

Expression of a *Gsx* Parahox Gene, *Cnox-2*, in Colony Ontogeny in *Hydractinia* (Cnidaria: Hydrozoa)

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ABSTRACT The ontogeny of colonial animals is markedly distinct from that of solitary animals, yet no regulatory genes have thus far been implicated in colonial development. In cnidarians, colony ontogeny is characterized by the production of a nexus of vascular stolons, from which the feeding and reproductive structures, called polyps, are budded. Here we describe and characterize the *Gsx* parahox gene, *Cnox-2*, in the colonial cnidarian *Hydractinia symbiolongicarpus* of the class Hydrozoa. *Cnox-2* is expressed in prominent components of the colony-wide patterning system; in the epithelia of distal stolon tips and polyp bud rudiments. Both are regions of active morphogenetic activity, characterized by cytologically and behaviorally distinct epithelia. Experimental induction and elimination of stolon tips result in up- and down-regulation, respectively, of *Cnox-2* expression. In the developing polyp, *Cnox-2* expression remains uniformly high throughout the period of axial differentiation. The differential oral–aboral *Cnox-2* expression in the epithelia of the mature polyp, previously described for this and another hydrozoan, arises after oral structures have completed development. Differential *Cnox-2* expression is, thus, associated with key aspects of patterning of both the colony and the polyp, a finding that is particularly striking given that polyp and colony form are dissociable in the evolution of Hydrozoa. *J. Exp. Zool. (Mol. Dev. Evol.)* 306B:460–469, 2006. © 2006 Wiley-Liss, Inc.

Colonial organisms undergo continuous growth and pattern formation throughout the life of the colony; a feature markedly different from solitary organisms whose development is typically confined to discrete periods of embryology and/or metamorphosis. Development in a colonial organism may be conveniently thought of in terms of three pattern-forming systems. The first system is the conventional one leading from the zygote to a larval form. A second system is the formation of the zooid of the colony (termed polyps in cnidarians), the first instance of which is produced by the metamorphosing larva and thereafter by budding. A polyp gathers foods, bears reproductive structures, and/or performs various defensive functions. Polyps are interconnected by tube-like structures called stolons (or rhizomes). The interconnectivity of these structures to the polyps effectively defines the colony as a single physiological unit. The third system is that of colony-wide patterning, involving morphogenetic processes that define the placement of polyps relative to one another and relative to the stolonial tissues.

Phyla comprising predominately colonial taxa include the Porifera, Cnidaria, and Bryozoa, as well as the chordate class Urochordata. Homeoboxes have been identified in all these taxa (Holland, '91; Murtha et al., '91; Schierwater et al., '91; Naito et al., '93; Kruse et al., '94; Seimiya et al., '94; Degnan et al., '95; Di Gregorio et al., '95; Kuhn et al., '96; Ma et al., '96; Manuel and Le Parco, 2000; Passamaneck and Halanych, 2004; Tiozzo et al., 2005), but thus far, no gene has been implicated in colony-wide patterning. Within the Cnidaria, the most extensively characterized homeobox gene is *Cnox-2*, the homolog to the parahox gene *Gsx* (Finnerty and Martindale, '99).

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Gsx homologs have been recovered from the colonial hydrozoans *Sarsia* (Murtha et al., '91), *Hydractinia* and *Eleutheria* (Schierwater et al., '91), and *Podocoryne* (Yanze et al., 2001), the solitary hydrozoan *Hydra* (Schummer et al., '92; Naito et al., '93; Shenk et al., '93a), the coral *Acropora* (Hayward et al., 2001), the sea anemones *Metridium* and *Nematostella* (Finnerty and Martindale, '97), and the scyphozoan jellyfish *Cassiopeia* (Kuhn et al., '99).

