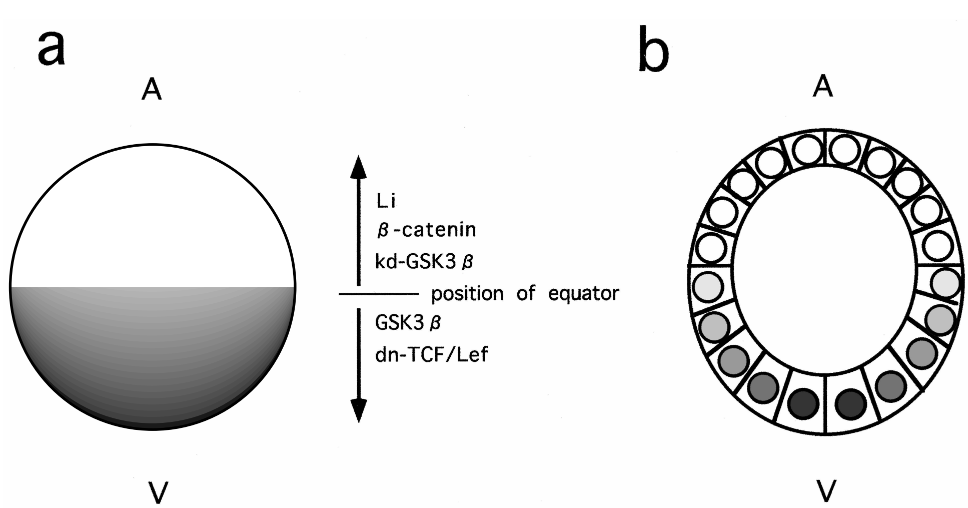


Fig. 7. Animal vegetal axis patterning. (a) Factors involved in animal vegetal axis patterning. Li; Li ion, kd-GSK3β; GSK3β lacking kinase domain, dn-TCF/Lef; TCF/Lef lacking β-catenin binding domain. A; animal pole, V; vegetal pole. (b) Animal-vegetal asymmetry in the levels of nuclear β-catenin. A; animal pole, V; vegetal pole.

expression of Wnt8 is also first detected after 16 or 32-cell stage (Wikramanayake personal communication). However, no asymmetrical distribution of nuclear β-catenin is observed before the 8 or 16-cell stage, thus mechanisms other than the conventional Wnt-signaling pathway must be present in order to establish the animal-vegetal axis in the egg. Maternally inherited RNAs and/or proteins could be responsible for the formation of animal-vegetal axis in the egg.

Transcription factors involved in primary mesenchyme cell differentiation

Micromeres, which are located at the vegetal pole in 16-cell stage embryos, divide at the 5th cleavage into 4 large micromeres and 4 small micromeres. Descendants of the large micromeres immigrate into the blastocoel and give rise to PMCs, which are responsible for skeletogenesis in the pluteus larvae (reviewed by Davidson *et al*., 1998), and play a key role in the cell fate specification and axis determination during sea urchin embryogenesis. Previous data suggested that the large micromeres are autonomously specified to become PMCs by maternally inherited determinants (Okazaki, 1975; Kitajima and Okazaki, 1980). An important question in sea urchins embryogenesis is the identity and function of the proposed maternal determinants. To gain a toehold for the elucidation of the cascade of micromere specification initiated by maternally inherited factors, we have isolated transcription factors responsible for PMC-specification. We found that *HpEts*, which encodes the Ets-related transcription factor of sea urchin, *Hemicentrotus pulcherrimus*, play an important role in the differentiation of PMCs (Kurokawa *et al*., 1999). *HpEts* is expressed exclusively in the PMCs after the blastula stage (Fig. 8a). The overexpression of *HpEts* by injecting the mRNA into fertilized eggs results in the alteration of the fate of all the embryonic cells into migrating cells, which is characteristic of PMCs, and the migrating cells expressed spicule matrix protein SM50 (Fig. 8b). Also the animal cap (mesomere) assay revealed that isolated mesomeres, which is fated to form ectoderm in the normal embryo, are transformed into skeletogenic PMCs when the animal cap is derived from eggs injected with *HpEts* mRNA (Fig. 8c). On the other hand, embryos injected with a truncated *HpEts* mRNA, which encodes the ets-DNA binding domain lacking the N terminal region, develop normally except for the skeletogenic PMCs. Spicule formation was repressed in these embryos (Fig. 8d). Quantitative reverse transcribed polymerase chain reaction used for monitoring the expression of various cell type specific marker genes revealed that the embryos injected with *HpEts* mRNA showed a low level of *HpArs* (aboral ectoderm specific) and *HpEndo16* mRNA (endoderm specific) in comparison to uninjected embryos. On the other hand, the level of primary mesenchyme specific *HpSM50* mRNA increased significantly in comparison to that in uninjected embryos. Embryos injected with the truncated *HpEts*-mRNA showed a normal level of *HpArs* (aboral ectoderm specific) and *HpEndo16* mRNA (endoderm specific), but the level of PMC specific *HpSM50* mRNA decreased significantly in comparison to uninjected embryos (Fig. 8). We also demonstrated that the ets binding site located in the upstream region of SM50 (Kurokawa *et al*., 1999) and SM30 (Akasaka *et al*., 1994, Kurokawa *et al*., 1999, Yamasu and Wilt, 1999) is a positive transcriptional element. These findings suggested that the *HpEts* gene is involved in sea urchin PMC differentiation. However, HpEts does not seem to be involved in the organizer activity of primary mesenchyme cells, since the embryos expressing dominant negative HpEts form archenteron and SMCs. Recently, we have isolated a sea urchin homolog of *T-brain*, a transcription factor containing T-domain. We referred the gene as *HpTb*. *HpTb* is exclusively expressed in the PMCs. When the expression of *HpTb* is repressed by injecting with anti-sense morpholino oligo, formation of archenteron and the SMCs are not observed in the injected embryo (submitted). We suggest that *HpTb* is involved in the organizer activity of PMCs.

