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Retinoic acid perturbs *Otx* gene expression in the ascidian pharynx

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Abstract In vertebrate embryos, ectopic application of all-*trans* retinoic acid (RA) alters the expression of *Otx* genes in the developing midbrain. In conjunction with RA-induced misexpression of other regulatory genes this leads to a loss of anterior CNS. In the ascidian *Herdmania curvata*, RA primarily inhibits the development of the juvenile pharynx. An ascidian *Otx* gene, *Hec-Otx*, is expressed largely in this tissue, associated stomodeal structures and the anterior endostyle of the juvenile. Treatment with 10^{-6} M RA reduces *Hec-Otx* mRNA levels in the juvenile to about 12% of normal and is correlated closely with the loss of pharyngeal structures. During embryogenesis the expression of *Hec-Otx* becomes restricted to cell lineages fated to give rise to the anterior-most nervous system and the stomodeal component of the primordial pharynx. In hatched larvae *Hec-Otx* transcripts are detected only in the sensory (brain) vesicle. RA reduces *Hec-Otx* expression in the tailbud stomodeal pharynx primordium/anterior nervous system cell line but not in the larval sensory vesicle, suggesting that RA regulation of *Hec-Otx* expression is restricted to pharyngeal tissues throughout embryonic and postlarval development. RA does not affect expression of *Hec-Pax2/5/8*, which is normally expressed within the developing nervous system immediately posterior to *Hec-Otx* at the tailbud stage, lending support to the proposition that RA does not impact CNS axial patterning. These data combined with those from other chordates suggest that RA regulation of *Otx* expression in the anterior nerve cord and pharynx is a primitive chordate feature which has been maintained predominantly in pharyngeal tissues in the ascidian.

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Introduction

Ascidians, as representative urochordates, provide a simple system in which to study a number of chordate-specific developmental processes (see Satoh and Jeffery 1995; Di Gregorio and Levine 1998). While the ascidian embryo displays determinative development and is comprised of a small number of cells, the expression patterns of conserved regulatory genes are similar to those occurring in vertebrates (e.g. *brachyury*, Yasuo and Satoh 1994; Corbo et al. 1997; *HrHox1*, Katsuyama et al. 1995; *HrLim*, Wada et al. 1995; *Hroth*, Wada et al. 1996; *CiHox5*, Gionti et al. 1998; *HrPax-2/5/8*, Wada et al. 1998). Comparisons between vertebrates and ascidians suggest that a genetic regulatory system controlling the development and patterning of chordate-specific tissues was established prior to the divergence of the chordate subphyla and the evolution of the diverse embryological processes exhibited by chordates.

Unlike other chordate taxa, ascidians (and thaliaceans) have a biphasic life history common among marine invertebrates (Gilbert and Raunio 1997). Embryogenesis results in a tadpole larva with notochord, dorsal hollow nerve cord and axial musculature. During metamorphosis these tissues degenerate, and morphogenesis of the ectodermal and endoderm primordia begins, forming, amongst other juvenile organs, the feeding apparatus which includes the mouth, pharyngeal basket, endostyle and posterior gut tube (Berrill 1950; Satoh 1994). The pharyngeal basket is considered the homolog of the vertebrate pharynx and the gill slits of amphioxus (Berrill 1955; Romer 1962).

We and others, have previously shown that ectopic all-*trans* retinoic acid (RA) affects embryonic and postlarval development in ascidians (DeBernardi et al. 1994; Katsuyama et al. 1995; Hinman and Degnan 1998; Katsuyama and Saiga 1998). In *Herdmania curvata*, RA

induces the loss of anterior pharyngeal basket in the juvenile and appears to have a less significant impact on larval anterior CNS development (Hinman and Degnan 1998). Here we determine whether some of the same genes known to be regulated by RA during vertebrate embryogenesis are also regulated during ascidian development.

In vertebrates, ectopic application of RA results in the misexpression of a number of homeobox genes which leads to defects, including the loss of forebrain and midbrain, fusion or mispatterning of rhombomeres, craniofacial defects and homeotic shifts and fusions (Morriss-Kay et al. 1991; Kessel 1992; Conlon 1995 and references therein). The anterior boundary of *Hox1* (e.g. mouse *Hoxb-1*) and the posterior boundary of *Otx2* expression in the midbrain/hindbrain boundary are shifted anteriorly (Conlon 1995 and references therein). *Pax2* expression in the hindbrain shifts rostrally to fuse with its anterior domain in the midbrain (Avantaggiato et al. 1996). The identification of retinoic acid response elements in the promotor region of a number *Hox* genes and the presence of RA receptors during appropriate development stages provide strong support for the direct role of RA in A-P patterning through regulation of specific homeobox genes (Marshall et al. 1996 and references therein).

Ectopic RA perturbs the development of a number of invertebrates but is not known to regulate A-P patterning via homeobox gene expression in any non-chordates (Shimeld 1996). It is still uncertain whether RA impacts on the development of the CNS of the invertebrate chordates, although expression of *Hox1* genes in the ascidian *Halocynthia roretzi* and amphioxus are extended anteriorly upon RA treatment (Katsuyama et al. 1995; Holland and Holland 1996). The recent isolation of a RA receptor, *PmRAR*, from the budding ascidian *Polyandrocarpa misakiensis* (Hisata et al. 1998) and from *H. curvata* (Devine, Hinman and Degnan, unpublished) suggests that RA also has an endogenous role in ascidians.

Here we investigate the effect of ectopic RA on the expression of an *Otx* homologue in the ascidian *H. curvata* (*Hec-Otx*) during postlarval development (metamorphosis) and embryogenesis. *Otx* homologues have been isolated from disparate taxa, and in representative vertebrates, cephalochordates, ascidians, arthropods, annelids and platyhelminthes these homologues are expressed in the anterior CNS (Finkelstein and Boncinelli 1994; Katsuyama et al. 1996; Bruce and Shankland 1998; Williams and Holland 1998b; Umesono et al. 1999). *Otx* expression is also detected variously in visceral endoderm, sensory structures and anterior gut and pharynx in some vertebrates (Simeone et al. 1993, 1995; Ang et al. 1994; Kablar et al. 1996; Williams and Holland 1996, 1998b; Tomsa and Langeland 1999) and amphioxus.

During normal development of *H. curvata*, *Hec-Otx* is expressed predominantly in the juvenile pharynx, associated prepharyngeal structures and endostyle. *Hec-Otx* mRNA abundance in the juvenile and postlarva is dramatically reduced upon RA exposure, correlating with the RA-induced loss of anterior pharynx (Hinman and Degnan 1998). *Hec-Otx* embryonic and larval expression matches closely with that originally reported for *Hroth* (Wada et al. 1996). Our results indicate that RA-induced perturbation of *Hec-Otx* expression during embryogenesis occurs in the cell lineage that gives rise to the stomodeal component of the primordial pharynx. This suggests that RA impacts on gene expression in the pharynx but not in the CNS. To further test this hypothesis we examined the effect of RA on *H. curvata Pax2/5/8* gene expression which normally is localised within the CNS, immediately caudal to *Hec-Otx* (as with *Hr-Pax2/5/8* and *Hroth* in *H. roretzi*; Wada et al. 1998). *Hec-Pax2/5/8* is not impacted by RA treatment, further supporting the contention that RA is impacting on gene expression in the pharynx and the stomodeal primordium and not the larval nervous system. This suggests that while the expression patterns of ascidian and vertebrate neural tube genes such as *Otx* and *Pax2/5/8* may be very similar, the processes by which they are regulated may be different.