Shenk et al. ('93a) found *Cnox-2* to be expressed at low levels in the epithelia of oral regions and at high levels in aboral epithelia of the freshwater, solitary cnidarian, *Hydra*. Moreover, they demonstrated that experimental alteration of the oral-aboral axis results in subsequent alteration in *Cnox-2* expression (Shenk et al., '93b). Cartwright et al. ('99) documented *Cnox-2* expression in the polyps of a colonial relative of *Hydra*, *Hydractinia symbiolongicarpus* and found that expression in feeding polyps of *Hydractinia* mirrors that of the solitary *Hydra* polyp. Thus, the pattern of *Cnox-2* expression in *Hydra* and *Hydractinia* feeding polyps appears conserved between these two distantly related (Collins, 2002) hydrozoans.

A subsequent study by Yanze et al. (2001) documented expression of the *Gsx* gene in larval stages of *Podocoryne carnea*, a close relative to *Hydractinia*. They found this gene to be strongly expressed in the anterior endoderm of the planula larvae. This axial expression pattern is consistent with that found in *Hydra* (Shenk, '93a) and *Hydractinia* (Cartwright et al., '99) in that the anterior end of the planula, upon metamorphosis, becomes the aboral pole of the primary polyp in both *Podocoryne* and *Hydractinia*. Interestingly, the expression pattern of the *Gsx* homolog is reversed in anthozoans, which display a spatially restricted expression in the posterior ends of the planula (Hayward et al., 2001; Finnerty et al., 2003) and oral poles of the polyp (Finnerty et al., 2003).

These studies suggest that the *Gsx* gene, *Cnox-2*, may play important roles in two of the three pattern-forming systems in the cnidarian life cycle, the larva, and the polyp. This prompted us to characterize *Cnox-2* in the third system, that of colonial development, in *Hydractinia*. Here we report the expression of *Cnox-2* in *H. symbiolongicarpus* in developing polyps and in prominent components of the central colony-wide patterning tissue, the stolonal nexus. Characterization of its expression in developing and experimentally manipulated tissue suggests that this gene may play a role in colony-wide patterning.

MATERIALS AND METHODS

Life cycle

The life cycle of *Hydractinia* is typical of many colonial hydrozoans, consisting of early embryonic development, metamorphosis of the larva to the primary polyp, and development of the stolonal nexus from which budded polyps emanate (Fig. 1). Fertilization is external, cleavage yields a coeloblastula, which gastrulates by synchronous multipolar delamination to ultimately yield a planula larva (Bunting, 1894). The *Hydractinia* planula displays marked A-P differentiation (Fig. 1A) with the anterior end destined to become the aboral pole of the primary polyp and the posterior end destined to become the oral pole (hypostome and tentacles) upon metamorphosis (Weis et al., '85). Stolons emanate from the aboral pole of the primary polyp (Fig. 1B). Stolons are fluid-filled

