DEVELOPMENTAL BIOLOGY **193,** 139–145 (1998)
ARTICLE NO. DB978802

Structure and Function of a Sea Urchin Orthodenticle-Related Gene (*HpOtx*)

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Two distinct types of orthodenticle-related proteins (HpOtx$_{E/L}$) have been implicated as transcription activators of the aboral ectoderm-specific arylsulfatase (*Ars*) gene. Here, we describe the structure of HpOtx gene and present evidence that mRNAs of *HpOtx*$_{E/L}$ are transcribed from a single HpOtx gene by altering the transcription start site and by alternative splicing. By transactivation experiments, we have also demonstrated that HpOtx$_L$ activates the *Ars* promoter in the gastrula-stage embryo. © 1998 Academic Press

INTRODUCTION

Proteins that regulate transcription play key roles in early developmental events. The sea urchin embryo offers a relatively simple model system for study of the mechanisms of cell lineage-specific gene regulation during development. Sea urchin also offers the exceptional experimental accessibility, both for functional assays of gene regulatory systems and for characterization of transcription factors.

The blastomeres are segregated into five territories of specific embryonic fate in sea urchin development: the oral ectoderm, the aboral ectoderm, the vegetal plate, the skeletogenic mesenchyme, and the small micromeres. The specification processes by which the territorial founder cells are defined result in differential spatial presentation of sets of transcription factors (reviewed by Davidson, 1986, 1989; Cameron and Davidson, 1991).

One molecular marker for the aboral ectoderm is the arylsulfatase gene (*HpArs*) which 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.*, 1990). 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 the *HpArs* gene (Sakamoto *et al.*, 1997).

It has been demonstrated that two isoforms of HpOtx proteins bind to the enhancer element. The first type of Otx protein, referred to as early-type Otx (HpOtx$_E$), appears in early development and gradually decreases by the gastrula stage. The second type of Otx protein, referred to as late-type Otx (HpOtx$_L$), appears at the blastula stage and remains until gastrula stage (Sakamoto *et al.*, 1997). The time course of expression of HpOtx$_L$ is similar to that of *Ars* gene. Thus, HpOtx$_L$ is very likely to be involved in the activation of the *Ars* gene.

Two distinct types of HpOtx cDNA clones for HpOtx$_E$ and HpOtx$_L$ have been isolated. 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).

It has been suggested that the SpOtx gene of *Strongylocentrotus purpuratus* (*HpOtx$_E$* is the homologue of *SpOtx*) is involved in aboral ectoderm differentiation by activating aboral ectoderm-specific genes, such as *Spec1*, and that modulating SpOtx expression can lead to changes in cell fate (Mao *et al.*, 1994, 1996; Gan *et al.*, 1995). In this present study, we describe the genomic organization of the *HpOtx* and the function of HpOtx$_L$. We report here the HpOtx$_E$ and HpOtx$_L$ mRNAs are transcribed from a single HpOtx gene by altering transcription start site and by alternative splicing. We also demonstrate that HpOtx$_L$ activates the

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0012-1606/98 $25.00

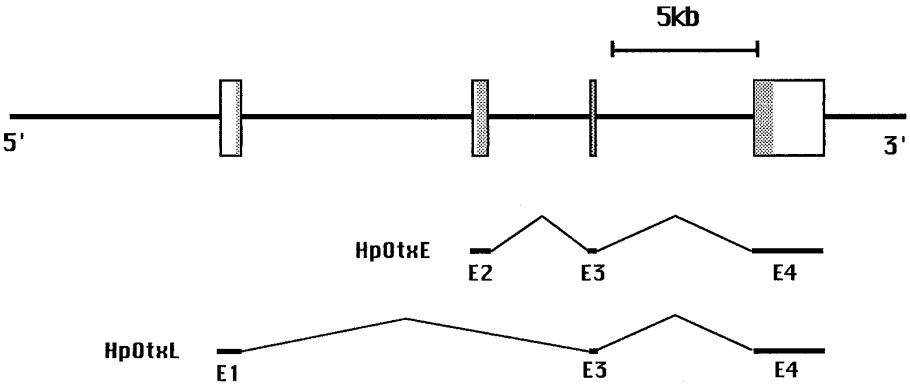

FIG. 1. Structure of the HpOtx gene. Boxes indicate exons. Open reading frames are shown as shaded boxes. The structure of the primary transcripts is shown as determined by comparison of the genomic DNA to the cDNA clones. The connecting thin lines depict the splicing of the introns.

