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学位請求論文

Maternal factors related to body plan of sea urchin
（ウニ形態形成に関わる母系因子の研究）

１９９９年

広島大学大学院理学研究科遺伝子科学専攻

小川麻里

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Asymmetrical distribution of mitochondrial rRNA into small micromeres of sea urchin embryos

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Sox and other regulatory elements in the promoter region of the sea urchin arylsulfatase gene

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Development Growth and Differentiation （投稿準備中）

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主論文

Maternal factors related to body plan of sea urchin
（ウニ形態形成に関わる母系因子の研究）

広島大学大学院理学研究科遺伝子科学専攻

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General Abstract

Expression of genes underlying various developmental processes such as morphogenesis, body plan, etc. is regulated by cascades of transcription factors. Factors or cell fate determinants of maternal origin are supposed to play important roles to drive these cascades. Sea urchin is one of the most useful material to study maternal factors and transcriptional cascades, because of simplicity of its developmental pattern and body plan and of easiness to get synchronized embryos in a large amount. In addition, the simple and quick procedure to introduce exogenous genes into a large number of sea urchin eggs has been developed in our laboratory.

The study in the present thesis has been undertaken to identify some of the maternal factors of sea urchin eggs and to elucidate their role in sea urchin development. In Part 1 of this thesis, detection and identification of maternal factors, poly(A)+ mitochondrial ribosomal type RNA (mt 12S rRNA and mt 16S rRNA), asymmetrically enriched in small micromeres are described. These two mt rRNAs are localize outside of mitochondria and distinguished from intramitochondrial rRNAs. Based on this findings, the author speculates possible role of extramitochondrial mt rRNAs in sea urchin development.

In Part II, the author has detected other maternal factor, HpSox protein, in sea urchin eggs, and found that by binding to a Sox motif in the promoter of the arylsulfatase gene HpSox protein regulates expression of arylsulfatase protein that is related to build up body plan of pluteus larvae.

General Introduction

The body plan of multicellular organisms consists of various tissues and organs, that are organized from many genetically identical but differently differentiated cells derived from only one fertilized egg. Expression of genes underlying the processes of morphogenesis is regulated by cascades of transcription factors including those of maternal origin. Many maternal informations, such as maternal mRNA, maternal protein, and so on, are accumulated in eggs during oogenesis and utilized in early development. It is suggested that some of the maternal factors act as the initial determinants for cell fate determination or for cell differentiation. A series of study by Kobayashi and Okada of Tsukuba University showed that mitochondrial large rRNA that is of maternal origin and detected outside mitochondria in germ plasm of *Drosophila* is intimately involved in formation of germ line cells (Kobayashi and Okada, 1989; Kobayashi *et al.*, 1993; Kobayashi *et al.*, 1994; Amikura *et al.*, 1996; Kashikawa *et al.*, in preparation). Extramitochondrial mt large rRNA is also detected from germ plasm of *Xenopus* (Kobayashi *et al.*, 1994; Kobayashi *et al.*, 1998). It is also reported that extramitochondrial mt large rRNA distributes asymmetrically in ascidian embryos though their role in early ascidian development is not known (Nishida *et al.* personal communication).

In sea urchin, many attempts have been performed to find molecules that is distributed in a gradient from either pole of a egg or an embryo, but no successful report has been published that provides clear evidence for identity of these molecules. In the Part 1, the author attempted to screen RNA enriched in micromeres of 8- to 16-cell embryos of sea urchin by RNA differential display, and identified poly(A)+ mt 12S rRNA. It is also detected that poly(A)+ mt 16S rRNA is also asymmetrically distributed

along an animal-vegetal axis enriching in micromeres. Both of them localizes extramitochondrially as they are in embryos of *Drosophila* and *Xenopus*. Based on their asymmetrical distribution in sea urchin embryos and from their restricted accumulation in small micromeres of 60-cell embryos, these mt rRNA molecules are intimately involved in cell fate of small micromere descendants in body plan of adult sea urchin.

Part 2 of this thesis is devoted to elucidation of a role of maternal factor as a component of cascade of transcription factors that regulates expression of genes for cell differentiation to build up a body plan. A gene for arylsulfatase (*Ars* gene) that is expressed temporally as well as spatially restricted manners during early development of sea urchin embryos (Sasaki *et al.* 1988; Akasaka *et al.* 1990a, b) and is related to formation of body plan of pluteus larvae (Mitsunaga-Nakatsubo *et al.* 1998) was selected as a terminal structural gene to elucidate its transcription cascade. In this study, it is found that Sox protein that is maternally produced and store in eggs function as a strong activator of the Ars gene by its interaction with Sox-binding site in the promoter of the Ars gene.

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Part 1

Asymmetrical distribution of mitochondrial rRNA into small micromeres of sea urchin embryos

Abstract

Blastomeres of the 16-cell stage embryos of the sea urchin, *Hemicentrotus pulcherrimus*, were separated by an elutriator. By differential display, several RNA species that are enriched in micromeres are detected and their cDNA was cloned. One of the cloned cDNA encodes mt 12S rRNA. cDNA for mt 16S rRNA was also cloned from the cDNA library of unfertilized eggs. Two mt rRNAs contain poly(A) tails in their 3' ends. Both mt rRNAs distribute asymmetrically along a vegetal-animal axis of the 16-cell embryos and are enriched in micromeres, and this is also confirmed by whole mount *in situ* hybridization as well as electron microscopic *in situ* hybridization. As development proceeds, these mt rRNAs become more enriched in small micromeres. Results of electron microscopical *in situ* hybridization reveal both mt rRNAs localize extramitochondrially. Though at present we have no evidence on the role of the extramitochondrial mt rRNAs in sea urchin development, it is speculated considering roles of extramitochondrial mt 16S rRNA in *Drosophila* development that extramitochondrial mt rRNA may be implicated in development of sea urchin embryos.

Introduction

In a variety of animal groups, molecules localized asymmetrically in egg cytoplasm play an important role in the specification of topologically-restricted cell lineages (Davidson, 1986; Gilbert, 1997). The sea urchin embryo offers a relatively simple model system to study early cell type specification. It was revealed more than a decade ago that the early cleavage planes of sea urchin embryo are invariant with respect to the animal-vegetal axis (Dan, 1979; Dan *et al.* 1983; Dan, 1987). The subsets of blastomeres aligned perpendicular to the animal-vegetal axis of early embryos acquire distinct cell fates. It has been proposed that molecules distributing in a gradient along animal-vegetal axis provide positional information to define micromeres, and the cell type specification in the entire embryo occurs through cell-cell interaction initiated by the micromeres (Boveri, 1901; Runnström, 1928 and 1975; Hörstadius, 1928 and 1973; Davidson, 1989). To assess how the animal-vegetal polarity is established during embryogenesis, we have focused our study on the molecules asymmetrically localized along the animal-vegetal axis.

It has been reported in the two phylogenetically distinct animals, *Drosophila* and *Xenopus*, that mitochondrial (mt) 16S rRNA is localized in a histologically distinct region of egg cytoplasm, called germ plasm, which contains factors required for germ line establishment (Kobayashi *et al.*, 1994; Kobayashi *et al.*, 1998; Illmensee and Mahowald, 1974, 1976; Ikenishi *et al.*, 1986; Togashi *et al.*, 1986; Smith, 1966; Okada *et al.*, 1974). Indeed, mt 16S rRNA is able to induce germ line progenitors in the UV-irradiated *Drosophila* embryos (Kobayashi and Okada, 1989), and is implicated in pole

cell formation (Iida *et al.* 1998). Ultrastructural studies in *Drosophila* reveals that both mt 16S and mt 12S rRNA are present outside mitochondria in germ plasm during the cleavage stage, and are localized in the distinctive organelles in germ plasm, or in germinal granules (Kobayashi *et al.*, 1993; Amikura *et al.*, 1996; Kashikawa *et al.*, in preparation). Similarly, in *Xenopus* embryos, extramitochondrial mt 16S rRNA is enriched in the germinal granules during discrete stages from 4-cell to blastula stages (Kobayashi *et al.*, 1994; Kobayashi *et al.*, 1998). Here we report that in sea urchin embryos mt 12S and 16S rRNA localize outside mitochondria and distribute asymmetrically among blastomeres along a vegetal-animal axis. As the development proceeds extramitochondrial mt rRNAs become enriched in micromeres, and by the 60-cell stage distribution of these RNAs becomes restricted to small micromeres. The present study provides the first report showing that mt rRNAs, germ plasm components in *Drosophila* and in *Xenopus*, are also present in echinoderm embryos and are partitioned into discrete blastomeres.

Materials and Methods

Dissociation of embryo into blastomeres

Gametes of the sea urchin, *Hemicentrotus pulcherrimus*, were shed by the conventional KCl procedure. Eggs were fertilized and developed in artificial seawater (ASW; Jamarin U, Jamarin laboratory, Japan) at 15°C until the 16-cell stage. The fertilization membrane was removed using 5mM *p*-aminobenzoic acid (PABA, Nasir *et al.* 1992) . Four-cell embryos were transferred to CFSW (Ca-free seawater) just before the 16-cell stage, then they were transferred to CMFSW (Ca, Mg-free seawater) at 16-cell stage, and blastomeres were dissociated by gently shaking.