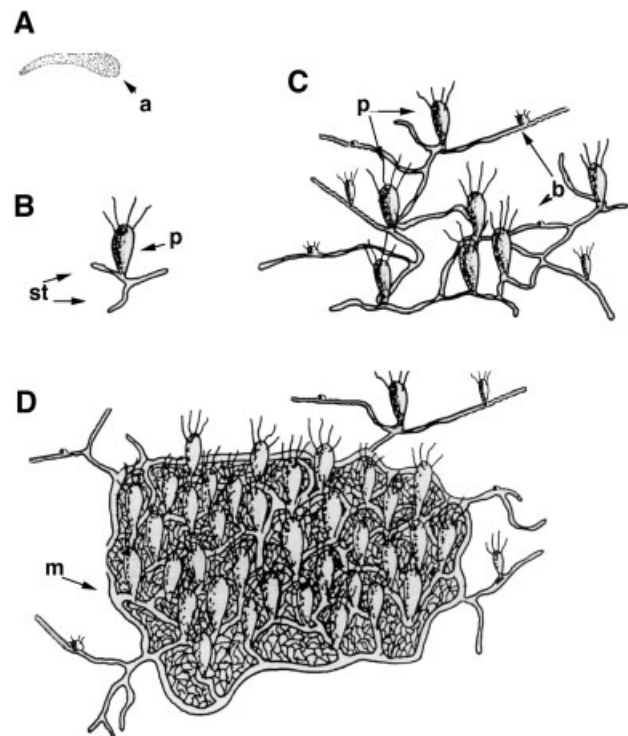


Fig. 1. *Hydractinia* colony ontogeny. (A) Planula larva displaying anterior (a)–posterior differentiation. (B) Schematic drawing of primary polyp (p) of *Hydractinia*, with stolons (st) beginning to elongate over substratum; (C) a later stage in colony ontogeny, showing the development of an anastomosing stolonal nexus and numerous polyps (p) and polyp buds (b) arising from elongating stolons; and (D) a later stage in colony ontogeny, where the basal ectoderm of polyps has extended over the stolonal nexus to form the stolonal mat (m). Modified from Blackstone ('96) and Weis et al. ('85).

tubes, the lumen and tissue layers of which are a continuation of the gastric cavity and tissue layers of the polyp. Stolons lie adherent to the substratum, elongate, and periodically branch by the production of new stolonial tips. Polyp bud rudiments arise from the apical surface of stolons and, as they grow, undergo characteristic axial differentiation into oral and aboral regions (Fig. 1C). Later ontogenetic events in the life cycle of *Hydractinia* include the development of polymorphic polyps, specialized for reproduction and defense, as well as the development of a stolonial mat, which is an extension of the ectodermal layers of the polyp body column to encase the endodermal stolonial canals between a common ectodermal sheet (Fig. 1D). Polymorphic polyps and a stolonial mat, while not uncommon in many hydrozoan lineages, are not universal features of colonial organisms and, hence, are not considered further here (but see Cartwright et al., '99 and Cartwright and Buss, '99). Many hydrozoans possess an additional component to their life cycle, the production of free-living medusae, which are asexually budded from polyps and produce sexually mature gonophores. This stage in the life cycle is reduced in *Hydractinia*, with gametes maturing in gonophores that remain fixed to specialized polyps.

Animal care

Colonies of *H. symbiolongicarpus* encrusting gastropod shells occupied by the hermit crab *Pagurus longicarpus* were collected at Yale Peabody Museum Marine Station, Guilford, CT, and at Lighthouse Point, New Haven, CT. Colonies were surgically explanted onto microscope slides and/or coverslips, placed in racks in seawater (REEF CRYSTALS, Aquarium Systems, Mentor, OH, USA) aquaria maintained at either 15°C or 22°C, and fed to repletion with 3–5-day-old nauplii of *Artemia salina* every 2 days. Further details on standard culture conditions are given in Blackstone and Buss ('91). Colonies were starved at least 3 days before nucleic acid isolation and fixation for immunohistochemistry.

Cloning of the *Cnox-2* gene

cDNA *Cnox-2* was isolated by PCR from cDNA using sequence information from the *Hydractinia Cnox-2* homeobox fragment reported in Schierwater et al. ('91). To obtain total RNA, whole colonies were homogenized in guanidium thiocyanate buffer and purified according to manufac-

turer's instructions (Total RNA Isolation Kit, Stratagene, La Jolla, CA, USA). First-strand cDNA was prepared as described previously by Dick and Buss ('94). The cDNA sequence located downstream of the *Cnox-2* homeobox fragment was isolated by RACE (Frohman, '90). Outer and inner gene-specific primers were designed from the homeobox fragment and used in conjunction with Frohman's ('90) RACE primers, (R_0 and R_1 , respectively). PCR amplification was performed in a Perkin-Elmer Thermal cycler, model 480, with conditions as previously described (Dick and Buss, '94). The cDNA sequence located upstream of the *Cnox-2* homeobox fragment was isolated by ligation-anchored PCR (Troutt et al., '92) using gene-specific primers designed from the homeobox fragment reported in Schierwater et al. ('91). Products from RACE and LA-PCR were visualized by electrophoresis and 1 μ l was cloned directly into pCRII vector (Invitrogen, Carlsbad, CA). RACE and LA-PCR clones were sequenced at the Keck Biotechnology Resource Lab, Yale University, by automated DNA sequencing.

