Making very similar embryos with divergent genomes: conservation of regulatory mechanisms of *Otx* between the ascidians *Halocynthia roretzi* and *Ciona intestinalis*

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Summary

Ascidian embryos develop with a fixed cell lineage into simple tadpoles. Their lineage is almost perfectly conserved, even between the evolutionarily distant species *Halocynthia roretzi* and *Ciona intestinalis*, which show no detectable sequence conservation in the non-coding regions of studied orthologous genes. To address how a common developmental program can be maintained without detectable cis-regulatory sequence conservation, we compared in both species the regulation of *Otx*, a gene with a shared complex expression pattern. We found that in *Halocynthia*, the regulatory logic is based on the use of very simple cell line-specific regulatory modules, the activities of which are conserved, in most cases, in the *Ciona* embryo. The activity of each of these enhancer modules relies on the conservation of a few repeated crucial binding sites for transcriptional activators, without obvious constraints on their precise number, order or orientation, or on the surrounding sequences. We propose that a combination of simplicity and degeneracy allows the conservation of the regulatory logic, despite drastic sequence divergence. The regulation of *Otx* in the anterior endoderm by Lhx and Fox factors may even be conserved with vertebrates.

Key words: *C. intestinalis*, *H. roretzi*, Ascidian, Otx

Introduction

In recent years, the ascidian larva has often been regarded as an organism close to the ancestral form of chordates, and has served as an interesting and informative model system for understanding the genesis of a simple chordate body plan (Satoh et al., 1996; Satoh et al., 2003). *Halocynthia roretzi* and *Ciona intestinalis* are representative species of the two orders, the Pleurogona and the Enterogona, which constitute the class Ascidiacea. Although these two classes probably diverged deep in the history of ascidian evolution, their embryos show remarkable similarity, with almost perfect conservation of the lineages up to the early gastrula stages. In addition, the recent cloning of a large number of genes in both species has shown a remarkable conservation of their embryonic expression profiles. This applies in particular to homeobox genes such as the *Otx*, *Pax* and *Hox* genes, which are expressed along the anteroposterior axis in the larval central nervous system (CNS) with distinct expression domains; these expression domains are also very similar to their vertebrate orthologs (Wada et al., 1998).

The conservation of expression domains between ascidian species, and with vertebrates, raises the possibility of the conservation of regulatory logics within the chordate lineage. However, cross-species analysis of the activity of the regulatory regions of *Ci-Hox3* in the mouse suggested lack of conservation (Locascio et al., 1999). Even between ascidians, two observations have been made that suggest a possible divergence of regulatory networks in spite of strikingly similar embryonic development. First, existing cDNA/EST and genomic data suggest a very poor sequence conservation between *Halocynthia* and *Ciona*. For example, the coding sequences of *Halocynthia* and *Ciona Brachyury*, a T-box gene specifically expressed in the notochord of both species, are remarkably different (Marcellini et al., 2003). Second, a previous report has suggested that *Brachyury*, may be regulated by very different mechanisms in *Halocynthia* and *Ciona* (Takahashi et al., 1999).

To readdress the question of the conservation of the regulatory logic among ascidians, and with vertebrates, we chose *Otx* as a model, as it is one of the most phylogenetically conserved developmental genes. *Otx/otd* genes have been isolated from various animal species, including cnidaria, *Drosophila*, ascidians and vertebrates (Bally-Cuif et al., 1995; Finkelstein and Perrimon, 1991; Hudson and Lemaire, 2001; Li et al., 1994; Pannese et al., 1995; Simeone et al., 1993; Smith et al., 1999; Wada et al., 1996). They have the same expression domain, in the anterior part of embryos, suggesting the evolutionary conservation of essential roles in the

formation and patterning of anterior embryonic territories. Consistently, *Drosophila*, mouse or ascidian embryos mutated or knocked down for *Otx/otd* genes exhibit defects in head structures, such as deletion or differentiation deficiency in the anterior central nervous system (CNS) (Acampora et al., 1996; Acampora et al., 1995; Ang et al., 1996; Finkelstein and Perrimon, 1991; Matsuo et al., 1995; Satou et al., 2001, Wada et al., 2004).

In mouse embryos, the expression pattern of *Otx* genes is complex and very dynamic. *Otx2* is first expressed in the anterior visceral endoderm, then in the anterior epiblast during gastrulation. Later, both *Otx2* and *Otx1* are expressed in the developing fore- and mid-brain (Simeone et al., 1993). Although the regulatory sequences driving late expression of *Otx* after gastrulation have received attention in both mouse and the puffer fish (Kimura et al., 1997; Kimura-Yoshida et al., 2004), very little is known about the transcriptional logic driving the onset of expression of *Otx* in the endodermal and neural lineages in vertebrates.

The detailed expression patterns of ascidian *Otx* genes have been characterized (Hudson and Lemaire, 2001; Wada et al., 1996) (the *Otx* gene of *Halocynthia roretzi* has been designated *Hroth* but, hereafter, we rename the gene *Hr-Otx* according to the recent agreement at the Urochordate Meeting, Marseille, 2003). They are reminiscent of the expression pattern of mouse *Otx* genes, and are very similar between *Ci-Otx* and *Hr-Otx*. Ascidian *Otx* genes are first expressed in the endoderm precursors and the anterior CNS precursor cells prior to gastrulation. In addition, they are expressed during cleavage stages in mesodermal cells, including in some muscle and trunk lateral cell precursors. The anterior nervous system and dorsoanterior epidermal cells also express *Otx* during neurula and tailbud stages. Previous reports have shown that 4 kb of *Halocynthia* genomic sequences upstream of the translation initiation sites are sufficient to drive late expression of *Hr-Otx* at the tailbud stages (Oda-Ishii and Saiga, 2003), and that a similar region in *Ciona* recapitulates *Ci-Otx* expression during cleavage stages (Bertrand et al., 2003). Here, we present a careful comparative analysis of the regulatory logic of *Hr-* and *Ci-Otx* before gastrulation, which reveals a strong conservation of the regulatory logic between ascidians in spite of poor sequence conservation in the identified enhancers. Our results also point to a shared regulatory logic in the endodermal territories between ascidians and vertebrates.

Materials and methods

Ascidians

Adult ascidians Halocynthia roretzi were purchased from fishermen near the Asamushi Marine Biological Station, Tohoku University, Aomori, or the International Coastal Research Center of the Ocean Research Institute, University of Tokyo, Iwate, Japan.

Adult ascidians Ciona intestinalis were obtained from the Station de Biologie Marine in Roscoff.

Preparation of reporter constructs

All reporter constructs were prepared by inserting genomic DNA fragments isolated from the 5′-upstream region or the first intron of Hr-Otx into the multicloning site of the pPD46.21 vector, a variant of pPD1.27 (Fire et al., 1990), which harbors the lacZ gene with a nuclear localization signal. The genomic DNA fragments without the putative endogenous promoter of Hr-Otx were inserted into the multicloning site of pMApro reporter, which includes HrMA4a basal promoter region. The plasmid of pMApro was prepared by inserting the basal promoter region of HrMA4a, 36 bp of the 5′ upstream region and 58 bp of the 5′ UTR, into the BamHI/SmaI sites within pPD46.21. The reporter construct p5402-1473 was described previously (Oda-Ishii and Saiga, 2003). Constructs of p812-24MA, #1, #2, #3, #4, #5 and #6 were prepared by inserting PCR products into the SalI/BamHI sites within the pMApro plasmid DNA. To prepare #3ΔT-box, #4ΔLhx/Fox, #5ΔT-box, #6ΔFox, #6ΔLhx and #6ΔLhx/Fox mutations were introduced into the putative binding sites for the transcription factors in the constructs of #3, #4, #5 and #6 using a Gene Editor™ in vitro site-directed mutagenesis system (Promega). Constructs of Lhx/Fox-BS and T-box-BS were prepared by inserting double-stranded synthetic oligonucleotides into the SalI/BamHI sites within the pMApro plasmid DNA.

