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Ets identified as a *trans*-regulatory factor of amphioxus *Hox2* by transgenic analysis using ascidian embryos

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Abstract

Although the functions of Hox genes in anterior–posterior patterning and their clustered organization are well conserved among metazoans, some Hox genes have lost their original function, as exemplified by *zen*, *ftz* and *bicoid* in *Drosophila*. The *Hox2* gene of amphioxus has also lost its original function and instead is expressed specifically in the preoral pit. As new *cis*-elements governing its expression in the preoral pit must have been essential for retention of *AmphiHox2*, we analyzed the transcriptional regulation of *AmphiHox2*. Although it is possible to make transgenic amphioxus, several technical limitations restrict their practical use; thus, we analyzed the *cis*-regulatory region surrounding *AmphiHox2* in transgenic ascidians (*Ciona intestinalis*). We found that Ets binding sites of *AmphiHox2* functioned in the ascidian embryo. As the amphioxus *Ets1/2* homologue is expressed in the preoral pit, we concluded that *AmphiHox2* is activated by Ets1/2 in the preoral pit. These analyses demonstrate the utility of *Ciona* embryos as a transgenic system for analyses of *cis*-elements from animals whose embryos are relatively inaccessible, such as amphioxus and hemichordates.

Keywords: Amphioxus; Hox2; Ets; *Ciona*; Preoral pit; Transcriptional regulation

Introduction

Hox genes have conserved developmental roles in anterior–posterior patterning in multicellular animals, as well as a conserved clustered organization on the chromosome in which the genes are arranged in a colinear manner with their functional position along the body axis. It is widely accepted that the common ancestors of the metazoans (with the possible exception of sponges) had several Hox genes that formed a genomic cluster and functioned to establish regional identity along the antero-posterior axis (Carroll, 1995; Slack et al., 1993).

Despite the conserved nature of the Hox genes, some have lost their original functions related to conferring positional identity. Such genes have taken one of two evolutionary routes: they were either lost after becoming pseudogenes or were retained after acquiring a new function (neofunctionalization). Examples of the former situation have been observed in nematodes, urochordates and vertebrates. In nematodes, four Hox genes (*abd-A*, *Ubx*, *Dfd* and *pb*) were lost at an early evolutionary stage (Aboobaker and Blaxter, 2003). Furthermore, the lineage leading to *Caenorhabditis* lost two more genes, *hox-3* and *Antp* (Aboobaker and Blaxter, 2003). Similarly, two members of the urochordates, ascidians and larvaceans, have lost several Hox genes (Ikuta et al., 2004). Vertebrates experienced two rounds of genome duplication that gave rise to four Hox clusters (Holland et al., 1994; Garcia-Fernandez and Holland, 1994). Subsequently, many of the paralogous genes became redundant, and some of the Hox

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genes have disappeared during vertebrate evolution (Holland, 1992). Examples of neofunctionalization of Hox genes can be found among insect Hox genes. The *Drosophila* gene *zerknult* (*zen*) encodes a protein that shows amino acid similarity to the *Hox3* product of vertebrates and other arthropods, and *zen* occupies the chromosomal position between the *Hox2* and *Hox4* homologues (*proboscipedia* and *Deformed*) (Damen and Tautz, 1998; Falciani et al., 1996; Telford and Thomas, 1998). However, *zen* does not function in patterning positional identity along the anteroposterior axis of the embryo. Instead, *zen* has roles in dorsoventral specification and extra-embryonic tissue formation (Rushlow and Levine, 1990). *Bicoid*, another *Drosophila* gene, is also believed to be derived from *Hox3* and survived after acquiring a new role in the development of the anterior part of the body (Stauber et al., 1999). *Ftz* is derived from *Hox5* and acquired a new function as a pair-rule gene (Lohr et al., 2001; Telford, 2000).

Amphioxus is among the closest relatives of vertebrates and shows a body plan that is essentially similar to that of vertebrates, possessing a notochord and a dorsal tubular nerve cord. However, amphioxus separated from the vertebrate lineage before the genome duplication events that occurred in the ancestors of the vertebrates and thus possesses only a single Hox cluster (Holland et al., 1994; Garcia-Fernandez and Holland, 1994). Therefore, amphioxus is generally regarded as retaining some aspects of the primitive body plan of the chordates, although not all amphioxus characteristics can be regarded as primitive a priori (Minguillon et al., 2003). The amphioxus Hox genes have been shown to be expressed colinearly in the neural tube (Wada et al., 1999). The anterior expression boundaries of *Hox1*, *Hox3* and *Hox4* in amphioxus exhibit two-segment periodicity. The colinear expression of these amphioxus Hox genes can be observed in epidermal cells as well, where peripheral nerves are scattered (Schubert et al., 2004). Therefore, it has been suggested that Hox genes function to produce anterior–posterior patterning in both the central and peripheral nervous systems. In contrast, the expression of *AmphiHox2* has not been detected in the neural tissue or epidermis but is found instead in the preoral pit, which is regarded to be homologous to the vertebrate adenohypophysis (Wada et al., 1999). Therefore, we reasoned that *AmphiHox2* has lost its function as a typical Hox gene but has survived as a transcribed gene because it has acquired another function in the preoral pit. As the expression of *AmphiHox2* was essential for its retention and because its expression is likely to be governed by distinctive *cis*-regulatory elements, we examined the evolution of the *cis*-regulatory elements responsible for the expression of *AmphiHox2* in the preoral pit.