Materials and methods

Animal collection, embryo rearing and retinoic acid treatments

Gravid *H. curvata* were collected from Heron Reef, Great Barrier Reef. Embryos, larvae and postlarvae were cultured at 25°C and treated with all-*trans* RA as previously described (Degnan et al. 1996, 1997; Hinman and Degnan 1998).

Molecular cloning of Hec-Otx and Hec-Pax2/5/8

Partial sequence of the *Hec-Otx* gene was previously attained by reverse-transcription polymerase chain reaction (RT-PCR; Degnan et al. 1996). The full-length cDNA sequence was isolated following nested rapid amplification of cDNA ends (Frohman et al. 1988) to generate separate 5' and 3' PCR products. Poly(A)$^+$ RNA was extracted from late tailbud embryos using a biotin-labelled oligo $(dT)_{20}$ probe and streptavidin magnetic particles following manufacturers instructions (Boehringer-Mannheim). Double-stranded cDNA was synthesised from 1 µg poly(A)$^+$ using the reverse transcriptase primer (5' GGCCACGCGTCGACTAGTACT$_{(15)}$; BRL), and double-stranded adaptors were ligated to the cDNA ends (Clontech). A series of nested PCRs were performed to amplify the 5' and 3' ends of the cDNA using primers that anneal to *Hec-Otx*, the adaptors and the oligo (dT) adaptor. *Hec-Otx* specific primers used to amplify the 3' and 5' ends of the cDNA were Otx-5 (5' ACACAAGAATATGCCATTCGGTGCT) and Otx-1 (5' GCTCTT TTCGCCAAGACCCGG), and Otx-2 (5' CCTGAGTCCAGAGT TCAGGTC), respectively. From the adaptor-ligated double-stranded cDNA 1 ng was PCR-amplified under the following conditions: 94°C for 2 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s and 68°C for 4 min. Subsequent nested PCRs used identical conditions with 100- to 200-fold dilutions of the previous reaction. Gel purified PCR products were cloned and sequenced using the ABI prism dye terminator kit.

A partial cDNA of the *H. curvata* homologue for the Pax2/5/8 gene (*Hec-Pax2/5/8*) was isolated via RT-PCR using degenerate primers designed to the QTIVDLAHQ and TMFAWEIRD motifs within the paired domain of the *H. roretzi* homologue, *HrPax-2/5/8*. PCR was performed on 50 ng cDNA from tailbud embryos using the following conditions 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 45°C for 45 s and 72°C for 45 s. Primers specific for *H. curvata Pax2/5/8* homologue, Hec-Pax258-1 (5' CCCGCCAGCTAAGAGTGTCG) and Hec-Pax258-2 (5' GGATT

TTGCCGTTTGTATTCACAT) were designed to anneal just inside the sites of the degenerate primers.

Phylogenetic analyses

Otx protein sequences from a number of deuterostome taxa were aligned using Clustal X (Thompson et al. 1994) and adjusted by eye using MacClade 3.06 (Madison and Madison 1992). As has been noted previously (Williams and Holland 1998a), a number of conserved domains were apparent and these were grouped into a contiguous sequence of 180 amino acids for subsequent analysis. PAUP* 4.0 (Swofford 1998) was used to estimate molecular distances assuming maximum parsimony using the two sea urchin *Otx* homologues (*HpOtxE* and *SpOtx*) as a monophyletic outgroup. The confidence at each node was assessed by 100 bootstrap pseudoreplications.

Northern and Southern blot hybridisation

Total RNA was extracted via acid guanidinium thiocyanate method (Chomczynski and Sacchi 1987) with an additional precipitation in 4 M LiCl (Sambrook et al. 1989). From the poly(A)$^+$ RNA 2 µg was size-fractionated on a 1.4% agarose formaldehyde gel and transferred to Hybond N nylon membrane (Sambrook et al. 1989). A radiolabelled riboprobe was synthesised from a linearised pBKS plasmid containing a 660-bp insert, which corresponds to positions 1245–1905 in the 3' untranslated region of *Hec-Otx*.

Genomic DNA was extracted from sperm of an individual ascidian as described previously (Degnan and Lavin 1995). From the restriction enzyme digested genomic DNA 2 µg was fractionated on a 0.7% agarose gel and transferred to Hybond N nylon membrane. The membrane was probed with ^{32}P-random primed-labelled pBKS plasmid containing the first 348 bp of *Hec-Otx* cDNA.

Membranes were hybridised using standard procedures (Sambrook et al. 1989), with final washes being in 0.1 ×SSC, 0.1% SDS at 65°C.

PCR analyses

RT-PCR was performed on total RNA extracted from staged embryos, larvae and postlarvae. Primer sets used were (a) Otx-5 with Otx-2, which amplified the 5' open reading frame (ORF) and homeobox across an intron, (b) Pax258-1 with Pax258-2 which amplified a region of the paired domain of *Hec-Pax2/5/8* across an intron and (c) Ubq-1 (5' GTCAAGACCCTGACTGGAAGG) with Ubq-2 (5' CCACCTCTAAGACGGAGGAC), which amplified ubiquitin cDNA. From the cDNA 50 ng was amplified in a PCR reaction containing 0.5 µM of each primer (where in some cases 0.1 µM of the sense primer was end labelled with ^{32}P-ATP using T4 polynucleotide kinase) under the following conditions: 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 45 s and 72°C for 1 min.

Intron locations were determined via a comparison of PCR products derived from either cDNA or genomic DNA. The exact intron site was determined by sequencing of PCR products derived from genomic DNA.

Hec-Otx and *Hec-Pax2/5/8* RNA abundance was quantified using a competitive RT-PCR technique (Siebert and Larrick 1992; Riedy et al. 1995). The *Hec-Otx* competitor was generated following the technique of Sun et al. (1996) by PCR on cDNA using Otx-2 and a modified Otx-5 (5' ACACAAGAATATGCCATT CGGTGCTGAGAACCACATTCAC) which generated a product with priming sites for Otx-5 and Otx-2 on either end but with a 38-bp deletion. A competitor for *Hec-Pax2/5/8* was generated in a similar fashion using Pax258-2 and a modified Pax258-1 (5' CCCGCCAGCTAAGAGTGTCGTGAAATGGGAAGTATC) which resulted in a product with priming sites for Pax258-1 and Pax258-2 with a 35-bp deletion. In vitro RNA transcripts of the smaller target were synthesised in the sense orientation, quantitated spectrophotometrically and analysed by gel electrophoresis. Of the total RNA 100 ng–1 µg, spiked with five serial dilutions from 5 fg to 200 fg of competitor RNA, was reverse transcribed using either Otx-2 or Pax258-2 primer. PCR was then performed as previously described, for 35 cycles, using either Otx-2 and Otx-5 or Pax258-1 and Pax258-2 primers as appropriate. The gel photographs were scanned into Adobe Photoshop (2.5.1) and the intensity of the bands analysed using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image). The estimate of amount of target was then determined via regression analysis, as the value of $\log_{10}$ (competitor) for which $\log_{10}$ (target/competitor) was equal to zero. Each analysis was performed three times from at least two independent RNA extractions. Student's *t* test was used to determine the significance of the differences in transcript abundance between normal and RA-treated and embryos, larvae, postlarvae and juveniles.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation (WMISH) was performed using digoxigenin-labelled antisense RNA probes transcribed from linearised pBKS plasmids. The *Hec-Otx* probe was transcribed from either a 660- or 470-bp insert corresponding to positions 1245–1905 in the 3' ORF and UTR or 1905–2376 in the 3' UTR, respectively. The probe for *Hec-Pax2/5/8* corresponded to the region of the paired domain flanked by the primers Pax258-1 and Pax258-2.

