Ascidian Tail Formation Requires caudal Function

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Although the tail is one of the major characteristics of animals of the phylum Chordata, evolutionary aspects of the molecular mechanisms involved in its formation are not clear. To obtain insights into these issues, we have isolated and investigated the caudal gene of an ascidian, one of the lower animal groups among chordates. Ascidian caudal is expressed from the midgastrula stage onward in the lateral walls of the posterior neural tube cell lineage and also in the posterior epidermal cells from the neurula stage. Thus, ascidian caudal expression is restricted to the ectoderm of a tail-forming region throughout embryogenesis. Suppression of caudal function by an antisense oligonucleotide or a dominant negative construct caused inhibition of the cell movement required for tail formation. Overexpression of wild-type caudal mRNA in an ascidian animal cap, an animal half explant prepared at the eight-cell stage, caused elongation of the cap. Furthermore, Xenopus embryos injected with dominant negative ascidian caudal exhibited defects in elongation, suggesting a conserved caudal function among chordates. These results indicate that caudal function is required for chordate tail formation and may play a key role in its evolution.

Key Words: ascidian; tail formation; chordate evolution; caudal.

INTRODUCTION

Mangold (1933) showed that transplantation of the organizer to the ventral side of a host animal induced a different part of the body depending on the timing of explanation and the part of the organizer used. The anterior part of the organizer or the dorsal lip of an early gastrula induces a head, the middle part of the organizer induces a trunk, and the posterior part of an organizer or the dorsal lip of a late gastrula induces a tail. These experiments suggest that the three parts of vertebrate body along the anterior–posterior (A–P) axis are generated by distinct developmental mechanisms. Much information about developmental mechanisms of the head region has been accumulated (Lumsden and Krumlauf, 1996), while those of posterior regions have been less well investigated (Tucker and Slack, 1995). Morphology of the posterior body is characteristic for each animal group. For example, an insect body has an abdomen with digestive and reproductive openings at the posterior end, whereas a vertebrate body has a tail without such openings at the posterior end. In other animal groups, such as molluscs and echinoderms, a comparable structure is not evident. The tail of chordates contains bilateral muscles, the notochord in the midline and the neural tube dorsally. These are hallmark organs for the phylum (Willmer, 1990; Satoh and Jeffery, 1995). To obtain insights into the origin and developmental mechanisms of the chordate tail, an ascidian, a primitive chordate and a sister group of vertebrates, offers a useful experimental system, because of its phylogenetic position and the simplicity of its embryonic structure and genome (Satoh et al., 1996). Regionalization of the body along the A–P axis is a common phenomenon of animal embryogenesis. It has been shown that conserved gene expression is observed in this process. For example, it is well known that Hox genes specify a medial region and orthodenticle/Otx and empty spiracle/Emx genes specify an anterior region in Drosophila, vertebrates, and some other animals (Holland et al., 1990; Manak and Scott, 1994; De Robertis, 1994). Previously we showed that the ascidian Hox-1 and Otx genes exhibit conserved expression patterns along the A–P axis during embryogenesis (Katsuyama et al., 1996). To further understand regionalization mechanisms in ascidian development, we decided to analyze expression and function of an ascidian caudal homeobox gene (Bürglin, 1994). The caudal gene was originally identified in Drosophila, where it is required for normal posterior development. Its transcripts and protein exhibit characteristic distributions along the A–P axis, rich in the posterior of the early embryo (Mlodzik and Gehring, 1987). Vertebrate caudal class genes...
have been isolated from several species. Xcad-1 and -2 of Xenopus were isolated as homeobox genes expressed in Spemann’s organizer (Blumberg et al., 1991). Chox-cad of chick has been reported to be involved in early regionalization of the endoderm (Frumkin et al., 1993). Mouse Cdx-1 expression correlates with regionalization of ectodermal and mesodermal cells (Meyer and Gruss, 1993). All of these caudal class genes exhibit expression in the posterior part of each embryo in each tissue. More recently, Xenopus Xcad genes have been shown to play a role in normal posterior development (Epstein et al., 1997; Isaacs et al., 1998).