The *cis*-regulatory elements of the amphioxus genomic region surrounding *AmphiHox1* to *3* have been previously surveyed using transgenic mouse and chicken embryos (Manzanares et al., 2000). One of the previously studied genomic fragments (construct 2B in Manzanares et al., 2000), which covers 3 kb of the 5′ flanking region, the coding region and 0.6 kb of the 3′ flanking region of *AmphiHox2*, has been shown to be sufficient to drive reproducible expression in the trigeminal and facial ganglia of transgenic mice (Manzanares et al., 2000). Thus, we tested the idea that this genomic fragment contains the *cis*-elements required for expression of *AmphiHox2* in the preoral pit. Recently, Yu et al. (2004) reported pioneering work producing transgenic amphioxus embryos by microinjection. However, there are still some technical limitations to the production and use of transgenic amphioxus; amphioxus eggs can be obtained in only a few places in the world, and spawning is limited to 2 months each year and is not easily controlled. Consequently, we decided to use an ascidian (*Ciona intestinalis*) as the host for transgenic analysis of the *cis*-regulatory systems of *AmphiHox2*. There are some similarities in the expression patterns of the Hox genes in amphioxus and ascidians; in both groups, colinear expression is observed in the neural tissue and the epidermis (Ikuta et al., 2004; Schubert et al., 2004; Wada et al., 1999). In addition, both ascidians and amphioxus possess a single set of Hox genes, although the ascidian genes have lost the single cluster organization (Garcia-Fernandez and Holland, 1994; Ikuta et al., 2004). In the present study, we found that construct 2B contains *cis*-elements that can drive expression of a reporter gene in the anterior nervous system of *Ciona*, probably by responding to Ets1/2. As amphioxus *Ets1/2* is expressed in the preoral pit where native *Hox2* is expressed, we suggest that the expression of *AmphiHox2* in the preoral pit is controlled by Ets1/2. Thus, amphioxus *Hox2* was retained after acquisition of an Ets-responsive element involved in its new function in preoral pit development.

Materials and methods

Preparation of the constructs

The promoter constructs were prepared as described by Manzanares et al. (2000). Amphioxus genomic DNA fragments were linked to the β-galactosidase reporter gene together with the mouse β-globin minimal promoter. The deletion constructs were produced by either restriction enzyme digestion or PCR using oligonucleotides with flanking restriction sites. Mutant constructs were prepared by using QuickChange XL-Site-Directed Mutagenesis Kit (Stratagene).

Ciona electroporation

Electroporation of *C. intestinalis* eggs was performed following the methods of Corbo et al. (1997) with slight modifications. In brief, 300 μl of dechorionated fertilized eggs was placed in 500 μl of 0.77 M mannitol containing 50 μg circular plasmid DNA in 0.4-cm cuvettes and pulsed

once with 50-V pulse for 20 ms on a T820 electroporator (BTX, San Diego, CA, USA). The electroporated eggs were immediately placed in agar-coated dishes with sufficient artificial sea water (Marine Art for Invertebrates, Senju Seiyaku, Japan) and reared for development. The *cis*-regulatory activity of each construct was tested in more than 50 embryos from more than two electroporation batches.

In situ hybridization and X-Gal staining

The in situ hybridization of the *Ciona* embryos was performed according to the method of Yasuo and Satoh (1994). The probe for the *Ciona ets/pointed2* was produced using a cDNA clone from a *Ciona* cDNA collection (Satou et al., 2002).

Embryos of *Branchiostoma floridae* and *Branchiostoma belcheri* were collected in Tampa (Florida) and Qingdao (China), respectively, and fixed for in situ hybridization. In situ hybridization was performed following the methods of Yasui et al. (1998).

The *Ets1/2* gene from *B. belcheri* was isolated by PCR using the following primers: forward primer F: 5′-TGGAC-NGGNGAYGGNTGGGA-3′; reverse primer R: 5′-TTRT-GDATDATRTTYTTRTC-3′. Longer cDNA fragments were obtained from a 3′-RACE library (Clontech). The nucleotide sequence for *BbEts1/2* was submitted to Genbank/EMBL/DDBJ under accession number AB219528. *Ets1/2* from *B. floridae* was recovered from NCBI megaTrace database by Blast search using *BbEts1/2* sequence as a query. A DNA fragment of *BfEts1/2* was amplified by PCR using primers (F: 5′-GATGGTGAAACAAGATTACTTAATTTTTCT-3′, and R: 5′-TTGTGAATCACAATACTGTGAATGGTT-TCC-3′).

