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Ascidian *otx* Gene *Hroth* Activates Transcription of the Brain-Specific Gene *HrTRP*

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ABSTRACT **The brain (sensory vesicle) of the ascidian larvae is thought to be homologous to the vertebrate forebrain and midbrain and, thus, is proposed as a simplified model to investigate mechanisms of brain formation in vertebrates. However, the genetic circuitry that governs formation of the sensory vesicle is largely unknown. To address this issue, we investigated the transcriptional regulation of the sensory vesicle–specific gene *HrTRP* by *Hroth*, the *otx* gene of the ascidian *Halocynthia roretzi*. A 133-bp 5′-flanking region of *HrTRP*, identified as a promoter that can drive expression of the reporter gene in the sensory vesicle, contains two otx binding consensus sites. When the two otx sites were deleted or mutated, the promoter activity of this region was decreased. *Hroth* overexpression can transactivate this promoter in an otx site–dependent manner. Transactivation of *HrTRP* promoter by *Hroth* overexpression was mimicked by overexpression of *Hroth/VP16*, which encodes a fusion protein of Hroth and the activator domain of VP16, and is suppressed by coexpression with *Hroth/En*, which encodes a fusion protein of Hroth and the Engrailed repressor domain. Finally, translational interference of *Hroth* by a morpholino oligonucleotide resulted in the reduction of *HrTRP* expression in the ascidian embryos. These results suggest that *Hroth* acts as a direct activator of *HrTRP* transcription during sensory vesicle development.**

Key words: ascidian; brain; *otx*; transcription; tyrosinase-related protein

INTRODUCTION

In recent years, larvae of ascidians (members of the subphylum Urochordata in the phylum Chordata) have attracted increasing attention as a unique animal model for studying developmental mechanisms of vertebrates (Corbo et al., 2001; Satoh, 2001). The anterior part of the neural tube of ascidian larvae is bulged and, therefore, called the brain or the sensory vesicle, because major sensory neurons and interneurons of the larval ascidian neural tube are located there (Nicol and Meinertzhagen, 1991; Meinertzhagen et al., 2000). The sensory vesicle is composed of approximately 260 cells, containing two types of sensory organs, the otolith (a gravity sensor) and the ocellus (a photoreceptor), both of which have a melanin-containing sensory pigment cell (Nicol and Meinertzhagen, 1991; Meinertzhagen et al., 2000). Comparative studies on the neural tubes of ascidian larvae and vertebrates have proposed that the sensory vesicle is homologous to the vertebrate forebrain and midbrain (Wada et al., 1998). Therefore, the sensory vesicle of the ascidian larvae is proposed as a simplified model to investigate developmental mechanisms of the vertebrate forebrain and midbrain (Okamura et al., 1993; Meinertzhagen and Okamura, 2001).

Although several genes expressed in the neural tube of the ascidian embryos have been reported (Satoh et al., 1996), little is known about a genetic circuitry that governs formation of the sensory vesicle. To address this issue, analysis of mechanisms of transcriptional regulation of sensory vesicle–specific genes is essential. For such an analysis, we have chosen *HrTRP* (a gene that encodes tyrosinase-related protein, see below) (Sato et al., 1999) because it is the only characterized gene that is expressed exclusively in a large domain of the sensory vesicle and, therefore, is regarded as the most suitable one known to date among ascidian genes. *HrTRP* is a single counterpart of the vertebrate *TRP-1* and *TRP-2* genes, which encode a melanogenic enzyme similar to tyrosinase and function in the melanin biosynthesis pathway (Tsukamoto et al., 1992; Kobayashi et al., 1994; Sato et al., 1999). During normal embryogenesis, *HrTRP* is expressed exclusively in dorsal and lateral parts of the sensory vesicle, including both the sensory pigment cells and non-pigment cells, and this expression starts at the early gastrula stage (Sato et

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al., 1999). *HrTRP* is probably involved in the melanin biosynthesis in the sensory pigment cells and/or may play a detoxification role in non-pigment lineage cells (Sato et al., 1999).