Elutriation

Size-dependent separation of blastomeres were performed by the centrifugal elutriation according to Nasir *et al.* (1992) with a slight modification. Segregated blastomeres were fixed with ethanol and stored at 4°C. Fixed embryos were resuspended in autoclaved CMFSW and centrifuged in an elutriator rotor (Beckman JE-5.0 elutriation system standard chamber, Beckman, Tokyo) at 4°C with a flow rate of 24ml/min at 2krpm. Homogeneity of each blastomere fraction separated by elutriator was about 100%, 100%, and 84% for micromeres, macromeres, and mesomeres, respectively (Fig.1).

RNA Differential display

Differential display was carried out using RNAmap™ (GenHunter corporation, MA) according to the manufacturer's instruction. RNA were

extracted from stored blastomeres with ISOGEN (Nippon gene, Japan), digested with DNaseI (Boehringer Mannheim, Germany) for 15min at 37°C, and extracted by phenol/chloroform followed by ethanol precipitation. cDNA was synthesized by reverse transcription of the total blastomere RNA with MMLV reverse transcriptase for 2hr at 37°C, and was amplified by PCR using Taq DNA polymerase (Recombinant Taq DNA polymerase, Takara Shuzo, Japan) and ^{35}S-dATP(redivue™ LABELLED NUCLEOTIDES, Amersham, UK) with T_{12}MG (5'-TTTTTTTTTTTTMG-3')and AP-2 (5'-GACCGCTTGT-3') as primers. After electrophoresing the PCR products in 6% DNA sequencing gel, the gel was dried on a filter paper (Whatman 3MM paper, Whatman Int. Ltd., England), and covered with wrap film (Saran Wrap, Asahikasei, Japan) to subject to autoradiography.

DNA extraction from gel bands

cDNA bands enriched in the micromere were excised from the gel, and DNA was extracted by soaking the gel pieces in TE (1mM EDTA in 10mM Tris-HCl at pH8.0) overnight at 4°C followed by 15min boiling. DNA was ethanol-precipitated from the extract, and reamplified by PCR using the same set of primers. The PCR products were electrophoresed on agarose gel and subcloned.

RT-PCR for isolation of poly(A)+ mt 16S rRNA

RT-PCR was carried out using TaKaRa RNA PCR Kit (AMV) Ver.2 (TaKaRa Shuzo) according to the manufacturer's instruction using the specific primers (described below) designed for each clones.

The presence of poly(A) tails in the PCR products were surveyed using an

oligo(dT) primer and a specific forward primer. One μg of total RNA was reverse transcribed with 2.5μM oligo(dT)$_{20}$-M4 Adaptor primer (5'-GTTTTCCCAGTCACGACTTTTTTTTTTTTTTTTTTT-3') for 30min at 50°C. After boiling, the PCR reaction was carried out by addition of the PCR mixture containing 0.2μM mt 16S rRNA-specific forward primer (16F; 5'-AGTCCTGCCTGCCCAGTGAC-3'). After initial denaturation at 94°C, 2min, PCR was performed for 30 cycles using the following parameter, 94°C for 30sec, 55°C, 30sec and 72°C, 1.5min, and the product was electrophoresed on 1.5% agarose gel.

Quantitative RT-PCR

Mt rRNA from each three types of blastomere was quantified by quantitative RT-PCR using following primers. For mt 12S rRNA; 5'-GGGACTTACTGCTGAATCCAATTTC-3' (reverse,12R) and 5'-GACCGCTTGTATACCATCGTCG-3' (forward, 12F1), and for mt 16S rRNA, 5'-CGGTCTGAACTCAGATCAGGTAG-3' (reverse, 16R) and 5'-AGTCCTGCCTGCCCAGTGAC-3' (forward, 16F).

cDNA screening

PCR products were labelled with ^{32}P-dCTP (NEN, USA) by the random-labelling procedure. Using these oligonucleotides as the probe, cDNA library of the unfertilized egg was screened at a high stringency (hybridization in 5X SSPE, 1% SDS, 5% ICL at 65°C; washing with 0.1X SSC, 1% SDS at 65°C).

Northern hybridization

Two μg each of the total RNA and poly(A)+ RNA from the 16-cell stage embryos was electrophoresed in 1% agarose gel containing formaldehyde, and RNA bands were transferred to nylon filter (NY 13N NYTRAN: Schleicher & Schuell, Germany). The membranes were prehybridized for 3hr at 65°C in 5X SSPE containing 1% SDS, 5% ICL and 100μg/ml salmon sperm DNA, hybridized with the probe DNA overnight at 65°C, and washed under a high stringency condition. The random-primed ^{32}P-labelled DNA probe for mt 12S rRNA was prepared by PCR-labelling method using both 12R reverse primer and 12F2 forward primer (5'-CCAGGATTAGATACCCTGTTATAC-3'). Primers used to prepare the probe for mt 16S rRNA were 16R and 16F.

Whole mount *in situ* hybridization

Whole mount *in situ* hybridization was performed basically as described by Ransick *et al.* (1993). The 16-cell stage embryos freed from fertilization membranes were fixed in S.T.F.(Streck Tissue Fixation : Streck Laboratories, Inc, USA) for several hours at 4°C and then for 2 days at 4°C. Fixed embryos were washed successively with ASW, a series of graded ethanol, xylene, and ethanol and stored at -80°C until use.

The medium was gradually replaced by PBST(phosphate-buffered saline containing 0.1% Tween 20), and the specimen was prehybridized in the hybridization buffer (50% formamide containing10% PEG#6000, 0.6M NaCl, 5mM EDTA, 20mM Tris-HCl pH7.5, 500μg/ml yeast RNA, 2X Denhardt's and 0.1% Tween 20) to prehybridize for 3hr at 46°C, and hybridization with the digoxigenin(DIG)-labelled antisense RNA probe (0.2μg/ml) for about 18hr at 46°C. The DIG-labelled probes were synthesized with MEGA script™ T3 or T7 kit (AMBION, USA) and digoxigenin-11-2'-deoxyuridine-5'-triphosphate

(Boehringer Mannheim) according to manufacturer's instruction. The antisense probe for mt 16S rRNA was alkaline hydrolyzed to a length of about 500bp before use. After hybridization, the sample was washed once with PBST, three times with 1X SSC-0.1% Tween 20 at 60°C, and non-hybridized RNA was digested with 50μg/ml RNase A for 30min at 37°C. DIG signals were visualized using anti-digoxigenin conjugated alkaline phosphatase (Boehringer Mannheim) and observed under an optical microscope.

Electron microscopic *in situ* hybridization

The specimen was hybridized by the post-embedding method (Kobayashi *et al.* in press). The 16-cell stage embryos were fixed for 1hr with 2.5% glutaraldehyde in the 0.3M NaCl-0.2M cacodylate buffer, embedded in Lowacryl HM20 resin and sectioned. The sections were hybridized with DIG-labelled double strand DNA probe (1μg/μl) in the hybridization buffer (50% formamide, 5X SSC, 100μg/ml Sonicated salmon sperm DNA, 50μg/ml heparin, 0,1% Tween 20) at 45°C for 5hr, and then incubated with a drop of 10nm-gold-conjugated anti-digoxigenin antibody (BioCell, UK, 200-fold diluted in TBST) for 1hr at room temperature. DIG-labelled sections were fixed with 0.5% glutaraldehyde and stained with uranyl acetate-lead citrate.

Results

mt 12S rRNA and mt 16S rRNA are unequally distributed in 16-cell embryos

To find RNA species enriched in micromeres of the 16-cell sea urchin embryos, each blastomeres (micromeres, macromeres and mesomeres) were collected separately from the embryos using an elutriator, and the total RNA from three distinct blastomeres was compared by RNA differential display reactions (Fig. 2). Among several RNA species enriched in micromere fraction, we cloned cDNA of the most distinct band (indicated by an arrowhead in Fig. 2) from the cDNA library of unfertilized sea urchin eggs. Its nucleotide sequence (EMBL access. No. AJ130797) was 95% similar to that of mitochondrial 12S ribosomal RNA (mt 12S rRNA) of other sea urchin species, *Strongylocentrotus drobachiensis* and *S. intermedius* , indicating that the cloned cDNA encodes mt 12S rRNA of *H. pulcherrimus*.

Since it is known in *Drosophila* that mt 16S rRNA as well as mt 12S rRNA exist extramitochondrially and are components of germ plasm localized in the posterior pole region of the cleavage embryos (Kobayashi *et al.*, 1993; Kashikawa and Kobayashi, unpublished), we cloned mt 16S rRNA from cDNA library of unfertilized *H. pulcherrimus* eggs. The nucleotide sequence of Hp mt 16S rRNA (EMBL access. No. AJ130798) was 90% homologous to that of *S. purpuratus*.

Quantitative RT-PCR analysis confirms that both mt 12S rRNA and mt 16S rRNA are enriched in micromeres of the 16-cell embryos (Fig. 4a).

Nucleotide sequence analysis and Northern blotting of poly(A)+RNA from the 16-cell embryos (Fig. 4b) show that sea urchin mt 12S and 16S rRNAs

contain poly(A) tracts at their 3' ends as in the case of several animal species (Wolstenholme, 1992).