WMISH was performed using adaptations of Harland (1991), Wada et al. (1995) and Holland et al. (1996). Embryos, larvae and juveniles were fixed in 4% paraformaldehyde in 0.1 M (*N*-2-morpholino)propane sulfonic acid (pH 7.5), 2 mM MgSO4, 1 mM ethyleneglycoltetraacetic acid, 0.5 M NaCl for 1–3 h at 23°C and washed several times in ethanol before storage at –20°C. Prior to use, embryos, larvae and juveniles were rehydrated into phosphate-buffered solution (PBS) with 0.1% Tween20. The chorion and test were removed manually from embryos and larvae, prior to proteinase K treatment, which was 20 µg/ml in PBS plus 0.1% Tween20 at 37°C for 10 min (embryos and juveniles) or 20 min (larvae). Specimens were prehybridised for 3 h in 50% formamide, 5×SSC, 5 mM EDTA, 1×Denhardt's solution, 100 µg/ml heparin, 100 µg/ml tRNA, 0.1% Tween 20 at 48°C, and hybridised in the same solution with the addition 0.25 µg/ml digoxigenin-labelled transcript for 14–20 h at 48°C. Following hybridisation, specimens were washed at 50°C (embryos and larvae) or 45°C (juveniles) twice in 50% formamide, 4× SSC, 0.1% Tween-20, then twice in 50% formamide, 2× SSC 0.1% Tween-20, then twice in 50% formamide, 1× SSC 0.1% Tween-20, for 15 min each, and then stepped into 0.1 M maleic acid, pH7.5, 0.15 M NaCl, 0.1% Tween 20. Antibody detection of digoxigenin-labelled probe was performed as in Shain and Zuber (1996). Prior to photography, embryos were cleared in benzyl benzoate:benzyl alcohol (2:1 v/v), and larvae and juveniles were cleared and mounted in 70% glycerol.

Whole-mount immunocytochemistry

Whole-mount immunocytochemistry was performed using protocols adapted from Panaganiban et al. (1997). Larvae were fixed for 30 min at 23°C in 9% formaldehyde in PBS with 50 mM ethyleneglycoltetraacetic acid. They were blocked for 1 h at 23°C in PBS with 0.1% Triton X-100 (PBSTx) with 5% DMSO, 5% sheep serum and 2% bovine serum albumin, and incubated in 1:1000 in anti-serotonin (Sigma) in PBSTx with 5% sheep serum, 2% bovine serum albumin at 4°C for 24 h. They were then washed several times in PBSTx and incubated as for primary antibody in 1:300 anti-rabbit antibody conjugated to horseradish peroxidase (Sigma). They were then washed several times in PBSTx, and the colour reaction was initiated via standard means with cobalt-enhanced diaminobenzidine (Sigma) for approximately 10 min. Larvae were cleared and mounted in 70% glycerol.