Staining for detection of β-galactosidase expression in *Ciona* embryos using X-Gal was performed according to the methods of Hikosaka et al. (1993).

Results

We tested the *cis*-regulatory activity of construct 2B that contains 3 kb of the 5′ flanking region, the coding region and 0.6 kb of the 3′ flanking region of *AmphiHox2* (Fig. 1A; sequence accession number AB050887; Manzanares et al., 2000) as a transgene during embryogenesis of *C. intestinalis*. When electroporated into fertilized *Ciona* eggs, construct 2B drives reproducible expression of the reporter gene in cells of presumptive anterior neural tissues, the adhesive palp and the oral siphon. These cells are all derivatives of the a6.5 blastomere of the 32-cell stage (Nishida, 1987), although expression is observed only after the neurula stage (Figs. 2A–C).

Deletions in the 5′ flanking and coding regions did not affect the *cis*-regulatory activity of construct 2B. From experiments using a series of deletion constructs, we found that 113 bp at the 3′ end of the construct, designated as region A, were sufficient to drive expression in tissues derived from a6.5 (Fig. 1A). Although these constructs drive ectopic expression, most of this is observed in mesenchyme cells where many constructs drive ectopic expression in *Ciona* (e.g. Locascio et al., 1999; Russo et al.,

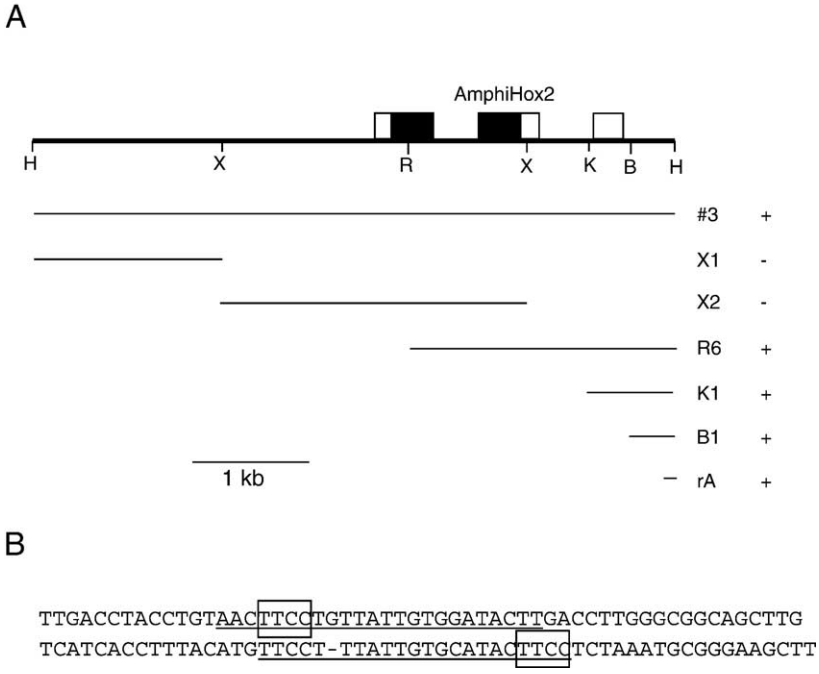

Fig. 1. Mapping of the *cis*-elements responsible for the expression of *AmphiHox2*. (A) Genomic structure of construct 2B and the deletion constructs. Deletion analyses indicated that 113 bp of the 3′ end of 2B was sufficient for expression of the reporter gene. H, *Hind*III site; X, *Xba*I site; R, *Eco*R I site; K, *Kpn*I site; B, *Bgl*II site. (B) Nucleotide sequence of region A. The core sequences of the putative Ets binding sites are boxed. The repeat sequence is underlined.