Genomic fragment: We prepared genomic *EcoRI* and *HindIII* libraries from *H. symbiolongicarpus* in phage (λ NM 1149). The library was screened at high stringency with a 166 bp *Cnox-2-Hs* homeobox probe. Positive clones were subcloned into pBS/K+ and grown in *E. coli* DH5 alpha (for further details, see Schierwater, '95). The plasmid, pBSC*Cnox-2*, contained an *EcoRI* genomic fragment that includes the complete coding sequence of the *Hydractinia Cnox-2* gene. The plasmid was purified with QIAGEN Plasmid Kit, and both strands were sequenced at the Keck Biotechnology Resource Lab, Yale University, by automated DNA sequencing and primer walking.

Specificity of anti-*Cnox-2* antibodies

Anti-*Cnox-2* polyclonal antibodies were produced and purified as described in Cartwright et al. ('99). *Cnox-2* protein was synthesized in *E. coli* to determine specificity of anti-*Cnox-2* antibodies on a Western blot. The entire *Cnox-2* cDNA was cloned into pET-23a (Novagen, Darmstadt, Germany) in order to generate a 32 kD hexahistidine/*Cnox-2* protein (His*Cnox-2*). *E. coli* BL21 cells were transformed with pET-*Cnox-2* plasmid and induced to express His*Cnox-2* in the presence of IPTG. The cells were sonicated and the His*Cnox-2* protein was partially purified by centrifugation at 10,000g in the presence of 8M

urea and fractionated by SDS-PAGE gels. The His*Cnox-2* protein was electroluted from gel slices.

For dot blots, proteins were diluted in carbonate buffer, pH 9.0, and added directly to the membrane. For Western blots, proteins were fractionated on a 15% SDS-PAGE gel at 40 mA and semi-dry blotted on PVDF transfer membrane (Millipore, Billerica, MA, USA). For Westerns and dot blots, membranes were blocked in 5% blocking reagent (Amersham, Uppsala, Sweden) and incubated in protein A purified anti-*Cnox-2* antibodies and pre-immune controls, diluted to 2 µg/ml in blocking reagent. All Westerns and dot blots were subsequently performed using the ECL detection method (Amersham) according to manufacture's instructions.

Whole mount immunolocalization

All observations reported here were repeated in multiple replicate trials with several unrelated *Hydractinia* colonies. Colonies grown on coverslips were relaxed with menthol and fixed in 4% paraformaldehyde at 4°C overnight. *Cnox-2* whole mount immunolocalization was performed as described in Cartwright et al. ('99).

The initiation of polyp bud rudiments and the anastomosis of stolons are continuously occurring processes in a growing *Hydractinia* colony. Colonies were chosen at the earliest stage at which polyp bud rudiments could be detected to arise from free stolons by visualization at 50× in a dissecting microscope. Taking this point as time point zero, this and subsequent timed stages were fixed for immunohistochemistry. Similarly, stolon tips were observed at 50× in a dissecting microscope and the point at which stolon tips first contacted an adjacent stolon was scored as time zero, from which subsequent timed stages were determined.

Stolon tips were induced by surgically removing a small piece of stolon tissue in a region of the stolon that does not normally express *Cnox-2*. Stolon tips form at the sites of incision. Thereafter, we monitored *Cnox-2* expression in the region of tip induction and in unmanipulated regions of free stolons in the same colony.

RESULTS

Isolation and characterization of *Cnox-2* cDNA and genomic sequence from *Hydractinia symbiolongicarpus*

The *Hydractinia Cnox-2* cDNA sequence, obtained from cDNA pools by RACE and LA-PCR, is

1,206 nucleotides in length, excluding the polyadenylation site. There are 104 bps of untranslated leader sequence, an open reading frame of 783 bps, and 319 bps downstream of the predicted stop codon, followed by a poly(A) stretch. The predicted protein consists of 261 amino acids. The cDNA sequence had been deposited in Genbank under accession number AF31953. A 2,279 base pair clone (pBSC*Cnox-2*) was isolated from an EcoR1/pBS genomic library. The coding region consists of two exons, 490 and 293 bps, respectively, interrupted by a single intron 379 bps in length. The genomic sequence has been deposited in GenBank under accession number DQ298519.