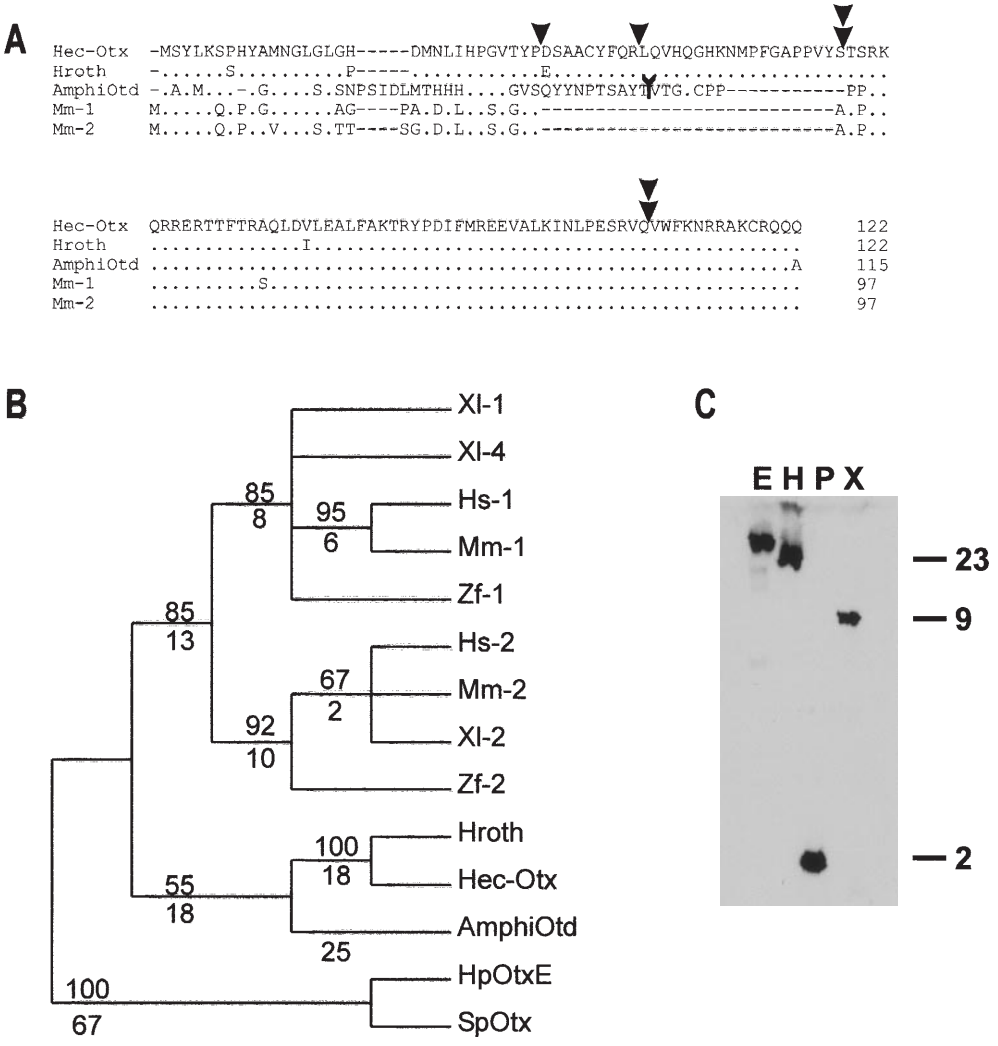

Fig. 1A–C *Hec-Otx* structure and phylogenetic relationship. **A** Alignment of the N terminus and homeodomain region of Hec-Otx with some chordate Otx proteins. The N terminus is more conserved between Hec-Otx and Hroth than is the carboxyl terminus (not shown), with the two ascidian proteins being 97% identical over the first 122 amino acids. Otx proteins from both ascidians have a unique stretch of 27 amino acids (positions 31–57 in Hec-Otx). The *Hec-Otx* gene possesses two conserved intron positions (*double arrowheads*) and two novel introns (*single arrowheads*). The arrow tail shows the location of a unique *AmphiOtd* intron (Williams and Holland 1998a). **B** Relationship of deuterostome Otx homologues. *Numbers above line* indicate percentage of times the node was supported by 100 bootstrap pseudoreplications; *numbers below line* show number of changes. Sequences are from the NCBI GenBank database and a publication (Kablar et al. 1996). *Amphi* Amphioxus; *Hp Hemicentrotus pulcherrimus*; *Hr H. roretzi*, *Hs* human; *Mm* mouse; *Sp Strongylocentrotus purpuratus*; *Xl Xenopus*; *Zf* zebrafish. *Hec-Otx* accession number: AF129932. **C** Southern blot analysis with genomic DNA restriction digested with either *Eco*RV (*E*), *Hin*dIII (*H*), *Pvu*II (*P*) or *Xba*I (*X*). *Right* size in kilobases

Results

Sequence analysis of Hec-Otx cDNAs

Rapid amplification of cDNA ends PCR of tailbud RNA indicated that *H. curvata* contains two *Otx* transcripts, called *Hec-Otx-A* and *Hec-Otx-B*, for the long and short mRNAs, respectively. These were probably produced by differential use of polyadenylation signals that were 452 nt apart. The two transcripts had the same ORF of 409 amino acids, with the homeodomain being closest to the N terminus. The predicted Hec-Otx amino acid sequence was 75% identical to an Otx protein from another pyurid ascidian, *H. roretzi* (*Hroth*; Wada et al. 1996), differing by only one amino acid in the homeodomain. The N-terminus and homeodomains were structurally similar in all chordates examined, although the Otx proteins from both ascidians had a unique stretch of 27 amino acids that was not present in the vertebrate Otx proteins (Fig. 1A). Analysis of intron positions within the *Hec-Otx* gene by sequence analysis of PCR products derived from genomic DNA revealed that this gene had four introns. The unique 27 amino acid stretch was flanked by two introns, one conserved and one novel, and contained a third intron within it, suggesting that this ascidian-specific domain was the product of the splicing of two unique exons (Fig. 1A).

We compared the Hec-Otx derived amino acid sequence with other deuterostome Otx proteins to further examine the evolution of this gene family (Fig. 1B). The two vertebrate orthologues formed well supported clades (bootstrap values of 85 and 92 for the Otx1 and Otx2 groups, respectively). The ascidian and amphioxus homologues formed a sister group to both of the vertebrate paralogues, strongly suggesting that vertebrate *Otx* genes duplicated after their separation from the ascidians and cephalochordates. While the cephalochordate and ascidian homologues clearly did not group within the vertebrate paralogues, the closer grouping of AmphiOtd with the ascidian rather than other vertebrate Otx protein homologues was only weakly supported via bootstrap analysis.

Southern blot analysis of genomic DNA from a single individual digested with either *Eco*RV, *Hin*dIII, *Pvu*II or