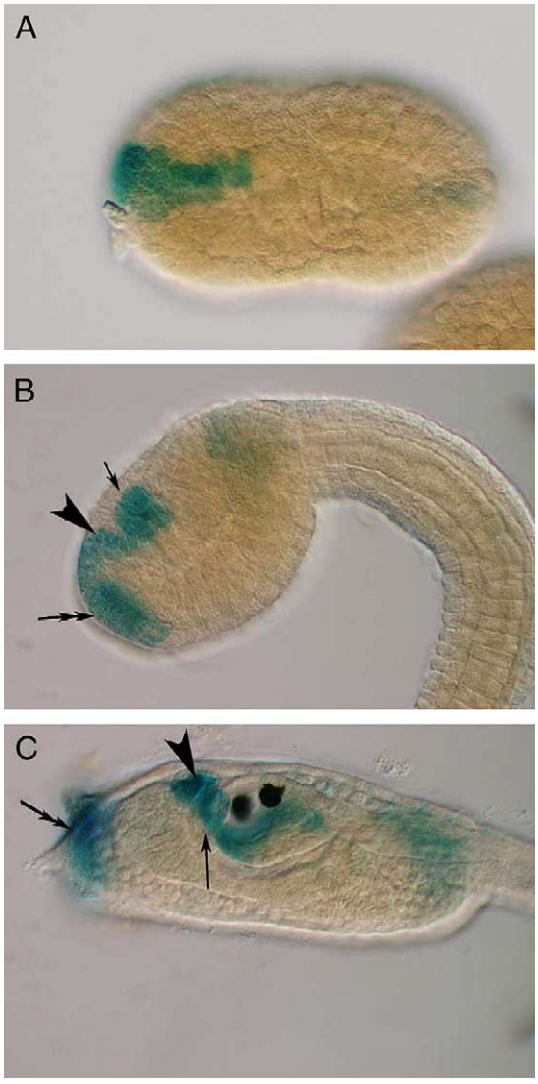

Fig. 2. Expression of the reporter gene driven by region A. Expression of the reporter gene was examined by detection of β-galactosidase activity, which was observed in tissues of neurula stage embryos derived from the a6.5 blastomere (A). The positive cells are destined to form the anterior neural tube (arrow), pharynx (arrowheads) and palp (double arrowheads) in the tailbud embryo (B) and tadpole larva (C).

2004; HW, unpublished observation). The basal promoter from mouse β-globin alone does not drive any expression in *Ciona*. Deletion of an additional 20 bp from either the 5′ or 3′ end of region A abolished the *cis*-regulatory activity in a6.5 derivatives. Thus, there are at least two essential *cis*-elements at the ends of the region A. Examination of the sequence of region A revealed direct repeats of almost identical 20–21-bp nucleotide sequences at both ends, designated repeat 1 and 2, respectively (Fig. 1B). Thus, we reasoned that these repeat sequences may be essential for *cis*-regulatory activity. To test this hypothesis, we systematically inserted mutations into these repeat sequences and tested the resulting *cis*-regulatory activities (Fig. 3). We found that the 5′ end of repeat 1 and the 3′ end of repeat 2 were essential for the *cis*-regulatory activity of region A. Interestingly, both of these essential sequences contained consensus binding sites for Ets. Bertrand et al. (2003) reported that the *cis*-regulatory elements of *Otx* in *Ciona* (*CiOtx*) contain binding sites for Ets and that these elements drive expression in the a6.5 blastomere of *Ciona* embryos in a pattern very similar to that observed in the present study. Thus, we believe that the expression of the reporter gene by construct 2B in *Ciona* embryos was probably controlled by Ets. It is worth pointing out that mutations in the 5′ flanking sequence (T/AAC) of the core binding sites for Ets (TTCC) abolished the expression of the reporter. This flanking sequence is also conserved in one of two Ets binding sites of *CiOtx*, mutation of which results in a more marked decrease in *cis*-regulatory activity than does mutation of the corresponding flanking sequence of the other Ets binding site (Bertrand et al., 2003).

Although we could not confirm that the Ets binding sites and the flanking repeat sequences of *AmphiHox2* function in amphioxus, the organized nature of these *cis*-regulatory elements in a small genomic region (the 113 bp of region A) supports the notion that these elements are under selective pressure and thus are likely to be functional in amphioxus. If so, one can predict that amphioxus *Ets* is expressed in advance of *AmphiHox2*, in cells where *AmphiHox2* is expressed. Thus, we examined the spatio-temporal expression of amphioxus homologue of *Ets1/2* during embryogenesis. The earliest zygotic expression of *Ets1/2* of *B. belcheri* (*BbEts1/2*) was observed in the posterior–ventral wall of the first somites of the neurula stage (Figs. 4A, B). Subsequently, expression of *BbEts1/2* was upregulated in the pharyngeal endoderm and in the preoral pit (Fig. 4C). We confirmed that *Ets1/2* orthologue is expressed in the preoral pit of *B. floridae*, from which genomic DNA utilized for Hox2 enhancer analysis was isolated (Fig. 4D). Therefore, *Ets1/2* is indeed expressed in the cells where *AmphiHox2* will subsequently be expressed.