Developmental changes in the distribution of mt 12S rRNA and mt 16S rRNA in the embryos

The temporal and spatial distribution of mt 12S and 16S rRNAs was determined by whole mount *in situ* hybridization using antisense mt rRNA probes (Fig. 5, a, b c and d for mt 12S rRNA; e, f, g and h for mt 16S rRNA). Two mt rRNAs showed a similar pattern of spatial distribution in the early embryos. Asymmetrical distribution of two mt rRNAs was observed in uncleaved fertilized eggs (Fig. 5 a and e) as well as in the 8-cell embryos (Fig. 5 b and f). Asymmetrica distribution of these rRNAs became more evident at 16-cell stages in which mt 16S rRNA is localized specifically in micromeres alone (Fig. 5g) while mt 12S rRNAs distributed in a gradient along a vegetal-animal axis (Fig. 5c). As development proceeds up to the 60-cell stage, distribution of mt rRNAs became clearly restricted to small micromeres, the descendants of micromeres (Fig. 5 d and h). After the 60-cell stage, any intense signal was no longer detectable. A probe synthesized from pBluescript vector failed to generate signals in the 16-cell embryo (data not shown).

We found that the distribution of mt rRNAs did not simply reflect that of mitochondria in the embryos. The distribution of mitochondria among blastomeres monitored by $DiOC_2(3)$, a fluorescent dye for vital staining of mitochondria (Zalokar & Sardet, 1984), was significantly different from that of the mt rRNAs (Fig. 5 i to l). The $DiOC_2(3)$ signal was distributed almost evenly throughout the cytoplasm of the fertilized eggs indicating

even distributions of mitochondria among blastomeres. Thus up to 60-cell stage embryos are found no difference among blastomeres in the intensity of $DiOC_2(3)$ staining. Validity of this observation can be assessed by that $DiOC_2(3)$ stained granular structures similar in size to mitochondria (inset in Fig. 5k). This result suggests that the mt rRNA signal we detected by whole mount *in situ* hybridization did not result from intramitochondrial signals but from extramitochondrial molecules.

In *Drosophila*, mt 16S and 12S rRNA are present outside mitochondria only in posterior polar plasm of cleavage embryos (Kashikawa et al., unpublished). To confirm if mt rRNAs are really localized outside mitochondria in blastomeres of sea urchin embryos as they are in *Drosophila* embryos, intracellular distribution of mt rRNAs were surveyed by electron microscopical *in situ* hybridization using cDNA probes for mt rRNAs, and localization of these RNA was visualized by gold particles (Fig. 6). Significant amounts of mt 12S rRNA (Fig. 6a) and mt 16S rRNA (Fig. 6b) were localized extramitochondrially. Densities of extramitochondrial mt rRNA in blastomeres of 16-cell stage embryos were estimated by counting the number of gold particles per $20\mu m^2$ of the cytoplasmic area (Fig 6c). The result shows that extramitochondrial 12S and 16S mt rRNAs distribute asymmetrically among blastomeres enriching in the vegetal-most region of the 16-cell embryos, while intramitochondrial mt rRNA distributes evenly among three types of blastomeres (data not shown). The asymmetrical distribution is much clear for mt 16S rRNA than for mt 12S rRNA. Electron micrograms show that extramitochondrial mt rRNA are not associated with cytoplasmic organelles (Fig.6 a and b).

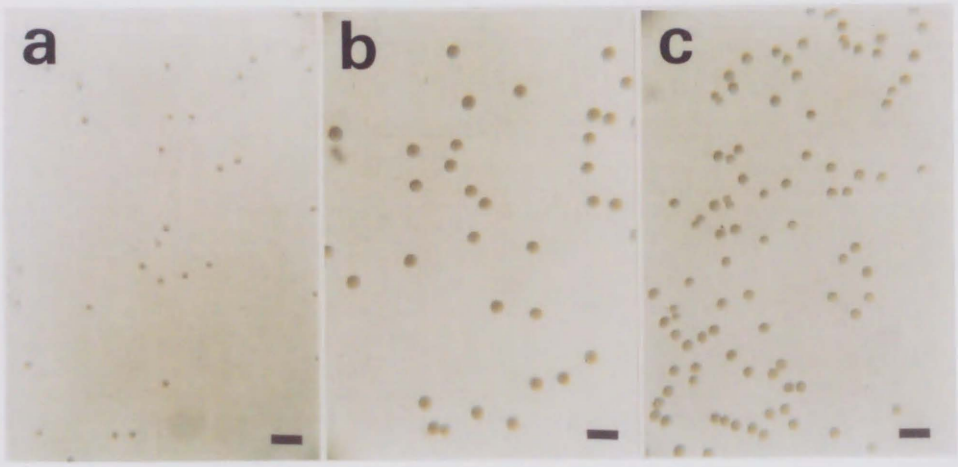

a
b
c

Fig. 1 Blastomeres isolated from 16-cell stage sea urchin embryos by centrifugal elutriation. Purity of micromere (a), macromere (b), mesomere (c) was about 100%, 100%, 84%, respectively. Bars=100μm

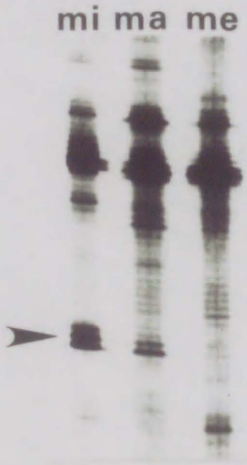

mi ma me

Fig. 2 Differential display of RNA from blastomeres of 16-cell stage embryos. Total RNAs from three types of blastomeres were subjected to differential display analysis using T_{12}MG primer and AP-2 primer as described in MATERIALS AND METHODS. An arrowhead indicates a band corresponding to mt 12S rRNA. mi; micromere, ma; macromere, me; mesomere.

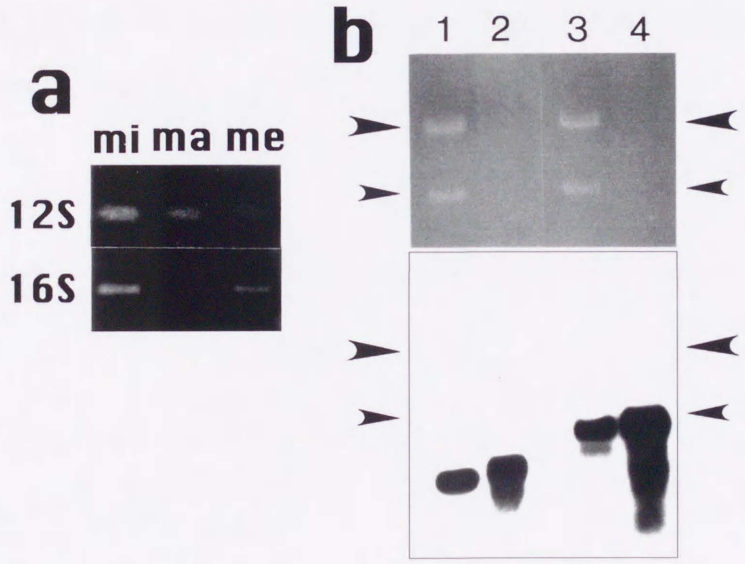

a
mi ma me
12S
16S
b
1 2 3 4

Fig. 3 (a) Quantitative RT-PCR of mt 12S and 16S rRNAs in three different blastomeres. Total RNA from micromere (mi), macromere (ma) and mesomere (me) fractions was used as the template in PCR. Conditions for RT-PCR are described in MATERIALS AND METHODS. 12S; mt 12S rRNA, 16S; mt 16S rRNA.

(b) Enrichment of mt rRNA in poly(A)+RNA fraction of the 16-cell embryos. Procedures for electrophoresis and Northern blotting are described in MATERIALS AND METHODS. Upper panel: RNA staining by ethidium bromide. Lower panel: Northern blotting probed with cDNAs of mt 12S rRNA (lanes 1 and 2) and of mt 16S rRNA (lanes 3 and 4). Total RNA; lanes 1 and 3, poly(A)+RNA; lanes 2 and 4.