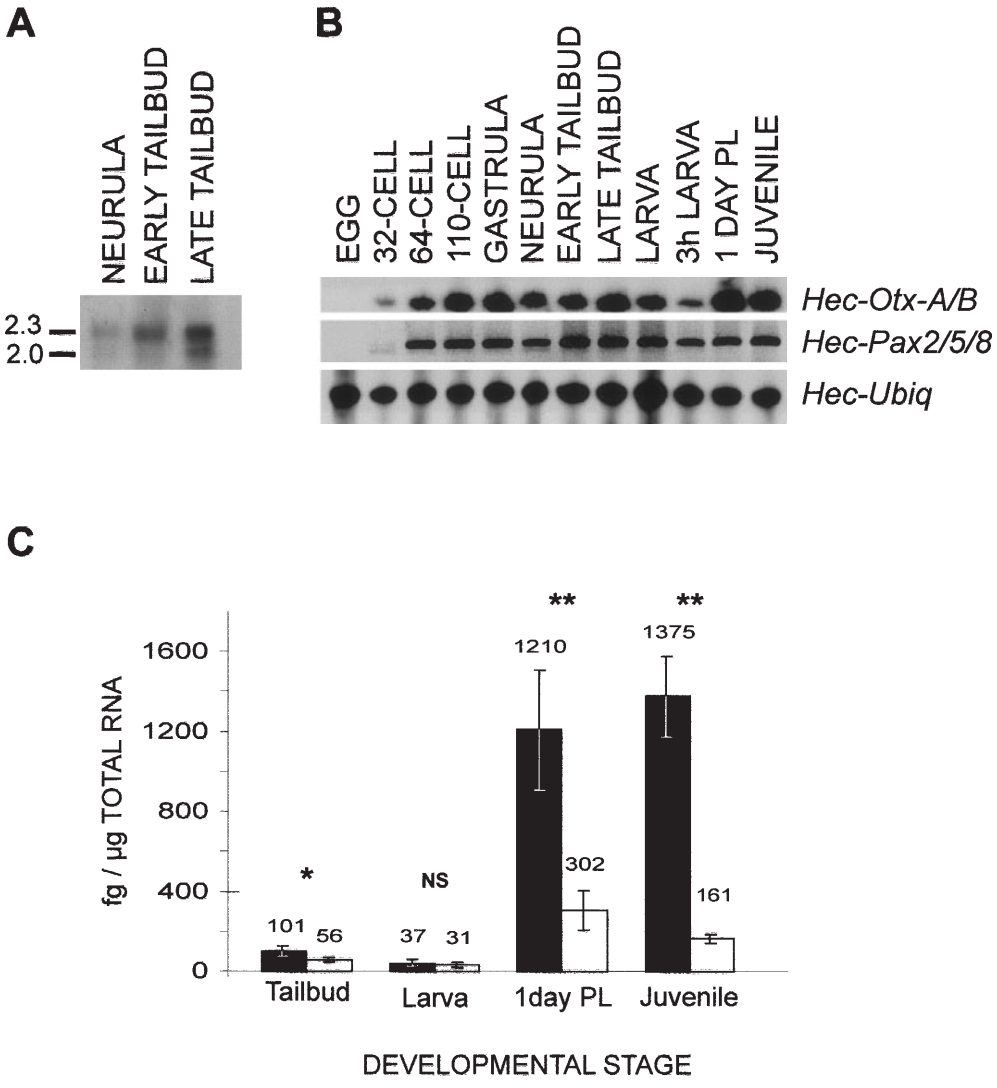

Fig. 2A–C Analysis of *Hec-Otx* and *Hec-Pax2/5/8* expression. **A** Northern blot analysis at three stages of embryonic development showing two expressed *Hec-Otx* transcripts (2.0 and 2.3 kb). *Left* Size in kilobases. **B** RT-PCR analysis of *Hec-Otx*, *Hec-Pax2/5/8* and *Hec-Ubiq* (polyubiquitin) expression. Both *Hec-Otx* and *Hec-Pax2/5/8* transcripts are detected from the 32-cell until at least the 5-day-old juvenile. *PL* postlarva. **C** Abundance of *Hec-Otx* transcripts during normal and RA-perturbed development. Tailbud and hatching embryos are treated with 10^{-6} M RA at 2-cell stage. One-day postlarvae (*PL*) and juveniles are treated with 10^{-6} M RA at the 3-h-old hatched larvae, just prior to induction of metamorphosis. *Numbers above columns* show transcript levels in femtograms of *Hec-Otx*/µg total RNA. *Filled bars* Normal; *open bars* RA-treated. *Error bars* ±1 SD. Student's *t* test was used to determine the significance of differences between normal and RA-treated transcript abundance at each stage of development. **P*<0.05, ***P*<0.01, *NS*, not significant

*Xba*I yielded bands ranging in size from 2 kb (*Pvu*II) to 23 kb (*Eco*RV; Fig. 1C). A single predominant band was detected for each restriction enzyme digestion and sequence analysis revealed that there were no restriction sites for these enzymes in the *Hec-Otx-A* and *Hec-Otx-B* cDNAs, indicating that we had detected a single Otx gene in the *H. curvata* genome.

Retinoic acid reduces *Hec-Otx* transcript prevalence in embryonic and postlarval development but not in the larva

Northern blot analyses performed on poly(A)$^+$ RNA isolated from neurula, early tailbud and late tailbud embryos demonstrated that long and short transcripts were present in these stages (Fig. 2A). RT-PCR analyses indicated that *Hec-Otx* transcripts were first expressed at the 32-cell stage and expressed throughout embryonic, larval and postlarval development until at least the 5-day-old juvenile (Fig. 2B).

Competitive RT-PCR demonstrated that during normal development *Hec-Otx* transcript abundance was highest during postlarval development, with expression in the juvenile (1375 fg *Hec-Otx* mRNA/µg total RNA) being almost 14- and 37-fold greater than in tailbud embryos (101 fg *Hec-Otx* mRNA/µg total RNA) and larvae (37 fg *Hec-Otx* mRNA/µg total RNA), respectively (Fig. 2C). RA treatment significantly reduced *Hec-Otx* mRNA transcript abundance in postlarvae with levels being 25% and 12% of normal abundance in 1-day-old postlarvae and 5-day-old juvenile, respectively (Fig. 2C). Transcript abundance in RA-treated embryos decreased significantly by almost 2-fold while no significant differences were noted between normal and RA-treated larvae.

Hec-Otx is expressed in juvenile pharyngeal tissues

During metamorphosis of *H. curvata* and other solitary ascidians, the endodermal primordium rotates anterodorsally (in relation to the larval body plan) and metamorphoses into the branchial basket and other gut structures (Hirano and Nishida, 1999). After this torsion is completed, the adult body axes are evident, with

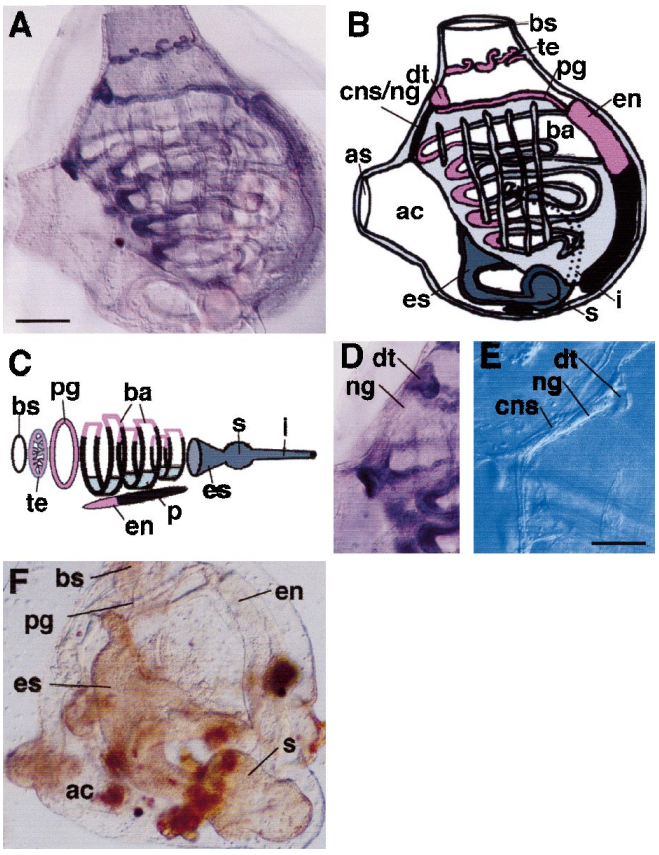

Fig. 3A–F *Hec-Otx* expression in the 5-day-old juvenile. All micrographs show lateral views with anterior (branchial siphon, *bs*) *to the top* and posterior (atrial siphon, *as*) *to the left*. The CNS and neural gland (*cns/ng*) are considered dorsal and the endostyle (*en*) lies ventrally. **A**,**B** WMISH (**A**) and schematic (**B**) of *Hec-Otx* expression (*pink*) in branchial arches (*ba*, stigmata), pharynx, prebranchial groove (*pg*), anterior endostyle (*en*), branchial tenticles (*te*) and dorsal tubercle (*dt*). *Hec-Otx* transcripts in the pharynx was limited to the cells in the most dorsal quarter of the first five stigmatal arches; mRNAs were not detected in the newly formed sixth arch. Within the portion of the stigmatal arches, transcripts appeared to be predominantly localised to the inner ciliated band lining the stigmata. *ac* Atrial cavity; *es* oesophagus; *i* intestine; *s* stomach. **C** Schematic representation of the gut of a juvenile ascidian drawn unlooped to demonstrate the homology of the A-P axis with that in other chordates; *pink Hec-Otx* expression. Anterior *to the left*; dorsal *top*. **D**,**E** *Hec-Otx* expression in the dorsal tubercle (**D**) and Normarski micrograph (**E**) of live animal to show clearly the dorsal neural ganglia and ventral neural gland which opens via the dorsal tubercle. **F** RA-treated (with 10^{-6} M RA, 23 h after hatching) juvenile with a pharynx lacking branchial arches, a comparatively normal endostyle (*en*), and a greatly expanded oesophagus (*es*) and stomach (*s*). *Scale bars* **A**,**F** 200 µm; **D**,**E** 150 µm

the larval anterior becoming the adult ventral side. The body plan of the adult ascidian is characterised by the predominance of endodermal structures and is highly ventralised relative to other chordates. The A-P axis can be related to the gut tube with anterior at the branchial siphon and the posterior at the atrial siphon; the endostyle is on the ventral side (Fig. 3A, B). If the twisted gut loop is linearised, the branchial tentacles, prebranchial groove and pharynx (with associated endostyle) are anterior of the oesophagus, stomach and intestine (Fig. 3C).