The analysis of the *cis*-regulatory elements required for the spatiotemporally regulated expression of *HrTRP* had been started before this study. This analysis has revealed that a reporter construct containing a 133-bp 5′-flanking region of *HrTRP* is capable of driving the expression in the sensory vesicle (Toyoda et al., submitted manuscript). We noticed that this region contains two otx homeoprotein binding consensus sites. This finding is intriguing, because it is consistent with the possibility of regulation of *HrTRP* transcription by the ascidian *otx* gene, which has been suggested by previous studies (see below).

otx genes are evolutionarily conserved genes that specify anterior body regions and sense organs and that have been isolated from a wide range of animals (Finkelstein and Boncinelli, 1994; Klein and Li, 1999). In higher vertebrates, two types of *otx* genes have been identified and shown to be involved in multiple aspects of rostral brain formation (Simeone, 1998; Acampora et al., 2001). In ascidians, a single *otx* gene has been isolated. *Hroth* is the *otx* gene we have isolated from *Halocynthia roretzi* (Wada et al., 1996). The *otx* gene also has been isolated from other three ascidian species: *Herdmania curvata* (Hinman and Degnan, 2000), *Ciona intestinalis* (Hudson and Lemaire, 2001), and *Ciona savignyi* (Satou et al., 2001b).

Expression of *Hroth* is detected in the sensory vesicle precursors and the adhesive organ precursors between the 32-cell stage and the early gastrula stage and then in the sensory vesicle lineage and anterior part of the epidermis up to the swimming larva stage (Wada et al., 1996). Thus, *Hroth* expression starts before the onset of *HrTRP* expression, and the expression domain of *HrTRP* is included in that of *Hroth*. More importantly, overexpression of *Hroth* mRNA induces ectopic expression of *HrTRP* in originally epidermal cells (Wada and Saiga, 1999). These results, together with the presence of two otx binding consensus sites in *HrTRP* promoter, give rise to the possibility that *Hroth* acts as a direct activator of *HrTRP* transcription. In the present study, we tested this possibility. First, we analyzed promoter activity of modified forms of the 5′-flanking region of *HrTRP* and show that the two otx binding sites are required for the normal level of sensory vesicle–specific activation of the promoter. We then demonstrate that *Hroth* overexpression can transactivate the *HrTRP* promoter in an otx binding consensus site–dependent manner. Finally, we carried out translational inhibition of endogenous *Hroth* by a morpholino antisense oligonucleotide and show that *HrTRP* transcription actually depends on *Hroth* activity. These findings all support the possibility of direct activation of *HrTRP* transcription by *Hroth* during sensory vesicle development of ascidian embryos.

RESULTS

Two otx binding consensus sites in the 133-bp 5′-flanking region of *HrTRP* are important for the normal level of *HrTRP* expression in the sensory vesicle. The preceding analysis of *cis*-regulatory elements of *HrTRP* transcription identified a 133-bp 5′-flanking region of *HrTRP* as a promoter that can drive expression of the reporter in the sensory vesicle (Toyoda et al., submitted manuscript). This region contains two copies of the otx binging consensus sequence (5′-TAATCC-3′): the distal sequence is located between the −113 and −108 nucleotide residue upstream from the transcription start site (+1) in the forward direction, and the proximal sequence is between −90 and −85 in the reverse direction (Fig. 1). In the first set of experiments, we investigated whether these otx binding consensus sites are necessary for the promoter activity of *HrTRP* in the ascidian embryos. Our primary construct for this investigation was pHrTRP133/LacZ, which contains a 133-bp 5′-upstream sequence and a 5′-untranslated region of the *HrTRP* fused with the *LacZ* reporter gene. When 1.6 pg of the intact pHrTRP133/LacZ was injected into fertilized eggs, more than 90% developed into normal embryos at the tail bud stage and nearly 100% of normally developed embryos exhibited positive X-Gal staining in the sensory vesicle (Table 1). The strong staining was detected in four or fewer cells at the dorsal side of the sensory vesicle, and the weaker staining was detected in cells at the dorsal and lateral sides (Fig. 1A,A′). Thus, the expression pattern of the reporter was essentially identical to that of endogenous *HrTRP* (compare Fig. 1A or A′ with Fig. 5B) (Sato et al., 1999).

We first tested the promoter activity of reporter plasmids with various deletions. When a distal 50-bp fragment of the 5′-flanking region of *HrTRP* containing both the otx binding sites was deleted from pHrTRP133/LacZ (pHrTRP83/LacZ), only approximately 45% of normally developed embryos expressed the reporter gene in the sensory vesicle (Table 1). When an internal 30-bp deletion (between −113 and −84), which removes the two otx binding sites, was introduced, the number also declined to approximately 60% (Table 1). Next, we examined the promoter activity of mutated plasmids. It has been shown that mutations in the fifth and sixth nucleotides of 6-bp otx binding consensus sequences abolish the binding of bicoid class proteins (Hanes and Brent, 1991). When such two-base mutations (5′-TAATCC-3′ to 5′-TAATAG-3′) were introduced in both of the two otx binding sites of pHrTRP133/LacZ (pHrTRP133Δotx1&2/LacZ), only approximately 60% of normally developed embryos expressed the reporter gene in the sensory vesicle (Table 1). Mutation of the proximal or distal otx binding site alone had little effect on the promoter activity. X-gal staining upon injection with the reporter plasmid in which the two otx binding sites were deleted or mutated