Microinjection of reporter constructs into Halocynthia roretzi fertilized eggs and detection of lacZ transcripts by whole-mount in situ hybridization

Microinjection of reporter gene constructs into fertilized eggs and whole-mount in situ hybridization were carried out as described previously (Oda-Ishii and Saiga, 2003). Construct plasmid DNAs of the circular form were dissolved in 1 mM Tris-HCl, 0.1 mM EDTA (pH 8.) at the concentration of 3 to 20 ng/μl, and about 90 pl was injected into a fertilized egg. For each construct, 20-70 embryos were injected, cultured, and examined for *lacZ* transcripts, and at least two independent experiments were carried out.

Electroporation of reporter constructs into Ciona intestinalis fertilized eggs and detection of lacZ transcripts by whole-mount in situ hybridization

Electroporation of reporter constructs into fertilized eggs of *Ciona intestinalis* and whole-mount in situ hybridization were carried out as described (Bertrand et al., 2003). For each construct at least 100 electroporated embryos were screened and experiments were performed at least twice.

Sequence comparison and binding sites prediction

Sequence comparisons between the *Ciona* and *Halocynthia* upstream regions were performed using general alignment programs (local: DNAsis, Blast 2 sequences, Dot plot; global: ClustalW) or cis-regulatory-devoted comparison programs (Vista, zPicture, Pipmaker and Family Relations). Binding site predictions were performed with Matinspector (GATA and Ets) or TFsearch (Fox), or by searching for published consensus sequences: T-box (Erives and Levine, 2000) or Lhx (Bridwell et al., 2001; Mochizuki et al., 2000).

Results

Halocynthia and *Ciona* upstream regions recapitulate *Otx* early expression when introduced into *Halocynthia* embryos

Endogenous expression of *Hr-Otx* starts at the 32-cell stage in a part of the animal a-line (precursors of the anterior nervous system and anterior epidermis), b-line (precursors of the dorsal neural tube) and vegetal B-line (precursors of the posterior mesendoderm) (Fig. 1B,B′). By the 44-cell stage, expression become undetectable. At the 64-cell stage, however, transcription of *Hr-Otx* is reactivated in the a-, b- and B-line cells, as well as in some A-line cells (precursors of the anterior mesendoderm) (Fig. 1D,D′) (Wada et al., 1996). To identify cis-regulatory regions that recapitulate *Hr-Otx* transcription during cleavage stage, we injected fertilized eggs with a construct, p5402-1473, in which the upstream region, 5402-1473 bp, previously shown to recapitulate the tailbud

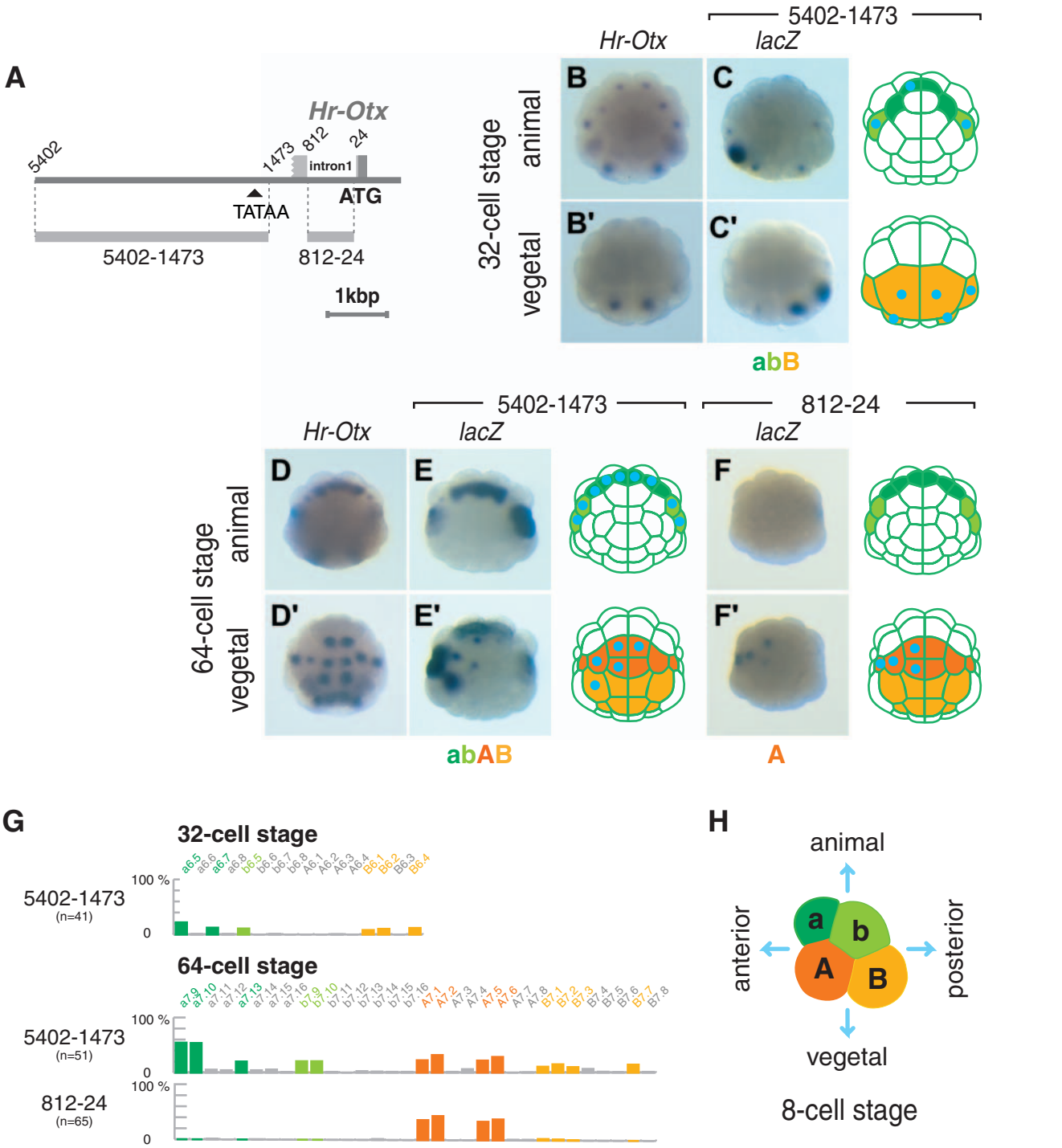


Fig. 1. The upstream (5402-1473 bp) and the first intron (812-24 bp) regions are capable of directing *Hr-Otx* transcription during cleavage stages. (A) The structure of 5′ region of *Hr-Otx*, including the putative endogenous promoter (TATAA) and intron 1. Boxes on the gray line indicate exons; ATG indicates the translation start site, which is located in the second exon. The light gray lines are the genomic regions examined for their transcription-driving activity. (B,B′,D,D′) Expression of *Hr-Otx* detected by whole-mount in situ hybridization. (C,C′,E-F′) Transcription of *lacZ* in embryos injected with the reporter construct harboring the upstream region 5402-1473 (C,C′,E,E′), or the intron region 812-24 (F,F′), as visualized by in situ hybridization. In injected embryos, not all *Hr-Otx*-expressing cells are stained because of the mosaic inheritance of injected DNA. Specimens at the 32-cell stage (B-C′) and 64-cell stage (D-F′) are shown. (B-F) Animal view; (B′-F′) vegetal view. A schematic representation of in situ hybridization is shown on the right side of each specimen (C,C′,E-F′). Cells expressing *Hr-Otx* are colored, according to their derivation from the 8-cell embryo (H), as follows: a-line, dark green; b-line, light green; A-line, orange; and B-line, yellow. Blue dots indicate the cells in which *lacZ* transcripts were detected. Below the specimens of whole-mount in situ hybridization, the identity of the *lacZ*-transcribing cell line is indicated. (G) Frequency of *lacZ* -positive cells for a given cell line in embryos injected with the construct harboring the region 5402-1473bp or 812-23bp, examined at the 32-cell stage (top) and 64-cell stage (bottom). Color codes are as in H. Gray indicates ectopic expression. Number in parenthesis indicates number of embryos examined.