In the present report, we examined expression and function of a caudal gene during embryogenesis of the ascidian, Halocynthia roretzi. Expression of Halocynthia caudal (Hrcad) is detected from the midgastrula stage onward in the lateral walls of the posterior neural tube cell lineage and also in the posterior epidermal cells from the neurula stage. Thus, expression of Hrcad is observed in the ectodermal tissues of the tail throughout embryogenesis. Then we examined the function of Hrcad gene in ascidian embryogenesis by employing antisense oligonucleotide and dominant negative methods. Suppression of Hrcad function resulted in inhibition of tail formation. Furthermore, we found that overexpression of wild-type Hrcad mRNA in the ascidian animal cap caused elongation of the cap. Also, we have demonstrated that injection of Xenopus embryos with dominant negative Hrcad interfered with tail formation. These results suggest that caudal function is required for tail formation through controlling ectodermal cell movement in ascidian embryos and may have a conserved role in a variety of chordate embryos.

MATERIALS AND METHODS

Ascidian. Adult ascidians, H. roretzi, were collected or purchased from fishermen in the Asamushi, Wakkanai, Kesen-numa, and Sanriku seaside areas of Japan. Naturally spawned eggs were fertilized with a sperm suspension collected from nonself individuals. Eggs were washed twice with fresh sea water to remove excess sperm and reared in filtered sea water (FSW) at 11–13°C. Batches of eggs in which embryogenesis proceeded synchronously were used.

Screening and sequencing of caudal cDNA of the ascidian. A partial caudal homeobox fragment was identified during the course of isolation of Hox gene fragments from the ascidian genome (Katsuyama et al., 1995). Using this fragment as a probe, 102 clones of a cDNA library of Halocynthia larva were screened, which yielded two positive clones. The insert DNA of each phage clone was subcloned into the Smal site of pBluescript SK- plasmid. The nucleotide sequence of these clones was determined using a Taq dye primer cycle sequencing kit (Applied Biosystems) and an ABI 373A DNA sequencing apparatus (Applied Biosystems).

Northern and Southern hybridization. Preparation of ascidian genome DNA and embryonic RNA, blotting, and hybridization were carried out as described previously (Katsuyama et al., 1995).

Whole-mount in situ hybridization. Whole-mount in situ hybridization was carried out as described by Wada et al. (1995). For synthesis of digoxigenin-labeled RNA probes, a CDNA clone, Hcd-3 (Fig. 1A) was used as a template. Whole-mount in situ hybridization specimens were cleared by soaking in glycerol. HrHox-1, Hrth, Hrlim, and neural tubulin probes used in this paper were the same as used in previous works (Katsuyama and Saiga, 1998; Miya et al., 1997). Ascidian muscle myosin fragment was amplified by PCR from ascidian cDNA, subcloned, and used as a template for the RNA probe synthesis (Okamura, unpublished data).

Antisense oligonucleotide experiments. Experiments using antisense oligonucleotides were carried out as described by Swalla and Jeffery (1996). The phosphorothioated oligonucleotides used in this report are asCAD-1, GGGCATGCCGATAGCCGC; asCAD-2, GTCCTTTTCTGATCCAACTCC; and ssCAD-2, GG-GATTGGATCGAAAAAGCG. These were purified by HPLC, quantified by TOF/MS system (Hokkaido System Science Co.), dissolved in distilled water at 10 mM, and stored at −20°C. Antisense oligonucleotides were diluted in FSW just before use. One-twentieth volume of ascidian embryo suspension was added to it. Embryos were reared in plastic culture dishes.

Plasmid construction. The coding region of the Hrcad gene cDNA sequence was amplified by PCR using primer oligonucleotides GCCGGCCCTTTCTACGGAGCG and ATATACCTT-CACCTAAGGG. This PCR product was digested by BamiHI and NotI and ligated into RN3 plasmid (Lemaire et al., 1995) between BgiII and NotI sites.

To construct dnCAD plasmid, the repressor region of the Engrailed gene was amplified from the genomic DNA of the Oregon R strain of Drosophila using primers GCCCTTTCTGAGAGCGGUAAGCTTGCGCTTGCGCTGCGACCG and TGAATTCACGGATCCAGACGACGATTGT and subcloned in pCR 2.1 plasmid (Invitrogen). Then its nucleotide sequence was confirmed. Hrcad sequence was amplified by PCR using primers TTTGGCCGCGCTTTTCTACGGAGCGACGACGATGATTGT and digested with NotI and BgiII, and ligated into RN3 vector and treated with EcoRI, digested with Blunting High (Toyobo), digested with NotI, and ligated to Hrcad sequence. This plasmid was designated RN3-CadXho. This plasmid was amplified using primers AAGATCTAGGCCTCATCGGACCAC and AAGCAGCGAGACGACGACGACGATGATTGT and digested with NotI and BgiII sites.