Ars promoter. Both N-terminal and C-terminal domains of $HpOtx_L$ may be required for the transactivation.

MATERIALS AND METHODS

Genomic clones. The genomic library made in λ phage EMBL3 (Stratagene) using sperm DNA from a single *Hemicentrotus pulcherrimus* male was screened with cDNAs for $HpOtx_E$ and $HpOtx_L$. Two overlapping clones were isolated, 16 and 18 kb, and then were restriction-mapped by standard methods and used for all further experiments reported here. Various restriction fragments were subcloned into pBluescript using *Escherichia coli* strain XL-1 as the host cell. Sequence analysis was performed by exonuclease III digestion and by the chain termination method (*Taq* DyeDeox Terminator Cycle Sequencing Kit and ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit, Perkin–Elmer).

S1-nuclease protection assay. For S1-nuclease protection experiments, the 5′ end of $HpOtx_L$ (−117 to +64) and the 5′ end of $HpOtx_E$ (−96 to +72) were subcloned into M13 vector. Internal-labeled antisense DNA probes were generated with sequenase and specific primers in the presence of [^{32}P]dCTP. The S1 probes were hybridized to 30 μg of total RNA isolated from unhatched blastula and mesenchyme blastula, respectively. Hybridization and S1-nuclease digestion were performed according to Hames and Higgins (1993). The digests were electrophoresed on 8% polyacrylamide gels under denaturing conditions.

DNA constructs. The original HpOtx cDNA ($HpOtx_E$ and $HpOtx_L$) was isolated from *H. pulcherrimus* (Sakamoto *et al.*, 1997). For the transactivation experiments, $HpOtx_L$ flanking a SV40 intron–poly(A) signal sequence was generated with ligating with the C15 (*Ars* enhancer)–Ars252 (*Ars* promoter) fragment for driving the $HpOtx_L$ gene. *Gal4–*$HpOtx_E$*/*$HpOtx_L$ fusion expression constructs were generated by ligating the sequence coding Gal4 (1–147) DNA binding domain (Clontech) in the correct reading frame to $HpOtx_E$/$HpOtx_L$ flanking a SV40 intron–poly(A) signal sequence and with the C15 (*Ars* enhancer)–Ars252 (*Ars* promoter) fragment for driving the *Gal4–*$HpOtx_E$*/*$HpOtx_L$ fusion genes (Fig. 4a). The luciferase reporter (*C15–Ars252–luc*) of *Ars* promoter activity constructed for the experiment of transactivation by the $HpOtx_L$ was generated by ligating *Ars* enhancer–promoter (C15–Ars252) with pGL-2 basic (Promega). The luciferase reporter (*UAS–Ars194–luc*) of *Ars* promoter activity constructed for the experiment of transactivation by the Gal4-HpOtx fusion proteins was generated by ligating five times tandemly repeated Gal4-binding site: UAS × 5 (Clontech) with pGL-2 basic (Promega) driven by the Ars194 basal promoter fragment.

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 was determined by Dual-Luciferase Reporter System (Promega) as described in the instruction manual. The firefly luciferase activity driven by activation of *Ars* promoter was normalized based on the activity of *Renilla* luciferase. For the transactivation experiment, an average of five copies of *C15–Ars252–*$HpOtx_L$, *C15–Ars252–Gal4–*$HpOtx_E$*/*$HpOtx_L$, *C15–Ars252–luc*, and *UAS–Ars194–luc*, and 10 copies of pRL-CMV were introduced into each fertilized eggs and 10^5 embryos were used for an experimental samples.