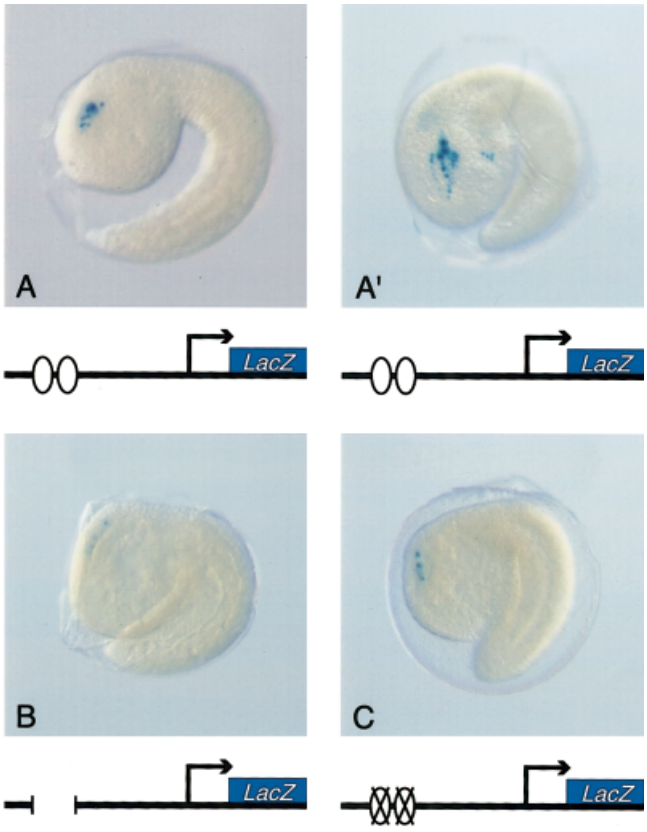

Fig. 1. Reporter gene expression in embryos injected with pHrTRP133/LacZ and its variants visualized by histochemical staining for β-galactosidase at the stage equivalent to the middle tail bud stage. **A:** An embryo injected with pHrTRP133/LacZ. **A′:** Another example of an embryo injected with pHrTRP133/LacZ. **B:** An embryo injected with pHrTRP133Δ133-84/LacZ. **C:** An embryo injected with pHrTRP133Δotx1&2/LacZ. Anterior is to the left. In B and C, X-gal staining was weaker than that in A and A′. Note that, although embryos exhibiting weakly positive staining for X-gal are presented in B and C for reference, the majority of embryos injected with pHrTRP133Δ133-84/LacZ or pHrTRP133Δotx1&2/LacZ were negative for X-gal staining (Table 1). A scheme of the injected plasmid is represented below each picture: ellipses indicate the otx binding consensus sites, and arrows indicate the transcription start site.

TABLE 1. Reporter Gene Expression of pHrTRP133/LacZ and Its Variants

Reporter plasmid examined	Number of embryos that showed X-gal staining in the sensory vesicle/number of injected embryos that developed normally (%)
pHrTRP133/LacZ	63/64 (98)
pHrTRP83/LacZ	27/61 (44)
pHrTRP133Δ113-84/LacZ	18/31 (58)
pHrTRP133Δotx1&2/LacZ	16/27 (59)
pHrTRP133Δotx1/LacZ	34/34 (100)
pHrTRP133Δotx2/LacZ	19/23 (83)

seemed weaker than that with the intact plasmid. Also, the number of cells expressing the reporter gene tended to be reduced (Fig. 1B,C). These findings suggest that the two otx binding consensus sites,

Fig. 2. Schematic representation of synthetic mRNA used in this study. Open reading frame and untranslated regions of *Hroth* are colored red and orange, respectively. The open reading frame is disrupted by a frame shift mutation (asterisk) in the negative control molecule *mHroth*^{SphI}. The *Engrailed* repressor region (En) and the VP16 activator region (VP16) are colored gray and pink, respectively. The target site of HrothMO1 is indicated by the small green rectangle.