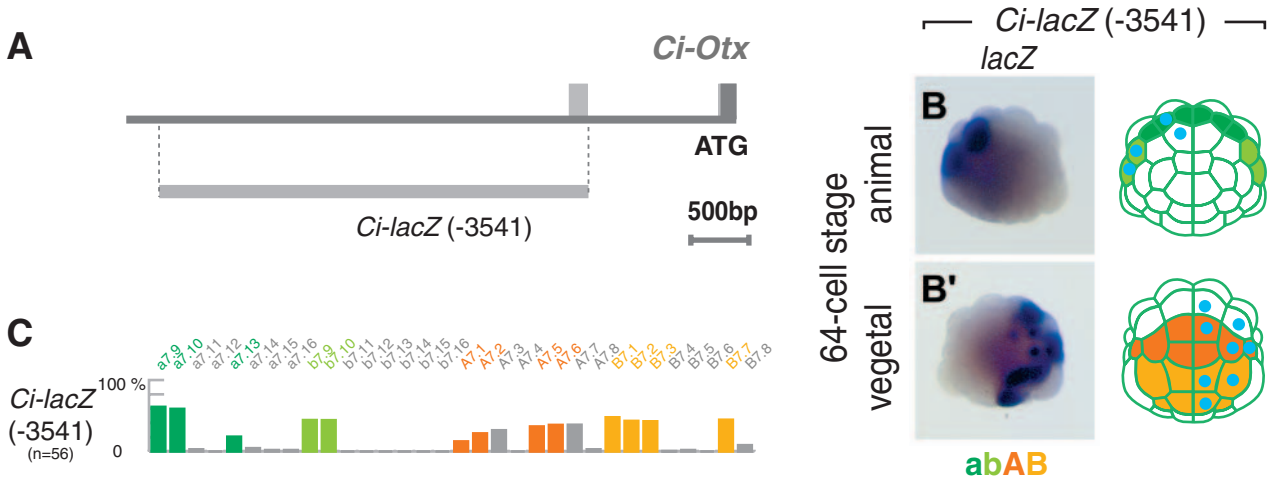


Fig. 2. The 3.5-kb upstream region of *Ciona Otx* is capable of reproducing endogenous *Otx* expression pattern in *Halocynthia* embryos. (A) The structure of the 5′ region of *Ci-Otx*. Boxes on the gray line indicate exons. ATG indicates the translation start site, which is located in the second exon. The light gray line represents the upstream region included in the construct –3541 (Bertrand et al., 2003). (B,B′) Transcription of *lacZ* in 64-cell stage embryos injected with the reporter construct –3541, as visualized by whole-mount in situ hybridization. Animal (B) and vegetal (B′) views are shown. Schematic representation of the in situ hybridization specimen is shown right (B,B′). Cells with endogenous *Hr-Otx* expression are colored as in Fig. 1. Blue dots indicate the cells in which *lacZ* transcripts were detected. Below the in situ hybridization specimens, the *lacZ* -positive cell line is indicated. (C) Frequency of *lacZ*-transcript-positive cells for the given cell line in embryos injected with the –3541 construct, examined at the 64-cell stage.

expression of *Hr-Otx* (Oda-Ishii and Saiga, 2003), was combined with *lacZ* reporter gene. Injected embryos were analyzed for *lacZ* transcripts by whole-mount in situ hybridization at the 32- or 64-cell stage. *lacZ* transcripts were detected exclusively in the cells of injected embryos, which express endogenous *Hr-Otx* (Fig. 1C,C′,E,E′). Thus the 5402-1473 bp upstream region is capable of reproducing the expression pattern of *Hr-Otx* at cleavage stages.

The first intron, which is located immediately upstream of the translation start sites of *Hr-Otx* (812-24bp), also contained regulatory elements active during cleavage stage. Embryos injected with p812-24MA, in which the first intron (812-24 bp) was placed upstream of the HrMA4a basal promoter driving *lacZ*, expressed *lacZ* transcripts in the A-line cells expressing endogenous *Hr-Otx* from the 64-cell stage (Fig. 1F,F′,G). Slightly later, starting after 110-cell stage onward, p812-24MA was capable of directing ectopic transcription in all a-line cells (anterior ectoderm precursors) (data not shown).

While we have previously shown that the first intron of *Ci-Otx* has no enhancer activity in *Ciona* before the onset of gastrulation, a reporter construct, –3541, in which 3.5 kb upstream of the first exon was placed upstream of the *lacZ* reporter gene (Fig. 2A), recapitulated the early expression of *Otx* in *Ciona* (Bertrand et al., 2003). To test whether the global transcriptional logic driving *Otx* early expression in the two species is conserved, we introduced the *Ci-lacZ* (–3541) construct into the fertilized eggs of *Halocynthia roretzi* by microinjection and examined the resulting embryos for *lacZ* transcripts at the 64-cell stage (Fig. 2). This construct directed *lacZ* transcription in mostly the same pattern as it does in *Ciona* (Bertrand et al., 2003) with two exceptions. First, the construct also directed *lacZ* transcription in the notochord precursors (A7.3, A7.7), in which *Otx* transcription is observed in neither species (Fig. 2B′,C). This may represent an artefactual expression. Second, *lacZ* transcription was also detected in the a7.13 blastomeres, which do not normally express *Ci-Otx* (Fig. 2C). In *Halocynthia*, however, these blastomeres express *Hr-Otx* (Wada et al., 1996). This suggests that the expression of endogenous *Hr-Otx*, but not *Ci-Otx*, in the a7.13 is not due to differences in the promoter regions in these two species, but rather to the way the embryos interpret a common cis-regulatory logic.

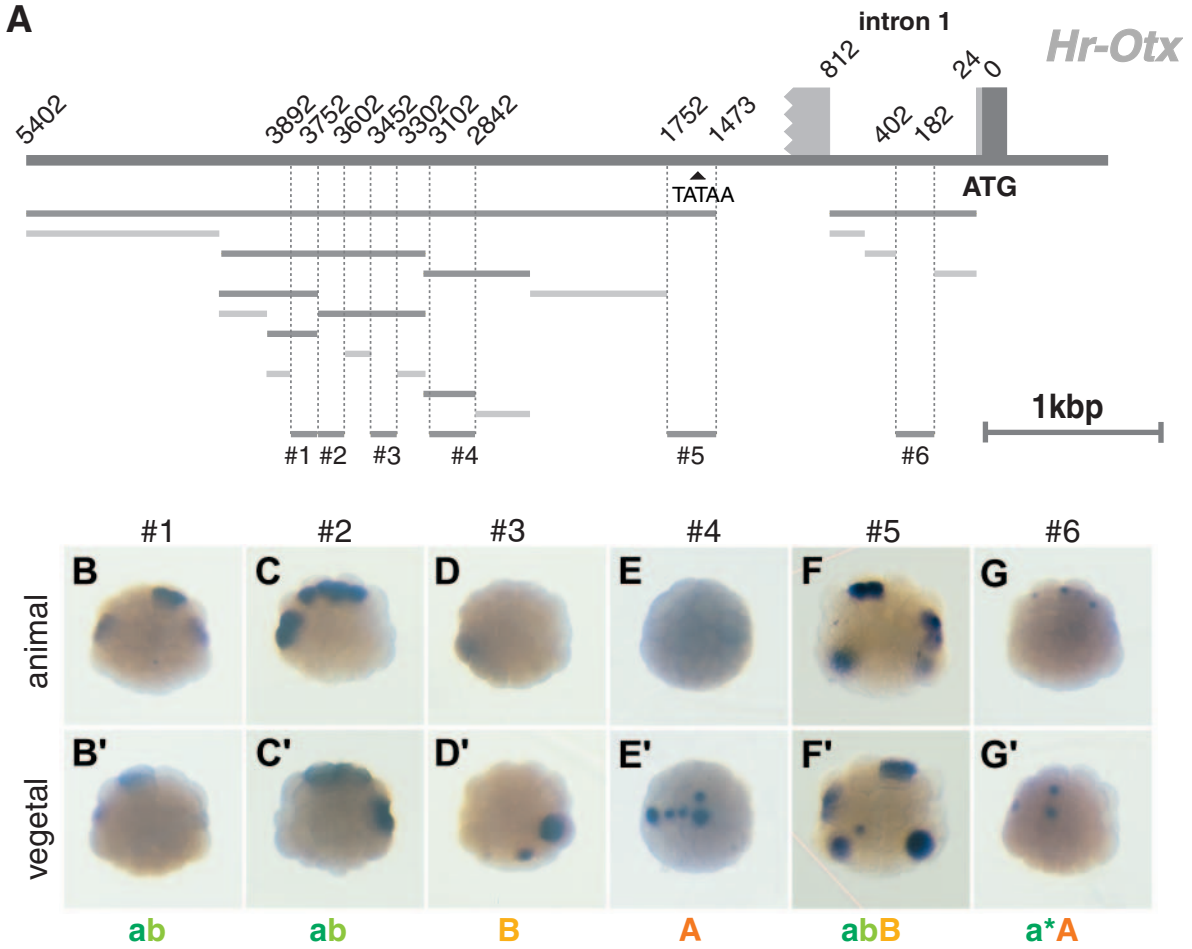
Taken together, the results presented here support the conservation of the regulatory logic driving *Otx* expression in *Halocynthia* and *Ciona*. Surprisingly, comparison of the *Halocynthia* and *Ciona* upstream sequences down to the sixth coding exon did not reveal any block of sequence conservation outside of transcribed sequences (see Fig. S1 in supplementary material).