RNA injection. Template plasmids, RN3-cad, RN3-dnCAD, and RN3-EnR, were linearized using SfiI (Promega). mRNA was synthesized using the mMESSAGE mACHINE kit (Ambion). Synthesized mRNA was precipitated with lithium chloride, dissolved in RNase-free water at 1 mg/ml, and stored at −70°C. Fast green was added (0.2–0.05%) to mRNA solutions to facilitate injection and to estimate a volume of injected RNA solution.

Fertilized ascidian eggs were immersed in sea water containing 0.05% actinase E to make the surface of the egg chorion sticky. The eggs were extensively washed with FSW and transferred into a plastic dish, where the eggs were attached to the bottom. Microinjection was carried out using an injection apparatus (M-152, Narishige). A RNA solution of approximately 1/10 vol of an egg was injected.

Fertilized Xenopus eggs were immersed in 2% cysteine solution and washed in 0.5% MBS. 3% Ficoll thoroughly. Xenopus embryos were injected with 12 nl RNA solution at the two-cell stage into both blastomeres using an injection apparatus (MN-333, Narishige) and Transjector 5246 (Eppendorf). One hour after injection,
the embryos were transferred into 0.05 M B5S, 3% Ficoll, and 1 h later the medium was changed. Embryos were cultured at 18–22°C.

Embryo manipulation. The chorion membrane of ascidian embryo was manually removed using tungsten needles. Ascidian embryos were dissected using a glass knife. Manipulated ascidian embryos were reared on 1% agarose in sterile artificial sea water (Jamarine Co. Ltd.).

RESULTS

Cloning of Ascidian caudal Gene

We identified an ascidian homeobox fragment with similarity to the caudal/Cdx family gene among PCR products using degenerate primers designed for amplification of Hox gene fragments and Halocynthia genomic DNA as template. Using this PCR fragment as a probe, two cDNA clones were isolated from an ascidian larva cDNA library. Nucleotide sequencing and conceptual translation of the cDNAs revealed that these cDNAs were derived from an ascidian caudal gene, which we designated Hrcad (Fig. 1A).

Hrcad shows the greatest similarity to other caudal family proteins in the homeodomain and the flanking regions (Fig. 1B; Marom et al., 1997). The phylogenetic tree constructed using caudal homeodomains shows clustering of vertebrate genes, which is consistent with the previous grouping of caudal genes by Marom et al. (1997) and that Hrcad locates outside of the cluster. Hrcad is even more remote from vertebrate caudal than fly caudal, which is, however, frequently observed with ascidian proteins. The hexapeptide, which is loosely conserved among some homeodomain

FIG. 1. Sequence of Hrcad, the ascidian caudal, and comparison with those of other caudal family genes. (A) The composite nucleotide sequence from cDNA clones and deduced amino acid sequences of the ascidian caudal gene, Hrcad. The homeobox and the homeodomain are shaded and the hexapeptide is boxed. Nucleotide sequences used for antisense oligonucleotide experiments are indicated by underlining; asCAD-1 and asCAD-2 sequences are complementary to those indicated by single and double underlining, respectively. (B) Comparison of the homeodomain and the flanking regions of Hrcad with those of other caudal family genes. Identical residues are indicated by dashes. (C) A phylogenetic tree of caudal homeodomains. The tree is constructed using the NJ method by a computer program (Genetyx, Software Development Co. Ltd.). Fly fushi tarazu (ftz) homeodomain is used as an outer group sequence. Numbers on the branches indicate the values of expected substitution of amino acid residues. Grouping of vertebrate caudal is according to Marom et al. (1997). The database Accession No. for Hrcad is AB031032.
protein families including Hox, ems, and TCL (Bürglin et al., 1994), is observed in Hrcad and vertebrate caudal, but not in the nematode and Drosophila genes, suggesting relatedness of Hrcad to vertebrate caudal in this respect.

Using a homeobox fragment of Hrcad as a probe, Southern hybridization to Halocynthia genome DNA was carried out, in which only a single band was detected under standard or reduced stringency hybridization conditions (data not shown), indicating that no other sequence similar to caudal homeobox is present in the Halocynthia genome. These analyses suggest that Hrcad is a single copy gene and might have diverged before multiplication of caudal genes during chordate evolution.

Expression Pattern of caudal during Ascidian Embryogenesis

The temporal expression pattern of Hrcad in embryogenesis was examined by Northern hybridization using the 1.8-kbp cDNA (Hcd-3 clone) as a probe, in which a single band was detected at 2.7-kb from the gastrula stage onward (data not shown), indicating that no other sequence similar to caudal homeobox is present in the Halocynthia genome. These analyses suggest that Hrcad is a single copy gene and might have diverged before multiplication of caudal genes during chordate evolution.