Notch signaling is involved in SMC induction by PMC

It is well known that the micromeres have the capacity to induce a second axis if transplanted to the animal pole, and the absence of micromeres at the vegetal pole results in the failure of macromere progeny to specify SMCs. This suggests that micromeres have the capacity to induce SMCs (Fig. 1).

The evolutionarily conserved Notch intercellular signaling pathway has been shown to play an essential role in the segregation of a diverse array of cell types in both invertebrate and vertebrate embryos (Kimble and Simpson, 1997). The sea urchin homolog of the Notch receptor displays dynamic patterns of expression within both the presumptive SMCs and endoderm during the blastula stage, the time at which these two cell types are thought to be differentially specified (Sherwood and McClay, 1997). The activation of Notch by injecting eggs with mRNA encoding a constitutively active form of Notch results in an increase in SMC specification, while loss or reduction of Notch signaling by overexpression of dominant negative forms of Notch eliminates or significantly decreases SMC specification (Sherwood and McClay, 1999). Transplantation studies show that much of the vegetal hemisphere is competent to receive the induction signal. They suggested that the micromeres induce SMCs, most likely through direct contact with macromere progeny, or at most with cells only a cell diameter away (McClay *et al*., 2000). The induction is quantitative in that more SMCs are induced by four micromeres than by one. Furthermore, they demonstrated that micromeres requires nuclear β-catenin to exhibit SMC induction activity (Sherwood and McClay, 1999). They suggest that the macromeres first must transport β-catenin to their nuclei in order to be receptive to the micromere inductive signal, and as one consequence the Notch pathway becomes competent to receive the micromere induction signal, and to transduce that signal (Fig. 9). Temporal studies showed that the induction signal is passed from the micromeres to macromere progeny between the eighth and tenth cleavage. The is consistent with the observation previously reported by Minokawa and Amemiya (1999).