Spatial expression of *Hec-Otx* was examined using WMISH. No differences in expression were detected between a probe specific for *Hec-Otx-A* and one for a region common to *Hec-Otx-A* and *Hec-Otx-B* (not shown), and subsequently WMISH was performed using a combination of the two probes. *Hec-Otx* mRNA was localised predominantly to anterior stomodeal and pharyngeal structures in the 5-day-old juvenile (Fig. 3A, B). This included the branchial tentacles, prebranchial groove, anterior third of the endostyle and dorsal edges of five of the forming stigmata (branchial arches). There was a distinct posterior boundary of expression along the endostyle that was correlated closely with second and third stigmatal arch (the most anterior arch is the first; Fig. 3A, B). In addition *Hec-Otx* was localised to the dorsal tubercle (Fig. 3A, B). This is the most anterior portion of the neural gland and is derived from the endoderm (Hirano and Nishida, in press). The neural gland lies adjacent to and ventral of (i.e. beneath) the CNS (Fig. 3D, E).

Treatment of embryos or postlarvae with RA prevented the morphogenesis of the pharynx and promoted the apparent expansion of the oesophagus and stomach (Fig. 3F; Hinman and Degnan 1998). We were unable to detect *Hec-Otx* transcripts in RA-treated juveniles by WMISH. This was probably the result of an 88% reduction in *Hec-Otx* transcript levels (Fig. 2C) and the loss of pharyngeal structures where *Hec-Otx* mRNA was detected in normal juveniles by WMISH (Hinman and Degnan 1998).

Retinoic acid reduces *Hec-Otx* expression in stomodeal pharynx primordium/anterior nervous system cell line but not the sensory vesicle

During normal embryogenesis *Hec-Otx* transcripts were detected in the same mesendodermal and ectodermal cell lineages as has been reported for *Hroth* in *H. roretzi* (Wada et al. 1996), except that generally *Hroth* transcripts were detected in *H. roretzi* lineages at an earlier stage than *Hec-Otx* in *H. curvata*. In the 110-cell (Fig. 4A), *Hec-Otx* mRNA was localised to blastomeres that were predicted to give rise in the larva to almost the entire endoderm, some of the mesenchyme, sensory vesicle, and primordial stomodeum/anterior nervous system (Nicol and Meinertzhagen 1991; Nishida 1987). No difference in *Hec-Otx* expression was noted between RA-treated and normal embryos prior to gastrulation (not shown), as for *Hroth* in *H. roretzi* (Katsuyama and Saiga 1998).

In the early tailbud stage *Hec-Otx* mRNA in both normal and RA-affected tailbuds remained localised to CNS progenitors posterior to the neuropore and the anterior ectoderm (Fig. 4B–D). The posterior boundary of *Hec-Otx* expression in the neural lineage in both normal and RA-treated embryos corresponded with the boundary between the underlying trunk endoderm and noto-

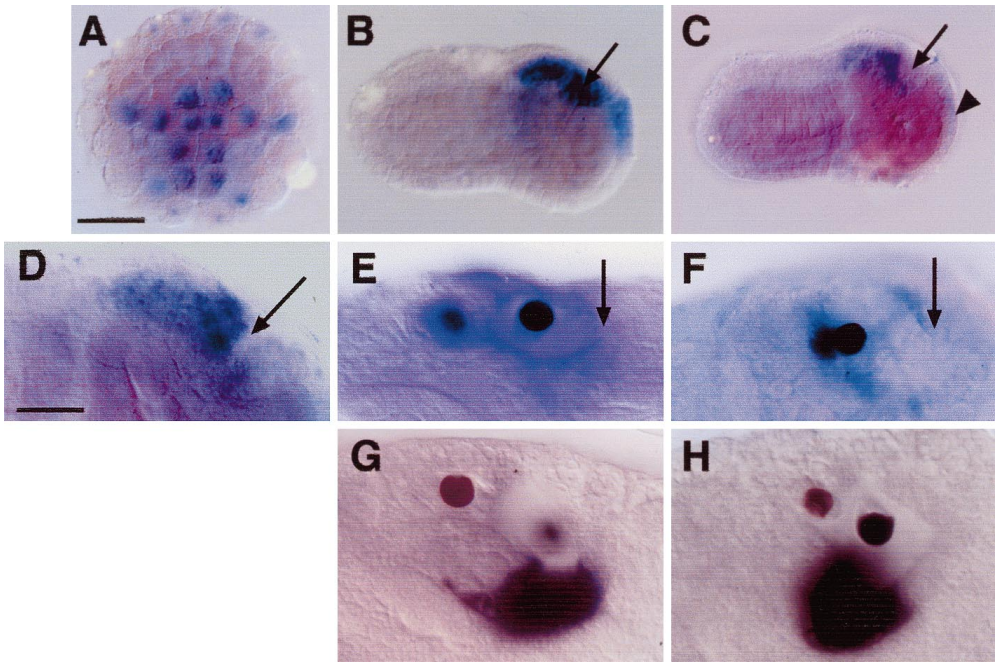

Fig. 4 WMISH analysis of *Hec-Otx* expression in normal (**A**,**B**,**E**) and RA-treated embryos and larvae (**C**,**D**,**F**). **B**–**H** Lateral views; anterior *to the right*, dorsal *up*. RA-treated embryos and larvae were cultured in 10^{-6} M RA from the 2-cell stage until fixation. **A** 110-cell embryo showing *Hec-Otx* mRNA localised to blastomeres that give rise to almost the entire endoderm (A7.1, A7.2, A7.5, B7.1, B7.2 and B7.5), some of the mesenchyme (A7.6 and B7.7), the sensory vesicle (a8.17 and a8.19) and primordial pharynx/anterior nervous system or neurohypophysis (a8.19); the B7.5 blastomere also gives rise small portion of larval muscle (see Conklin 1905; Satoh 1994 for naming of blastomeres). Due to the transparency of the cleared embryo the two pairs of anterior-most cells (a8.17 and a8.19) on the animal surface can be seen on the vegetal surface. **B** Early tailbud embryo shows expression in the ectoderm, and cell lineages predicted to give rise to stomodeal component of the pharynx primordium/anterior CNS (anterior wall of neuropore; *arrow* neuropore), sensory vesicle (posterior of neuropore) and the brainstem. **C**,**D** Tailbud embryos treated with 10^{-6} M RA, with D being a higher magnification. *Hec-Otx* transcripts are detected in anterior ectoderm (*arrowhead* in **C**), and sensory vesicle and brainstem cell lineages. Transcripts are not detected in cells anterior wall of the neuropore (*arrow* in **C**,**D**). **E** The sensory vesicle of a normal larva at hatching, containing the posterior ocellus and the anterior otolith. *Hec-Otx* expression is restricted to the cells of the sensory vesicle. *Arrow* Anterior boundary of expression in the sensory vesicle. **F** Sensory vesicle in 10^{-6} M RA-treated larva showing a posterior shift of the otolith relative to the ocellus. *Hec-Otx* expression is present around and throughout the sensory vesicle similar to normal larvae. *Arrow* Anterior boundary of expression in the sensory vesicle. **G**,**H** Expression of serotonin around the sensory vesicle of normal (**G**) and RA-treated (**H**) larvae as monitored with an anti-serotonin antibody. In both, expression is restricted to cells ventral to the otolith. *Scale bars* **A**–**C** 60 µm; **D**–**H** 25 µm