Expression Pattern of caudal detected by in situ hybridization. Shown are embryos at the mid gastrula stage (A); at the late gastrula stage (B); at the neural plate stage (C); at the midneural stage, in which weak but significant nuclear staining is observed in the posterior epidermis (D); at the late neurula stage (E); at the early tailbud stage (F); and at the midtailbud stage (G). The names of Hrcad-positive blastomeres are indicated in A–C. Dorsal view (A to G) or lateral view (E) is shown. Anterior is to the top for all pictures. In (E), dorsal is to the left. All pictures are at the same magnification. Scale bar, 100 μm. No significant signal was obtained with a sense strand probe (data not shown).

Hrcad-expressing cells, which are precursors of the lateral walls of the posterior neural tube (Nishida, 1987), move posteriorly and become aligned parallel to the dorsal midline (Fig. 2D). By the beginning of neurulation, expression of Hrcad has also become detectable in posterior epidermal cells (Fig. 2D). Epidermal expression was enhanced during neurulation and all posterior epidermal cells express Hrcad by the tailbud stage (Fig. 2E). At the early tailbud stage, a transient reduction in expression was observed in posterior-most epidermis (Fig. 2F). In midtailbud embryos, expression of Hrcad was evident in the lateral walls of the posterior neural tube (Fig. 2G). The anterior borders of Hrcad expression in the neural tube and epidermis were roughly at the same level, which was at the junction of the trunk and the tail (Figs. 2E, 2F, and 2G). In the larva, expression of Hrcad was evident by Northern analysis (data not shown), but not by in situ hybridization. In Halocynthia embryos, it is known that in situ hybridization under the present conditions is severely interfered with by the presence of thick tunic especially in the larval tail region. Therefore, it is highly likely that expression of Hrcad continues in the larval tail.

Suppression of caudal Function by Antisense Oligonucleotides

The restriction of Hrcad expression to the tail-forming region during ascidian embryogenesis suggests a role in tail formation. To investigate this possibility, we examined
caudal function in ascidian embryos using antisense oligonucleotides (Okamura et al., 1994; Swalla and Jeffery, 1996). We synthesized phosphorothiolated oligonucleotides, asCAD-1 and asCAD-2, complementary to the nucleotide sequence encompassing or close to the first methionine-encoding residues (Fig. 1A). Embryos were cultured in the presence of the antisense oligonucleotides until control sibling embryos became larvae. After testing a series of concentrations, we used asCAD-2 at 2.5 μM, which gave a reproducible phenotype as described below. By contrast, asCAD-1 did not cause any morphological difference or any alteration in molecular marker expression from control embryos.

Abnormalities were first evident in asCAD-2-treated embryos at the early neurula stage. At this stage, normal embryos complete gastrulation, during which the blastopore almost closes and the folding movement begins in the dorsal midline to form the neural tube from the posterior direction to the anterior direction (Fig. 3A). In the asCAD-2-treated embryos, the blastopore remained for longer time and the neural plate did not fold completely (Fig. 3B). At the tailbud stage, tail formation starts in normal embryos; the posterior half of the embryo becomes narrower and elongates, curving ventrally, within which the notochord cells form a line along the anterior–posterior (A–P) axis (Fig. 3C). In the asCAD-2-treated embryos, narrowing of the posterior half was less evident and the body bent to the dorsal side unlike normal embryos (Fig. 3D). In normal tailbud stage Halocynthia embryos, the tail elongates rapidly by more than sevenfold in length within 24 h (from approximately 120 to 900 μm) (Fig. 3E). By contrast, asCAD-2-treated embryos exhibited poor tail elongation, which was never longer than twofold (Figs. 3F, 4, 5). They did not possess the pigment cells and adhesive papillae (Fig.

FIG. 3. Effect of antisense oligonucleotides on morphology of the ascidian embryos. (A,C,E) Control embryos reared in the presence of 2.5 μM asCAD-1. These embryos exhibit normal morphology. (B,D,F) Embryos reared in the presence of 2.5 μM asCAD-2. Note that normal embryos curve ventrally, while asCAD-2-treated embryos bend to the dorsal side. (A,B) Embryos at the neurula stage. Dorsal view is shown. (C,D) Embryos at the tailbud stage. Lateral view is shown. (E,F) Larvae before hatching are shown. For all pictures, anterior is to the top. Scale bar, 100 μm.