Recently, sea urchin *delta* homolog is found to be ex-

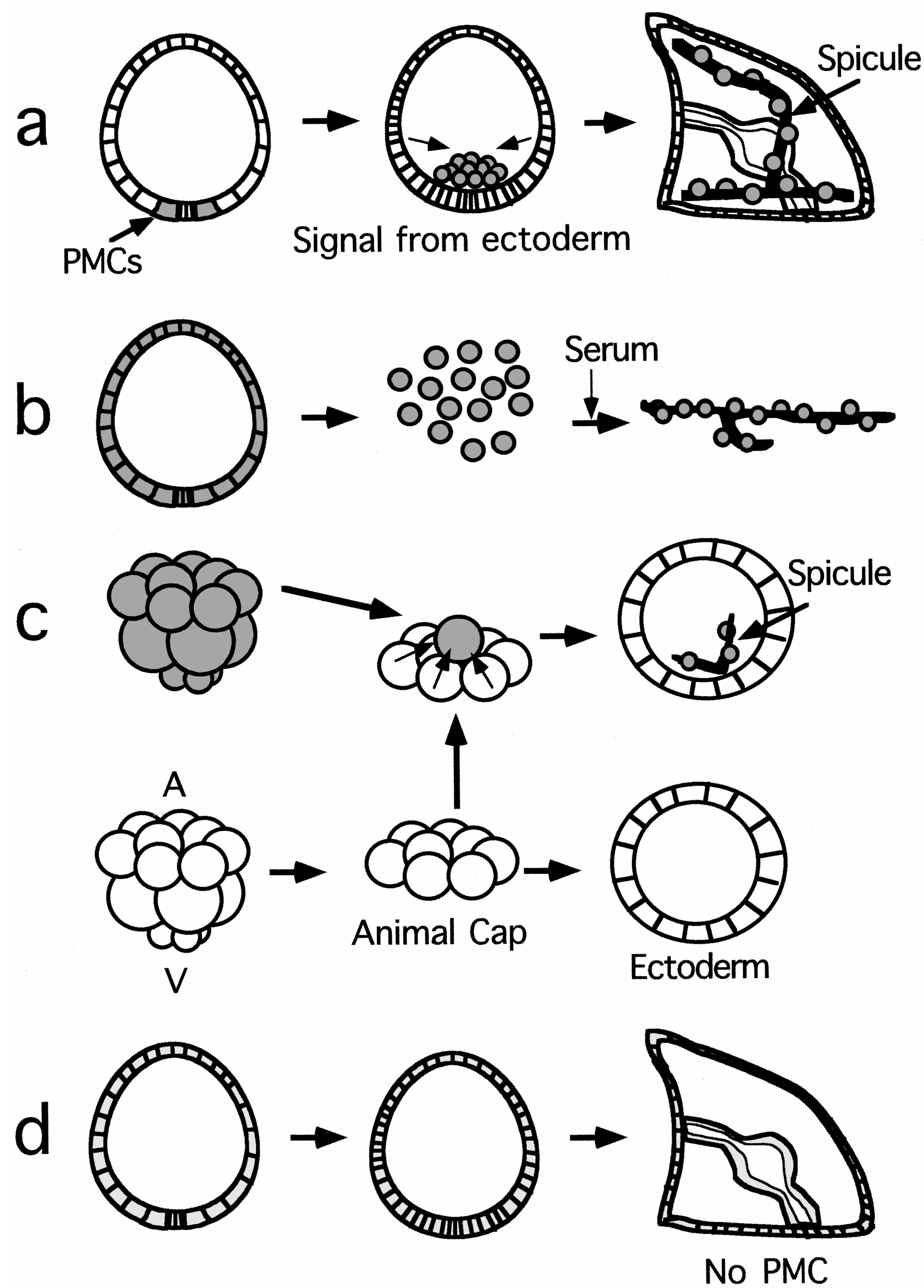


Fig. 8. Function of HpEts in sea urchin development. (a) HpEts expressing cells are indicated by the dark gray. Signals from ectoderm are though to be required for the formation of spicule. (b) Ectopic expression of HpEts results in the alteration of all the embryonic cell-fate into migrating PMCs. The transformed cells produce spicules in vitro in the presence of serum. (c) A chimera composed of one mesomere isolated from an embryo injected with HpEts mRNA, which is indicated with dark gray, and eight mesomeres isolated from a normal embryo. The mesomere progeny injected with HpEts mRNA form spicules. Animal cap (mesomeres) is fated to form ectoderm. (d) Dominant negative expression of HpEts which lacks the activation domain results in a normal prism embryo except for the formation of skeletogenic PMCs. The cells which express the truncated form of HpEts are indicated by light gray.

pressed exclusively in PMCs when the PMCs have the capacities to induce SMCs (Ettenshorn, personal communication). *delta* is known to be a ligand for Notch (Morrison *et al*., 2000; Bally *et al*., 2000). It is possible that delta is a SMC inducing factor emanated from PMCs.