RESULTS

Sequence Analysis of the HpOtx Gene

The *HpOtx* gene consists of four exons with three introns (Fig. 1). The region of the cloned genomic fragment corresponding to $HpOtx_E$ and $HpOtx_L$ was sequenced and is shown in Fig. 2. The splice sites show typical splice consensus sequences. The open reading frames encode proteins of 295 amino acids ($HpOtx_L$) and 371 amino acids ($HpOtx_E$) which are very similar to the previously described $HpOtx_L$ and $HpOtx_E$ cDNAs, respectively (Sakamoto *et al.*, 1997). One of 371 amino acids in the $HpOtx_E$ has undergone conservative substitutions caused by a single substitutions in the open reading frame. There are also one and four silent substitutions in the open reading frame of $HpOtx_L$ and $HpOtx_E$, respectively. There are more frequent base changes in the 3′-untranslated regions. Whether the difference between HpOtx cDNAs and HpOtx is because of polymor-

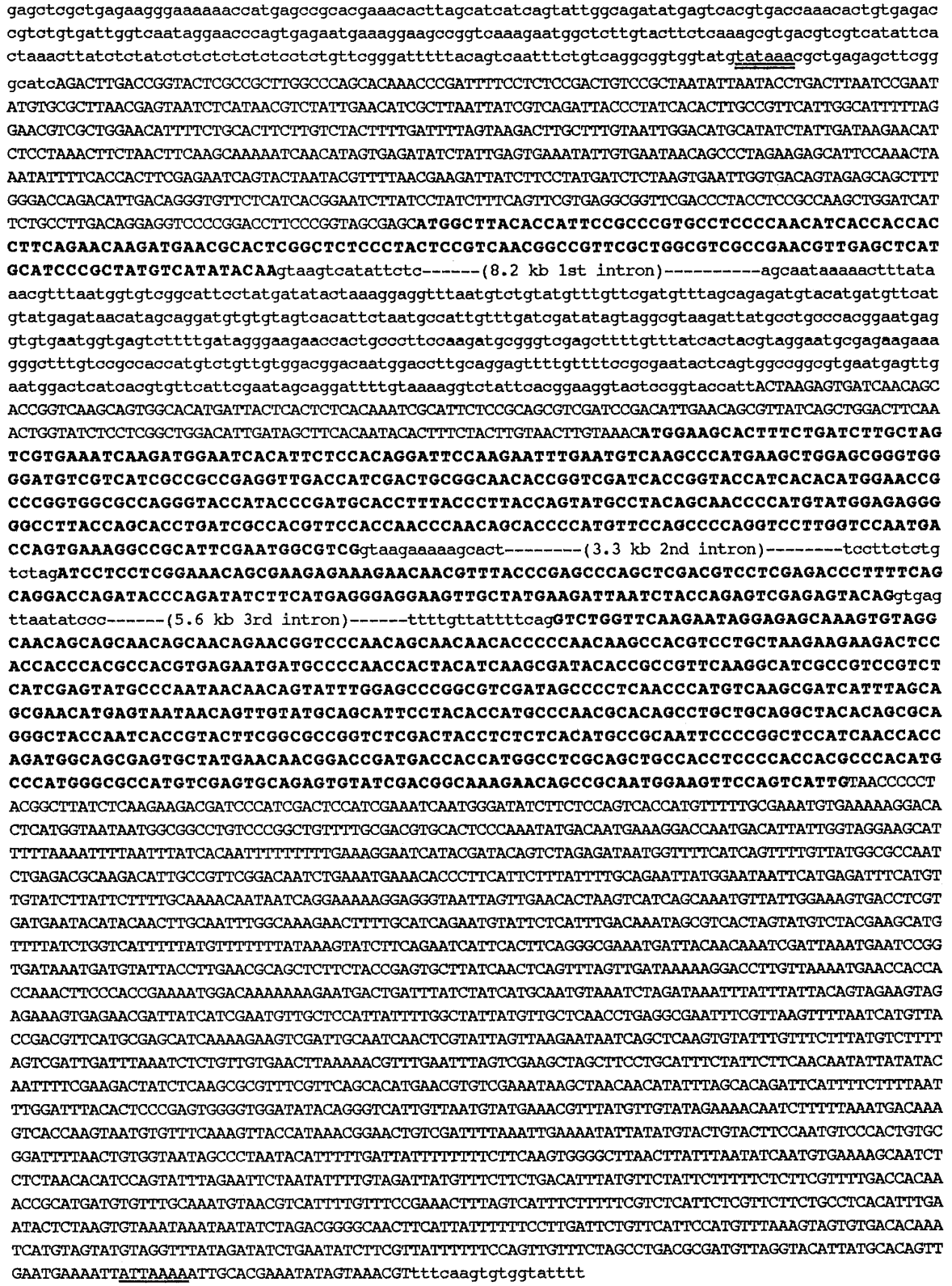

FIG. 2. Sequence of *HpOtx*. The nucleotide sequence of the *HpOtx* based on sequencing of its exons is presented. A putative TATA box at position −26 to −30 is double-underlined and a poly(A) consensus addition site is underlined. Capital letters represent the exon sequences and lowercase letters represent the flanking and intron sequences. Bold characters indicate coding regions. Numbers in parentheses indicate approximate length of the introns.