FIG. 4. Reduction of Hrcad expression by antisense oligonucleotide treatment demonstrated by in situ hybridization. Control embryos (A,C,E) and embryos treated with 2.5 μM asCAD-2 antisense oligonucleotide (B,D,F) were examined for Hrcad expression by in situ hybridization. (A,B) Dorsal view of late gastrula embryos. (C,D) Lateral view of neurula stage embryos. Dorsal is to the left. (E,F) Ventral view of tailbud stage embryos. For all pictures anterior is to the top. Scale bar, 100 μm.
but were covered by tunic (Fig. 5N), and hatched out from the chorion at the same time as in normal embryos (data not shown).

To see whether these abnormalities are asCAD-2 specific, we added a series of concentrations of the sense oligonucleotide ssCAD-2 that is complementary to asCAD-2 to the culture medium containing 2.5 μM asCAD-2. As the concentration of ssCAD-2 increased, the larval phenotype became weaker. When oligonucleotides were added at equimolar concentration, all embryos in the batch developed normally (data not shown). Phenotypes observed here differ from those reported in other experiments using antisense oligonucleotides (Swalla and Jeffery, 1996; Olsen and Jeffery, 1997). This suggests that the phenotypes obtained in our experiment are specific for suppression of caudal function.

Under the above conditions, expression of Hrcad was examined by in situ hybridization in asCAD-2-treated embryos. The signal intensity of a pair of spots at the blastopore edge of gastrulating embryos was significantly reduced (Figs. 4A and 4B). Expression in epidermis was not detected at the neurula stage (Figs. 4C and 4D), but it became detectable from the midtailbud stage (Figs. 4E and 4F). Thus, under the present conditions, asCAD-2 reduces

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**FIG. 5.** Effect of antisense oligonucleotide treatment on expression of regional and cell-type-specific markers. Control (A,C,E,G,I,K, M,O,Q) and antisense oligonucleotide-treated (B,D,F,H,J,L,N,P,R) embryos were examined for marker expression. Expression of an anterior region marker, Otx (A–D), a proximal tail region marker, Lim-1 (E–H), and a medial posterior region marker, Hox-1 (I–L), is shown. Expression patterns were examined at the neurula stage (A,B,E,F,I,J) or at the tailbud stage (C,D,G,H,K,L). (M,N) Larvae are covered by tunic that is a marker for epidermal differentiation. Blue staining is for alkaline phosphatase activity, a marker for differentiation of endoderm. (O,P) Notochord differentiation monitored by expression of an Islet gene of H. rorezi, in the mid tailbud stage embryos. In a control embryo, the Halocynthia Islet gene is also expressed in the sensory neurons (O. Katsuyama et al., in preparation). (Q,R) Differentiation of neurons monitored by neural tubulin gene expression in early tailbud stage embryos. (R) In an antisense oligonucleotide treated embryo, the neural folds remain unclosed (stained area) and expression is not detected in the anterior terminus at which the papilla sensory neurons would otherwise be found. Anterior is to the top for all pictures. (A,B,E,F,Q,R) Dorsal view is shown. (I,J) Ventral view is shown. (C,D,G,H,K,L and M–P) Lateral view is shown and dorsal is to the right for these pictures. All pictures are of the same magnification. Scale bar in (R), 100 μm.
or eliminates Hrcad transcription up to the midtailbud stage. Recovery of Hrcad expression in the asCAD-2-treated embryos at the midtailbud stage seems to be reflected by partial narrowing of the posterior half of the embryo at this stage (compare Fig. 3D with Fig. 4F).

**Suppression of Hrcad Affects Posterior Regional Marker Gene Expression but Not Posterior Tissue Differentiation**

To further characterize the phenotype caused by the antisense oligonucleotide, we examined expression of regional markers along the A-P axis. Expression of an anterior marker, ascidian Otx (Wada et al., 1996), appeared to be enhanced by suppression of caudal function at the neurula stage (Figs. 5A and 5B). At the mid tailbud stage, Otx expression was at normal levels, although the expression domain appeared to be restricted to the dorsal trunk region (Figs. 5C and 5D). An ascidian LIM homeobox gene Hrlim marks the cell lineage of the visceral ganglion which is a proximal part of the posterior neural tube from the late gastrula stage onward (Figs. 5E and 5G; Wada et al., 1995) and cells of the sensory vesicle which is an anterior part of the neural tube from the early tailbud stage onward (Fig. 5G; Wada et al., 1995). In the asCAD-2 treated embryos, expression of Hrlim was undetectable at the neurula stage (Fig. 5F) but became detectable at the tailbud stage in the dorsal trunk, but not the putative visceral ganglion region (Fig. 5H). Expression of the ascidian Hox-1 begins at the early neurula stage and is maintained in the middle ectoderm of the embryo from the tailbud stage onward (Figs. 5I and 5K). In the asCAD-2 treated embryos, Hox-1 expression was not detected at the neurula stage (Fig. 5J) but recovered to control levels at the mid tailbud stage (Figs. 5K and 5L). Thus, suppression of caudal gene function causes reduction of expression of the middle to posterior, but not anterior regional markers.