Finally, we asked whether *Hox2* of *Ciona* (*CiHox2*) is regulated in a similar manner by Ets. In *Ciona*, although *Hox1*, *Hox3* and *Hox5* have previously been reported to be expressed in a colinear manner in the central nervous system (Gionti et al., 1998; Ikuta et al., 2004; Katsuyama et al., 1995; Locascio et al., 1999), *Hox2* is not expressed in these tissues. Rather, it is expressed in the trunk lateral cells of the larva (Ikuta et al., 2004). Thus, similar to *AmphiHox2*, *CiHox2* has been retained after acquiring a new role in trunk lateral cells. Two *Ets1/2* homologues have been identified in the *Ciona* genome (Yagi et al., 2003). The product of one of these, *ets/pointed2* (Yagi et al., 2003), was shown to be responsible for the activation of *CiOtx* (Bertrand et al., 2003). Maternally derived expression of *ets/pointed2* was detected throughout the embryo during the early stages of *Ciona* embryogenesis (data not shown), and this maternal product was probably responsible for expression of the reporter gene in the a6.5 blastomere (Bertrand et al., 2003). Zygotic expression of *ets/pointed2* has been published by Imai et al. (2004) (ciad008k16: http://ghost.zool.kyoto-u.ac.jp/tagtscriptg.html), and we have investigated it in more

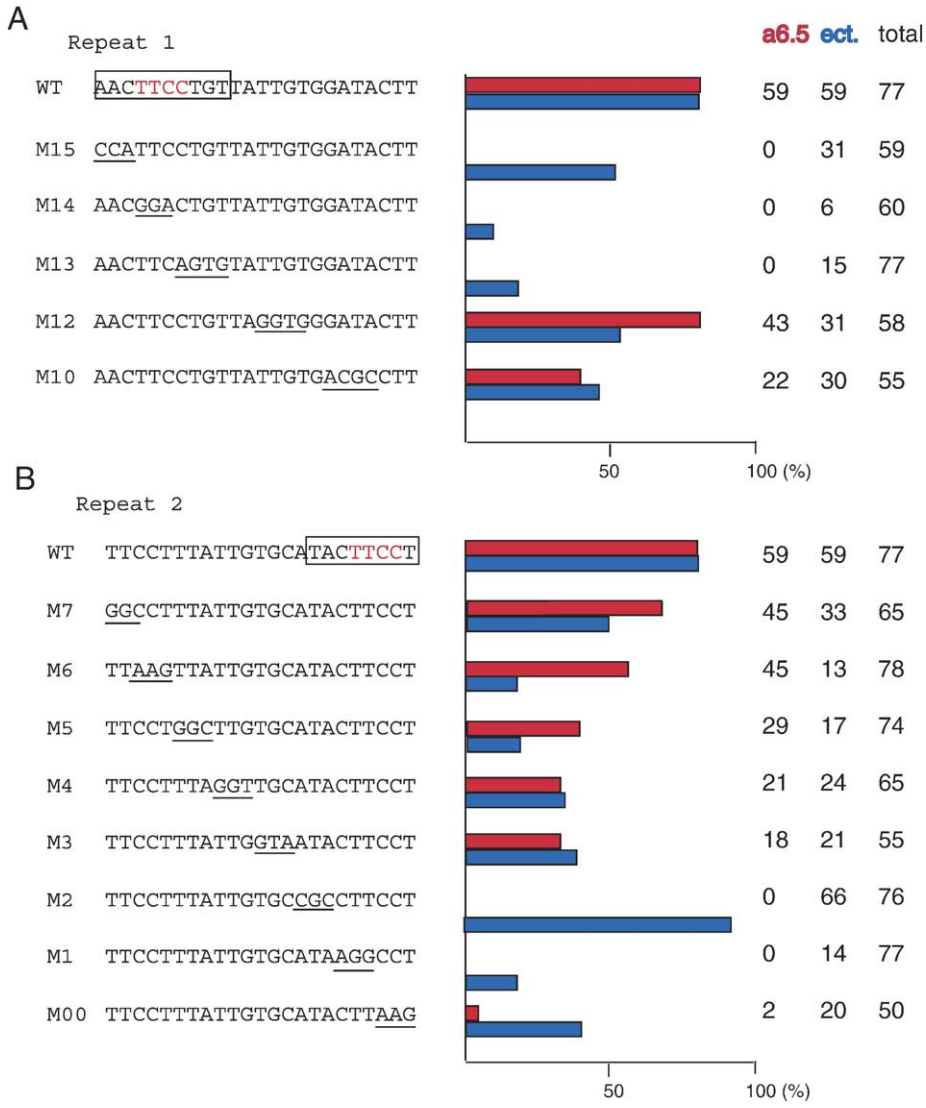

Fig. 3. *Cis*-regulatory activities of the mutant constructs. Mutations were inserted in repeat 1 (A) and repeat 2 (B) of region A. Bars in the graph on the right represent the percentage of embryos in which the reporter was expressed in tissues derived from the a6.5 blastomere (red bars) and in ectopic locations most of which are in mesenchymal cells (blue bars). The core binding sites of Ets are indicated by red characters in the wild-type sequence. Boxed characters indicate nucleotides for which mutations abolished the expression of the reporter gene in tissues derived from the a6.5 blastomere.