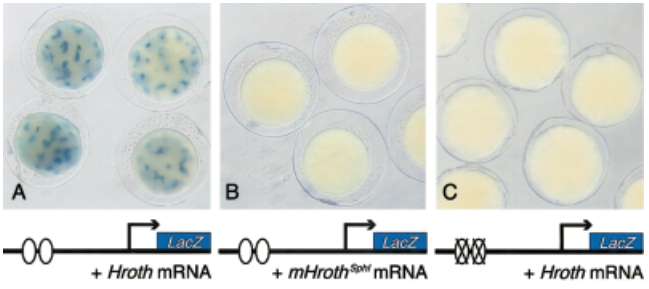

Fig. 3. Transactivation of pHrTRP133/LacZ by *Hroth* mRNA in cleavage-arrested embryos. Reporter gene expression was visualized by histochemical staining for β-galactosidase at the stage equivalent to the late tail bud stage. **A:** X-gal staining in cleavage-arrested embryos injected with pHrTRP133/LacZ and *Hroth* mRNA. Because the β-galactosidase used as a reporter in this study contained nuclear localization signal, X-gal staining was detected in multiple nuclei of cleavage-arrested one-cell embryos by treatment of cytochalasin B. **B:** X-gal staining in cleavage-arrested embryos injected with pHrTRP133/LacZ and negative control *mHroth*^{SphI} mRNA. In the absence of exogenous *Hroth* activity, pHrTRP133/LacZ was never activated in cleavage-arrested embryos. **C:** X-gal staining in cleavage-arrested embryos injected with pHrTRP133Δotx1&2/LacZ and *Hroth* mRNA. *Hroth* no longer activates *HrTRP* promoter when the two otx binding sites in the promoter were mutated. A scheme of the injected plasmid is represented below each picture: ellipses indicate the otx binding consensus sites, and arrows indicate the transcription start site.

which may compensate for one another, are required for the normal level of *HrTRP* expression.

Hroth Overexpression Activates pHrTRP133/LacZ in an otx Binding Consensus Site–Dependent Manner

The above-mentioned results raise a possibility that the Hroth protein activates the *HrTRP* promoter by means of its binding to the two otx binding consensus sites in the 133-bp 5′-flanking region of *HrTRP*. To test this possibility, we carried out transactivation experiments by using *Hroth* mRNA as an effector and the intact or mutated pHrTRP133/LacZ as a reporter. For these experiments, we used cleavage-arrested embryos, in which cleavage was completely inhibited by addition of cytochalasin B from the one-cell stage onward (Hirano and Takahashi, 1984). We used cleavage-

TABLE 2. Transactivation of pHrTRP133/LacZ or Its Variants by Wild-Type or Modified *Hroth* in Cleavage-Arrested Embryos

Reporter plasmid injected	mRNA injected	Number of embryos that showed X-gal staining/number of embryos injected (%)
pHrTRP133/LacZ	None	0/35 (0)
	Hroth	27/48 (56)
	mHroth^{SphI}	0/35 (0)
	Hroth/En	0/38 (0)
	Hroth + *Hroth/En*	8/32 (25)
	Hroth/VP16	27/45 (60)
pHrTRP133Δ113-84/LacZ	None	0/47 (0)
	Hroth	0/24 (0)
pHrTRP133Δotx1&2/LacZ	None	0/43 (0)
	Hroth	0/52 (0)

arrested embryos because pHrTRP133/LacZ is not activated in them unless *Hroth* is overexpressed simultaneously (see below), making it easier to judge whether the reporter plasmid is activated or not. Another reason for this choice was because a possible indirect effect on reporter gene expression due to abnormal morphology of the embryo induced by overexpression of *Hroth* can be avoided.

Fertilized eggs were injected with a mixture of a reporter construct and effector RNA, reared in the presence of cytochalasin B, and were examined for β-galactosidase activity at the stage equivalent to the middle tail bud stage. In negative control experiments, in which 1.6 pg of pHrTRP133/LacZ was injected either alone or together with 54 pg of *mHroth*^{SphI} (mutated *Hroth* with a frameshift; Fig. 2) mRNA, expression of the reporter gene was never detected in injected/cleavage-arrested embryos (Fig. 3B; Table 2). By contrast, when 1.6 pg of pHrTRP133/LacZ together with 54 pg of *Hroth* mRNA was injected, expression of the reporter gene was detected in approximately 55% of injected/cleavage-arrested embryos (Fig. 3A; Table 2). However, when pHrTRP133Δ113-84/LacZ or pHrTRP133Δotx1&2/LacZ was used instead of the intact pHrTRP133/LacZ, the reporter gene was no longer activated by *Hroth* overexpression (Fig. 3C; Table 2). Therefore, response of the reporter plasmid to *Hroth* overexpression seemed to depend on the presence of the two otx binding consensus sites in a 133-bp 5′-flanking region of *HrTRP* in the plasmid. These findings suggest that transcription of *HrTRP* can be activated by the Hroth protein and that binding of Hroth protein to the two otx binding consensus sites in the 133-bp 5′-flanking region of *HrTRP* is needed for the activation.