phism or because they are different alleles cannot be determined at this time.

Transcription Start Site of the HpOtx Gene

The start sites of transcription of $HpOtx_L$ and $HpOtx_E$ were mapped by S1-nuclease assay (Fig. 3). The A residue identified as position +1 was utilized as the start point to $HpOtx_L$ construct Fig. 2. There is a putative TATA box 26 bases upstream from the transcription start site of $HpOtx_L$. The cDNA for $HpOtx_L$ lacks the entire region of the second exon. The $HpOtx_E$ mRNA is transcribed from the 5′ end of the second exon. The S1 nuclease assay reveals that the transcription of $HpOtx_E$ starts at multiple sites. The A residue showing relatively intense signal identified as position +1 was utilized as the start point to construct Fig. 1. The typical TATA box does not exist in the promoter region of $HpOtx_E$. The putative splicing patterns of $HpOtx_E$ and $HpOtx_L$ are shown in Fig. 1.

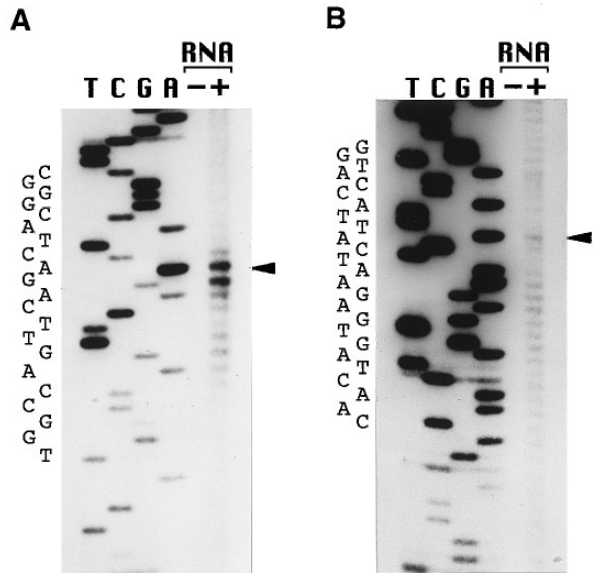

FIG. 3. S1-nuclease analysis of transcription start site. S1 mapping of $HpOtx_L$ (A) and $HpOtx_E$ (B). The arrowheads indicate the major initiation sites. The sizes of their products were determined by comparing them with sequencing ladder of probes.