detail (Fig. S1). The expression was detected in the central nervous system, in presumptive palp cells and presumptive heart cells (trunk ventral cells) before the neurula stage (Figs. S1A, B), and it was maintained in a small population of CNS, palp cells and heart cells of tailbud embryos (Figs. S1C, D). However, even in late-tailbud stage, no expression was observed in the trunk lateral cells (Fig. S1E). Expression of the other *Ets1/2* homologue, *ets/pointed1*, has been reported in mesenchymal cells of the B7.7 and B8.5 lineages (Imai et al., 2004) (cibd061j11). Thus, the expression of *CiHox2* in the trunk lateral cells, which are derived from a7.6, is not likely to be regulated by *Ets1/2* homologues. Therefore, although *Hox2* has lost its "Hox gene" functions in both *Ciona* and amphioxus, the genes in each case might have survived by independently acquiring new roles. However, as the expressions of *CiHox2* or *ets/pointed* genes have not been completely surveyed, such as in metamorphosing juveniles or adult tissues, we could not exclude the possibility that *ets/ponied* orthologues are involved in the transcriptional regulation of *CiHox2*. In addition, it is also possible that some other Ets family genes regulate transcription of *CiHox2*.

Discussion

In this study, we analysed the *cis*-regulatory region controlling *AmphiHox2* expression using a transgenic approach by electroporation of *Ciona*. Using this methodology, we found that Ets binding sites in the 3′ flanking region of *AmphiHox2* were responsible for the *cis*-regulatory activity in the anterior neural tissues, pharynx and

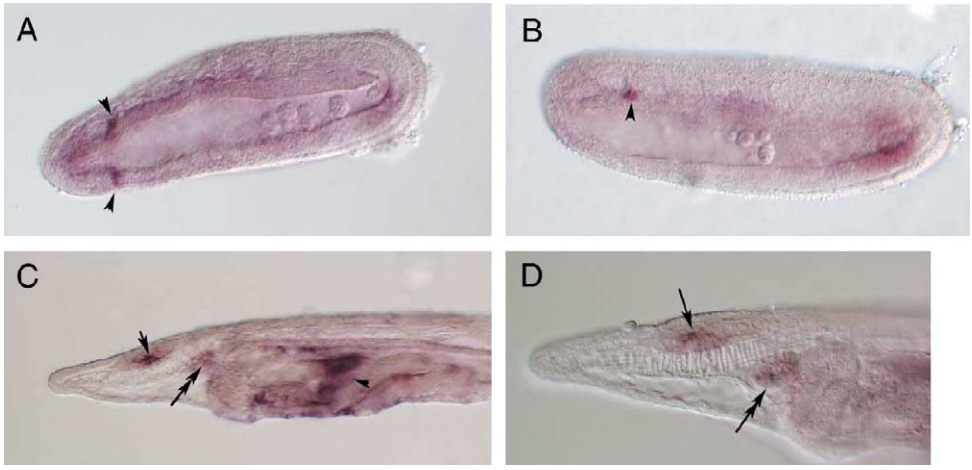

Fig. 4. Expression patterns of amphioxus *Ets1/2*. (A, B) In the neurula stage, *BbEts1/2* was expressed in the posterior–ventral wall of the first somites (arrowheads). Dorsal (A) and lateral (B) views of the neurula embryo. (C) In larvae, *BbEts1/2* expression was observed in part of the cerebral vesicle (arrow), pharyngeal endoderm (arrowheads) and preoral pit (double arrows). (D) Ets1/2 from *B. floridae* also show expression in part of the cerebral vesicle (arrow), pharyngeal endoderm (arrowheads) and preoral pit (double arrows).

palp of *Ciona* (all derived from the a6.5 blastomere) (Nishida, 1987). In addition, we also found that the abutting sequence of the Ets core binding sequence was also essential for the *cis*-regulatory activity. This abutting sequence is also conserved in one of the Ets binding sites of *CiOtx*, the mutation of which resulted in a more severe effect on the *cis*-regulatory activity than did mutation of the other Ets binding element in *CiOtx* (Bertrand et al., 2003). The nucleotides flanking the core binding site are known to influence the binding affinity or specificity of the Ets family members in vertebrates (Verger and Duterque-Coquillaud, 2002). Fourteen members of the *Ets* gene family have been identified in the *Ciona* genome (Yagi et al., 2003), and Bertrand et al. (2003) indicated that one of the *Ets1/2* homologues was responsible for the Ets *cis*-regulatory activity.