Transactivation of pHrTRP133/LacZ by *Hroth* Overexpression Is Mimicked by *Hroth/VP16* Overexpression and Suppressed by Coexpression with *Hroth/En*

To test the possibility that *Hroth* acts as an activator of *HrTRP* transcription further, we carried out a second set of transactivation experiments by using two distinct types of *Hroth* variants as an effector. One was *Hroth/En*, which encodes full-length Hroth protein fused to the repressor domain from *Drosophila* Engrailed (Fig. 2). Because this domain has been shown to exhibit strong repressing activity in embryos of various animals, including ascidians (Fujiwara et al., 1998), we suspect that this domain overrides the activating activity of *Hroth* and coerces it into behaving as a dominant negative version of *Hroth*. The other was *Hroth/VP16*, which encodes the full-length Hroth protein fused to the activator domain of VP16 (Fig. 2). Because this domain also has been shown to function well in ascidian embryos (Fujiwara et al., 1998), we suppose that the ability of *Hroth/VP16* to activate *HrTRP* transcription is stronger than or at least similar to that of *Hroth*.

When 1.6 pg of pHrTRP133/LacZ and 54 pg of *Hroth/En* mRNA were injected, expression of the reporter gene was never observed (Table 2). This finding suggests that *Hroth* in this form is no longer capable of activating the reporter plasmid. We then injected a mixture of 1.6 pg of pHrTRP133/LacZ, 54 pg of *Hroth* mRNA, and 108 pg of *Hroth/En* mRNA. Less than half of embryos expressed the reporter gene compared with the number obtained when 54 pg of *Hroth* mRNA alone was used as an effector (Table 2). Thus, *Hroth/En* seemed to act as a dominant negative molecule, which prevents *Hroth* from activating transcription of pHrTRP133/LacZ. On the other hand, when 1.6 pg of pHrTRP133/LacZ together with 54 pg of *Hroth/VP16* mRNA was injected, expression of the reporter gene was detected in 60% of the injected/cleavage-arrested embryos (Table 2). This finding suggests that *Hroth/VP16* can activate transcription of pHrTRP133/LacZ. That *Hroth/En* antagonized wild-type *Hroth* while *Hroth/VP16* mimicked it supports the idea that *Hroth* functions as the activator of *HrTRP* transcription.

Translational Interference of *Hroth* Leads to a Defect in Sensory Vesicle Formation and Reduction of *HrTRP* Expression

To demonstrate the importance of endogenous *Hroth* in transcriptional regulation of endogenous *HrTRP*, we next carried out morpholino antisense oligonucleotide-based translational inhibition of *Hroth*. This technique

Fig. 4. Phenotype of larvae injected with a morpholino oligonucleotide that targets translation of *Hroth* mRNA. Anterior is to the right. Fertilized eggs were injected with morpholino oligonucleotides to achieve a final concentration of 10 μM. **A:** Phenotype of a larva injected with HrothMO1. Note that the larva lacks both the the adhesive organ and the sensory pigment cells.**B:** A larva injected with the standard control oligo. Note that the larva developed normally and formed protrusions of the adhesive organ (arrows) and the sensory pigment cells (arrowheads).

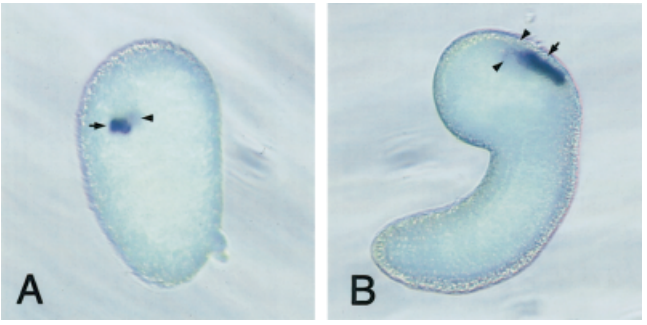

Fig. 5. Expression of *HrTRP* in embryos injected with HrothMO1 and visualized by whole-mount in situ hybridization at the stage equivalent to the middle tail bud stage. Anterior is to the top. **A:** Dorsal view of an embryo injected with HrothMO1. Note that only two cells showed strong staining (arrow) and that a few cells showed weak signal (arrowhead). **B:** Lateral view of an uninjected control embryo. Note that strong staining was found in four cells at the dorsal side of the sensory vesicle (an arrow) and that weaker signal (arrowheads) was detected in dorsal and lateral sides.

was shown to be an effective tool for loss-of-function experiments in the ascidian embryos (Satou et al., 2001a), in which a function of *Cs-otx* (*Ciona savignyi otx*) has been demonstrated (Satou et al., 2001b).

