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 Ascidiaeae. 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 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 Sall/BamHI sites within the pMAPro plasmid DNA. To prepare #3AT-box, #4ALhx/Fox, #5AT-box, #6AFox, #6ALhx and #6ALhx/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 EditorTM 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 Sall/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). 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). 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,E′) Transcription of lacZ in embryos injected with the reporter construct harboring the upstream region 5402-1473 bp, or the intron region 812-24 bp, 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,E′). 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 5402-1473 bp or 812-24 bp, 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.
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* expression pattern of at cleavage stages. Regulated modules active during cleavage stage. Embryos analyzed for *lacZ* expression pattern of *Hr-Otx* expression at the 32- or 64-cell stage.

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 microinjected with p812-24MA, in which the first intron (812-24 bp) was placed upstream of the HrMA4a basal promoter, directed transcription 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 (animal 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 notochordal 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.

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**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-Fgr9/16/20 (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) (Shimaiuchi et al., 1997). Consistent with the possible 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 (#6AF/L/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 expressing 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),
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 \( \text{lacZ} \) transcription in the \textit{Halocynthia} B-line cells (Fig. 4C,D). This requirement for T-box BSs extended to \textit{Ciona} embryos, as mutation of those BSs reduced #3 expression in \textit{Ciona} B-line cells (Fig. 5G). Taken together, T-box proteins are direct regulators of \textit{Otx} transcription in the ascidian B-line cells.

**Fig. 5.** The regulatory modules of \textit{Hr-Otx} are capable of directing line-specific transcription in \textit{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 \textit{Ciona} fertilized eggs, and the resulting embryos were examined for \textit{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 \textit{lacZ}-expressing cell line in the \textit{Ciona} embryo and a schematic representation of the in situ hybridization specimen. (G,H) Frequency of \textit{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.
Development

distantly related ascidian species found in developmental modules seems to be plastic (Struhl, of TF-binding sites are important, the arrangement of sites differentiation enhancers in which the orientation and spacing of activity (Arnosti, 2003). Last, in contrast to some terminal causing the expression of the corresponding gene in its territory Fourth, modules are often redundant, the deletion of one to different classes. Third, the activity of the modules requires to different 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-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.

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 Brachury regulatory sequences

As in the case of Otx, no local conservation was found between the flanking sequences of the Ciona and Halocynthia Brachury 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 Brachury 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 Brachury 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 Brachury regulatory modules identified in Halocynthia and Ciona, in spite of being both located close to the transcription start site, may fulfill different functions. In particular, while the onset of activity of the Ciona element mirrors that of Brachury 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 Brachury 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 Tbx5 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 and vertebrates (e.g. *Halocynthia* and Enterogona (e.g. *Ciona*), which are the two orders that constitute the class Ascidicaea, 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 (antero-mesoderm 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 L.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

**References**


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