These results indicate significant similarities between the *cis*-regulatory mechanisms of *AmphiHox2* and those of *CiOtx*, and this similarity helps us to characterize the *cis*-elements of *AmphiHox2*. However, there are also several differences in these regulatory systems. Although comparison of the activities of foreign *cis*-elements of *AmphiHox2* to native ones of *CiOtx* may not be entirely appropriate, some useful information can be gained for an understanding of the function of *Ets* in *Ciona*. The first notable difference is in the specificity of the activities in the a6.5 and b6.5 blastomeres. In the case of *CiOtx*, the *cis*-regulatory activities in the a6.5 and b6.5 blastomeres are not well separated, and no *CiOtx* construct drives specific expression in the a6.5 lineage, although some constructs can drive expression in tissues derived from b6.5, but not in those derived from a6.5 (Bertrand et al., 2003). In this aspect, an interesting observation is that, when we replaced the sequences of the 3′ flanking region of the repeat 1, where mutations were induced in the constructs #10 and #12, with CTCGAG, higher numbers of embryos drive expression of lac-Z in a6.5 derivatives (Fig. S2; #10-CTC: 35/60, #12-CTC: 27/68). Ets often functions with specific partners in transcriptional regulation (Verger and Duterque-Coquillaud, 2002). Additional transcription factors may be able to interact with ets/pointed2 in these mutant constructs, and subsequently reporter expression was driven in derivatives of b6.5. Other minor differences between the *cis*-regulatory activity of region A of *AmphiHox2* and the *cis*-element of *CiOtx*, including differences in the timing of the earliest detection of β-galactosidase activity (neurula stage for *AmphiHox2* and gastrula for *CiOtx*), may be explained by differences in the minimal promoters; the endogenous promoter was used in the analysis of *CiOtx* (Bertrand et al., 2003), whereas the mouse β-globin promoter was used in the analysis of *AmphiHox2* in the present study.

As a first step to identifying the *cis*-elements in region A, we recognized a direct repeat of 20–21 bp and identified at least three *cis*-elements in the repeat. However, the Ets binding sites were located at different ends of the repeats. Although the Ets core binding sequence (TTCC) can be recognized in the 5′ end of the repeat 2, mutation in this sequence did not abolish the *cis*-regulatory activity in tissues derived from the a6.5 blastomere. Thus, we could not obtain any evidence to explain the existence of the direct repeat sequences from the functional aspect. Rather, it is also possible that the repeats may be remnants of a tandem duplication of a small DNA fragment during the evolution of the organized *cis*-elements in the ancestors of amphioxus.

The construct 2B (Manzanares et al., 2000) has been shown to drive reproducible expression in the trigeminal and facial ganglia of transgenic mice (Manzanares et al., 2000). Since Ets1 has been shown to be expressed in the neural crest cells from anterior hindbrain (Maroulakou et al., 1994), it is possible that the expression in mouse ganglia is driven by Ets.

Although the 3′ flanking region of *AmphiHox2* is functional in *Ciona*, we could not prove that this region functions in amphioxus itself. In addition to testing the *cis*-

regulatory activity of construct 2B in *Ciona*, we also tested all other regions spanning *AmphiHox1* to *3*, which we had examined in transgenic mice in a previous study (Manzanares et al., 2000). Of the tested regions, construct 2B is the only one that can drive expression of a reporter gene in *Ciona*; the other regions, including those that function in chicks and mice (Manzanares et al., 2000), did not exhibit any reporter expression in *Ciona* (Wada et al., in preparation). This suggests that only some of the functional *cis*-elements of amphioxus can work in the *Ciona* embryo, and that non-functional sequences are not likely to drive expression in *Ciona*. Moreover, in region A, two Ets binding sequence and one repressive element are tightly packed within 120 bp. We think it is quite unlikely that these organized *cis*-elements have been produced simply by genetic drift and that they are not really functional in amphioxus. In support of this notion, amphioxus *Ets1/2* is expressed in the pharyngeal endoderm and in the preoral pit where *AmphiHox2* is expressed. Therefore, our results suggest that amphioxus Ets is involved in the transcriptional regulation of *AmphiHox2* in the preoral pit.