Expression of *Cnox-2* is specific to the ectoderm of post-metamorphic tissues in *Hydractinia*

To examine *Cnox-2* expression in *Hydractinia*, we raised antisera to a synthetic peptide of the *Cnox-2* protein (NC*Cnox-2*). To confirm that the IgG purified antibodies detected the *Cnox-2* protein, we performed dot blot and Western blot analysis against the recombinant *Cnox-2* protein produced in *E. coli* (His*Cnox-2*), and peptide conjugated to an alternative carrier protein on a dot blot (KLH-NC*Cnox-2*). The IgG purified fraction recognized both KLH-NC*Cnox-2* and His*Cnox-2* on a dot blot and binding was detected from pre-immune serum on dot blots (not shown). The antibodies also recognized the 32 kD His*Cnox-2* protein and a band of predicted size in a total *Hydractinia* protein fraction. Neither of these bands appeared in the pre-immune serum (not shown).

Cnox-2 expression was detected by whole mount immunolocalization with the IgG purified immune fractions of polyclonal antibodies, and visualized by high-intensity staining of bright green or yellowish-green, depending on the particular filter (FITC). In immunoreactive cells, high-intensity staining was localized to the nuclei, as expected of a putative transcription factor. A high density of immunoreactive nuclei was qualitatively assessed to have high *Cnox-2* expression, and few or no immunoreactive nuclei were assessed to have low *Cnox-2* expression. The pre-immune serum showed no nuclear staining (not shown).

Cnox-2 expression was limited to nuclei of ectodermal epithelial cells in *Hydractinia*. No *Cnox-2* protein was detected in the endodermal cells as determined by visualizing radial sections of whole mounts (not shown). The antibodies also stained nuclei in post-metamorphic tissues of the

species *Hydractinia echinata*, but displayed no cross-reactivity to the closely related hydractiniid hydroid genera *Podocoryne* and *Stylactaria* (not shown, J. Bowsher, pers. comm.).

Cnox-2 is expressed in developing polyps

To characterize *Cnox-2* expression in developing polyps, we examined polyps at different intervals from the time when a polyp bud rudiment was first detectable on a stolon until the polyp became competent to feed. Polyps develop on free stolons at a predictable distance from the tip, i.e., in regions of free stolon where *Cnox-2* is not being expressed. In developing polyps, *Cnox-2* was expressed at the first detectable indication of polyp bud formation (Fig. 2A) and continued to be expressed at high intensities throughout the epithelia of the elongating bud. By 40 hr, the young polyp has developed its axial organization,

with a hypostome and tentacles at the oral region. At this time, there are still continuous high levels of *Cnox-2* expression throughout the length of the developing polyp ($n = 14$) (Fig. 2B and C). Between 40 and 80 hr, *Cnox-2* expression begins to decrease in the oral region, but continues to be expressed at high levels in the body column. About 61% of the polyps examined displayed this region-specific expression, the remainder maintained the continuous pattern of *Cnox-2* expression throughout the length of the polyp ($n = 23$). After 80 hr, all polyps examined displayed decreased *Cnox-2* expression in the oral region (hypostome and tentacles) (Fig. 2D and E) and high continuous levels in the polyp body column (Fig. 2D and F) ($n = 69$). The pattern of expression in mature polyps is as previously described for *Hydractinia* (Cartwright et al., '99) and for *Hydra* (Shenk et al., '93a).