Boundary of chromatin

A great number of genes are arranged on a single DNA molecule. Since enhancer elements are able to activate promoters at great distances and in an orientation independent manner (Atchison, 1988), the enhancer elements in one gene could influence the promoter of other genes. The chromatin

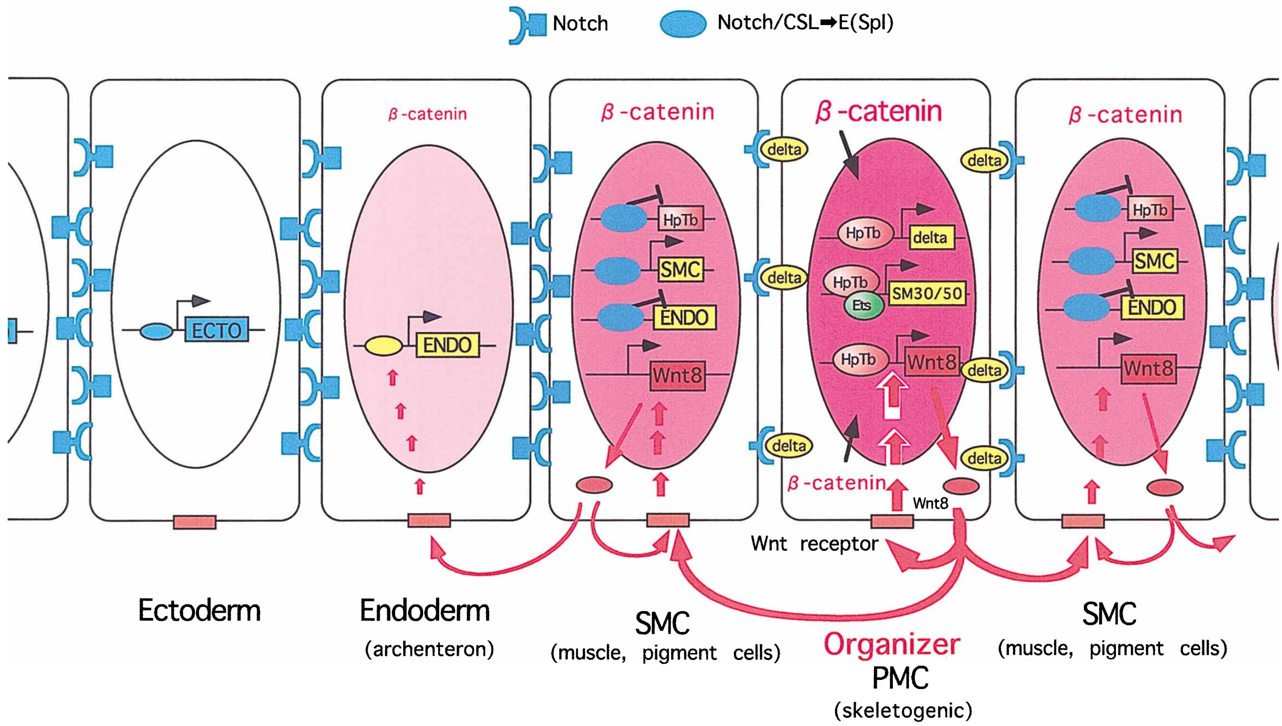


Fig. 9. Model for endoderm and SMC induction by micromere progeny. The intensity of color in the nucleus indicates the concentration of nuclear β-catenin.

fibers of the eukaryotic genome are thought to be organized into discrete domains. Boundaries may serve to confine enhancers and silencers to act only within a domain, as well as to insulate the genes within a domain from the cis-regulatory elements or chromatin structure of an adjacent domain. A boundary designated as an insulator is defined as a DNA fragment which blocks the activity of enhancer when it is located between the enhancer and the promoter of the gene (Kellum and Schedl, 1992, Chung *et al.*,1993), and which confers position independent transcription on transgenes stably integrated into the chromosome (Kellum and Schedl, 1991; Kalos and Fournier, 1995) (Fig. 10a).

We have been studying the mechanism of expression of *Ars* gene, which is expressed in a tightly regulated manner (Sasaki *et al.* 1987, Sasaki *et al.*, 1988; Akasaka *et al.*, 1990a, b) during sea urchin development. The *Ars* gene is actively transcribed during post gastrula stages, and the accumulation of arylsulfatase mRNA may reach about 1% of total mRNAs (Sasaki *et al.*, 1988); the C15 enhancer in the first intron is apparently very strong. Therefore, the *Ars* enhancer could influence the promoter of the neighboring genes. We assumed that a boundary of chromatin is located in the flanking region of such an actively transcribed *Ars* gene. In order to find putative insulator elements in the upstream region of *Ars*, we used the well-characterized *Ars* enhancer C15 (225 bps), which contains Otx binding sites as major positive elements (Iuchi *et al.*, 1995; Sakamoto *et al.*, 1997; Kiyama *et al.*, 1998), together with *Ars* promoter (–252 bp to +38 bp) (Akasaka *et al.*, 1994; Morokuma *et al.*, 1997) to drive a luciferase reporter gene. The constructs were introduced in the fertilized eggs by particle gun and the transcriptional activity was determined at gastrula stage when the *Ars* gene is actively transcribed. We found that a fragment spanning from –2686 bp to –2113 bp of *Ars* gene acts as an insulator (Fig. 10b). Gel mobility shift assay revealed that at least two different proteins extracted from sea urchin embryos bind to the *Ars* insulator sequence.