Next, we asked whether differentiation of posterior tissues is affected by suppression of caudal function. For this, we examined tunic formation for epidermis differentiation (Figs. 5M and 5N), cell morphology (Nakatani and Nishida, 1994), islet gene expression for notochord differentiation (Katsuyama, unpublished data; Figs. 5O and 5P), and tubulin expression for neuron differentiation (Figs. 5Q and 5R; Miya et al., 1997). Taking these as indices, it appears that posterior tissue differentiation was not inhibited by suppression of caudal function using the antisense oligonucleotide. Thus, the suppression of caudal function leads to defects in tail formation without inhibiting posterior tissue differentiation.

**Suppression of caudal Function by Injection with Dominant Negative Molecule**

To further investigate the function of ascidian caudal, we injected mRNA coding for a dominant negative molecule. We made a dominant negative construct (designated dnCAD) by fusing Hrcad and the repressor region of Drosophila Engrailed (EnR). Fertilized eggs were injected with dnCAD mRNA at a series of concentrations before the first cell cleavage and cultured until siblings developed into larvae. Within the range of concentrations we examined, morphological abnormality was observed from the late stage of gastrulation. When embryos were injected with mRNA coding for EnR only (control embryos), neither
A morphological abnormality nor alteration of gene expression was observed (Fig. 6A and data not shown).

Embryos injected with 5 ng/μl mRNA had a shortened tail containing muscle and notochord cells, and the tail length varied from 1.5- to 3-fold the length of the trunk (Fig. 6B; n = 18). All embryos injected at this concentration bent dorsally, papillae did not form at the anterior tip, and the brain (the sensory vesicle) protruded outside, suggesting...
incomplete formation of the neural tube. Embryos injected with 12.5 ng/μl dnCAD mRNA had a tail no longer than the trunk length (Fig. 6C; n = 23). Injection of 25 ng/μl dnCAD almost completely suppressed tail formation so that even narrowing of the posterior region of the embryo was not observed (Fig. 6D; n = 36). Thus, embryos injected with dnCAD exhibited suppression of gastrulation movement, neural tube formation, and narrowing of the posterior half of the embryos, the severity of which was dependent on the concentration of injected mRNA. Embryos injected with 12.5 ng/μl dnCAD mRNA were examined for molecular marker expression, which yielded similar results as described for antisense oligonucleotide treatment (data not shown).

**Overexpression of caudal Induces Elongation in Ascidian Animal Caps**

Suppression of function experiments described above led us to hypothesize that caudal is required for control of ectodermal cell movement during tail formation. If this is the case, overexpression of caudal in naive ectodermal cells should induce alteration in cell behavior. We examined behavior of the ectodermal cells injected with wild-type caudal mRNA. For this, we employed an ascidian animal cap, that is, four animal blastomeres isolated in combination from the eight-cell-stage ascidian embryos. When cultured until control embryos become larvae, the animal explant differentiates into epidermis (Nishikata et al., 1987) forming a bulbous structure (Fig. 7A) in which it has been shown that HrHox or other regional ectoderm markers such as HrHox-1 are not expressed (Wada et al., submitted for publication). Thus, the animal cap can be considered to form naive epidermal tissue. Animal caps prepared from embryos injected with 25 ng/μl wild-type caudal mRNA at the one-cell stage were indistinguishable from un.injected (n = 9) or EnR mRNA-injected (n = 12) control animal caps until sibling intact embryos reached the tailbud stage. After this, however, caps from caudal mRNA-injected embryos gradually elongate, forming a single-cell-layered tubular structure which was covered by tunic, a marker for epidermal differentiation (Fig. 7B; n = 32). Coinjection of equimolar amounts of dnCAD and wild-type caudal mRNA did not cause elongation (n = 5; data not shown), indicating the specificity of caudal function in elongation of animal caps. Furthermore, a vegetal half explant that consists of mesodermal and endodermal lineages did not elongate upon overexpression of wild-type caudal (n = 7). Therefore, a function of the ascidian caudal is to control cell movement and this is specific for the ectoderm.