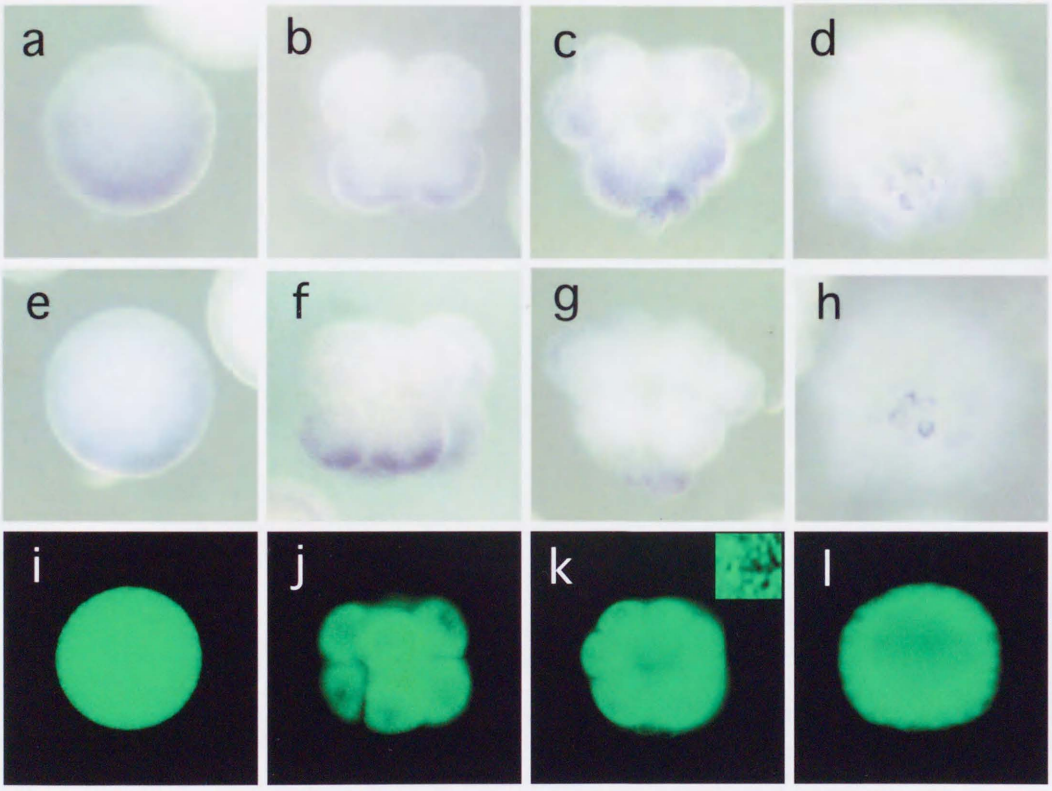
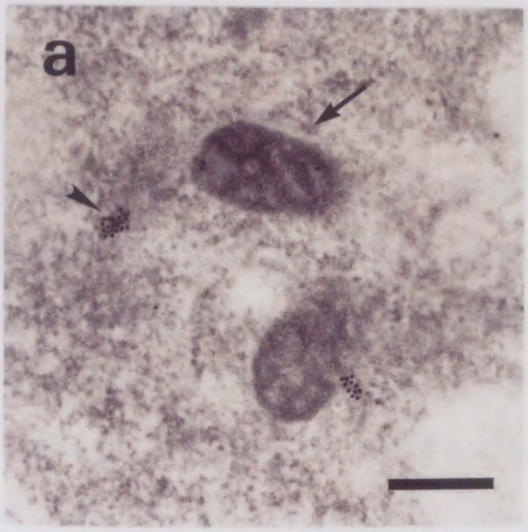
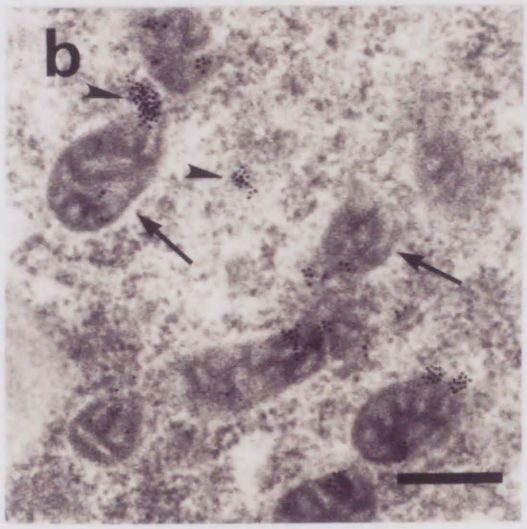
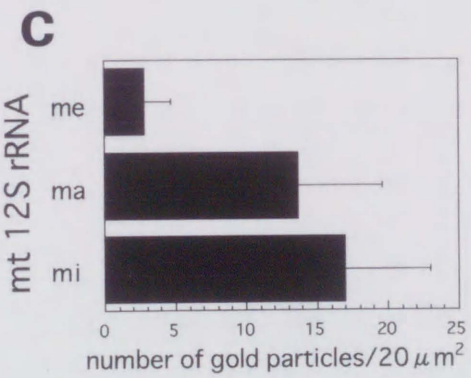

a
b
c
d
e
f
g
h
i
j
k
l

Fig. 4 Whole mount *in situ* hybridization of fertilized eggs and the embryos with mt 12S rRNA (a, b, c, d) and with mt 16S rRNA probes (e, f, g, h). i, j, k and l; stained with $DiOC_2(3)$. a, e and i; fertilized egg, b, f and j; 8-cell stage, c, g and k; 16-cell stage, d, h and l; 60-cell stage. Inset in k; a higher magnification of the surface of embryo shown in k. Bars=50μm


a


b


c
mt 12S rRNA
me
ma
mi
0
5
10
15
20
25
number of gold particles/20 μm²

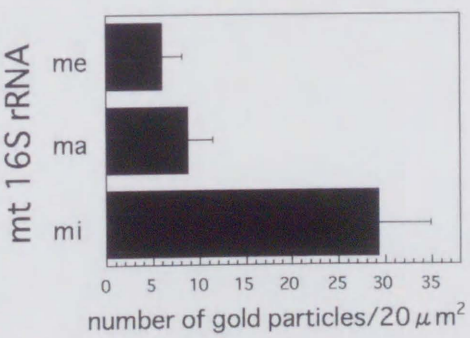

mt 16S rRNA
me
ma
mi
0
5
10
15
20
25
30
35
number of gold particles/20 μm²

Fig. 5 Electron microscopic *in situ* hybridization of mt 12S rRNA (a) and mt 16S rRNA (b). Arrows indicate mitochondria, and arrowheads indicate the signals outside mitochondria. Bars=200nm. (c) Distribution of mt 12S and 16S rRNAs outside mitochondria in three types of blastomeres of the 16-cell stage embryos. The number of gold particles in the total area of 100-180 μm^2 in each blastomeres (1-3 embryos) were counted and the results expressed by the average numbers of gold particles per 20μm^2. Bars indicate the standard error.

Discussion

Mt 12S and 16S rRNAs are known to be transcribed from a mitochondrial genome and become components of ribosomes within mitochondria. However, asymmetrical distribution of mt rRNAs among blastomeres in early embryos does not coincide with distribution of mitochondria themselves that are evenly distributed among blastomeres. The result of electron microscopical *in situ* hybridization shows that a significant fraction of mt rRNAs are localized outside mitochondria (Fig. 6) distributing asymmetrically along a vegetal-animal axis of 16-cell embryos (Fig. 6). Asymmetrical distribution of mt rRNA is also detected by whole mount *in situ* hybridization (Fig. 5), suggesting that the present whole mount *in situ* hybridization protocol detects extramitochondrial mt RNAs alone. This is probably because the riboprobes hardly penetrate inner mitochondrial membranes as reported by Kobayashi *et al.* (1993) and Amikura *et al.* (1996).

In *Drosophila*, mt 16S rRNA has been identified as a factor that induces the germ line progenitors in UV-irradiated embryos (Kobayashi *et al.*, 1989). Ultrastructural analysis reveals that extramitochondrial mt 16S rRNA is tightly associated with germinal granules, distinctive organelles of germ plasm, during a short period from oviposition to pole cell formation (Kobayashi *et al.*, 1993; Amikura *et al.*, 1996). In *Xenopus* embryos, germ plasm is localized in the vegetal pole region of the early embryos and contains germinal granules that are very similar to polar granules in *Drosophila* (Mahowald and Hennen, 1971; Czolowska 1972; Beams and Kessel, 1974; Ikenishi *et al.*, 1974; Eddy, 1975; Ikenishi and Kotani, 1975). The extramitochondrial mt 16S rRNA is present in the germinal granules in

Xenopus embryo from 4-cell to blastula stage (Kobayashi *et al.*, 1994; Kobayashi *et al.*, unpublished). Thus, the extramitochondrial mt 16S rRNA is a common component of germ plasm in *Drosophila* and *Xenopus* (Kobayashi *et al.*, 1994). As in the case of *Xenopus*,, extramitochondrial mt 16S rRNA is enriched predominantly in the vegetal pole region of the early sea urchin embryos (Fig. 5). In addition, the mt rRNA is partitioned into four blastomeres locating in the vegetal-most position of the embryos as development proceeds, while germ plasm is partitioned into four blastomeres by the first 2 cleavage division in *Xenopus*. Although these four blastomeres of *Xenopus* embryos continue to divide 10 more times during the rest of the cleavage mitosis, the number of the germ plasm-bearing cells remains four because germ plasm is included only in one of the two daughter cells (Whitington and Dixon, 1975), and the extramitochondrial mt 16S rRNA distribution coincides with the distribution of germ plasm. A similar situation is observed in sea urchin embryos. Unequal cleavage of four micromeres produces four large micromeres and four small micromeres (Dan, K. 1979, Dan, K. *et al.* 1983, Dan, K. 1987), and mt 16S rRNA enriched in four micromeres is partitioned into four small micromeres with only a trace in fouremaining r large micromeres (Fig. 5 d and h).

Developmental fate of small micromeres has been partly described (Endo, Y, 1966; Pehrson and Cohen, 1986; Amemiya, 1989) and four small micromeres divide once into 8 cells by hatching and remain quiescent at the tip of the invaginating archenteron of gastrulae. In *Strongylocentrotus* plutei, the descendants of small micromeres are incorporated in the coelomic sac (Pehrson and Cohen, 1986) that is known to produce an echinus rudiment which forms adult body. Therefore, it is likely that

extramitochondrial mt rRNA enriched in small micromeres may play an important role for further differentiation of these micromeres. Recently Oka *et al.* found that mt large rRNA localizes in myoplasm of early ascidian embryos, though its localization inside or outside mitochondria is not known (personal communication). It is of interest that rRNA of mitochondrial type plays a role in early embryogenesis of a wide range of animals with different pattern of development.