To determine whether the *Ars* insulator isolated from sea urchin genome functions in other species when coupled with other regulatory elements, we tried the sea urchin insulator in *Drosophila* embryo by employing P element-mediated transformation. We introduced a plasmid construct containing the *even-skipped* stripe enhancer-promoter (Cai and Levine, 1995) with or without the insulator fragment. When the insulator fragment was placed between the enhancer of stripe 3 and stripe 2, the insulator fragment blocked stripe 3 expression from the *eve/lacZ* promoter, but allowed the stripe 2 enhancer to direct the expression of its promoter (Fig. 10c). The simultaneous use of two different stripe enhancers (*eve* stripes 2 and 3) indicate that the enhancer lying distal to the sea urchin insulator is selectively blocked in *Drosophila*.

When a gene is inserted close to a heterochromatic region in a gene transfer experiment, the transgene is subjected to repression by the chromatin environment. It is therefore of interest to determine if the insulator fragment insulates transgenes from repressing chromosomal position effect. We stably transfected HeLa cells with the constructs in which the neomycin-resistant gene driven by a SV40 enhancer-promoter is flanked by the insulator fragments (Fig. 10d). The number of geneticin-resistant colonies increased significantly when the neomycin resistant gene is flanked on both sides by the insu-

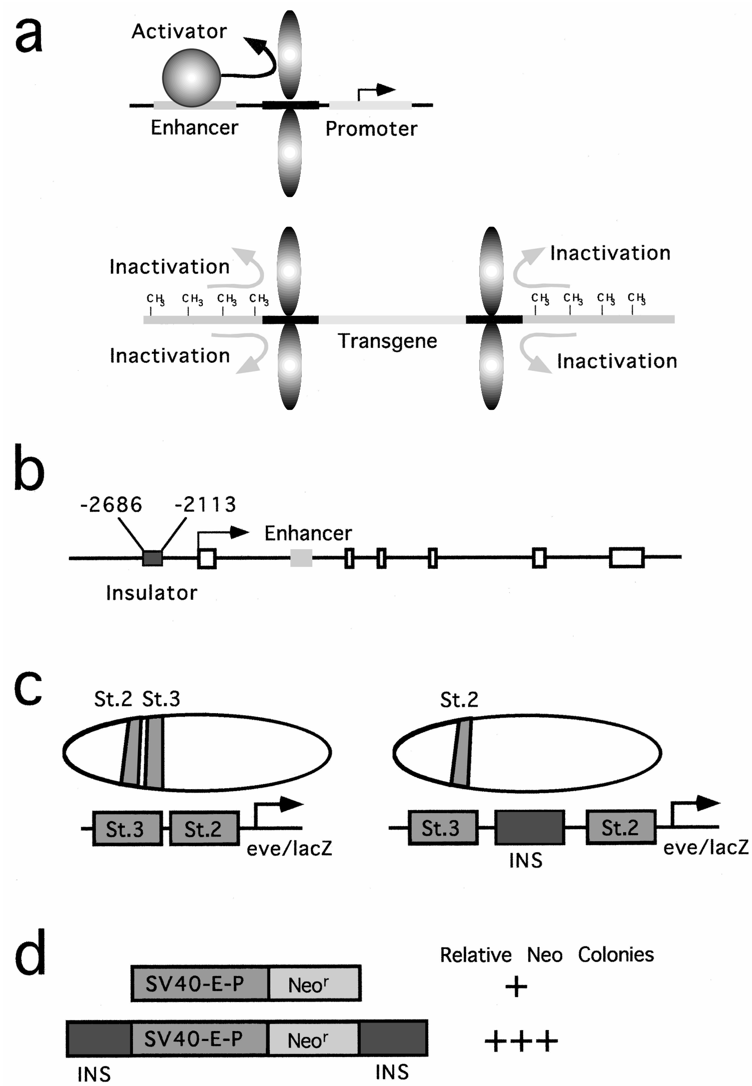


Fig. 10. *Ars* insulator. (a) Definition of insulator. (b) An insulator in the flanking region of the Ars gene. (c) The Ars insulator functions in *Drosophila* embryo. Upper panel indicates the expression pattern of the reporter gene. St.2; stripe 2, St.3; stripe 3. Lower panel indicates the structure of reporter constructs which are introduced into *Drosophila* embryos. St.2; stripe 2 enhancer, St.3; stripe 3 enhancer, INS; *Ars* insulator. (d) Inhibition of position effect by the *Ars* insulator. Structure of constructs which are introduced into HeLa cells and the expression. SV40-E-P; enhancer and promoter derived from SV40, Neo^r; neomycin resistant gene, INS; Ars insulator. The activity is represented as number of colonies/plate.