For retention of *AmphiHox2*, new *cis*-elements that drive expression in the preoral pit may have been necessary but would not be sufficient. For *AmphiHox2* to be retained, it must also have performed some developmental role that was under selective pressure. Hatschek's pit of amphioxus, which derives from the preoral pit, is generally regarded to be homologous to the vertebrate adenohypophysis (Gorbman, 1995). Genes that are involved in the differentiation of the adenohypophysis, such as *Ptx*, *islet* and *Pax6*, are expressed in the amphioxus preoral pit (Boorman and Shimeld, 2002; Christiaen et al., 2002; Glardon et al., 1998; Jackman et al., 2000; Yasui et al., 2000). *Ets1* is also involved in the development of the adenohypophysis and has been shown to activate the transcription of prolactin in the vertebrates (Bradford et al., 1997; Howard and Maurer, 1995). Thus, the expression of *AmphiEts1/2* in the preoral pit is consistent with the homology between the preoral pit and the adenohypophysis. If so, what developmental role does *AmphiHox2* perform in the preoral pit? At this time, we do not have a clear answer to this question and can only speculate. One possibility is that *AmphiHox2* is involved in some aspect of the preoral pit that is not seen in vertebrates. Such a feature of the amphioxus preoral pit can be identified. The preoral pit is formed as a combination of the pharyngeal ectoderm and the gut diverticulum of mesendodermal origin, while the counterparts in ascidians and vertebrates are solely of ectodermal origin (Schwind, 1928; but see Gorbman, 1983). On the other hand, *Drosophila* genes that have lost "Hox functions," such as *zen*, *ftz* and *bicoid*, are not involved in development of characters specific to *Drosophila* or their relatives. Rather, they are involved in general characters (anterior–posterior patterning, segmentation and extra-embryonic tissue differentiation) (Frohnhofer and Nusslein-Volhard, 1986; Rushlow and Levine, 1990; Wakimoto et al., 1984) that are shared by other insects or arthropods. Thus, these genes may have taken over developmental roles that were performed by other genes. Similarly, *AmphiHox2* may have taken over some roles in preoral pit differentiation.

It is worth noting that *Hox2* has lost its colinear expression pattern, not only in amphioxus, but also in two species of urochordates: *Oikopleura dioica* (larvacean) and *C. intestinalis* (Ikuta et al., 2004; Seo et al., 2004). However, the new expression of *CiHox2* in the trunk lateral cells may not be controlled by Ets. *Hox2* genes probably lost their "Hox functions" independently in amphioxus and in urochordates. It may simply be a coincidence that it is *Hox2* that has lost colinear expression in these two lineages; however, it might be linked to the absence of retinoic acid responsive elements (RARE) flanking *Hox2*. Retinoic acid performs important roles in the transcriptional regulation of Hox genes, especially in the 3′ Hox genes (Bel-Vialar et al., 2002; Gavalas and Krumlauf, 2000). Recently, it has been shown that RARE may be involved not only in the transcriptional control of the flanking genes, but also in more global regulation of the chromosome structure or location within the nucleus (Cambeyron and Bickmore, 2004). Conserved RARE are found in the flanking regions of *Hox1*, *Hox3* and *Hox4*, but not in the flanking region of *Hox2* in vertebrate clusters (Mainguy et al., 2003). We have found conserved RARE sequences in the flanking regions of *AmphiHox1* and *AmphiHox3*, but not in those of *AmphiHox2* (Manzanares et al., 2000; Wada et al. in preparation). The absence of RARE in the sequences surrounding the *Hox2* gene might account for the observation that *Hox2* loses colinear expression more readily.

In this study, we were able to analyze the *cis*-regulatory activity of an amphioxus gene using ascidian eggs and succeeded in mapping some of the responsible *cis*-elements. In contrast to the amphioxus, whose embryos can be obtained in only a few places and during only a few months of the year, *Ciona* eggs can be obtained around the world throughout most of the year. In addition, large numbers of transgenic *Ciona* embryos are easily obtained by electroporation (Corbo et al., 1997). Moreover, most of the transcriptional factors have been characterized in the *Ciona* genome, and their expression patterns are available in public databases (Imai et al., 2004). Therefore, *Ciona* is an excellent system for *cis*-regulatory analyses. This study indicated that *Ciona* embryos can be utilized, not only for *cis*-analyses of *Ciona* genes, but may also be useful for analysis of *cis*-elements in other species, such as amphioxus and acorn worms, whose eggs are relatively difficult to access. In addition, we indicated that mouse β-globin promoter is functional in *Ciona*. In many cases, the *forkhead* promoter has been utilized as a basal promoter in *Ciona* transgenic system (e.g. Harafuji et al., 2002; Keys et al., 2005). Since *cis*-elements of *Ciona* are often observed in close proximity of basal promoter, it is possible the basal promoter from *Ciona* genes may contain additional weak *cis*-elements for tissue specific expression that is not

sufficient alone to drive reporter expression. The mouse β-globin promoter may be utilized with other enhancer elements as a less-biased promoter. As transcription of *CiHox2* is probably not controlled by Ets, a homologous regulatory system may not be necessary for the foreign *cis*-elements to work in *Ciona*. On the other hand, we found that the RARE sequences from *AmphiHox1* or *AmphiHox3* cannot function in *Ciona*, even when combined with several different minimal promoters, including that of *CiHox1* (Wada et al., in preparation), although the *Ciona Hox* genes are also controlled by retinoic acid (Nagatomo and Fujiwara, 2003). Thus, although homologous systems operate in *Ciona*, foreign *cis*-elements are not guaranteed to function. However, in those cases in which the transgenic system functions, *Ciona* obviously provides a powerful tool for the analysis of *cis*-regulatory activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.06.018.

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