Transcription of *Hr-Otx* during the cleavage stage is regulated by line-specific regulatory modules functionally conserved in *Ciona*

To dissect regulatory regions directing *Hr-Otx* transcription, we prepared various deletion constructs of p5402-1473 and p812-24MA and examined embryos injected with them for *lacZ* transcripts at the 64-cell stage. These constructs are summarized in Fig. 3A. This approach allowed us to identify six regulatory modules of 140-280bp (#1 to #6) that are sufficient to drive expression in subdomains of *Hr-Otx*-expressing territories (Fig. 3, Fig. 4A-D). Regulatory modules #1 and #2 acted as enhancers, which, when placed upstream of the HrMA4a basal promoter, directed transcription in both a- and b-line cells expressing *Hr-Otx* (Fig. 3B-C′,H). By contrast, regulatory modules #3 and #4, placed in front of the same basal promoter, directed transcription only in the B- and A-line cells normally expressing *Hr-Otx*, respectively (Fig. 3D-E′,H). Regulatory module #5, which included the endogenous *Hr-Otx* promoter, directed transcription in the *Hr-Otx*-expressing cells

of the a-, b- and B-lines, but not in the A-line cells (Fig. 3F,F′,H). Finally, the intronic regulatory module #6, placed in front of the HrMA4a basal promoter, directed transcription in the A-line cells expressing *Hr-Otx*, as well as some ectopic expression in a-line cells (Fig. 3G,G′,H). These results suggest that the complex expression pattern of *Hr-Otx* during cleavage stage is the result of the combined activities of multiple transcription regulatory modules with distinct specificities, each, in most cases, working in one or two lines only.

We next tested the conservation of the activity of these modules in *Ciona* by introducing constructs #1 to #6 into the fertilized eggs of *Ciona intestinalis* by electroporation.



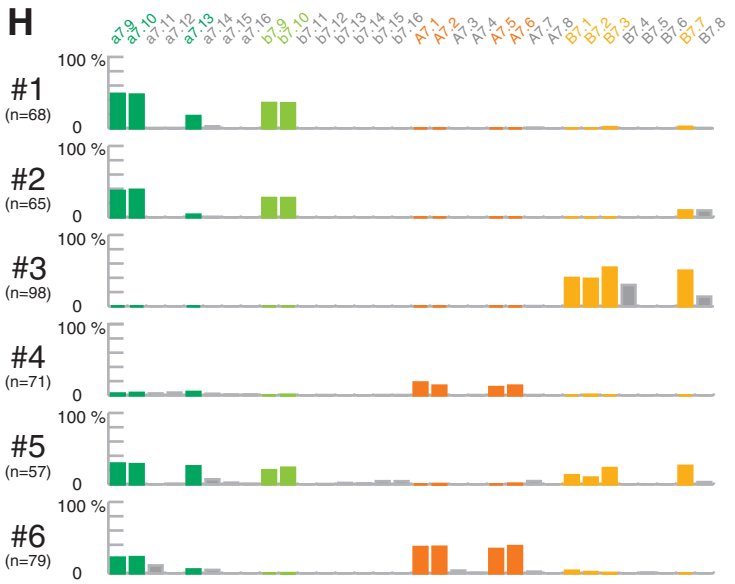
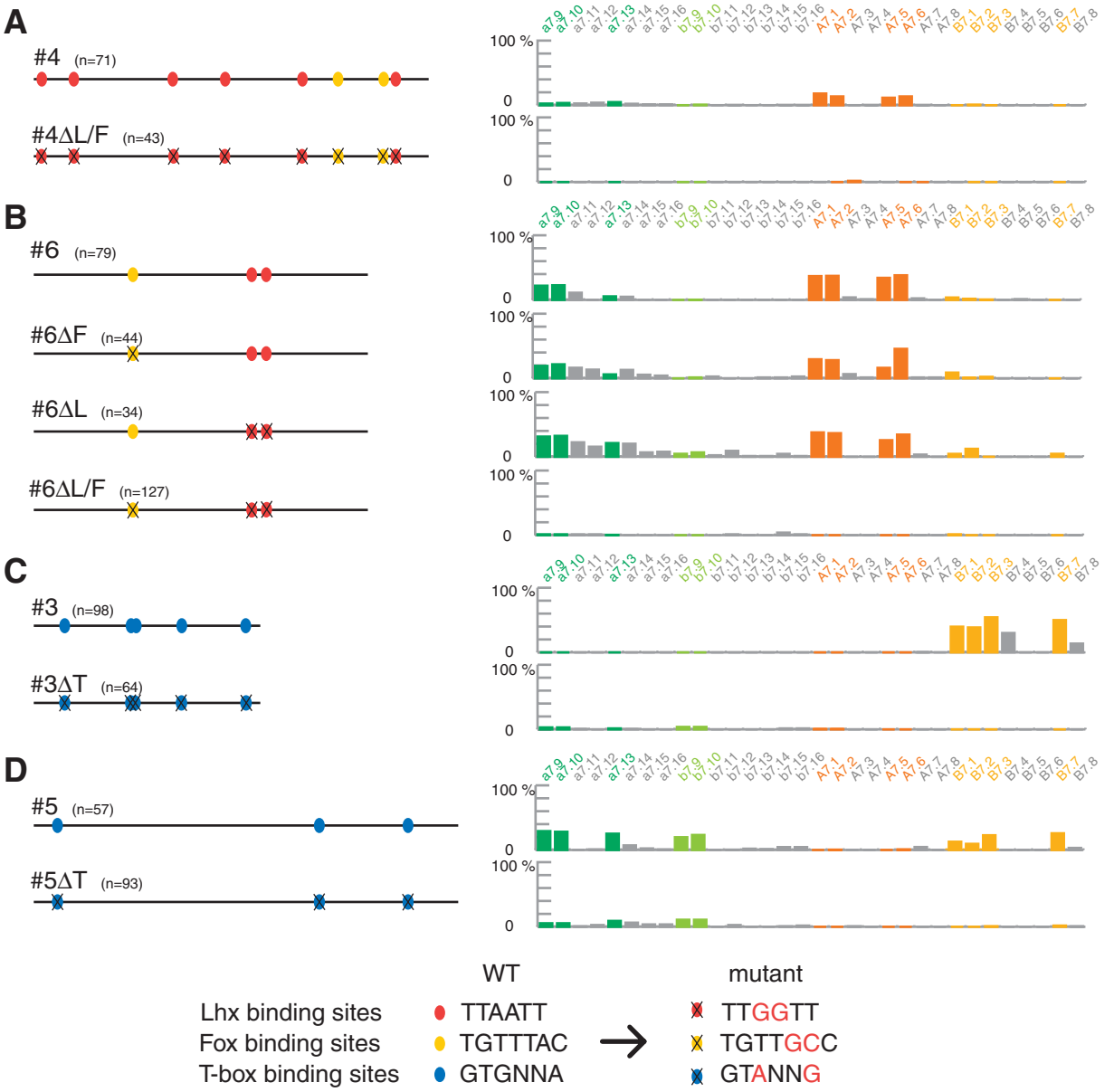
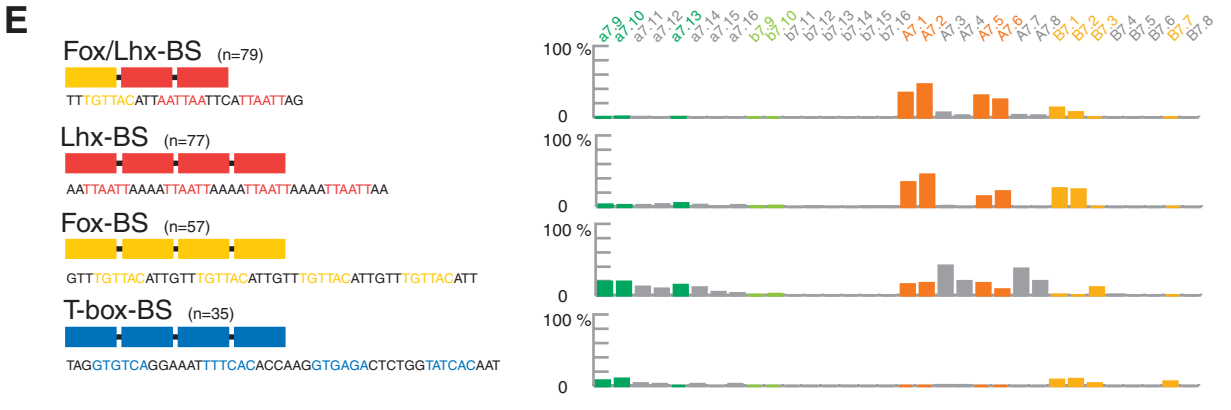