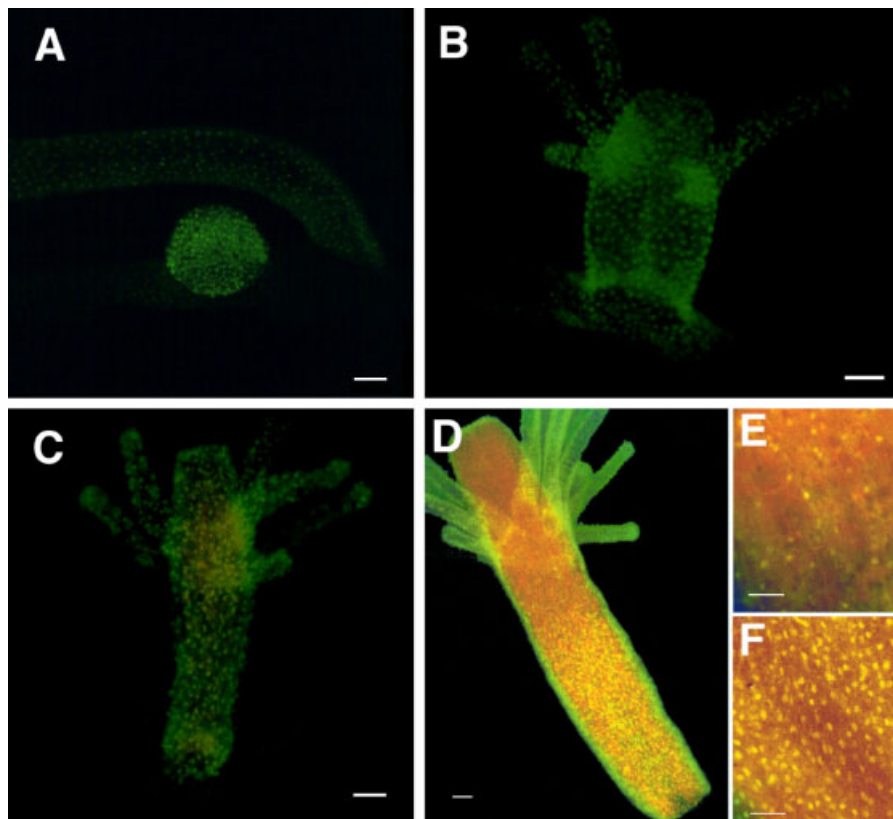


Fig. 2. Detection of *Cnox-2* protein at different stages of polyp ontogeny, using polyclonal antibodies generated to the *Cnox-2* gene product and visualized by immunofluorescence. Nuclei with the *Cnox-2* protein appear green or yellowish green. (A) Polyp bud expressing *Cnox-2*. Also shown in (A) is a newly branched stolon, expressing *Cnox-2*, located above the polyp bud. (B) Immature gastrozooid (1 day old) expressing *Cnox-2* throughout the length of the polyp. (C) Two-day-old gastrozooid expressing *Cnox-2* throughout the length of the

polyp. (D) Adult gastrozooid expressing *Cnox-2* in a region-specific manner, with low expression in the oral pole and high expression toward the aboral pole of the polyp. (E) Higher magnification of a representative region of the oral pole of the adult polyp showing low *Cnox-2* expression. (F) Higher magnification of a representative region of the aboral pole of the adult polyp showing high *Cnox-2* expression. Scale bars = 0.1 mm.

Cnox-2 is expressed in peripheral stolon tips and stolon buds

Cnox-2 is expressed differentially along the longitudinal axes of peripheral stolons. Expression is high in the region of the stolon tip, but is either low or undetectable in regions proximal to the tip (Fig. 3A). Stolons branch laterally by the production of a stolon bud at points proximal to the growing tips. Stolon branching begins by a localized dissolution of the periderm, which is an acellular sheath secreted at the stolon tip, followed by an apical elongation of ectodermal cells along an axis normal to the stolon from which it arises. *Cnox-2* expression was not detected during the period of apical elongation. Elongated ectodermal cells in this region then adopt the highly vacuolated state that characterizes the tip formation (Berrill, '61; Belousov, '73; Lange et al., '89). High *Cnox-2* expression coincides with the first detectable extension of the newly formed tip, i.e., at the time when the ectoderm tip acquires its diagnostic cytology (Fig. 3B). By contrast, *Cnox-2* expression is low or undetectable in the proximal stolon from which it budded, as evident when compared with a generalized nuclear stain (Fig. 3C).

