# Ars insulator protects transgenes from long-term silencing in sea urchin larva 

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#### Abstract

Reporter genes have been used as a powerful tool to analyze cis-regulatory elements responsible for temporal and spatial expression in the early development of sea urchin. However, here we show that the transgenes introduced into the sea urchin embryos undergo suppression in larval stage. The transgene silencing could be one of major obstacle in the analysis of regulatory regions in the late stages of development. We previously demonstrated that a DNA fragment (ArsI) located in the upstream region of sea urchin (Hemicentrotus pulcherrimus) arylsulfatase gene has the property of an insulator. We show that tandem ArsI prevents silencing of a transgene in sea urchin larvae when the ArsI is fused to the $5^{\prime}$ end of the reporter gene. Furthermore, we demonstrate that DNA of the reporter gene introduced into the sea urchin eggs is methylated during development and that the ArsI protects the transgene from the DNA methylation.


Keywords Instulator • Methylation • Transgene -
Sea urchin - Arylsulfatase

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## Introduction

The reporter genes have been used as a powerful tool to analyze cis-regulatory elements responsible for temporal and spatial expression. The exogenous reporter gene introduced into the fertilized sea urchin eggs is stably integrated into chromosomal DNA and the transgenes are expressed during the early development under the control of the cis-regulatory elements (Flytzanis et al. 1985). However, in this study, we show that the transgene gradually undergoes suppression after the early pluteus stage, and eventually, its expression is reduced to background levels by the late pluteus stage. Transgene silencing is one of major obstacles in the analysis of cis-regulatory elements in late larvae. Although the mechanism of the silencing remains to be elucidated, accumulating evidence suggests that epigenetic alterations of the transgene such as DNA methylation is a likely component (Nishino et al. 2006).

A chromatin insulator is a DNA sequence that serves as a boundary element between differentially regulated genes. The insulator has two conserved properties, one is an enhancer-blocking activity and the other is protection from a position effect (Burgess-Beusse et al. 2002). We previously demonstrated that the 537-bp fragment (ArsI) of sea urchin (Hemicentrotus pulcherrimus) arylsulfatase has typical features of an insulator such as the blocking of enhancer/promoter interactions in sea urchin embryos (Akasaka et al. 1999) and the antisilencing activity in mammalian culture cells (Akasaka et al. 1999; Hino et al. 2004; Tajima et al. 2006). Using mouse embryonic culture system, we also showed that the presence of ArsI in flanking regions of reporter genes increased the number of the transgene-expressing blastocysts and the proportion of blastocysts with high-level expression (Takada et al. 2000). To demonstrate the antisilencing activity of ArsI in vivo
using developing embryos, we introduced reporter genes with or without ArsI into sea urchin embryos and then determined the expression of the exogenous genes in different developmental stages. We used SM50 basal promoter, which is extensively analyzed (Makabe et al. 1995), to drive the reporter genes.

SM50-GFP is expressed under the control of the SM50 promoter from the blastula to the late gastrula stage; however, the expression is gradually suppressed after early pluteus stage, eventually almost no expression of the GFP is detected by the six-armed pluteus stage ( 10 days after fertilization). We show that ArsI has an ability to prevent silencing and DNA methylation of the transgene in sea urchin larvae. We suggest that the ArsI will be a useful tool to ensure stable expression of a transgene in developing larvae and that the quick silencing of transgene in sea urchin larvae offers the advantage of screening for novel insulator fragments.

## Materials and methods

Culture of embryos and larvae
H. pulcherrimus were collected from the seashore of Miura and Boso Peninsula, Japan. Gametes were obtained by coelomic injection of 10 mM acetylcholine chloride. Eggs were washed several times with Millipore filtered seawater (SW). Dry sperm were kept at $4^{\circ} \mathrm{C}$ until use. Eggs were fertilized and washed several times in SW containing penicillin $(50 \mathrm{mg} / \mathrm{ml})$ and streptomycin $(25 \mathrm{mg} / \mathrm{ml})$ and then cultured at $19^{\circ} \mathrm{C}$. From the second day after fertilization, Chatetoceros gracilis and Chatetoceros calcitrans were supplied to the cultures twice a week. Larvae developed into six-armed pluteus in 10 days after fertilization. The developmental process of sea urchin larva is illustrated in Fig. 1a.