Fig. 3. Line-specific regulatory modules responsible for regulating *Hr-Otx* transcription during cleavage stage. (A) Schematic representation of the regions, 5402-1473 bp and 812-24 bp. For analysis of their transcriptional activity, regions lacking the putative endogenous *Hr-Otx* promoter were placed upstream of the *HrMA4a* basal promoter. Dark gray lines indicate the region that exhibited *lacZ* transcription in *Hr-Otx*-expressing cells. The positions of the line-specific transcription regulatory modules (#1-#6) are indicated at the bottom. The regions marked by light gray lines directed *lacZ* transcription only weakly during cleavage stage. (B-G′) Transcription of *lacZ* in 64-cell stage embryos injected with reporter constructs harboring regulatory modules #1 (B,B′), #2 (C,C′), #3 (D,D′), #4 (E,E′), #5 (F,F′) and #6 (G,G′), as visualized by whole-mount in situ hybridization. Animal (B-G) and vegetal (B′-G′) views are shown. The identity of the *lacZ*-transcribing cell line is indicated below the in situ hybridization specimens. Asterisk indicates that module #6 also drives *lacZ* transcription in all a-line cells from the 110-cell stage onward. (H) Frequency of *lacZ*-positive cells for a given cell line in 64-cell embryos injected with constructs harboring modules #1-#6. For color codes see Fig. 1

Resulting embryos were examined for *lacZ* transcripts at the 64-cell stage. As shown in Fig. 5A-G, five out of the six regulatory modules exhibited transcription-directing activity in *Ciona* embryos. Regulatory module #1, which is capable of directing *Hr-Otx* transcription in the a- and b-line cells, failed to direct *lacZ* expression, but the regulatory modules,





#2, #3, #4, #5 and #6 directed the same line-specific *lacZ* expression in *Ciona* as that observed in *Halocynthia* embryos. With module #4, the activity was very low at the 64-cell stage (Fig. 5G) but became much stronger at the 76-cell stage (Fig. 5H).

Thus, the functional conservation of the full upstream region is due to the conservation of the activity of the small line-specific enhancer modules between the two species.

Putative binding sites for transcription factors in the regulatory modules of *Otx*

Regulatory modules required for *Ci-Otx* early expression in specific lines in *Ciona* have been identified previously (Bertrand et al., 2003). The functional conservation of *Hr-Otx* line-specific enhancer modules suggests that the regulatory logic driving *Otx* expression in each line is conserved between species without sequence conservation between the modules of the two species. To further test this hypothesis, we compared the putative binding sites present in regulatory modules of similar specificities in both *Ciona* and *Halocynthia*.

In *Ciona*, activation of *Otx* in the neural a- and b-lines results from the action of clustered ETS- and GATA-binding sites, which transduce the neural inducing activity of *Ci-Fgf9/16/20* (Bertrand et al., 2003). The two *Hr-Otx* regulatory modules capable of directing transcription in the ab-line cells of both *Ciona* and *Halocynthia* (#2 and #5) contain clustered binding sites for both Ets and GATA factors (#2, eight GATA sites, one Ets site; #5, four GATA sites, one Ets site; see Fig. S1 in supplementary material), suggesting conservation of the role of ETS and GATA factors in these lines.

In the vegetal lines, it has been demonstrated that *Ciona savignyi Otx* is positively regulated by β-catenin (Satou et al., 2001), and three additional β-catenin targets expressed earlier than *Otx* in the endodermal lineage have been identified, which are the Lim homeobox gene *Cs-Lhx3* and the winged helix genes *Cs-FoxD* and *Cs-Hnf3*. Similarly, the *Halocynthia* orthologs of *Cs-Lhx3* (*Hr-Lim*) and *Cs-Hnf3* (*Hr-FoxA5*) are expressed in the A-line before *Otx* (Wada et al., 1995) (Shimauchi et al., 1997). Consistent with the possibile involvement of these or similar factors in *Hr-Otx* regulation, the A-line modules #4 and #6 shared clustered putative binding sites (hereafter, a putative binding site will be referred to as a BS) for Lhx and Fox (#4, six Lhx sites, two Fox sites; #6, four Lhx sites, one Fox site; see Fig. S1 in supplementary material). In *Ciona*, consensus BSs for Lhx and Fox factors were also found in the module necessary for early A-line expression (Fig. 6, see also Fig S1 in supplementary material).

Finally, T-box proteins have been implicated in the specification of some B-line cells in *Ciona* and *Halocynthia* (Erives and Levine, 2000; Mitani et al., 1999). The regulatory modules driving the B-line expression (#3 and #5) possessed BSs for T-box proteins (#3, five T-box sites; #5, three T-box sites; see Fig. S1 in supplementary material). T-box binding motifs were also present in the *Ciona* sequences required for B-line expression of *Otx* (Fig. 6, Fig. S1 in supplementary material).

As the transcriptional regulatory logic of *Otx* in the vegetal hemisphere is less well understood than that driving expression in the animal lines, we focused on the transcription factors regulating *Hr-Otx* transcription in the A- and B-lines, and in particular on the role of the Lhx, Fox and T-box BSs identified above. For this, we next examined the effect of mutating these BSs on the transcriptional activity of the identified enhancers.

Putative Lhx and Fox binding sites have partially overlapping roles in *Hr-Otx* transcription in A-line cells

We introduced two point mutations into each of the BSs for Lhx and/or Fox in regulatory modules #4 and #6. Mutation of all Lhx BSs, or of all Fox BSs, had no significant effect on the activity of module #6 (Fig. 4B). However, combined mutations of all identified Lhx and Fox BSs (#6ΔL/F) led to inactivation of the module (Fig. 4B). Mutation of Lhx and/or Fox BSs in module #4 also led to its inactivation (data not shown, Fig. 4A). The lower intrinsic activity of this module (Fig. 4A) may account for its inactivation by single mutations. Consistent with a conserved regulatory logic between *Halocynthia* and *Ciona*, the activity in *Ciona* of modules #4 and #6 was also abolished by mutating all the Lhx and Fox BSs (Fig. 5G,H).

Next, we tested whether combinations of Lhx and Fox BSs were sufficient to drive expression in the A-line. First, we prepared a reporter construct, Lhx/Fox-BS (Fig. 4E), in which a 30-bp DNA fragment derived from the regulatory module #6 containing two BSs for Lhx immediately downstream of a single BS for Fox was placed upstream of the *HrMA4a* promoter driving *lacZ* (Fig. 4E). This construct was active and mainly drove *lacZ* transcription in the A-line *Hr-Otx*-expressing cells (Fig. 4E). Likewise, the construct Lhx-BS, containing four Lhx BSs but no Fox BSs in the same pMA-lacZ context, drove *lacZ* transcription in the A-line. This construct, however, also drove ectopic activation in the B-line (Fig. 4E). Finally, a construct containing four Fox BSs but no Lhx BSs directed *lacZ* transcription in the *Otx*-expressing A-line, as well as in the other A-line cells and in the a-line cells (Fig. 4E).