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Part 2

Sox and other Regulatory elements in the promoter region of the Sea Urchin Arylsulfatase Gene

Abstract

The region between -194 bp and -144 bp from the transcription start site of the sea urchin (*Hemicentrotus pulcherrimus*) arylsulfatase (*HpArs*) gene play a role in an activator of the temporal expression of this gene. Point mutation analysis indicates that the Sox (Sry-related HMG box) binding site in this region is responsible for the transcriptional activity, and that the region by this site is essential for complete activity. Gel mobility shift analysis reveals that the nuclear protein in of sea urchin binds to the Sox binding site , and that the *in vitro* synthesized *Strongyrocentrotus purpratus* Sox (HpSox) protein also binds there. Northern blot analysis shows the *HpSox* gene is maternally expressed, and that HpSox RNA keeps extremely large quantity during embryogenesis. This finding suggest that transcriptional factor, HpSox, is essential for the transcriptional regulation of many gene, and one of them is *HpArs* gene.

We also report that the negative *cis*-regulatory element in the region between -194 bp and -144 bp.

Introduction

The complex body plans of multicellular organisms are completed by cooperation of a great number of cells rising from a single fertilized egg. To differentiate each genetically equal cell appropriately, there is a complex process regulated by successive expression of various genes at appropriate stages and in appropriate embryonic regions. The gene expression systems are controlled by sequential functional cascades of the various transcription factors. In sea urchin embryo, many upstream regulatory cascades have been studied and linked to embryogenesis (see review in Davidson *et al.* 1998).

Expression of *arylsulfatase* gene of the sea urchin (*Hemicentrotus pulcherrimus*) embryo (*HpArs* gene) is also regulated in stage-specific and tissue-specific manners (Sasaki *et al.* 1988; Akasaka *et al.* 1990a, b). Its transcription begins at the blastula stage in whole embryo, and the HpArs mRNA accumulates as development proceeds. Thus *HpArs* gene is activated at hatching in all embryonic cells, while at the mesenchyme blastula stage HpArs mRNA becomes undetectable in the mesenchyme cells and cells in the vegetal plate. By the gastrula stage, accumulation of the HpArs mRNA becomes restricted to the aboral ectoderm.

Several cis- elements and trans-regulatory factors which appear to have a key importance in transcription of the *HpArs* gene have so far been found. Morokuma *et al.* (1997) found that a promoter region of the *HpArs* gene spanning from -100bp to +38bp (HpArs -100 promoter) contains the minimal information for temporal activation of the this gene, and that the region between -194bp and -144bp (AfNV) has a strong activity to stimulate HpArs

-100 promoter. It was shown that a 229bp fragment (C15 fragment) in the first intron of the *HpArs* gene acts as a strong transcriptional enhancer (Iuchi *et al.* 1995). Sakamoto *et al.* (1997) detected that a significant fraction of the enhancer activity is shared by Otx-binding motifs in C15 and identified the two Otx proteins ($HpOtx_E$ and $HpOtx_L$) that bind to this motif. Interaction of $HpOtx_L$ (a late type HpOtx that is expressed at the blastula stage) with Otx-binding motif enhances transcription of the *HpArs* gene (Sakamoto *et al.*, 1997; Kiyama *et al.*, 1998). Koike *et al.* (1998) showed that two regions, PU1 site (-72bp to -56bp), which is similar to SpZ-12 and/or Oct-1 motif, and PD1 site (+133bp to + 142bp), which is homologous to the binding sites of Rel family transcription factors and/or AGIE-BP1, regulate transcription of the *HpArs* gene and the enhancer activity of C15 fragment is mediated via these two sites.

In spite of accumulating information on the regulatory activity of various regions of the *HpArs* gene, more detailed anatomy of the function of promoter region between -194bp and -144bp is needed to understand transcriptional regulation of the *HpArs* gene. In the present study, I attempted to find cis-active elements in this region and to identify proteins that bind to these elements. By gel mobility shift assay and reporter assay, I report here that binding of Sox protein to a Sox consensus motif in this region stimulates the activity of the basal promoter of the *HpArs* gene, and that embryonic content of Sox protein changes ontogenically in accordance with the ontogenical change of the *HpArs* gene expression. Existence of a negative cis-element at near upstream of Sox motif is also reported. This cis-element depresses the activity of the *HpArs* promoter.

Materials and Methods

Embryo culture

Gametes of sea urchin (*H. pulcherrimus*) were obtained by coelomic injection of 0.55M KCl, and fertilized eggs were cultured at 16°C under constant aeration and stirring.

Fusion gene construct

The 5' flanking region of the *HpArs* gene was obtained from a λ recombinant clone isolated from the sea urchin (*H. pulcherrimus*) genomic library (Akasaka *et al.*,1994). The fusion gene pAL(ΔAf) (Morokuma et al, 1997) was constructed by ligating an *Ars* gene fragment spanning from -194bp(*Afl*II site) to +38bp to the upstream of the luciferase gene. The *HpArs* gene enhancer fragment, C15 (a 229bp fragment, Iuchi *et al.*,1995), inserted into the upstream of *Afl*II site of pAL(ΔAf) is referred to as C15-ΔAf.

Site-directed mutagenesis was carried out by the cassette method as described by Koike et al (1997). The pair of single stranded oligo DNA containing mutated AfNV sequences were annealed and phosphorylated by T4 polynucleotide kinase (Cloned T4 Polynucleotide Kinase, TaKaRa Shyzo, Japan). Then the double stranded DNA was ligated into *Afl*II and *Nsp*V site. The details of mutated sequences are described in the legend to Fig.1a.

Gel mobility shift analysis

Preparation of nuclear extracts of sea urchin embryos and gel mobility shift assay were performed as described by Sakamoto *et al.*,(1997). ^{32}P-

labelled DNA probes were prepared as described by Morokuma *et al.* (1997). Approximately 1ng of the labelled probe was mixed with 5μg of the nuclear extract, 1μg of poly(dI-dC):poly(dI-dC) (Pharmacia Biotech, Uppsala, Sweden) and 50ng of *Hin*dIII-digested λDNA, in 10μl of the binding buffer consisting of 20mM Hepes-KOH at pH7.9, 100mM KCl, 5mM $MgCl_2$, and 10mM DTT. After 30min incubation at 23°C, 2 μl of Ficoll-dye was added to the reaction mixture. Electrophoresis was performed in a 4% native polyacrylamide gel in TBE buffer at 13°C, and gel mobility shift bands were visualized by Fujix Bio Imaging Analyzer BAS3000 (Fuji Photo Film, Tokyo, Japan).

Reporter assay

Procedures for introduction of genes into sea urchin fertilized eggs by pneumatic particle gun, and assays for luciferase activities were performed at the prism stage as previously described from our laboratory (Akasaka *et al.* 1995; Morokuma *et al.* 1997; Kiyama *et al.* 1998).

In vitro translation of *Strongylocentrotus purpuratus* Sox (SpSox) cDNA

To generate SpSox protein, SpSox cDNA (a gift from L. Angerer of Rochester University) was subcloned into the pBluescript SK(-) vector (referred as pBSK(-)SpSox). The *in vitro* transcription and translation reactions were carried out in the TNT Coupled Reticulocyte Lysate System (Promega) using 1μg of purified closed circular plasmid as a template. Two μl of the reaction mixture was used in gel mobility shift assay.

Northern hybridization

The total RNA was extracted from *H. pulcherrimus* embryos at various developmental stages as described by Chomcznski and Sacchi (1987). The DIG-labelled antisense RNA probes were synthesized with pBSK(-)SpSox as a template using MEGA script™ T3 kit (AMBION, USA) and digoxigenin-11-2'-deoxyuridine-5'-triphosphate (Boehringer Mannheim) according to the manufacturer's instruction.

After electrophorezing 5μg of the total RNA extracted from embryos, gel bands was transferred to nylon filter (NY 13N NYTRAN: Schleicher & Schuell, Germany), and the membranes were prehybridized for 3hr at 65°C in 5X SSPE solution containing 1% SDS, 5% ICL and 100μg/ml salmon sperm DNA, and then hybridized with the DIG-labelled antisense RNA probe for over night at 65°C. After washing the filters under a high stringency condition, hybridized probes were visualized by using Fab fragments of anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Germany) and CSPD (TROPIX, MA, USA).

Result

Nuclear protein that binds to a Sox consensus motif in AfNV fragment

Previously Morokuma *et al.* (1997) showed that the 50bp fragment (AfNV fragment) between -194bp and -144bp functions as a cis-element to activate the basal promoter of the sea urchin Ars gene (HpArs-100 promotor), and found nuclear proteins that bind to this fragment. To identify the protein-binding site within AfNV fragment, I performed gel mobility shift assay using normal and mutated AfNV fragments. As shown in Fig.2, two closely located but distinct bands that bind to the wild type AfNV fragment were detected. The bands appeared before hatching and their intensities remained constant until the mesenchyme blastula stage, while the intensities of bands clearly decreased at the gastrula stage.

Because the AfNV fragment contains an AACAAAG sequence (a consensus sequence of Sry-type HMG box (Sox) protein binding site) between -168bp and -162bp (Fig.1a), a mutated AfNV fragment (mtSox) was constructed in which an AACAAAG motif was replaced by CCACCCT sequence by site-directed mutagenesis. As indicated by an arrowhead in Fig.2, the Sox-mutated AfNV fragment failed to produce any gel shift band with nuclear proteins from all stages of embryos surveyed. This indicates that it is a Sox-like motif that binds nuclear proteins. This result, together with ontogenic change of Sox-binding proteins, suggests Sox protein is also involved in regulation of transcription of the sea urchin Ars gene.