$HpOtx_L$ Activates C15–Ars Promoter-Directed Luciferase Activity

In order to demonstrate that the $HpOtx_L$ activates the *Ars* enhancer, we prepared the construct shown in Fig. 4a, which produces a native (nonfusion) $HpOtx_L$, driven by the *Ars* enhancer and promoter, and cointroduced it into the egg with the reporter luciferase gene which is driven by *Ars* enhancer–promoter. It is supposed that the $HpOtx_L$ driven by *Ars* enhancer–promoter is activated in an autoregulatory fashion. As a consequence, the expression of $HpOtx_L$ increases and the expression of the luciferase gene increases. The expression of the luciferase gene did increase; however, the increase in the gene activation by the exogenous $HpOtx_L$ was less than 1.5-fold (Fig. 4b).

Gal4–$HpOtx_L$ Fusion Protein Activates UAS–Ars Promoter-Directed Luciferase Activity

One explanation for the low level of transactivation observed with the native HpOtx protein is that endogenous HpOtx can activate the Ars promoter. This is a real possibility because introduction of exogenous DNA by the particle gun method introduces very small number of constructs (Akasaka *et al.*, 1995). An approach that should eliminate the contribution of the endogenous HpOtx to the expression of the reporter is the use of 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 binding site (UAS) (instead 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. We wished to know whether either early or late form of Otx could activate transcription of the *Ars* gene; therefore, the two alternate forms of Otx, early and late, were fused to a Gal4–DNA binding domain. The Gal4–Otx-driven luciferase activity was normalized by cointroducing *Renilla* luciferase construct driven by CMV promoter as an internal standard.

These different construct combinations were introduced into fertilized eggs, which were cultured to the gastrula stage (36 h postfertilization). The results of the normalized luciferase assays are shown in Fig. 4c. It is clear that the late form of Otx successfully activates the *Ars* promoter fourfold, but that the early form does not under the same conditions. A control experiment in which the Gal4–Otx fusion protein is not included in the luciferase reporter shows no activity over background. The Gal4–$HpOtx_L$ 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–$HpOtx_L$ fusion protein activates *Ars* promoter-directed gene activity at the UAS site (Fig. 4d).

Domains in HpOtx Required for Activation of Ars Promoter

In order to determine which domains of Otx are responsible for the activation of *Ars* promoter, we fused portions of $HpOtx_{L/E}$ with the Gal4–DNA binding domain and analyzed the ability of these fusion proteins to activate a luciferase reporter gene driven by UAS–Ars194. Figure 5 shows that the deletion of either N-terminal region or C-terminal region of $HpOtx_L$ diminishes the activity of the transactivation. It was confirmed that the C-terminal region of $HpOtx_E$