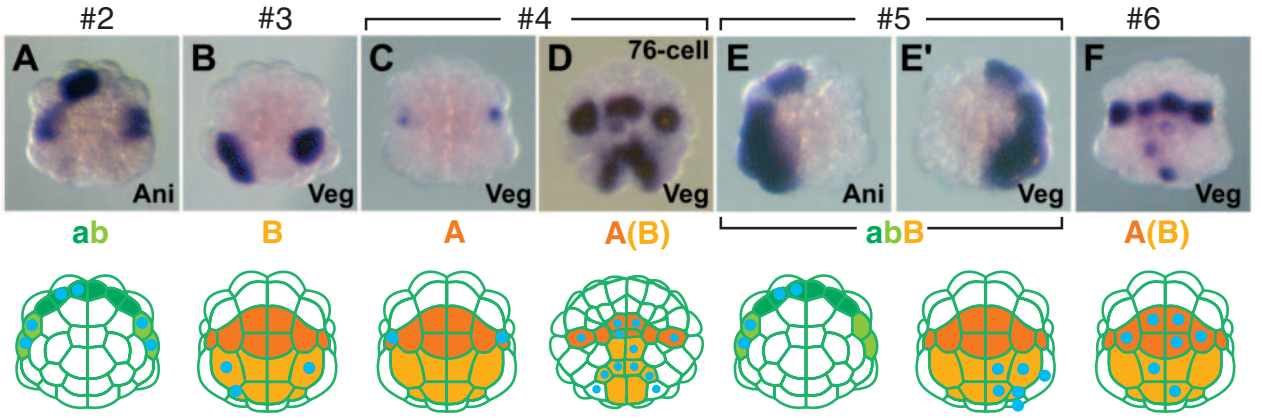
Taken together, the results from this section show that both Lhx and Fox BSs are important for the activity of the regulatory modules #6 and #4 in both *Ciona* and *Halocynthia*, suggesting that in both animals, *Otx* may be a direct target of Lhx and Fox proteins. These transcription factors show some redundancy of function, as a multimer of either Lhx or Fox sites was sufficient to drive A-line expression.

Putative T-box binding sites are required for directing *Hr-Otx* transcription in the B-line cells

The regulatory modules #3 and #5, capable of directing *lacZ* transcription in the B-line cells expressing *Hr-Otx* (Fig. 4C,D),

Fig. 4. Introduction of mutations into the BSs for Lhx, Fox and T-box proteins resulted in a loss of *lacZ* transcription in the vegetal hemisphere. (A-D) Frequency of *lacZ*-positive cells for a given cell in 64-cell embryos injected with reporter constructs harboring the regulatory modules indicated on the left side. Red, yellow and blue ovals represent the binding sites for Lhx, Fox and T-box proteins, respectively. (E) Frequency of *lacZ*-transcribing cells for a given cell lineage in 64-cell embryos injected with reporter constructs harboring the DNA fragments shown on the left side. DNA fragment Lhx/Fox-BS contains the single Fox-binding site and the two Lhx-binding sites from regulatory module #6. DNA fragments Lhx-BS and Fox-BS include four binding sites for Lhx and Fox, respectively, which are from regulatory module #6. The DNA fragment T-box-BS includes four copies of the T-box-binding site present in the regulatory module #3. The nucleotides marked yellow, red and blue represent the putative binding sites for Fox, Lhx and T-box proteins, respectively. For color codes in histograms, see Fig. 1.

contain five and three T-box BSs, respectively. When all of the T-box BSs were mutated (#3ΔT, #5ΔT), both regulatory modules lost their ability to direct *lacZ* transcription in the *Halocynthia* B-line cells (Fig. 4C,D). This requirement for T-box BSs extended to *Ciona* embryos, as mutation of those BSs reduced #3 expression in *Ciona* B-line cells (Fig. 5G). Taken together, T-box proteins are direct regulators of *Otx* transcription in the ascidian B-line cells.



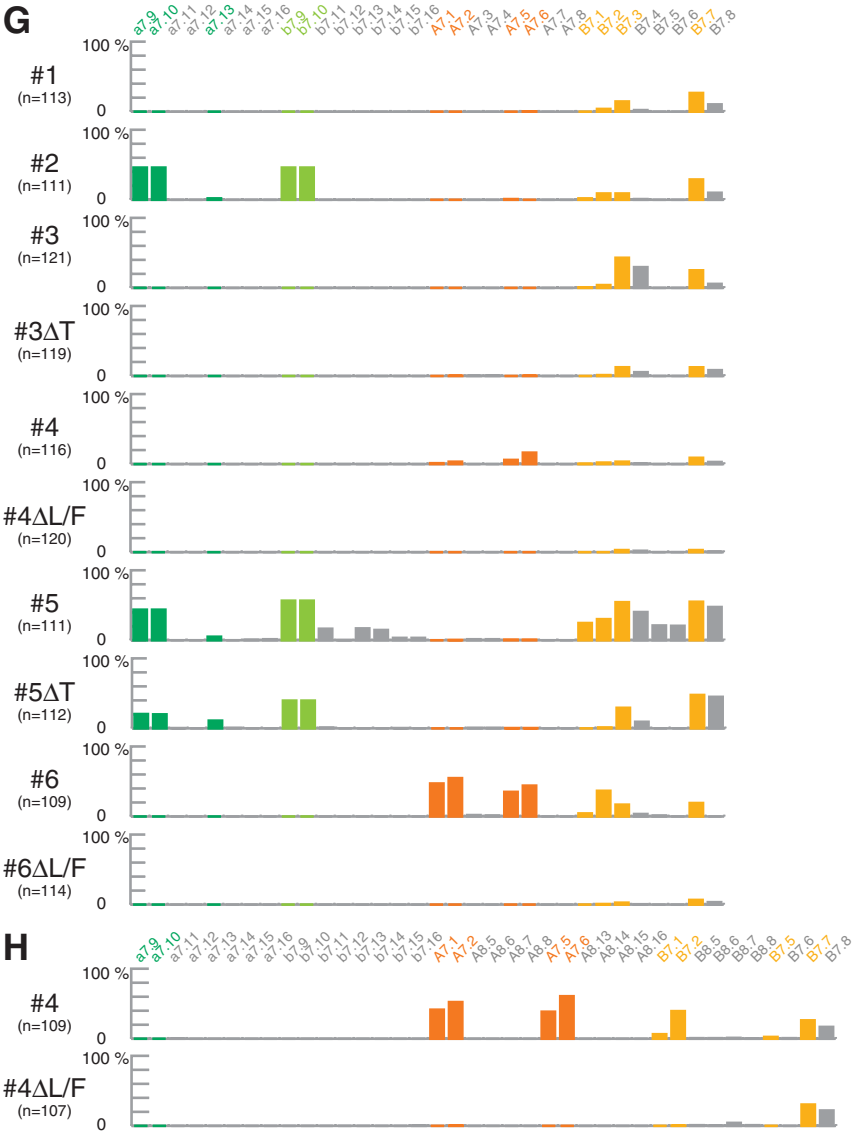


Fig. 5. The regulatory modules of *Hr-Otx* are capable of directing line-specific transcription in *Ciona intestinalis* embryos. The reporter constructs containing regulatory modules #2 (A), #3 (B), #4 (C,D), #5 (E,E′) and #6 (F) were electroporated into *Ciona* fertilized eggs, and the resulting embryos were examined for *lacZ* transcripts at the 64-cell (A-C,E,E′,F) or 76-cell (D) stage. Animal (A,E) and vegetal (B-D,E′,F) views are shown. Shown below the images are the identity of the *lacZ*-expressing cell line in the *Ciona* embryo and a schematic representation of the in situ hybridization specimen. (G,H) Frequency of *lacZ*-positive cells for a given cell line in 64-cell (G) or 76-cell (H) embryos injected with reporter constructs harboring the regulatory modules indicated on the left side. For color codes, see Fig. 1.