Other cis-active elements in the close upstream of Sox motif

In addition to a Sox motif, AfNV fragment contains other cis-active elements between -186bp and -172bp (Morokuma *et al.* 1997). In order to locate protein-binding sites in this region, gel mobility shift assay was carried out using three different AfNV fragments (mt-1, mt-2, mt-3) that have sequence relacement at three different sites. A sequence mutated in each fragment is depicted in Fig.1a. Fig.3 shows the result of gel mobility shift assays for nuclear extracts from crude mesenchyme blastulae using mutated AfNV fragments as the probe. While the shift band for Sox motif is clearly detectable with WT and mt-1 probes, it disappeared with mt-2 and mt-3 probes. This result suggessts that the nucleotide sequence close to the Sox motif positively cooperate for the nuclear proteins to bind to the Sox motif.

Sox protein really binds to the Sox motif in AfNV fragment

To confirm if the nuclear protein that binds to the Sox consensus motif in AfMNV fragment is really a Sox protein, the protein was synthesized in an in vitro translation system using the Sox (SpSox) cDNA of the other sea urchin species,*Strongylocentrotus purpuratus*, and applied to gel mobility shift assay with the AfNV fragment as a probe. In this experiment the SpSox4B cDNA was subcloned into pBluescript SK(-) vector at *Eco*RI site (pBSK(-) SpSox4B) so that its transcription by T7 RNA polymerase produces SpSox4B mRNA (Fig.1b). As shown in Fig.4, the SpSox4B protein generated in vitro produced two distinct byt closely located bands (indicated by an arrowhead) with the WT probe (lane3), while any significant band (lane 4) was not detected at these positions using sense mt-2 probe. Though in

vitro transcription of pBSK-SpSox4B using T3 polymerase generates nonsense RNA that produces no protein in an in vitro translation system, translation products of this nonsense system was used as a control for gel mobility shift assay. In this control, any specific gel shift bands except those produced by components of transcription and translation ststem were not observed (lane 5 and 6). This result indicate that it is a Sox protein that binds sequence-specifically to the Sox consensus motif in AfNV fragment.

Ontogenic change of Sox transcripts

To know the ontogenic change of Sox transcript in H. pulcherrimus, total RNA from embryos at various stages was northern blotted using a 2437bp fragment of SpSox cDNA as the probe (Fig. 5). Sox RNA of approx 4kb in size is detected abundantly from unfertilized eggs to pluteus larvae, suggesting that it is maternally expressed and then zygotically generated during development.

Sox motif is a positive cis-element for *HpArs* gene transcription

Reporter assay was attempted to study if the Sox-binding site in AfNV fragment functions as cis-element to regulate transcription of the Ars gene. For this purpose, fusion constructs were generated by ligating AfNV fragment at the 5' end of luciferase gene. In some fusion constructs sequence replacement was introduced at mt-1, mt-2, mt-3 sites and Sox motif (mtSox) of AfNV fragment (Fig. 6a). The fusion constructs were introduced into fertilized eggs of *H. pulcherrmus* by particle gun mediated gene transfer system and the eggs were developed up to prism stage. The

activity of luciferase was quantitated using the prism extracts. Fig.6a shows the result of reporter expression experiments.

Replacement of Sox sequence AACAAAG with the sequence CCACCCT (mtSox) resulted in a marked decrease in reporter expression. The level of expression of Sox mutated construct (mtSox) was only one sixth of the wild type construct and nearly equal to the one that lacks the sequence upstyream from -144.

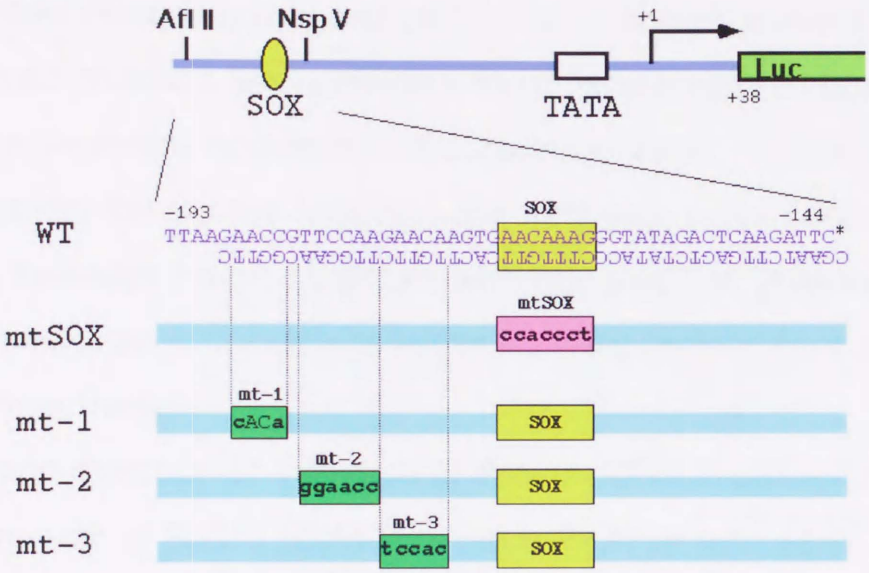
Cis-elements in the close upstream of Sox motif

Introduction of sequence replacement at mt-3 site clearly decreased the reporter gene expression, while that at mt-2 had little influence on expression of the reporter gene. In constrast to these two sites, mutation in mt-1 site remarkably increased the reporter expression. Thus it seems that mt-1 site functions as negative cis-element that depresses expression of the *HpArs* gene. Since the mutation in mt-1 has no influence on binding of Sox protein to downstream Sox consensus motif, it is assumed that mt-1 site is a new cis-element that depresses transcription of the Ars gene independently of the Sox motif. Because mutation in the mt-3 site, that is needed for binding of Sox protein to the Sox consensus motif (Fig.3), produced significant decrease in repoter expression, it is expected that the mt-3 site may constitute a part of Sox consensus motif. Though mt-2 site is also related to increase binding of Sox protein to the Sox motif, its role in regulation of the *HpArs* gene expression is not clear since the present result suggests that this site itself does not function as a cis-active element for the *HpArs* gene expression.

Activity of C15 enhancer is mediated via Sox motif

It is known that the enhancer activity of C15 fragment is mediated via a 17bp element between -72bp and -56bp and a 10bp element between +133bp and +142bp (Koike *et al*, 1998). A similar relationship between regulatory domains of SM50 (Yuh *et al.* 1998) has also reported. Considering these reports, the functional relationship between the C15 enhancer and Sox motif was surveyed by reporter assay using C15-ΔAf (see Materials and Methods) in which a 229bp C15 fragment was ligated into upstream of AfNv fragment of the reporter construct. As shown in Fig. 6b, C15 greatly increased the reporter expressio, while introduction of sequence replacement in the Sox motif in AfNV fragment downstream to C15 caused 50% decrease of the reporter expression. This result supports that the enhancer activity of C15 is mediated via Sox motif, because the extent of reduction of the reporter expression is much greater than that expected by assuming that C15 directly stimulates transcription of the Ars gene.

a


Afl II
Nsp V
SOX
TATA
+1
+38
Luc
-193
-144
SOX
WT
TTAAGAACCGTTCCAAGAACAAGTCAACAAAGGGTATAGACTCAAGATTC*
CGAATCTTGAGTCTATACCCTTTGTTCACTTGTTCCTTGGAACGTTG
mtSOX
mtSOX
ccaccct
mt-1
cACa
SOX
mt-2
ggaacc
SOX
mt-3
tccac
SOX
mtSOX
mt-1
mt-2
mt-3

b

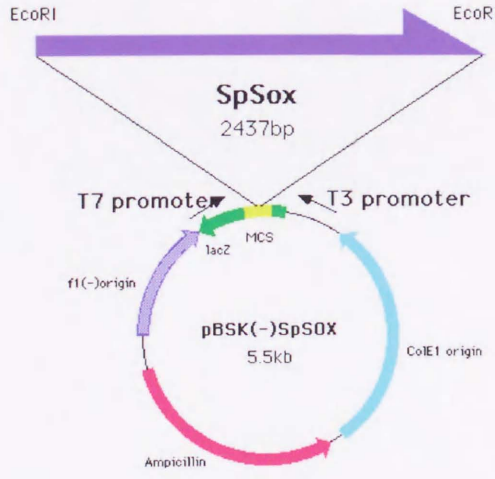

EcoRI
EcoRI
SpSox
2437bp
T7 promoter
T3 promoter
lacZ
MCS
f1(-)origin
pBSK(-)SpSOX
5.5kb
ColE1 origin
Ampicillin

Fig.1

(a) Schematic presentation of the Ars promoter-Luciferase fusion constructs.

Transcription start site with direction of transcription shown by a bent arrow. Yellow colored boxes and ellipse; Sox consensus motif, pink colored box; sequence-mutated Sox consensus motif, green colored boxes; site of sequence replacement upstream Sox consensus motif. Mutated nucleotides are indicated by lower case letters. Sox indicates a Sox consensus motif, and mtSox indicates fusion construct with mutated Sox sequence. mt-1, mt-2 and mt-3; fusion constructs with sequence replacement at mt-1, 2 and 3 sites, respectively.

(b) A site and direction of insertion of SpSOX cDNA in pBluescript SK(-) vector. The size of SpSox cDNA inserted is 2437bp with an 867bp ORF.