The possibility that the observed expression pattern might be an artifact of differential permeability of the periderm was addressed by staining colonies with antibodies against the histone H1 subunit (Leinco Technologies, Inc., St. Louis, MO, USA). The histone protein was localized to the nuclei at even, high intensities throughout the stolons (not shown), thus demonstrating that differential staining of the *Cnox-2* protein was not a result of periderm permeability to antibodies or other staining reagents.

Stolon tip formation and elimination yields concordant alterations in Cnox-2 expression

If an existing stolon tip is destroyed or physically separated from its proximal region, a new stolon

tip is formed at the site of the injury. We experimentally induced stolon tip formation by surgically bisecting a region of proximal stolon (Fig. 4A). We then observed stolon tip formation (Fig. 4B), and onset of *Cnox-2* expression at the site of incision (Fig. 4C). *Cnox-2* expression was detected as soon as the induced tip acquired its diagnostic cytology (apically elongated, vacuolated ectodermal cells). The pattern in induced stolons is identical to that seen in unperturbed lateral branches (Fig. 3A and indicated by an arrow in Fig. 4F).

Lateral stolons are transient structures in *Hydractinia* colony ontogeny. Branches from adjacent stolons contact one another and, upon contact, anastomose. Following anastomosis, the once differentiated tip tissues become a proximal component of an existing stolon, and lose their distinctive cytology. We examined *Cnox-2* expression during and after stolon tip anastomosis. Figure 4D shows a peripheral stolon and tip (t) and two approaching tips (at-1 and at-2). At the time of fixation, at-1 has just begun to anastomose and at-2 has been anastomosed for several hours (Fig. 4E). At-1 has a high level of *Cnox-2* expression, equivalent to what is seen at the tip of a peripheral stolon, whereas at-2 has decreased levels of *Cnox-2* expression (indicated by arrows in Fig 4F). After observing several timed stages, we found *Cnox-2* to be expressed in the former tip 3 hr. after anastomosis, but no expression is detectable in a former tip 10 hr after it has anastomosed.

DISCUSSION

Each of the three pattern-forming systems in the *Hydractinia* life cycle, the larva, the polyp, and the colonial stolonal nexus, require specification of axial positional information. Yanze et al. (2001) suggest that the *Gsx* parahox gene *Cnox-2*, functions in larval axial patterning. Our characterization of this gene suggests that it plays a

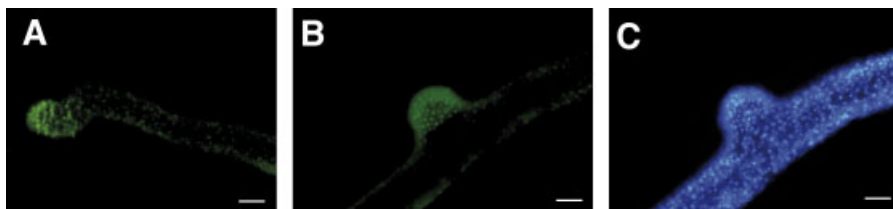


Fig. 3. Detection of *Cnox-2* protein in stolons of *Hydractinia* using polyclonal antibodies generated to the *Cnox-2* gene product and visualized by immunofluorescence. Nuclei with the *Cnox-2* protein appear green. (A) *Cnox-2* expression in the distal region of a peripheral stolon. (B) *Cnox-2* expression at an early stage of a stolon bud. (C) Same specimen as (B) showing nuclei stained with DAPI, which appear blue. Scale bars = 0.1 mm.