**Overexpression of Wild-Type and Dominant Negative HrCad in Xenopus Embryos**

Structural conservation of caudal family protein is limited to the homeodomain and a few amino acid residues nearby (Fig. 1B). To investigate conservation of caudal function among chordates, we asked whether HrCad is functional in Xenopus embryos. Xenopus embryos were injected with increasing concentrations of dominant negative HrCad (dnCAD) mRNA and were cultured until stage 35. Control embryos injected with EnR mRNA exhibited no obvious abnormality (Fig. 8A, n = 10). In the batch of embryos injected with 12.5 ng/μl dnCAD mRNA, some embryos (n = 2/15) exhibited slight bending of the body to the dorsal side and a slight abnormality in the dorsal fin (Fig. 8B). Other embryos in this batch appeared normal (n = 13/15). In the batch injected with 25 ng/μl dnCAD mRNA, the severity of phenotypes varied; in some embryos with milder phenotype, the bend of the body was apparently enhanced, but the length of the tail was similar to that of wild-type embryos (Fig. 8C; n = 3/23), whereas severely affected embryos had an evidently shortened tail (Fig. 8D; n = 20/23). In the batch injected with 50 ng/μl dnCAD mRNA, the tail was shortened in all embryos (n = 11). Most of the embryos were U-shaped, bending dorsally (Fig. 8E; n = 10/11), and in an extreme case the tail tip was touching the head (Fig. 8F). In all dnCAD mRNA-injected embryos, the cement gland and the eyes were well formed, indicating that development of the anterior part of these embryos was not affected. The bending of the embryos to the dorsal side suggests interference with the convergent extension movement in the dorsal ectodermal cells. Convergent cell movement is also involved in gastrulation and neural tube formation. Xenopus embryos overexpressing dnCAD exhibit delay or incompletion of these events.

Recently it has been reported that overexpression of a dominant negative Xenopus caudal gene, Xcad-3, suppresses development of the posterior region in Xenopus embryos (Isaacs et al., 1998) similar to the present results obtained by dominant negative HrCad. Isaacs et al. (1998) also reported that overexpression of Xcad-3 caused elimination of anterior structures of Xenopus embryos. Consistent with this, when 25 ng/μl wild-type HrCad mRNA was injected into Xenopus embryos, anterior structures, such as cement gland and eyes were eliminated (Figs. 8G and 8H). From these observations, we conclude that HrCad is functional in Xenopus embryos, suggesting conservation of the biological properties of caudal proteins.

**DISCUSSION**

In this report, we have described the isolation, expression pattern, and function of the ascidian caudal gene, HrCad. Our results suggest HrCad is involved in ascidian tail formation. We further suggest functional conservation of caudal by introducing wild-type or dominant negative HrCad mRNA into Xenopus embryos.

**Comparison of Expression of HrCad with Other caudal Genes**

HrCad is expressed from the gastrula stage onward and is restricted to the posterior ectoderm throughout embryogen-
Function of Hrcad in Tail Formation

In the present study, we employed two different techniques to suppress function of Hrcad. A difference between the two techniques is that an antisense oligonucleotide suppresses a function of the target gene at the RNA level, while the target of a dominant negative molecule is the protein product. We compared the results obtained by the two approaches. The phenotypes shared were reduction of the length of the tail, inhibition of neural tube formation, and delay or failure to complete gastrulation. We suggest the length of the tail, inhibition of neural tube formation, and/or a difference between the two techniques is that an antisense oligonucleotide suppresses a function of the target gene at the RNA level, while the target of a dominant negative molecule is the protein product. We compared the results obtained by the two approaches. The phenotypes shared were reduction of the length of the tail, inhibition of neural tube formation, and delay or failure to complete gastrulation. We suggest that Hrcad functions in tail formation through controlling ectodermal cell movement in ascidian embryos.

Since Hrcad expression starts at the midgastrula stage, Hrcad plays a role in the events after the onset of gastrulation, but not in the initiation of gastrulation. Expression of Hrcad is first observed in posterior lateral cells at the neural plate boundaries at the middle to late gastrula stage. In ascidian embryos, intercalating cell movement along the posterior dorsal midline has been described within neuroectoderm (Nicol and Meinertzhagen, 1988) and notochord (Miyamoto and Crowther, 1985) upon tail elongation. Furthermore, it has been reported that the change of cell shape from columnar to wedge-shaped is first observed in the cells at the lateral borders of the neural plate (Nicol and Meinertzhagen, 1988). The initial expression of Hrcad coincides with this cell shape change, which may be responsible for the subsequent dorsal convergence of ectodermal cell sheet. This correlation, together with the observation that elongation of animal caps was induced by overexpression of Hrcad, further supports a function of Hrcad in tail formation.