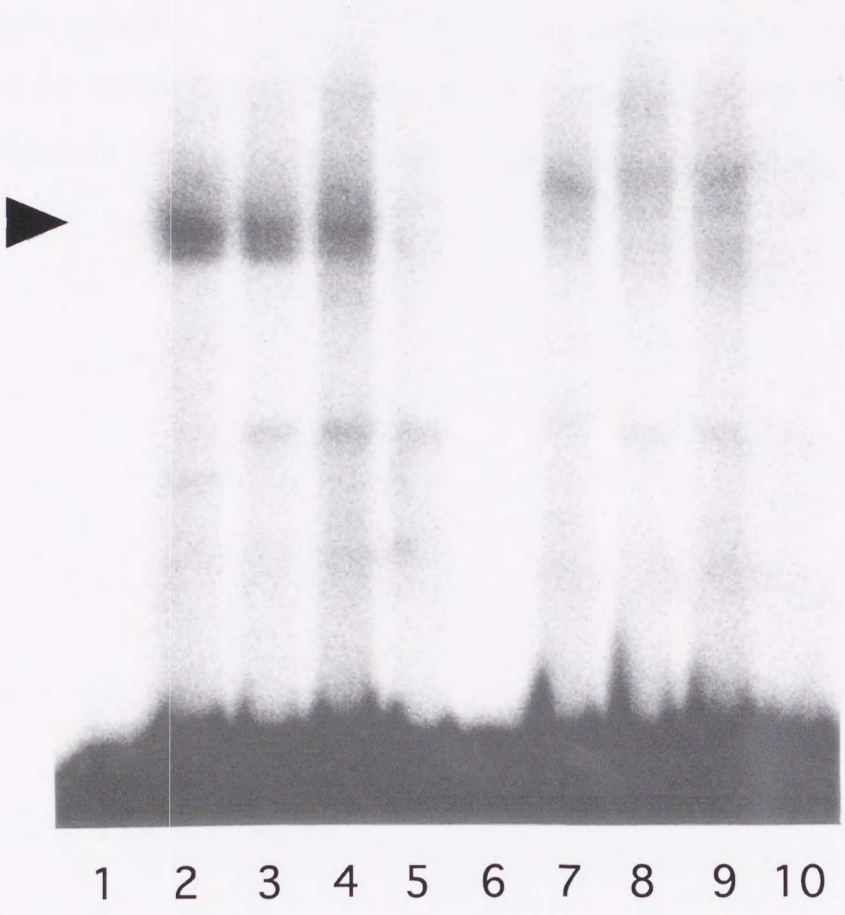

1 2 3 4 5 6 7 8 9 10

Fig.2

Ontogenic change of nuclear proteins that bind to Sox motif. Binding of nuclear proteins from embryos at various stages to Sox motif in AfNV fragment was detected by gel mobility shift assay. Lanes 1 to 5; probed with wild type AfNV fragment, lanes 6 to 10; probed with AfNV fragment in which Sox motif was replaced with nonsense sequence. Lanes1 and 6; without nuclear extracts, lanes 2 and 7; unhatched blastula, lanes 3 and 8; hatched blastula, lanes 4 and 9; mesenchyme blastula, lanes 5 and 10; gastrula. Possible Sox complex is indicated by an arrowhead.

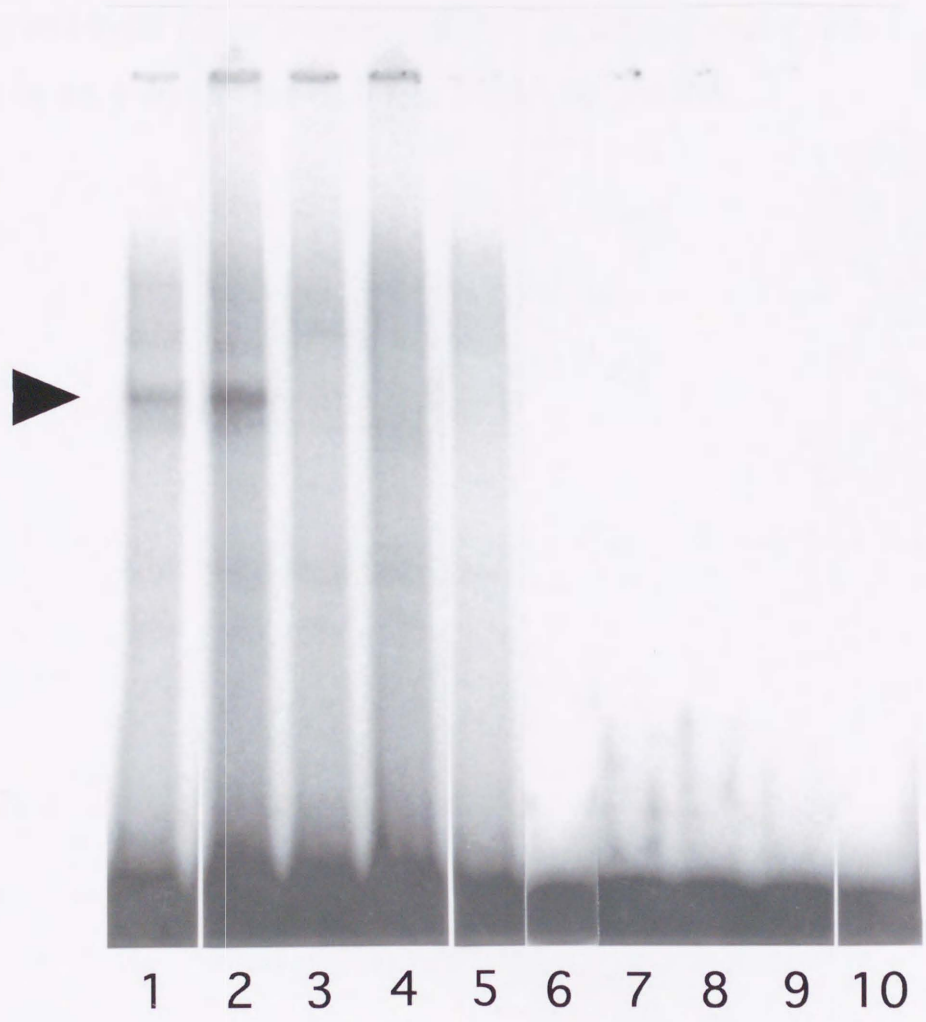

1 2 3 4 5 6 7 8 9 10

Fig.3

Effect of sequence replacement in the close upstream of the Sox consensus motif on binding of nuclear proteins. Lanes 1 to 4; gel mobility shift assay in the presence of nuclear extract from mesenchyme blastulae (MB), and lanes 6 to 10 in the absence of MB nuclear extract. Probe DNA used in lanes 1 and 6; wild type AfNV fragment (WT), in lanes 2 and 7; mt-1, lanes 3 and 8, mt-2; lanes 4 and 9; mt-3, lanes 5 and 10; mtSOX.

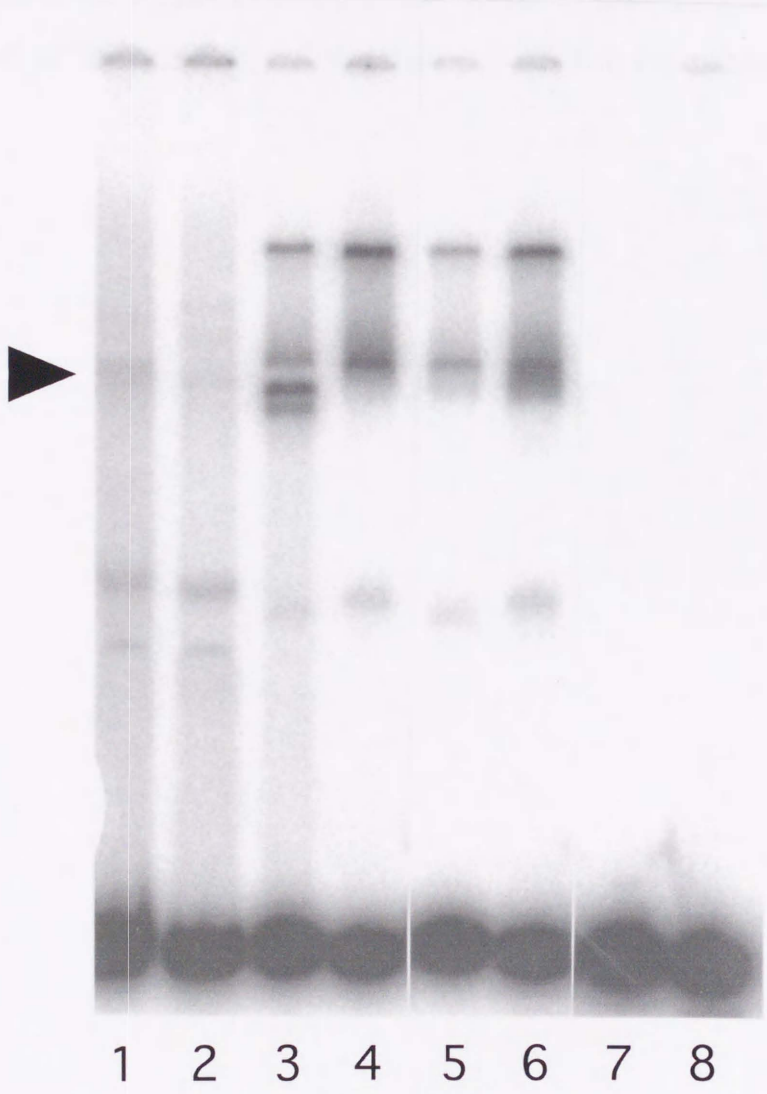

1 2 3 4 5 6 7 8

Fig.4

Gel mobility shift assay of *In vitro* synthesized SpSox protein. DNA probe in lanes 1, 3, 5and 7; wild type AfNV fragment (WT), lanes 2, 4, 6and 8; mtSOX. Lanes 1 and 2; nuclear extract of mesenchyme blastulae, lanes 3 and 4; Sox protein translated from sense Sox mRNA, lanes 5 and 6; Sox protein translated from antisense Sox mRNA, lanes 7 and 8; no protein.