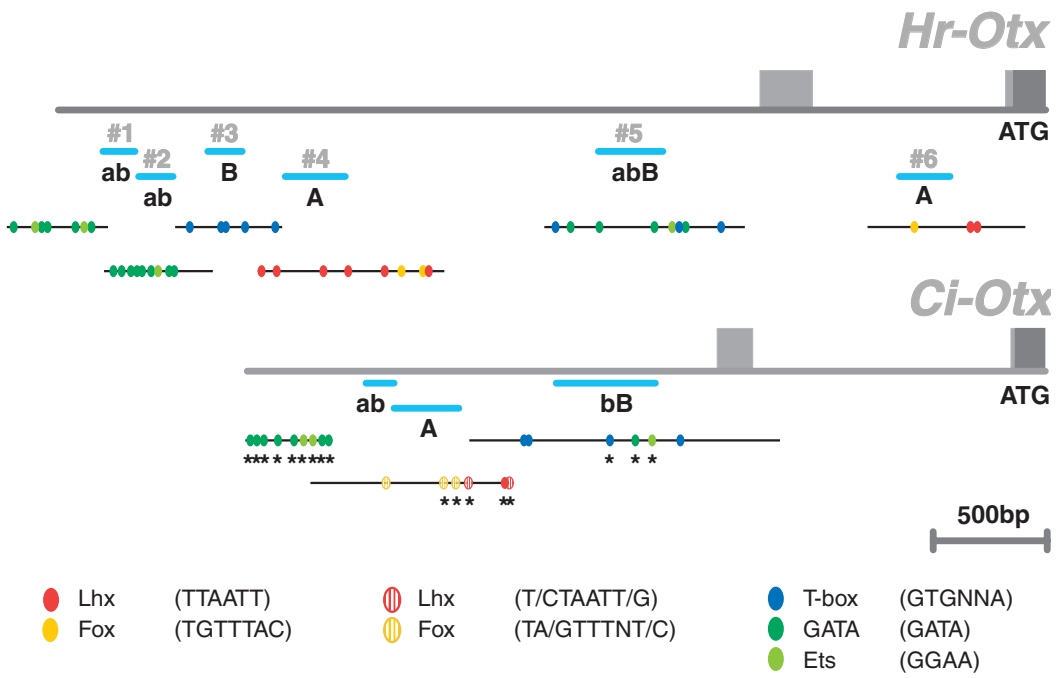


Fig. 6. Comparison of the *Otx* upstream region in *Ciona intestinalis* and *Halocynthia roretzi*. Gray lines indicate the 5′ genomic region of *Otx* genes. Boxes indicate exons; light and dark gray indicate the untranslated and translated regions, respectively. Blue lines indicate the transcription regulatory modules identified in each ascidian species; below the lines, the distribution of putative transcription factor-binding sites (colored small ovals) is indicated for each regulatory module. The name of the cell lineage in which *lacZ* transcripts were detected is indicated for each regulatory module. Color codes for transcription factors and the nucleotide sequences of their binding sites are indicated at the bottom. In *Ciona*, asterisks indicate sites conserved between *C. intestinalis* and *C. savignyi*.

However, a 40-bp DNA fragment, T-box-BS, including four T-box BSs from #3, scarcely directed transcription (Fig. 4E). Therefore, it is likely that T-box proteins collaborate with other factors to direct *Hr-Otx* transcription in the B-line cells.

Discussion

In the present study, we have demonstrated that the regulatory logic of *Otx* is remarkably well conserved between the distantly related ascidian species *Halocynthia roretzi* and *Ciona intestinalis*, relying on the presence of similar transcription factor (TF) binding sites in an otherwise poorly conserved environment, as summarized in Fig. 6 (see also Fig. S1 in supplementary material). In this section, we will first discuss the general properties of the system that allow flexibility before focusing on the factors that may bind the enhancer modules active in the different lines.

Features of the *Otx* cis-regulatory logic in ascidians

Lessons from other invertebrate and vertebrate systems led to the formulation of a set of rules guiding the properties of cis-regulatory modules active in developmental systems (Arnone et al., 1997; Davidson et al., 2002; Kulkarni and Arnosti, 2003). First, each module acts in a distance-independent manner on exogenous minimal promoters, and their activity is independent of the other modules. Second, characterized regulatory modules in flies, mouse and sea urchin bind, on average, close to five different transcription factors belonging to different classes. Third, the activity of the modules requires their binding by both transcriptional activators and repressors. Fourth, modules are often redundant, the deletion of one causing the expression of the corresponding gene in its territory of activity (Arnosti, 2003). Last, in contrast to some terminal differentiation enhancers in which the orientation and spacing of TF-binding sites are important, the arrangement of sites found in developmental modules seems to be plastic (Struhl, 2001). The regulatory modules identified in this study respect some of these rules, but depart from others.

As in other systems, the modules described here, have a modular nature and they are able to activate exogenous minimal promoters when placed immediately upstream. Hence, the precise distance that separates them from the promoter does not appear to be critical. Nevertheless, each of the ab, A and bB modules found in *Ciona* by deletion analysis correspond to a *Halocynthia* module of similar activity located in a similar position (#1+#2, #4 and #5, respectively; see Fig. 6). Whether there is any constraint on the position of the modules should be considered in future. In addition to these shared modules, two additional modules are present in *Halocynthia*, an upstream B-line module (#3) and a first intron A-line module (#6). The larger genome size of *Halocynthia* (Satoh, 1994) thus correlates with an increased redundancy of modules, rather than with the use of more complex modules. Comparative analysis of the cis-regulatory sequences of additional genes with a complex expression pattern will clarify whether this correlation is a general rule in urochordates.

As expected, each module harbors crucial binding sites for transcription factors of different classes. The ab-module contains ETS (Ets domain) and GATA (zinc-finger domain) BSs, the A-module, Lhx (homeodomain) and Fox (winged helix domain) BSs, the B-module T-box BSs, as well as as yet uncharacterised collaborating binding sites. The presence of these different classes of TF-binding sites is in keeping with previous work, but it should be noted that the ab- and A-modules are extremely simple. Their activity can be accounted for by the presence of only two types of TF-binding sites in each case, as shown by the reconstruction of their activity by multimers of isolated binding sites (this work) (see also Bertrand et al., 2003). In the A-line, the tetramer of Fox- or Lhx-BS directed expression in the endodermal A-line, and in the rest of the A- and a-lines (Fox-BS) or in the B-line (Lhx-BS). As the only common territory of activity between Lhx-

BS and Fox-BS corresponds to the A-line endoderm (Fig. 4E), we propose that the restriction of the activity of the A-modules to the endoderm is due to a synergy between the sites whose activities overlap solely in the A-line endoderm. A similar logic has been demonstrated in the ab-module, which is solely activated in territories where the activities of GATA- and ETS-binding sites overlap (Bertrand et al., 2003).

We have no evidence for the involvement of crucial repressors in the ab-line and A-line modules, as the activity of these modules can be reconstituted mainly by the combination of two binding sites mediating activation. This contrasts with the general experience from *Drosophila* and sea urchin, in which very precise expression patterns, such as the one described here, are the result of interplay between activators and repressors.

Finally, comparison of the sequence of the different regulatory modules found in *Ciona* and *Halocynthia* reveals a great plasticity within each type of module. Evolutionary plasticity was previously found within Drosophilids in the *eve* stripe 2 enhancer, in which conserved sites are spaced differently between species (Ludwig et al., 1998). Similar findings were uncovered in rhabditid nematodes (Ruvinsky and Ruvkun, 2003; Webb et al., 2002) and sea urchin (Romano and Wray, 2003). The plasticity observed in the ascidian *Otx* modules is much more extensive, probably reflecting greater evolutionary distance. The number of crucial sites, their order and relative distances all vary greatly both between similar modules in the two species, and between *Halocynthia* modules of similar activity (#1 and #2, #3 and #5, #4 and #6). This variation suggests that the regulatory syntax is highly degenerate, reflecting the lack of evolutionary pressure on these modules. A detailed analysis of the effect of shuffling the position, distance and orientation of important binding sites in one module would allow further testing of this hypothesis. The only possible hint of syntax in our study is that binding sites frequently repeated in a module (e.g. the GATA sites in the ab-modules, the Lhx sites in #4, the T-box sites in #3 and #5, and *Ciona* bB) tend to be evenly distributed along the module, rather than tightly clustered (see Fig. 6).