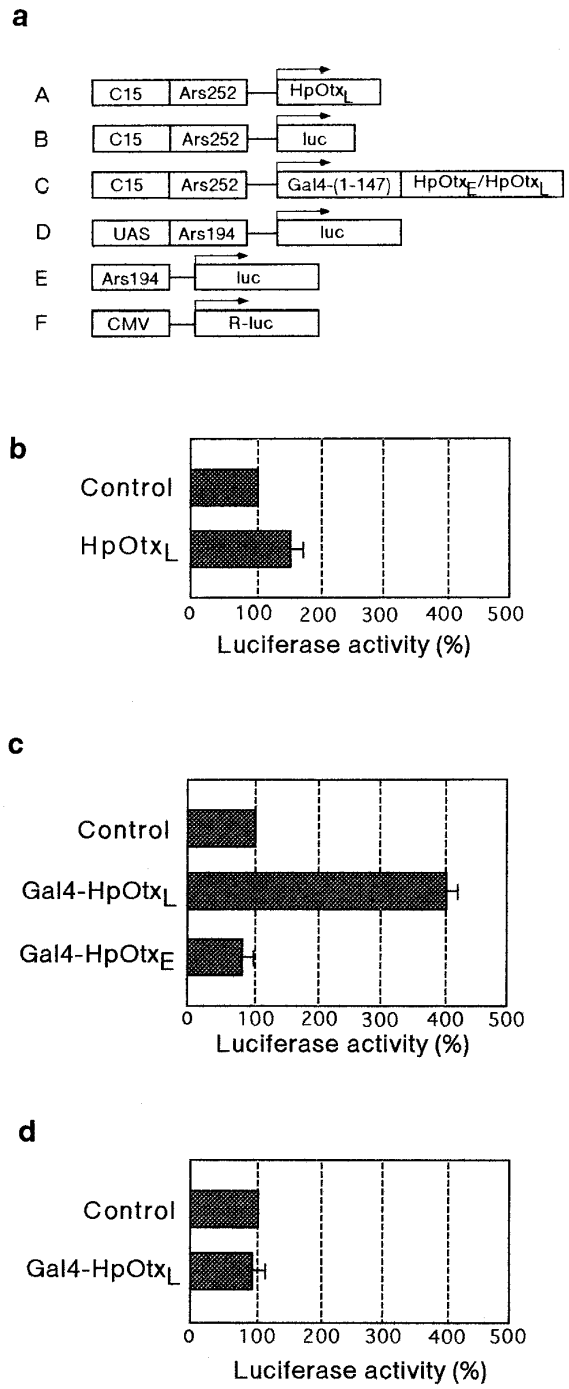

FIG. 4. (a) Diagram of fusion genes. A, *HpOtx$_L$* driven by *Ars* enhancer C15–*Ars* promoter Ars252 (−252bp to +38bp) produces HpOtx$_L$ in sea urchin embryos; B, firefly luciferase–reporter construct driven by *Ars* enhancer C15–*Ars* promoter Ars252; C, Gal4-HpOtx$_{E/L}$ fusion genes driven by *Ars* enhancer C15–*Ars* promoter Ars252 produce Gal4–HpOtx proteins in sea urchin embryos; D, firefly luciferase–reporter construct driven by UAS (5 tandemly repeated Gal4 binding sites) and *Ars* basal promoter Ars194 (−194bp to +38bp); E, firefly luciferase–reporter construct driven by *Ars* basal promoter Ars194; F, reference *Renilla* luciferase–reporter construct driven by CMV promoter. (b) Effect of *HpOtx$_L$* expression on C15–*Ars252–luc* activity. C15–*Ars252–luc* was introduced into 10^5 fertilized eggs. Additional construct *C15–Ars252–HpOtx$_L$* was cointroduced with *C15–Ars252–luc*. The embryos were harvested at the mesenchyme blastula stage. The relative firefly luciferase activities were represented based on that of *C15–Ars252–luc* (control), measured in the same experiment and assigned a value of 100. Bars indicate standard errors of nine independent experiments. (c) Effect of *Gal4–HpOtx$_E$/HpOtx$_L$* expression on *UAS–Ars194–luc* activity. *UAS–Ars194–luc* was introduced into 10^5 fertilized eggs. Additional fusion constructs *C15–Ars252–Gal4–HpOtx$_{L/E}$*(*Gal4–HpOtx$_L$*, *Gal4–HpOtx$_E$*) were cointroduced with *UAS–Ars194–luc.* The embryos were harvested at gastrula stage. The relative firefly luciferase activities were represented based on that of *UAS–Ars194–luc* (control), measured in the same experiment and assigned a value of 100. Bars indicate standard errors of 18 independent experiments. (d) Gal4–*HpOtx$_L$* fusion protein does not activate a reporter lacking the UAS sites. *Ars194–luc* were introduced with or without *C15–Ars252–Gal4–HpOtx$_L$*. The embryos were harvested at gastrula stage. The relative firefly luciferase activities of transactivated *Ars194–luc*(+Gal4–Otx$_L$) were represented based on that of *Ars194–luc* alone (control), measured in the same experiment and assigned a value of 100. Bars indicate standard errors of nine independent experiments.

does not have the activity (Fig. 5). It seems likely that both N-terminal and C-terminal domains of HpOtx$_L$ are required for the transactivation.