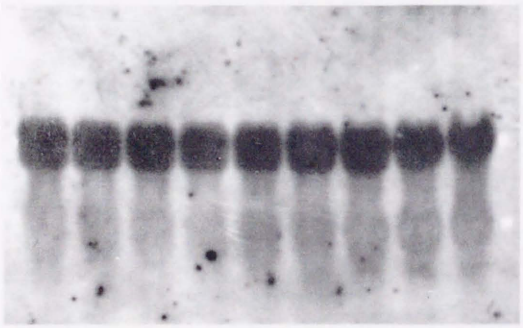
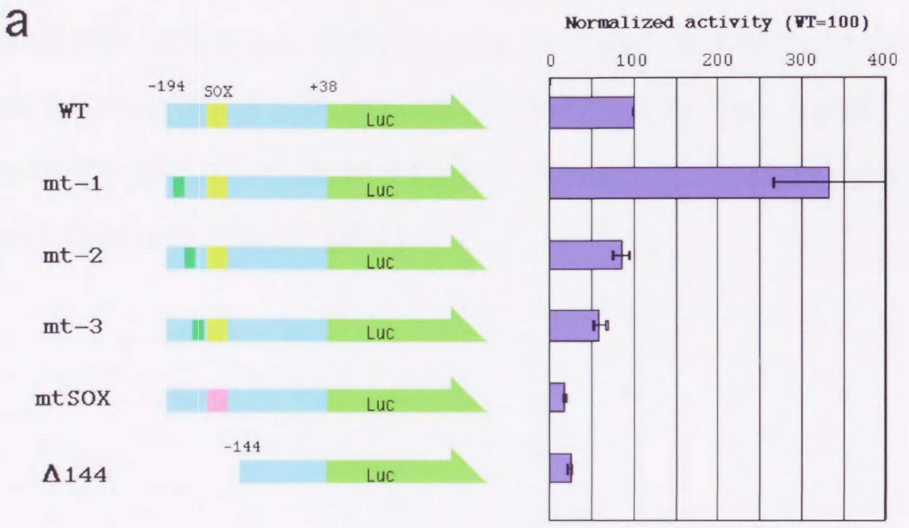

1 2 3 4 5 6 7 8 9

Fig.5

Ontogenic change of accumulation of HpSox mRNA as realed by Northern blotting with a 2437bp flagment of SpSox cDNA as a probe. Five μg of total RNA extracted from *H. pulcherrimus* embryos at various developmental stages was electrophorezed. Lane 1; unfertilized egg, lane 2; 16-cell, lane 3; molula, lane 4; unhatched blastula, lane 5; hatched blastula, lane 6; mesenchyme blastula, lane 7; gastrula, lane 8; prism, amd lane 9; pluteus larvae. Arrowheads indicate the position of 26S and 18S rRNA.


a
Normalized activity (WT=100)
0
100
200
300
400
-194
SOX
+38
WT
Luc
mt-1
Luc
mt-2
Luc
mt-3
Luc
mtSOX
Luc
-144
Δ144
Luc

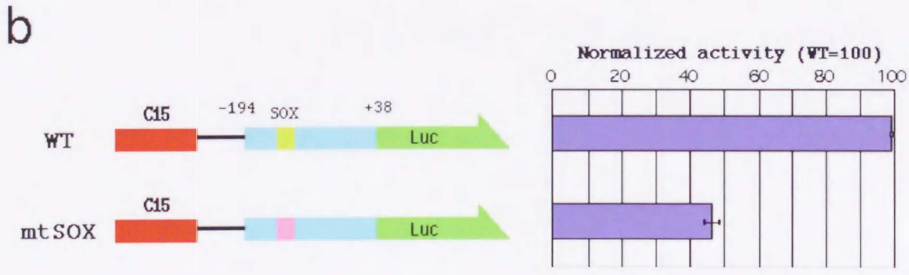

b
Normalized activity (WT=100)
0
20
40
60
80
100
C15
-194
SOX
+38
WT
Luc
C15
mtSOX
Luc

Fig.6

(a) Effect of seuence replacements in the promoter region (AfNV fragment) on expression of the fusion constructs. Firefly luciferase gene was used as a reporter of the *HpArs* gene expression. Activity of expressed luciferase was normalized to that of wild type fusion construct (uppermost panel; WT). Fusion constructs were transferred to fertilized eggs by a particle gun procedure and the luciferase activity was assayed at the prism stage.

(b) Enhancer activity of C15 fragment is mediated by Sox motif. The luciferase activity of Sox-mutated fusion construct (mtSox) is normalized to that of wild type construct (WT).

Discussion

The Ars gene of the sea urchin, *Hemicentrotus pulcherrimus*, is expressed in temporally as well as spatially regulated manners during development up to pluteus larvae. So far, several cis-elements and some transcription factors that bind to these cis-elements have been elucidated. A 229bp element (C15) in the first intron of this gene shows a strong enhancer activity, and a significant fraction of this activity is ascribed to Otx-binding sites and the nearby sequences including a CAAT motif. Two distinct HpOtx cDNAs that are generated from a single HpOtx gene by alternative splicing were cloned from cDNA library of *H. pulcherrimus* embryos. The promoter region of the *HpArs* gene was also studied and existence of several cis-active elements in this region was reported. Very recently Morokuma *et al* found a strong positive regulatory element between -186bp and -164bp. A Sox consensus motif spanning from -168bp to -162bp found in this study is another cis-positive element and nuclear protein was identified that binds to this motif by gel mobility shift assay. Though Sox protein used in the gel shift assay to confirm that it is a Sox protein that binds to the Sox consensus motif in AfNV fragment is derived from cDNA for Sox protein of other sea urchin species *Strongylocentrotus purpuratus*, detection of only a single band of 4kb in Northern blotting using SpSox cDNA as the probe and similarity of gel shift bands between nuclear protein pof *H. pulcherrimus* and *in vitro* synthesized SpSox protein provide sufficient rationals for the use of SpSox cDNA

Disappearance or marked decrease of gel shift bands in the assay using AfNV fragment with its Sox motif mutated also supports the existence of

Sox protein in nuclei of mesenchyme blastulae. As indicated in gel mibility shift assay in Fig. 2 and in Norther blotting in Fig. 5, Sox protein as well as Sox mRNA are of maternal origin, and embryonic contents of them remain unchanged up to mesenchyme blastulae but rapidly decline at gastrulation, These ontogenic change of Sox protein agrees well with the ontogenic change of expression of the *HpArs* gene (Sasaki *et al.* 1988), indicating that Sox protein plays an important role in temporally regulated expression of the *HpArs* gene.

Loss or marked decrease in gel shift bands after introduction of sequence replacements in mt-2 and mt-3 sites located upstream to Sox motif (Fig. 3) and marked decrease in the reporter expression of the fusion constructs containing sequence replacement in mt-3 site (Fig. 6a) suggest that mt-3 site itself is a cis-active element that facilitate binding of Sox protein to the Sox site. Since mt-2 site shows no cis-regulatory activity while its mutation has negative effect on binding of the Sox protein to the Sox site, it is likelt that mt-2 site may be considered to be a part of mt-3 site. A similar cooperation between two closely located cis-elements is also observed between Otx motif and Otx-like motif in C15 enhancer of the *HpArs* gene. In this case binding of $HpOtx_L$ protein to the Otx site is greatly reduced by deleting the nearby Otx-like motif (Sakamoto *et al*, 1997). The result of the reporter assay using fusion constructs containing C15 enhancer fragment in the upstream of AfNV fragment in Fig.6b suggests that the enhancer activity of C15 is mediated via Sox protein bound to the Sox motif, since reduction of reporter expression in Sox mutated fusion construct was much lower than that expected from the assumption that two elements, C15 and Sox, function independently to stimulate the transcription.

In *S. purpuratus* embryo, whole-mount *in situ* hybridization shows that SpSox mRNA is distributed in all embryonic cells up to the blastula stage, and as development proceeds, signals disappear from vegetal plate cells and their descendants (Kozlowski *et al.*, personal communication). This result suggests a possibility that the Sox protein plays a highly important role in regulation of other genes than the *HpArs* gene during sea urchin embryogenesis. Co-operated function of Sox and nearby motif is repeatedly reported in mammalian cells. It is thus known that Sox proteins alone cannot activate transcription and partner factors are required which bind to the nearby site within the same enhancer region (Kamachi and Kondoh 1993; Kamachi *et al.* 1995; Yuan *et al.* 1995; Lefevbre *et al.* 1997).

When sequence replacement was introduced in the mt-1 site located between -188bp and -185bp, transcription of the fusion construct was increased to approx 3 fold over that of wild type construct (Fig. 6a). This result suggests that the mt-1 site functions as a cis-element to depress transcription of the *HpArs* gene, though any known sequence motif is not detected by a computer search.

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