In summary, the major features of the cis-regulatory logic of *Otx* in ascidians are: (1) the general conservation across a large evolutionary distance of the cis-regulatory logic, based on very simple line-specific regulatory modules involving mainly BSs for transcriptional activators; and (2) a conservation of the position of some of these modules between species, which contrasts with the great plasticity of the arrangement of BSs within individual modules. This degeneracy, combined with the involvement of a few types of crucial TF-binding sites, is sufficient to explain how a conserved regulatory logic can be retained in the absence of detectable sequence conservation in the *Otx* flanking sequences.

Lessons from the comparative analyses of *Otx* and *Brachyury* regulatory sequences

As in the case of *Otx*, no local conservation was found between the flanking sequences of the *Ciona* and *Halocynthia Brachyury* genes coding for a T-box transcription factor (Takahashi et al., 1999). Although the identified *Ciona* and *Halocynthia* modules show a largely conserved activity in cross-species experiments, the types of crucial binding sites differed markedly between species. The authors thus proposed that the regulatory logic of *Brachyury* may differ in *Halocynthia* and *Ciona*. Comparison between two distantly related echinoderm, the starfish *A. miniata* and the sea urchin *S. purpuratus*, revealed that although some gene regulatory networks have persisted unaltered since the Cambrian period, others have extensively diverged (Hinman et al., 2003). The comparative analysis of the *Brachyury* and *Otx* regulatory logics between ascidians suggests that a similar phenomenon may also have occurred in this phylum. It should be noted, however, that the *Brachyury* regulatory modules identified in *Halocynthia* and *Ciona*, in spite of being both located close to the transcription start site, may fulfil different functions. In particular, while the onset of activity of the *Ciona* element mirrors that of *Brachyury* as demonstrated by a recent study (Yagi et al., 2004), little is known about the onset of activity of the *Halocynthia* element. The presence of a crucial T-box-binding site in this latter element suggests that it may have a maintenance function via a *Brachyury* auto-regulatory loop, rather than an activation function.

Transcription factors involved in the activity of the ab-line-specific transcription regulatory module of ascidian *Otx*

In *Ciona,* we have previously shown that *Otx* is activated in the animal hemisphere (a- and b-line) by the neural inducer *Ci-Fgf9/16/20*. This signal is mediated by the transcription factors Ci-GATAa and Ci-Ets1/2 via a cluster of GATA- and Ets-binding sites in the *Ci-Otx* ab-module [referred to as the a-element in Bertrand et al. (Bertrand et al., 2003)]. In *Halocynthia*, it has also been shown that *Hr-Ets*, the ortholog of *Ci-Ets1/2*, is required for *Otx* activation in the a- and b-line (Miya and Nishida, 2003), and here we have shown that the modules driving *Hr-Otx* expression in this line (#1, #2, #5) also contain clusters of GATA- and Ets-BSs. This suggests that the regulatory logic in this line is conserved between the two species. In addition, #2 and #5 also drive expression in a- and b-line cells when tested in *Ciona*. However, #1 does not drive expression in *Ciona*, indicating that the syntax of the module is not entirely degenerate and that it partly differs between the two species. Given the important sequence divergence of at least one of the factors binding to the a-module, Ets1/2 (50% amino acid identity between *Halocynthia* and *Ciona*), some co-evolution of the module and its binding factors is not unexpected.

Candidate transcriptional regulators for ascidian *Otx* in the A-line cells

We showed that the BSs for Lhx and Fox are crucial for the activity of A-line-specific transcriptional regulation. In *Halocynthia* and *Ciona* embryos, counterparts of *Lhx* and *Fox* have been identified; *Hrlim*, a member of *Lhx3* group of LIM homeobox gene in *Halocynthia* (Wada et al., 1995), five *Lhx* genes in *Ciona* (Imai et al., 2004; Satou et al., 2001), *Hr-FoxA5*, a member of *FoxA* subclass in *Halocynthia* (Shimauchi et al., 1997), and more than 20 *Fox* genes in *Ciona* (Di Gregorio et al., 2001; Imai et al., 2004; Satou et al., 2001). *Lhx3* and several *Fox* genes are co-expressed in the endoderm lineage at the 32- and/or 16-cell stage prior to the onset of *Otx* expression in the A-line cells and, therefore, represent good candidates for the activators of *Otx* transcription in the A-line cells.

It is known that expression of *Cs-Otx* and *Cs-Lhx3* is

upregulated in all blastomeres by β-catenin overexpression. Interestingly, at the 32-cell stage, transcription of *Cs-Lhx3* was already upregulated conspicuously, whereas that of *Cs-Otx* only occurred later (Satou et al., 2001), which further suggests that Lhx3 may act upstream of *Otx* in the ascidian A-line.

Candidates for a transcription regulator for ascidian *Otx* in the B-line cells

T-box protein BSs are required for directing *Otx* transcription in the B-line cells. In *C. intestinalis*, *Ci-VegTR*, a member of the *Tbx15/18/22* subfamily has been proposed to upregulate gene expression in B-line cells (Erives and Levine, 2000; Takatori et al., 2004). Additionally, three *Tbx6* subfamily genes are expressed in B-line cells (Takatori et al., 2004). These genes may contribute to the activation of *Ci-Otx* in this line. In *Halocynthia*, three T-box genes, *As-T* (*Hr-Bra*), *As-T2* and *As-mT*, have been isolated, their expression patterns described, and their putative functions estimated through overexpression experiments (Takada et al., 1998; Yasuo and Satoh, 1998). Among these, *As-mT* and *As-T2* could contribute to the transcriptional activation of *Hr-Otx* in B-line cells because their transcripts are present in B-line cells at the 32-cell stage (Takada et al., 1998; Yasuo et al., 1996). The reporter construct containing only the T-box BSs directed *lacZ* transcription in *Halocynthia* embryos only weakly (Fig. 4E). In addition, the module #5ΔT harboring the mutated T-box BSs retained some activity in *Ciona* embryos (Fig. 5G). These observations further support the involvement of additional transcription factors in the regulation of *Otx* in the B-line.

Comparison of regulatory mechanism of *Otx* genes between ascidians and vertebrates

The results of the present study led to the notion that the transcription regulatory logic of ascidian *Otx*, involving GATA-, ETS-, Lhx-, Fox- and T-box-transcription factors, might have been established in the common ancestor for ascidians of Pleurogona (e.g. *Halocynthia*) and Enterogona (e.g. *Ciona*), which are the two orders that constitute the class Ascidiacea, and thus the origin of the logic must be very old. To determine the extent of the conservation, we compared the regulatory mechanisms of *Otx* genes between ascidians and vertebrates.

The present study has suggested that a LIM homeoprotein and a Fox protein are responsible for regulating *Otx* transcription in the A-line cells (anterior mesendoderm precursors) of the pre-gastrula stage embryos. Interestingly, in mouse embryos, *Lhx1* and *FoxA* are co-expressed in the anterior visceral endoderm (AVE), in which *Otx2* is expressed (Perea-Gomez et al., 1999). Furthermore, the expression of *Otx2* during gastrulation is completely lost in $FoxA^{-/-};Lim1^{-/-}$ mutant embryos (Perea-Gomez et al., 1999), suggesting a possibility that *Lhx1* and *FoxA* are responsible for regulating *Otx2* transcription in mouse embryos in early development. From these results, we propose a hypothesis that LIM homeoproteins and FoxA proteins are responsible for regulating *Otx* gene transcription in both animals, and the regulatory mechanisms of *Otx* gene transcription in the endoderm might have been conserved during chordate evolution. Consistent with the hypothesis, regulatory regions responsible for the transcription of *Otx2* in the AVE have been identified (Kimura et al., 2000), and further analysis of the regulatory mechanism has revealed that a certain Fox BS in the regulatory region is crucial for the transcription in AVE (C. Kimura-Yoshida and I.M., personal communication). Thus *Otx* genes offer a unique paradigm to advance our knowledge on the evolutionary conservation or change in chordate embryonic transcriptional regulatory networks.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/7/1663/DC1

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