lator fragment. The findings suggest that the sea urchin *Ars* insulator could overcome the position-dependent repression of transgene expression in mammalian cells. Recently, we have demonstrated that the sea urchin Ars insulator functions in mouse embryo (Takada *et al*., 2000) and in plant cells (Nagaya *et al*., 2001). Currently, different boundary sequences have been reported (Kellum and Schedl, 1991; Chung *et al*., 1993; Michel, *et al*., 1993; Cai *et al*., 1995; Kalos and Fournier, 1995; Hagstrom *et al*., 1996; Zhong *et al*., 1997; Akasaka *et al*., 1999). However, no sequence similarity was observed among them. Some of the insulators, such as chicken β-globin insulator (Chung *et al*., 1993) and the sea urchin *Ars* insulator reported here, function in species other than those from which they originate. Given these findings, we expect that the mechanism(s) of action of insulators have been conserved throughout evolution, and that the nuclear proteins which bind to the boundary sequences may recognize higher order DNA structures rather than specific DNA sequences.

Concluding remards

It has been suggested that by Precambrian period early animals had already obtained almost all the genes responsible for the morphogenesis of animals alive today and that the evolution of different genetic regulatory programs is what produced divergent animals. Phylogenic studies have revealed that systems involved in morphogenesis are well conserved among divergent animals. However, in some genes, there seem to be certain variety in their usages. Sea urchin possesses homologs of *Otx, Lim, T-brain* and the *Hox* gene cluster, which are involved in head and segment formation in vertebrate development, although the sea urchin has not evolved a head or segments. We propose that the Precambrian was a period where these regulatory genes were utilized in many different combinations during animal development, leading to the evolution of a wide range of body plans, many of which were successful. We described here that sea urchin *Otx* is involved in various aspects of early development and that the Hox genes do not obey spatial colinearity in sea urchin embryo. The *Otx* and *Hox* genes seems to be used subsequently for head formation and determining the antero-posterior axis respectively during chordate evolution. The acquisition of an anterior-posterior axis of the body, especially a head at the most anterior end of the body, seems to be extremely advantageous for survived over a long evolution history. Thus only animals, for instance, arthropod and vertebrate, which used these genes to establish anterior-posterior axis of the body could increase their population and give rise to new species. The animals which did not use these genes for the establishment of the anterior-posterior axis, such as sea urchin, thus could not increase their number. On the other hand, the chromatin boundary, which is a basic mechanism responsible for the regulation of gene expression, seems to be conserved among all the variety of animals. The elucidation of the mechanisms of evolution of body plans is one of the most important problem we face. In order to understand the whole story of evolution of body plans, we need to investigate the functions of many different genes involved in morphogenesis in different marine invertebrates, which are thought of as the ancestral type of animals.

ACKNOWLEDGEMENTS

We thank the Editor-in-Chief of this journal, Professor Norio Suzuki, for recommending us to write this review. The authors wish to express their thanks to members of our laboratory, especially Kazuko Takata, Keiko Mitsunaga-Nakatsubo, Mamoru Ishii, Daisuke Kurokawa, Naoaki Sakamoto, Takae Kiyama. The authors also thank Dr. Brian Livingston (University of Missouri, Kansas City) for his advice in preparation and critical reading of the manuscript, and Dr. H. Katow (Asamushi Marine Biological Station) for supplying live sea urchins. A part of this work was carried out in the Center for Gene Research of Hiroshima University. We thank the Cryogenic Center, Hiroshima University for supplying cryogenÅiliquid nitrogen). This work was supported in part by Grants-in-Aid for Scientific Research (C2) (No. 11680724) and for Scientific Research on Priority Areas (No. 11152227) to K.A., and for Scientific Research (B) (No. 07458195) to H.S. from the Ministry of Education, Science, Sports and Culture, Japan, and Program for Promotion of Basic Research Activities for Innovative Biosciences.

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(Received March 9, 2001 / Invited Review)