chord cells. In RA-treated embryos *Hec-Otx* transcripts appeared more localised to the nuclei of these cells (Fig. 4D). *Hec-Otx* transcripts were detected in the anterior wall of the neuropore in normal tailbud embryos but not in RA-exposed embryos (compare Fig. 4B with 4C, D). Lineage experiments indicated that these cells (derived from the a8.19 lineage) will form the stomodeal component of the primordial pharynx and anterior nervous system (Nishida 1987). The anterior ectodermal staining appeared to be reduced in intensity and shifted to a more dorsal position in embryos cultured in presence of 10^{-5} M RA (not shown).

By hatching, expression was restricted to the cells around the sensory vesicle (Fig. 4E); the posterior boundary of expression coincided with the ocellus. In larvae that had been treated with RA, the pigment cells of the sensory vesicle tended to be closer together [in normal larvae the mean distance between ocellus and otolith was 52±1.9 µm (n=10) while in larvae treated with 10^{-6} M RA this distance was reduced significantly to 33±2.9 µm (n=10, t=5.7, df=18, P<0.001)]. Despite this change in sensory vesicle morphology, *Hec-Otx* transcripts remained restricted to cells around this vesicle as in normal larvae (Fig. 4F). An antibody against the neurotransmitter serotonin was immunoreactive to a set of cells ventral of the otolith (Fig. 5G). In RA-treated larvae these immunoreactive cells did not shift relative to the otolith (Fig. 5H).

Retinoic acid does not impact on *Hec-Pax2/5/8* in the developing nervous system

The derived Hec-Pax2/5/8 amino acid sequence is 100% identical to the *H. roretzi* homologue (80% identical at the nucleotide level; *Hec-Pax2/5/8* accession number AF189154). The *Hec-Pax2/5/8* gene has an intron located within the alanine residue corresponding to position 57 of the paired domain from *HrPax2/5/8* (Wada et al. 1998).

Hec-Pax2/5/8 transcripts were detected throughout *H. curvata* development from the 32 cell stage onwards (Fig. 2B). *Hec-Pax2/5/8* was localised to cells of the

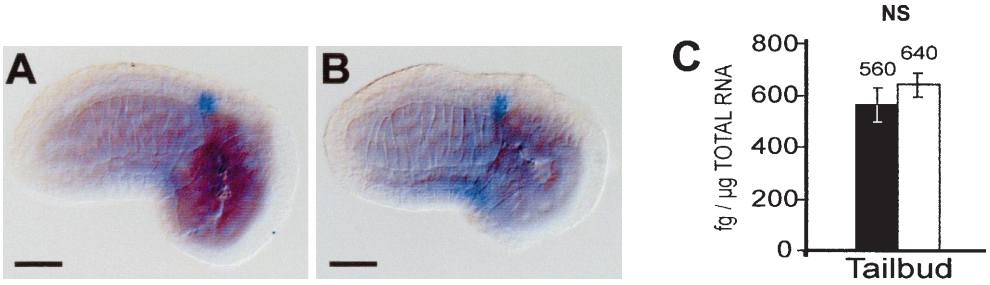

Fig. 5A–C WMISH and quantitative RT-PCR analysis of *Hec-Pax2/5/8* expression in normal and RA-treated embryos. **A** A tailbud larvae showing localisation of *Hex-Pax2/5/8* in a small number of cells within the anterior CNS. Expression is dorsal to the anterior-most notochord cells. **B** A tailbud larvae at the same stage that was treated with 10^{-6} M RA at the 2-cell stage. No differences in *Hec-Pax2/5/8* expression is noted. **C** Abundance of *Hec-Pax2/5/8* transcripts during normal and RA-perturbed tailbud development. *Numbers above the columns* indicate transcript levels in femtograms of *Hec-Pax2/5/8*/µg total RNA. *Filled bar* Normal; *open bar* RA-treated. *Error bars* ±1 SD. *NS* Student's *t*-test failed to find a significant difference between transcript prevalence. *Scale bars* 50 µm

CNS dorsal to the anterior-most cell of the notochord (Fig. 5A) and was therefore also immediately caudal to *Hec-Otx* expression in the CNS (*HrPax2/5/8* expression was also restricted to a small number of cells of the CNS immediately caudal to the posterior boundary of *Hroth* expression; Wada et al. 1998). In *H. curvata* embryos treated with 10^{-6} M RA (Fig. 5B), the expression domain did not alter relative to the notochord. Quantitative RT-PCR analysis of normal and RA-perturbed expression in tailbud embryos (Fig. 5C) also confirmed that there was no significant difference between transcript abundance with RA exposure.

Discussion

We have previously shown that the effect of ectopic RA on ascidian development is most dramatic in the postlarvae, where there is a graded loss of the pharynx and expansion of the posterior gut (Hinman and Degnan 1998). Here we demonstrate that the *H. curvata Otx* gene is normally expressed in the juvenile pharyngeal gill slits and is significantly down-regulated when postlarvae are treated with RA.

Work from vertebrates has suggested that endogenous RA regulates expression of a number of genes involved in axial patterning and is therefore involved in the correct spatial patterning of the CNS and other tissues. In RA-treated vertebrates the posterior boundary of *Otx2* expression is shifted anteriorly from the midbrain/hindbrain region (Bally-Cuif et al. 1995; Simeone et al. 1995), and the posterior expression domain of mouse Pax2 is shifted into the midbrain (Avantaggiato et al. 1996). Such changes in the expression of these and other genes in this region results in the loss and mispatterning of anterior CNS and structures derived from it (Bally-Cuif et al. 1995; Conlon 1995; Simeone et al. 1995). RA does not appear to regulate expression of homologous genes in non-chordates. A comparison of the effects of RA on *Hox1* gene expression in amphioxus, the ascidian *H. roretzi* and vertebrates suggests that this gene is regulated in chordates in an apparently homologous manner (Katsuyama et al. 1995; Holland and Holland 1996; see Shimeld 1996 for review). However, it has been difficult to establish a correlation between morphological changes and a modification of gene expression in amphioxus and *H. roretzi*. To date the best correlation between RA-induced modification of gene expression and morphological change in invertebrate chordates has been the reduction in *Amphi-Pax-1* expression in the amphioxus pharynx (Holland and Holland 1996) and *Hec-Otx* in the ascidian pharynx (this study).