Reverse transcriptase polymerase chain reaction

Total RNA was isolated from embryos or larvae using Trizol Reagent (Invitrogen). Complementary DNAs were synthesized with 800 ng of the total RNA as the template using SuperScript III RNaseH-Reverse Transcriptase (Invitrogen) and random 9-mer oligonucleotides. Reverse transcriptase polymerase chain reaction (RT-PCR) amplification was performed with EX Taq (Takara). All comparisons were performed in the linear range of amplification. PCR products were separated on agarose gel, and the DNA bands were stained with ethidium bromide.

## Construction of a reporter gene

We used the SM50 basal promoter ( -437 to +126 ), which is well characterized (Makabe et al. 1995), to drive the

a

## SM50-LUC <br> SM50-GFP

SM50 LUC/GFP

Arsl-SM50-LUC
Arsl-SM50-GFP


Arsl-Arsl-SM50-LUC
Arsl-Arsl-SM50-GFP

## ArsI Arsl SM50 LUC/GFP



## c

Fig. 1 Skeletogenesis and SM50 expression in H. pulcherrimus development and the design of constructs. a Skeletogenic cells and spicules are drawn in gray in $L G, 4$ plu, and $6 p l u$. b The schematic views of reporter constructs. c Expression of SM50 during $H$. pulcherrimus development. RT-PCR was performed as described in "Materials and methods." The PCR products were separated on an agarose gel, and the DNA bands were stained with ethidium bromide. FE, fertilized eggs; HB, hatched blastula; LG, late gastrula; 4plu, fourarmed pluteus; $6 p l u$, six-armed pluteus
reporter genes. SM50-GFP (green fluorescent protein) and SM50-LUC (luciferase) with or without the Ars insulator was constructed from Green Lantern-1 (GIBCO BRL) and pGL2-Basic (Promega), respectively. The SM50 basal promoter was inserted into the multicloning site in the plasmids (Fig. 1b, SM50-GFP/LUC). It has been reported that ArsI functions in an orientation-dependent manner and that the ArsI inserted in the - orientation (ArsI-) is more effective than that in the + orientation (ArsI + ) in preventing transgene from silencing in mammalian culture cells (Hino et al. 2004; Tajima et al. 2006). Furthermore, there is a report that a transgene expression is effectively protected from silencing by the surrounding two units of ArsI(Tajima et al. 2006). Thus, we fused ArsI- or two copies of ArsI- in tandem to the $5^{\prime}$ end of the SM50 promoter (Fig. 1b, ArsI-SM50-GFP/LUC, ArsI-ArsI-SM50-GFP/ LUC).

## DNA injection

The reporter constructs were linearized at the $5^{\prime}$ end of the SM50 basal promoter (SM50-GFP) or ArsI (ArsI-SM50$G F P$, ArsI-ArsI-SM50-GFP) with appropriate restriction enzymes. It has been reported that carrier DNA accelerates the integration of reporter constructs (Hough-Evans et al. 1988). We mixed $H$. pulcherrimus genome DNA fragments bearing compatible sticky ends with the linearized reporter constructs at a mass ratio of $5: 1$ as we reported before (Akasaka et al. 1995). The DNA fragments were dissolved in $40 \%$ glycerol at a concentration of $3 \mathrm{pg} / \mathrm{pl}$, and 2 pl of the solution was injected into each fertilized egg. The number of larvae expressing GFP was counted at the late gastrula, four-armed pluteus, and six-armed pluteus stages. Through all the developmental stage, the number of GFPexpressing larvae per total number of injected and surviving larvae were counted live by fluorescence microscopy (TE300, Nikon, Japan), and the larvae were cultured for observation at later stages.

## Luciferase assays

Introduction of the DNA constructs and the luciferase assays were carried out according to the method described by Akasaka et al. (1995) with slight modification. To normalize the luciferase activity, pRL-CMV (Promega) was cointroduced as a reference construct, and the expression of the constructs were determined by the Dual-LuciferaseTM Reporter System (Promega) as described in the instruction manual. The firefly luciferase activity driven by activation of SM50 promoter was normalized based on the activity of Renilla luciferase. There were $6 \times 10^{4}$ embryos/larvae collected and used for the luciferase assay.