The target of the morpholino oligonucleotide we designed (HrothMO1) corresponds to the initiation codon and its immediate downstream sequence of *Hroth* mRNA (Fig. 2). As a negative control, we injected the standard control oligo supplied by Gene Tools. When eggs were injected with HrothMO1 to achieve a final concentration of 10 μM, all injected eggs developed into larvae with severe defects in the anterior structures (Fig. 4A). Trunks were round and adhesive organs, sensory pigment cells, and the cavities of the sensory vesicle were missing. Analysis of marker gene expression revealed that this phenotype is not caused by the loss of the neural tube but rather by the failure of neural tube differentiation (Wada et al., manuscript in preparation). On the other hand, embryos injected with the standard control oligo at 10 μM developed normally (Fig. 4B). The phenotype generated by HrothMO1 injection seems essentially the same to that of *Ciona* embryos injected with a *Cs-otx* morpholino oligonucleotide (Satou et al., 2001b). Therefore, the phenotype we obtained by HrothMO1 injection is thought to be a certain, specific consequence of translational interference of the ascidian *otx*. Detailed analysis of HrothMO1-injected embryos will be reported elsewhere (Wada et al., manuscript in preparation); here, we concentrated our attention on the expression of *HrTRP* in them.

The effect of translational inhibition of *Hroth* on *HrTRP* expression was investigated by examining the expression by whole-mount in situ hybridization at the stage equivalent to the middle tail bud stage. In uninjected control embryos, strong expression was detected in four cells at the dorsal side of the sensory vesicle and weaker expression was detected in cells at the dorsal and lateral sides (Fig. 5B). By contrast, in embryos injected with 10 μM HrothMO1, only one or two cells at the dorsal side of the sensory vesicle showed strong expression and a few other cells of the sensory vesicle exhibited weak expression (Fig. 5A). These results suggest that *Hroth* activity is required for the normal level of *HrTRP* expression in ascidian embryos.

DISCUSSION

Hroth Acts as a Transcriptional Activator of *HrTRP*

The previous studies revealed the significant correlation between *Hroth* and *HrTRP* expression and prompted us to test the possibility that *Hroth* directly activates transcription of *HrTRP*. In the present study, we provide evidence that this possibility is the case: (1) Two otx binding consensus sites in a 133-bp 5′-flanking region of *HrTRP* are required for the normal level of sensory vesicle–specific expression of this gene; (2) Overexpression of *Hroth* activates pHrTRP133/LacZ in an otx binding consensus site–dependent manner; (3) Transactivation of pHrTRP133/LacZ by *Hroth* overexpression is mimicked by *Hrothfull/VP16* overexpression and suppressed by coexpression with *Hrothfull/En*; (4) Morpholino oligonucleotide-based translational interference of endogenous *Hroth* results in reduced *HrTRP* expression. On the basis of on these data, we propose that the Hroth protein acts as a transcriptional activator that enhances the activity of the *HrTRP* promoter by means of binding to the two otx binding consensus sites found in a 133-bp 5′-flanking region of *HrTRP*.

In vertebrates, otx proteins have been shown to act as a transcriptional activator in human (Bobola et al., 1999), mouse (Kelley et al., 2000), and *Xenopus* (Gam-

mill and Sive, 1997; Green et al., 2001). Therefore, it is highly likely that the ability to activate expression of target genes is a conserved property of otx proteins between vertebrates and ascidians. Consistent with this idea, the functional homology between mouse *Otx1* and human OTX2 (Acampora and Simeone, 1999), mouse *Otx1* and *Drosophila otd* (Acampora et al., 1998), human OTX1 and *otd* (Leuzinger et al., 1998), and *Hroth* and *otd* (Adachi et al., 2001) has been demonstrated by transgenic analyses.

Interestingly, most of the direct *otx* targets identified to date are not regulatory genes such as transcription factors and signaling molecules but genes that define the identity of the cell (Boncinelli and Morgan, 2001). Because *HrTRP* encodes a putative melanogenic enzyme that may be involved in the melanin biosynthesis in the sensory pigment cells and/or may play a detoxification role in non-pigment lineage sensory vesicle cells (Sato et al., 1999), the activation of *HrTRP* transcription by *Hroth* is in agreement with this mechanism.