DISCUSSION

Production of Two Isoforms of Otx from a Gene

In the earlier experiments, it was suggested that two different types of HpOtx mRNA, HpOtx$_E$ and HpOtx$_L$, are produced from the same gene, since the *HpOtx* is a single copy gene and the nucleotide sequence from the homeobox to 3′ end of cDNA, including the 3′ UTR, is almost identical between the different types of HpOtx mRNAs (Sakamoto *et al.*, 1997). Northern blot analysis showed that the transcripts of *HpOtx$_E$* exist in the unfertilized eggs, that *HpOtx$_E$* was transcribed during cleavage and blastula stages, and that the *HpOtx$_L$* begins to be expressed at the hatching blastula stage. The transcripts of Otx$_L$ are then accumulated until the gastrula stage (Sakamoto *et al.*, 1997). We have shown in this study that the HpOtx$_E$ mRNA consists of the second, third, and fourth exons lacking the first exon, whereas the HpOtx$_L$ mRNA consists of the first, third, and fourth exons lacking the second exon. Thus it is likely that HpOtx mRNA is transcribed from the 5′ end of the second exon in the oogenesis and cleavage stages; then after the blastula stage, it begins to be transcribed from the 5′ end of the first exon. Since there is no acceptor consensus sequence for splicing in the 5′ flanking region of the second exon, the first exon could be spliced to the third exon. Consequently, the second exon could be excised by the processing during the maturation of the HpOtx$_L$ mRNA.

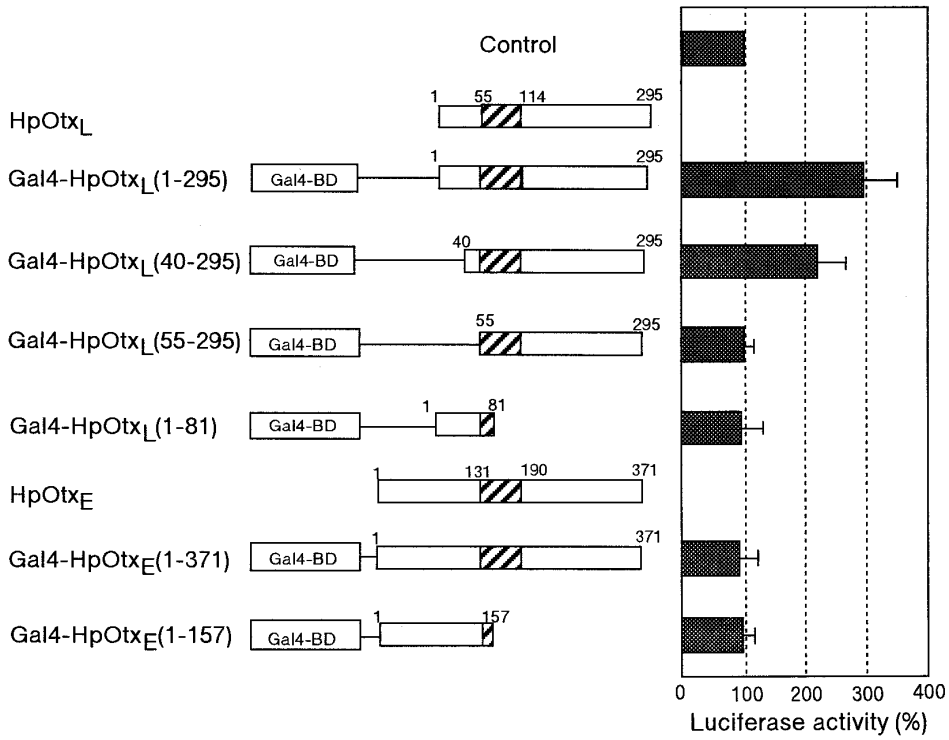

FIG. 5. Analysis of transcriptional activation domain for *Ars* promoter within HpOtx$_L$ using Gal4–HpOtx fusions in the sea urchin embryo. Hatched boxes refer to the HpOtx homeodomain from amino acids 55–114 (HpOtx$_L$) and 131–190 (HpOtx$_E$). The embryos were harvested at gastrula stage. The relative firefly luciferase activities were represented based on that of *UAS–Ars194–luc* (control), measured in the same experiment and assigned a value of 100. Bars indicate standard errors of 12 independent experiments.