## DNA methylation analysis

DNA was extracted with Qia Amp Kit (Qiagen, Germany) from $10^{5}$ embryos/larvae introduced with SM50-LUC with or without ArsI by the particle gun method. Three microgram of the DNA in $100 \mu \mathrm{l}$ of reaction mixture was digested completely with $M s p \mathrm{I}$ or HapII at a concentration of $300 \mathrm{U} / \mathrm{ml}$ at $37^{\circ} \mathrm{C}$ for 12 h . To estimate the amount of the $L U C$ fragment in the digested DNA specimen, semiquantitative PCR was performed. The primers were designed according to the sequence of the coding region of the reporter luciferase gene (Fig. 3b). The PCR primer sequences are as follows:

5' luciferase sense, 5'AAAGGCCCGGCGCCATTC TATCCTCTAGA3', and antisense, 5'GGACTTTCC GCCCTTCTTGGCCTTTATGA3'. One hundred nanograms of the digested or nondigested DNA was used for PCR.

The amplification reactions were as follows: 25 cycles of denaturation at $94^{\circ} \mathrm{C}$ for 30 s , annealing at $65^{\circ} \mathrm{C}$ for 30 s , extension at $72^{\circ} \mathrm{C}$ for 1 min . PCR products were separated on agarose gel, and the DNA bands were stained with ethidium bromide.

## Results and discussion

Ars insulator prevents silencing of transgenes in sea urchin larva

SM50 is exclusively expressed in the skeletogenic primary mesenchyme cells from blastula through to pluteus stage (Killian and Wilt 1989). The expression is also observed in adult spines and tube feet (Richardson and Benson 1989). We confirmed that SM50 is expressed during development of $H$. pulcherrimus until at least the six-armed pluteus stage by RT-PCR (Fig. 1c).


Fig. 2 Expression of GFP in embryos/larvae injected with reporter construct with or without ArsI. GFP is exclusively expressed in primary mesenchyme cells and spicules in embryos/larvae introduced with SM50-GFP. a Late gastrula introduced with ArsI-SM50-GFP. Bar $=50 \mu \mathrm{~m}$. b four-armed pluteus larva introduced with Ars-SM50$G F P$. Bar $=50 \mu \mathrm{~m}$. c Percentage of GFP-positive embryos/larvae introduced with SM50-GFP with or without ArsI. ArsI apparently suppresses the silencing of the reporter gene in the late larval stage. $L G$, late gastrula; $4 p l u$, four-armed pluteus; $6 p l u$, six-armed pluteus; $n$, number of embryos examined

SM50-GFP introduced into $H$. pulcherrimus eggs is exclusively expressed in skeletogenic primary mesenchyme cells during development (Fig. 2a,b). Approximately 90\% of embryos introduced with SM50-GFP constructs were GFP-positive regardless of the presence or absence of ArsI. In four-armed plutei, the percentage of GFP-expressing larvae decreased significantly to $30 \%$ in the SM50-GFP larvae (Fig. 2c). Approximately 50\% of larvae introduced with ArsI-SM50-GFP continued to express GFP. Decrease in the fraction of GFP-positive larvae introduced with ArsI-ArsI-SM50-GFP was not observed until the four-armed pluteus stage and most of the SM50-GFP larvae ceased their expression by the six-armed pluteus stage. However, a significant portion of larvae introduced with ArsI-SM50GFP (30\%) and ArsI-ArsI-SM50-GFP (55\%) expressed GFP although the number of GFP-expressing larvae decreased to some extent (Fig. 2c). It seems that ArsI suppresses the silencing of this transgene in a copy-number-dependent manner. The number of embryos exam-

Table 1 Effect of ArsI on spatial expression of SM50-GFP fusion gene

| Construct name | PMC-specific expression (\%) | Total no. $^{2}$ |
| :--- | :---: | :---: |
| SM50-GFP | 95 | 159 |
| ArsI-SM50-GFP | 98 | 134 |
| ArsI-ArsI-SM50-GFP | 97 | 216 |

${ }^{1}$ Percentage of embryos expressing $G F P$ exclusively in primary mesenchyme cells at late gastrula stage
${ }^{2}$ Scored embryos expressing GFP
ined is as follows ( $n=248$, ArsI-ArsI-SM50-GFP; $n=161$, ArsI-SM50-GFP; $n=223$, SM50-GFP). ArsI did not affect the specificity of the spatial expression in primary mesenchyme cells (Table 1).