The Manx gene is another factor implicated in ascidian tail formation. Manx codes for a transcription factor which is expressed maternally in ascidian species which develop a tailed larva but not in closely related species, which develop a tailless larva (Swalla et al., 1993). Manx transcripts are confined to the ectoderm lineage after fertilization (Swalla et al., 1993). Suppression of function of Manx results in inhibition of tail formation (Swalla and Jeffery, 1996). The phenotypes caused by suppression of function of Manx and Hrcad resemble each other but the abnormality is more severe in Manx than in Hrcad function-suppressed larvae. This suggests that caudal may be downstream of Manx. On the other hand, Nishida (1994) has demonstrated the presence of a region in the ascidian egg that is responsible for tail formation. Thus, it will be interesting to establish the relationship between this region and regulation of expression of caudal and/or Manx. This will allow us to clarify the relationship between morphogenesis and the maternal information spatially distributed in the ascidian egg.

Caudal and Chordate Tail Formation

Here we have shown conservation of caudal function in chordate tail formation using ascidian and amphibian embryos. In early vertebrate embryos the tailbud plays important roles in tail formation (Griffith et al., 1992; Gont et al., 1993; Tucker and Slack, 1995). The tailbud retains organizer activity and the inner cell mass of the tailbud produces the axial mesodermal and endodermal structures (Gont et al., 1993; Knezevic et al., 1998). By contrast, it has been demonstrated by fate mapping that a large amount of tail epidermis originates from the trunk (Tucker and Slack, 1995). Elongation of internal tissue appears to depend on differentiation and intercalation of axial mesodermal cells (Gont et al., 1993; Tucker and Slack, 1995). The ectodermal layer must undergo ordered morphogenesis to follow mesodermal extension. We suggest that caudal function may be required for this point. Caudal expression in the tail ectoderm may be regulated along the A–P axis by FGFs that are secreted from the underlying tailbud mesoderm (Pownall et al., 1992; Isaacs et al., 1992; Tannahill et al., 1992). Since ascidian animal caps elongate upon FGF treatment (Katsuyama, unpublished results), it will be interesting to examine the relationship between Hrcad and FGF in ascidian embryogenesis.

In ascidian embryos, there is no structure equivalent to the vertebrate tailbud and Hrcad is expressed only in ectoderm. However, as mentioned in the previous section, intercalating cell movement along the posterior dorsal midline has been described with the cells of the notochord (Miyamoto and Crowther, 1985) and the neuroectoderm (Nicol and Meinertzhagen, 1988) upon tail elongation. Similar cell movements are observed upon tail elongation or posterior shift of the tailbud in frog, chick, zebrafish, and mouse embryos (Gont et al., 1993; Catala et al., 1995;
Knezevic et al., 1998; Kanki and Ho, 1997; Wilson and Beddington, 1996). This suggests that such cell movements observed in posterior body formation are shared among chordate embryos. Taking this into account, we suggest that control of ectodermal cell movement may be a caudal function of early origin in chordates.

In mouse and frog embryos, it has been revealed that caudal genes regulate Hox gene expression (Subramaniyan et al., 1995; Epstein et al., 1997; Isaacs et al., 1998). In ascidian embryos, suppression of caudal function down-regulates Hox-1 expression, suggesting conservation of Hox gene regulation by caudal in the ascidian. To further clarify involvement of Hrcad in regulation of ascidian Hox gene expression, analyses of transcriptional regulation of Hox-1, including its promoter function, are needed.

Involvement of the caudal gene family in development of posterior parts is conserved among animals, from nematodes to insects and mammals. This suggests that the developmental mechanisms of posterior part formation that require caudal function is evolutionarily very old. However, the structures generated by these molecular mechanisms are different among animals, such as abdominal segments of insects and a long extended tail in chordates. The requirement of caudal function for posterior development may be a bottleneck in the evolution of animal development (Duboule, 1994). On the other hand, uniqueness in posterior body parts for each animal suggests that molecular events that reside downstream in posterior patterning may have diverged. Accordingly, target genes of caudal may be different among animal groups. During the course of chordate evolution, caudal might have acquired a set of genes to control cell behavior or to coordinate cell movement and this may be one of the critical molecular events that facilitated formation of the tail, one of the major characteristics of the phylum Chordata.

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