Using *S. purpuratus*, it has been also demonstrated that two isoforms of Otx, SpOtx (α) and SpOtx (β), are produced in sea urchins by differential promoter utilization and alternative splicing, and that three different types of transcripts, SpOtx (β-1), SpOtx (β-2), and SpOtx (β-3), encode the late form SpOtx (β) (Li *et al.*, 1997). However, we did not detect any other exon in the upstream region of *HpOtx$_L$* transcription start site, although we have performed 5′ RACE extensively. A single band was detected as a late type of HpOtx mRNA by Northern blot analysis which also suggests that a single type of HpOtx$_L$ transcript exists in *H. pulcherrimus* embryo (Sakamoto *et al.*, 1997). This disparity could be due to the difference of species.

Transactivation of HpArs by HpOtx Protein

In order to perform the transactivation assay, we introduced constructs into sea urchin eggs using the particle gun method. In this method, many fewer exogenous constructs are present and many more embryos are assayed when compared to the microinjection method (Akasaka *et al.*, 1995). As a consequence of the method, the activity provided by exogenously supplied Otx transcription factors may be less than their endogenous counterparts. Actually, in the present experiments the exogenous HpOtx$_L$, in addition to the endogenous factor activities, accounts for about 50% more activity than endogenous factors alone. It should be pointed out that the unmodified exogenous HpOtx$_L$ has activity with the *Ars*–reporter construct, despite the low level of transactivation.

The use of the Gal4/UAS–luciferase system allows the activating potential of transcription factors to be tested in the homologous system in the absence of interfering endogenous background. The introduction of low copy numbers of test constructs coupled with a highly sensitive reporter is likely to be a more reliable system than high copy number system.

It was shown that C-terminal region of the SpOtx as well as the N-terminal region was an activator domain by using a yeast Gal4 transcription system (Mao *et al.*, 1996). C-terminal regions of HpOtx$_E$ and HpOtx$_L$ are almost identical to that of SpOtx; thus it would be expected that the C-terminal region of HpOtx could also activate the transcription. However, apparently the C-terminal region of HpOtx$_{E/L}$ by itself does not activate the Ars promoter in the sea urchin embryo. It is possible that the difference is derived from the different assay systems of transactivation. Recently, it has been demonstrated that various mediators, which do not bind DNA, are involved in the regulation of transcription (Li *et al.*, 1994). The transactivation assay us-

ing the yeast system might miss some such putative mediator functions.

The temporal expression pattern of the HpOtxL is similar to that of *Ars* gene. In addition to the results of transactivation assay, this suggests that the $HpOtx_L$ is responsible for the activation of *Ars* promoter. However, it has been shown that a very low level of transcription of *Ars* starts prior to the appearance of $HpOtx_L$ (Sasaki *et al.*, 1988; Yang *et al.*, 1993). Our earlier studies show that the reporter construct driven by *HpArs*–promoter which does not contain the Otx site is activated at early blastula, significantly before the initiation of $HpOtx_L$ accumulation (Morokuma *et al.*, 1997). Thus, the initial low level of *Ars* gene activation may be due to factors other than Otx, although we cannot exclude the possibility that $HpOtx_E$ is involved in the activation of Ars gene at these early stages of development.

ACKNOWLEDGMENTS

The authors express their thanks to Dr. Fred Wilt for his advice in preparation and critical reading of the manuscript. A part of this work was carried out in the Center for Gene Research of Hiroshima University. This work was supported in part by Grants-in-Aid for Scientific Research (B) (No. 07458195) and for Scientific Research on Priority Areas (Nos. 07262211 and 08254212) to H.S., and for Scientific Research (C2) (No. 08680795) to K.A., from the Ministry of Education, Science, Sports and Culture, Japan. This research was also supported in part by a Grant Pioneering Research Project in Biotechnology given to H.S. by the Ministry of Agriculture, Forestry and Fisheries, Japan. Research support by a grant from the Mitsubishi Foundation to H.S. is also acknowledged.

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Received for publication May 23, 1997
Accepted November 12, 1997