A promoter/reporter cassette surrounded by insulators is expected to be isolated from the local chromosomal environment and therefore to be protected from positional effects. Watanabe et al. (2006) demonstrated that a transgene fused

Fig. 3 ArsI protects a transgene from silencing and DNA methylation in sea urchin larvae. a Expression of LUC in embryos/ larvae introduced with reporter construct with or without ArsI. Bars indicate standard errors of nine independent experiments. b MspI/HapII recognition sites in $L U C$ reporter gene. The numbers indicate the position of MspI/HapII recognition sites from the $5^{\prime}$ end of $L U C$. The primer binding sites for detecting $L U C$ fragments by PCR are indicated as hatched boxes. $F$ primer, forward primer; $R$ prim$e r$, reverse primer. c Analysis of DNA methylation of transgene using methylation sensitive HapII. The $L U C$ fragment was detected by PCR after digestion of the restriction enzymes. $M$, size marker; $N$, PCR products from nondigested DNA. The embryos/larvae were introduced with SM50-LUC with two copies of ArsI in tandem (ArsI×2), SM50-LUC with a single copy of ArsI (ArsI), and SM50-LUC alone (dash)

with ArsI to the both flanking ends is highly expressed in NIH3T3 in comparison with the transgene lacking the ArsI. We fused ArsI to the $5^{\prime}$ end of the promoter of the reporter constructs. Although the plasmid constructs do not contain ArsI on the $3^{\prime}$ end, the exogenous genes introduced into the sea urchin embryo concatenate and become incorporated into the chromosome in a directionindependent manner (Hough-Evans et al. 1988), and thus, the reporter constructs are expected to be bordered with the insulators.

ArsI retains the expression of transgenes in later developmental stages

To determine the expression level of the transgenes quantitatively, we used $L U C$ as a reporter. SM50-LUC constructs with or without ArsI were introduced into fertilized sea urchin eggs using a particle gun. The luciferase expression was assayed at late gastrula and four-armed pluteus stages. The expression of $L U C$ was higher in the embryos/larvae introduced with SM50-LUC bearing ArsI than that introduced with SM50-LUC alone in both stages examined (Fig. 3a). It has been demonstrated that ArsI does not enhance the promoter activity of transgenes (Akasaka et al. 1995; Nagaya et al. 2001; Hino et al. 2004) in different systems as well as in sea urchin embryos. It is likely that the ArsI prevents silencing of transgenes during development. The luciferase activity decreased as development proceeded in all constructs examined; however, tandem ArsI apparently prevented the silencing. Two copies of ArsI in tandem are more effective to suppress the silencing than a single copy of ArsI.

ArsI reduces DNA methylation level of transgene in developing sea urchin larvae

DNA methylation is one of the key mediators of transgene silencing (Pikaart et al. 1998). To determine whether the DNA methylation of the $L U C$ is prevented by ArsI in sea urchin larva, the methylation level was estimated by monitoring sensitivity of the reporter gene to $M s p \mathrm{I}$ and HapII. Cleavage of DNA by the restriction endonuclease HapII is prevented by the presence of a 5-methyl group at the internal C residue of its recognition sequence CCGG , whereas MspI (isoschizomer of HapII) cleaves DNA irrespective of the presence of a methyl group at this position. DNA extracted from the embryos/larvae, which were introduced with SM50-LUC, ArsI-SM50-LUC or ArsI-ArsI-SM50-LUC, by particle gun as described in "Materials and methods," was digested with HapII or MspI followed by PCR amplification using a sandwich primer set for $L U C$ (Fig. 3b), and the products were analyzed on by
agarose gel electrophoresis (Fig. 3c). Analysis of the PCR products by HapII restriction enzyme indicates that at least a portion of the exogenous DNA molecules introduced into sea urchin fertilized eggs is methylated during embryonic stages, and that tandem ArsI fused to the $5^{\prime}$ end of SM50$L U C$ prevents methylation of $L U C$ until late gastrula stage. At four-armed pluteus stage, SM50-LUC lacking ArsI was highly methylated, whereas a large portion of SM50-LUC molecules bearing ArsI were protected from DNA methylation. No visible band was produced by the PCR from the DNA digested with methylation insensitive $M s p$ I.

Sea urchin larvae can be used as a screening system for insulators

Insulators ensure stable expression of an ectopically introduced gene and would be useful for the production of transgenic animals and gene therapy. In spite of its usefulness, only a few insulators have been identified so far, perhaps because a major obstacle is the requirement for long-term cell culture for detecting silencing of transgenes. Generally, silencing of transgenes introduced into mammalian culture cells occurs several weeks after the integration into chromosomal DNA (Hino et al. 2004; Tajima et al. 2006). As we show that silencing of transgene in sea urchin embryo can be detected within 24 h after introduction of the reporter construct (Figs. 2c and 3 a ), we suggest that the combination of the particle gun gene introduction method and the quick silencing system in sea urchin larvae offers the advantage of screening for a novel insulator useful for protecting transgene from silencing.

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