Of the major chordate groups only ascidians and thaliaceans develop a larva that is morphologically distinct from the adult. Molecular phylogenetic data strongly suggest that this biphasic life history is derived from a directly developing free-living ancestor (Wada and Satoh 1994; Wada 1998). In the ascidian/thaliacean evolutionary lineage chordate-specific characters have been dissociated and partitioned between the different life history phases. Axial structures (dorsal hollow neural tube, notochord, tail muscle) form during embryogenesis, while visceral structures (pharynx, endostyle, gut) form during postlarval development.

During metamorphosis, *Hec-Otx* transcript levels dramatically increase, with an almost 37-fold increase between larval and juvenile levels. In sea urchins it has been suggested that such increases in abundance of a number of *Hox* gene transcripts during postlarval development is linked to more complex development in the postlarva and subsequent increased reliance on regulatory genes (Arenas-Mena et al. 1998). The increase in *Hec-Otx* transcripts in the juvenile is largely attributed to expression in the pharynx and anterior pharyngeal associated structures. Expression is found in both ectodermally and endodermally derived pharyngeal structures, but not in any part of the juvenile CNS. The expression patterns of *Otx* genes in some vertebrates and amphioxus suggest that this gene may be involved in patterning the pharynx in addition to the brain (Simeone et al. 1993; Ang et al. 1994; Kablar et al. 1996; Williams and Holland 1996, 1998b). In mouse and *Xenopus Otx* genes are expressed in the foregut and ectodermal and endodermal cells of the first branchial arch and pouch during early somatogenesis while $Otx2^{-/-}$ mice fail to develop foregut

and branchial arches (Ang et al. 1994; Acampora et al. 1995; Matsuo et al. 1995; Simeone et al. 1995). Strong expression in the endodermal cells surrounding the first pharyngeal pouch has also been detected in the lamprey (Tomsa and Langeland 1999).

In amphioxus *Otx* is expressed in the anterior mesendoderm during gastrulation, anterior endoderm during neurulation and persists in the endoderm of the pharynx until at least the late (36 h) larva (Williams and Holland 1998a, 1998b). Our detection of *Hec-Otx* gene expression in the ascidian pharynx implies that neural and pharyngeal expression of *Otx* is the primitive condition in chordates. This expression may indeed predate the origin of the chordate body plan, as the pharynx probably is inherited from a more distant deuterostome ancestor (Turbeville et al. 1994; Bromham and Degnan, in press). While the pharynx is a shared character of chordates and hemichordates, *Otx* expression in hemichordates has not yet been investigated. Recently *Pax1/9* genes have been shown to be expressed in the epithelia of the ascidian and hemichordate pharynx (Ogasawara et al. 1999).

Hec-Otx mRNA abundance in the 1- and 5-day-old postlarvae treated with 10^{-6} M RA is significantly reduced being approximately 25% and 12% of normal levels, respectively. These data suggest that the dramatic reduction in *Hec-Otx* by RA treatment during metamorphosis may be correlated with the loss of anterior pharyngeal structures. In addition to RA-induced reduction in neuroectodermal *Otx2* expression, Simeone et al. (1995) also report loss of foregut expression in middle- to late-streak stage mice. This suggests that regulation of *Otx* by RA may be a primitive chordate feature, but that in ascidians this regulation is predominantly in the juvenile pharyngeal tissues. In this study postlarvae are exposed to RA during metamorphosis, after *Hec-Otx* expression in the stomodeal primordium ceases. In these juveniles stomodeal structures (such as incurrent siphon and branchial tentacles) develop. RA-exposed embryos metamorphose into a highly abnormal juveniles with no discernible adult structures.

As our previous work has suggested that there is no RA-induced loss of CNS in ascidian embryos (Hinman and Degnan 1998), we determined whether RA impacts on *Otx* expression in the ascidian neural tube. *Otx* plays a key role in the spatial patterning of the vertebrate brain and is expressed in homologous regions of the amphioxus and ascidian neural tube (Katsuyama et al. 1996; Wada et al. 1996, 1998; Williams and Holland 1996, 1998b; this study). Normal embryonic and larval expression of *Hec-Otx* is very similar to *Hroth* gene expression in *H. roretzi* (Katsuyama et al. 1996; Wada et al. 1996, 1998; Katsuyama and Saiga 1998).

We demonstrate that at no stage of development is the posterior boundary of *Hec-Otx* expression in the CNS modified in RA-treated embryos or larvae as occurs in vertebrates (Bally-Cuif et al. 1995; Simeone et al. 1995). Instead, the expression of *Hec-Otx* in the anterior wall of the neuropore is lost upon RA exposure. These cells correspond to the stomodeal pharynx primordial/anterior CNS lineages, which are combined prior to neurulation (Nishida 1987). At hatching the intimate association of these two lineages is maintained by the hypophyseal tube, which derives from the neuropore and connects the pharynx primordium and the prosencephalon (Willey 1894; Katz 1983). This in fact may be the homologue of the vertebrate stomodeum/hypophyseal tube (Wada et al. 1998), where *Otx* expression has also been noted in some vertebrates (Kablar et al. 1996)

While we are not able to determine whether RA-induced repression of *Hec-Otx* is restricted to either or both of these lineages at the tailbud stage, a number of lines of evidence support the contention that the primordial stomodeum rather than nervous system lineage has been impacted. Firstly, *Hec-Otx* transcript abundance in RA-treated embryos decreases at the tailbud stage when the gene is expressed in both lineages, but not in the larva when expression is restricted to the sensory vesicle of the CNS. Secondly, *Hec-Otx* expression in the tadpole is confined to the sensory vesicle in both RA-affected and normal larva, although in treated tadpoles this vesicle is shortened. Thirdly, RA has only minor affects on the larval anterior CNS, with anterior chemosensory functionality and a general marker for the CNS being maintained (Hinman and Degnan 1998). Maintenance of serotonin immunoreactive cells in the sensory vesicle in RA-treated larvae also indicates that these cells have not been functionally respecified; in *H. roretzi* expression of *HrLim* and *TuNa1* in the visceral ganglia also are not affected by RA (Katsuyama and Saiga 1998). Fourthly, *Hec-Pax2/5/8* expression, which is immediately caudal to *Hec-Otx* (see also Wada et al. 1998 for *Hroth* and *Hr-Pax-2/5/8*), within the anterior developing nervous system is not impacted by RA.

In summary, *Hec-Otx* is expressed in the anterior of the larval neural tube and juvenile pharynx in a manner similar to that observed in representative taxa of the other chordate subphyla. RA impacts on *Hec-Otx* expression that is associated with pharyngeal tissues in both embryos and juveniles. Unlike the situation in vertebrates, RA does not appear to affect either *Hec-Otx* or *Hec-Pax2/5/8* expression in the CNS or to result in a loss or misspecification of CNS tissue. These data imply that while the expression patterns of *Otx* and *Pax2/5/8* genes in vertebrate and ascidian neural tubes are strikingly similar (which implies that this pattern existed in basal chordates), the process by which these patterns are regulated are likely to be different. RA regulation of *Otx* genes is probably an ancestral condition in chordates but is predominantly associated with pharyngeal rather than neuroectodermal expression in the ascidian.

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