Mouse *TRP2* is expressed in the developing forebrain in addition to retinal pigment epithelium and neural crest cells, just as *HrTRP* is expressed in not only the sensory pigment cells but also other cells of the sensory vesicle (Steel et al., 1992; Zhao and Overbeek, 1999; Hornyak et al., 2001). The *TRP2* promoter that can drive reporter gene expression in these cell populations has been identified (Budd and Jackson, 1995; Zhao and Overbeek, 1999). We noticed that this promoter contains a single otx binding consensus site. Moreover, we found that the 5′-upstream sequence of the human TRP2 gene registered on the database also contains a single otx binding site. It will be intriguing to test whether the otx binding site is important for promoter activity of vertebrate *TRP2*.

There are a few cases for which the *otx* gene acts as a transcriptional repressor (Gherzi et al., 1997; Morgan et al., 1999). The present results do not necessarily exclude the possibility that *Hroth* also acts as a transcriptional repressor in a different context. In the previous report, it was demonstrated that overexpression of *Hroth* results in suppression of expression of genes such as an epidermis-specific gene and a notochord-specific gene (Wada and Saiga, 1999). It should be determined whether the suppression of gene expression by *Hroth* is direct or indirect in future experiments.

Transcription of *HrTRP* Is Regulated by Multiple Factors

Although our data suggest that *Hroth* is involved in activation of *HrTRP* transcription, *Hroth* is not considered the only regulator of *HrTRP* transcription. In the present analysis of the *HrTRP* promoter, reporter gene expression was reduced rather than lost by deletion or mutation of the two otx binding sites of the pHrTRP133/LacZ. Similarly, in the translational inhibition experiments, *HrTRP* expression was severely reduced but not extinguished in HrothMO1-injected embryos. Together, these results infer the presence of factor(s) other than *Hroth* that promote *HrTRP* transcription in the sensory vesicle. With regard to this problem, results of analyses of other *cis*-acting elements of the *HrTRP* promoter will be reported soon (Toyoda et al., submitted manuscript).

One of the candidates for an activator of *HrTRP* transcription other than *Hroth* among known genes is *HrPax37*, an ascidian homologue of vertebrate *Pax3* and *Pax7* (Wada et al., 1997), because *HrPax37* is expressed in the sensory vesicle of both normal embryos (Wada et al., 1997) and HrothMO1-injected embryos (Wada et al., manuscript in preparation) and because vertebrate *Pax3* is a direct activator of the tyrosinase and *TRP1* genes (Watanabe et al., 1998; Galibert et al., 1999). It has been demonstrated that overexpression of *HrPax37* induces ectopic expression of the ascidian tyrosinase gene (Wada et al., 1997). In a future study, it will be necessary to test whether *HrPax37* also activates expression of *HrTRP*.

In summary, the present study establishes *Hroth* as one of the direct activators of *HrTRP* transcription and represents the first example of target genes of the ascidian *otx*. Further investigation about the regulation of *HrTRP* and function of *Hroth* during sensory vesicle development should provide clues to understand a genetic circuitry controlling brain formation conserved among vertebrates and ascidians.

EXPERIMENTAL PROCEDURES

Embryos

Adult ascidians, *Halocynthia roretzi*, were obtained from fishermen near Asamushi Marine Biological Station, Tohoku University, Aomori, Japan, and Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan. Naturally spawned eggs were fertilized with a suspension of sperm from other individuals. Fertilized eggs were raised at 11–13°C in Millipore-filtered seawater containing 100 μg/ml streptomycin and 100 units/ml penicillin.

Construction of Reporter Plasmids

pHrTRP133/LacZ was generated by subcloning a 541-bp *HrTRP* genomic fragment that includes the 133-bp 5′-upstream region and the 284-bp 5′-untranslated region disrupted by first intron of 124 bp, into *Hin*dIII/*Bam*HI-digested p46.21 vector, a variant of pPD1.27 (Fire et al., 1990). To generate pHrTRP83/LacZ, polymerase chain reaction (PCR) fragments were amplified from pHrTRP133/LacZ by using primers 80HUS and TRPDS, digested with *Hin*dIII plus *Sal*I and cloned into *Hin*dIII/*Sal*I-digested pHrTRP133/LacZ. To generate pHrTRP133Δ113-84/LacZ, the PCR fragment amplified from pHrTRP133/LacZ with the primers PPDAMPUS and 110DS and with 80MUS and TRPDS were digested with *Mlu*I, treated with S1 nuclease, digested with *Hin*dIII and *Sal*I, respectively, and cloned into *Hin*dIII/*Sal*I-digested pHrTRP133/LacZ. Primers were as follows:

80HUS, 5′-GGAAGCTTTAATTTCCTACAAATCCGA-3′; primer TRPDS, 5′-TAGTCGACCTTATAACTGAA-3′; PPDAMPUS, 5′-ACAGCTATGAACATGATTAC-3′; 110DS, 5′-GGACGCGTGACATCATAAGGGA-AGAAC-3′; and 80MUS, 5′-GGACGCGTTAATTTCCTACAAATC-CGA-3′. Mutated plasmids were generated by using GeneEditor in vitro Site-Directed Mutagenesis System (Promega, Madison, WI) with the following primers: primer for pHrTRP133Δotx1, 5′-ATGATGTCATAATA-GATTAATGAAATATTG-3′; and the primer for pHrTRP133Δotx2, 5′-TGAAATATTGTCCTATTAT-TAATTTCC-3′. To generate pHrTRP133Δotx1&2, pHrTRP133Δotx1 was digested with *Hin*dIII and *Ssp*I, pHrTRP133Δotx2 was digested with *Ssp*I and *Sal*I, and the DNA fragments containing the mutated site were cloned into *Hin*dIII/*Sal*I-digested pHrTRP133/LacZ.

Construction of Expression Plasmids for In Vitro Transcription of mRNA

All plasmids for in vitro transcription were generated by using pBluescriptRN3 (Lemaire et al., 1995) as an expression vector. The expression plasmids for *Hroth* and *mHroth*^{SphI} mRNA (pRN3/Hroth and pRN3/mHroth^{SphI}) were described previously (Wada and Saiga, 1999). To generate the expression plasmid for *Hroth*/*En* mRNA, the *Engrailed* transcriptional repressor region was amplified from *Drosophila* genomic DNA by PCR using primers ENUS and ENDS, digested with *Nhe*I, treated with S1 nuclease, digested with *Mlu*I, and cloned into *Bst*XI/*Mlu*I-digested pRN3/Hroth together with C-terminal part of *Hroth* translated region amplified by PCR with primers OTH1200 and OTHENJ, digested with *Xba*I, treated with S1 nuclease, and digested with *Bst*XI. To generate the expression plasmid for *Hroth*/*VP16* mRNA, pVP16 was digested with *Eco*RI, treated with Klenow fragment, and digested with *Xho*I to release the VP16 transcriptional activator region. The C-terminal part of the *Hroth*-translated region was amplified by PCR using primers OTH1200 and OTH-VPJ, digested with *Bst*XI and *Xho*I, and then the VP16 transcriptional activator region and C-terminal part of *Hroth* translated region were cloned into *Bst*XI/*Mlu*I (blunted)-digested pRN3/Hroth. Primers were as follows: ENU, 5′-TAGCTAGCGCCCTGGAG-GATCGCTGCA-3′; ENDS, 5′-AGACGCGTTCAG-GAT-CCCAGAGCAGATTTCTC-3′; OTH1200, 5′-TATCTTCCATCGATGCAGTTCCCA-3′; OTHENJ, 5′-CGTCTAGAGAACCTGAAACTTCCACGATGGCGT-3′; and OTHVPJ, 5′-CGCTCGAGAGAACCTGAAACT-TCCACGATGGCGT-3′. In vitro transcription was carried out by using Message Machine (Ambion, Austin, TX) as described previously (Wada and Saiga, 1999).

Design of *Hroth* Morpholino Antisense Oligonucleotide

Morpholino antisense oligonucleotides were obtained from Gene Tools (Philomath, OR). The nucleotide sequence of HrothMO1 is 5′-AGTGTGAGGATTTCAGA-TAAGACAT-3′ (underlining indicates the sequence complimentary to the putative initiation codon). The ability of HrothMO1 to inhibit translation of *Hroth* mRNA was assessed by examining the effect of HrothMO1 on translation of *Hroth*/*LacZ* mRNA (a chimeric mRNA, in which the 5′-UTR and the initiation codon of *LacZ* mRNA were substituted with the 5′-UTR and the first 48 nucleotides of *Hroth* mRNA coding region) in cleavage-arrested embryos (data not shown). As a negative control, standard control oligo (Gene Tools) was used.

Microinjection and Treatment with Cytochalasin B

Microinjection was carried out as described previously (Wada and Saiga, 1999). DNA and morpholinos to be injected were dissolved in 0.1× TE buffer, and synthetic RNA was dissolved in distilled water. Each set of microinjection experiments was conducted twice or more. Treatment with cytochalasin B was carried out as described previously (Hirano and Takahashi, 1984).

Histochemical Staining for β-Galactosidase and Whole-Mount In Situ Hybridization

Reporter gene expression was visualized by histochemical staining for β-galactosidase as described previously (Hikosaka et al., 1994). Marker gene expression was visualized by whole-mount in situ hybridization as described previously (Wada et al., 1995).

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