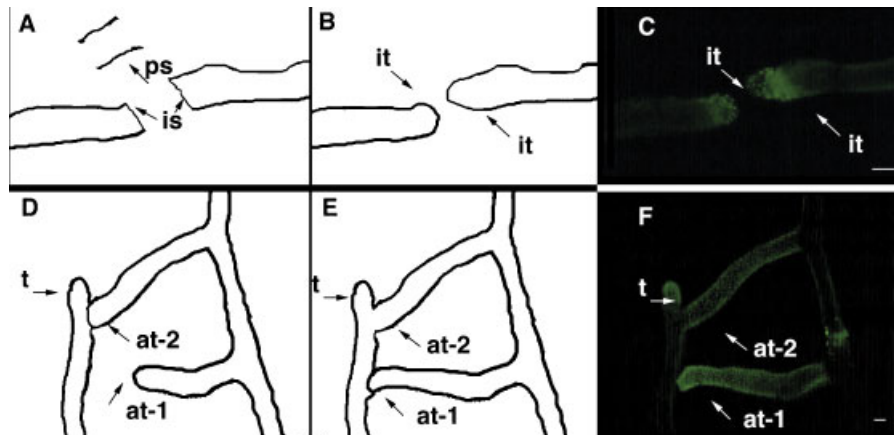


Fig. 4. Experimentally induced stolon tip formation (A and B) and elimination (D and E), and detection of *Cnox-2* protein using polyclonal antibodies generated to the *Cnox-2* gene product and visualized by immunofluorescence (C and F). Nuclei with the *Cnox-2* protein appear green. (A) Stolons tips were experimentally induced by removal of a region of proximal stolon (ps), leaving two sites of injured stolon tissue (is). (B) Induced stolon tips form at the sites of injury (it). (C) Immunolocalization of *Cnox-2* at the stage shown in (B). The *Cnox-2* protein is detected at the regions of newly formed induced tips (it). (D and E) Time series of two stolons (at-1 and at-2) anastomosing with an adjacent stolon. (D) at-2 is about

to anastomose near the tip (t) of the adjacent stolon, whereas at-1 is approaching the adjacent stolon. (E) The stolon tip, at-2, has fully anastomosed, becoming part of the adjacent stolon, whereas the stolon tip, at-1, has just begun to anastomose. (F) Immunolocalization of *Cnox-2* at the time period shown in (E). Following stolon tip anastomosis, the region of the anastomosed tip initially shows high *Cnox-2* expression (at-1) approximately equivalent to what is found at the tip of an unperturbed peripheral stolon (t), but expression fades in the older anastomosed tip (at-2) as it loses its diagnostic stolon tip cytology. Scale bars = 0.1 mm.

prominent, though distinct role in specification of axial positional information in the polyp and the colonial stolonal nexus.

The role of Cnox-2 in polyp patterning processes

The patterning processes that account for the initial formation of the polyp is distinct from the patterning processes in a mature polyp (Bode and Bode, '84). In *Hydractinia*, a polyp bud rudiment arises from the apical surface of stolons and undergoes axial differentiation to produce a hypostome and tentacles at the oral pole. The observation that oral structures develop prior to the detection of differential expression of *Cnox-2* (Fig. 2B and C) suggests that this gene is not involved in the regulatory circuitry required for setting up the initial oral–aboral axis in the developing polyp and that this initial patterning process is governed by an unidentified mechanism.

In the mature polyp, cells are constantly dividing in the body column and discarded at opposite ends (Muller, '64; Campbell, '67; Braverman, '69). These continuously moving cells must be able to transform to the identity of the region in which they enter, in order to maintain the oral–aboral differentiation in the adult polyp. A regulatory system is therefore required to provide positional

information as cells are displaced into new regions. As first suggested by Shenk et al. ('93a), the differential expression of *Cnox-2* in the mature polyp (Fig. 2D–F) is consistent with this gene having a role in maintaining aboral–oral positional information.

The down-regulation of *Cnox-2* in the oral region of the mature polyp suggests that oral signals act to inhibit the expression of this gene. The role of oral structures inhibiting *Cnox-2* expression is consistent with the studies of Shenk et al. ('93b). They reported a number of experiments from *Hydra*, in which body column tissue is converted into oral structures. These include experimental conversion of regions of body column identity to regions of oral identity, bisecting body column tissue to induce formation of multiple oral poles, and grafting tissues to the site of bud formation to induce oral pole formation. In all cases, they found that acquisition of oral pole identity was uniformly accompanied by a reduction of *Cnox-2* expression, suggesting that the presence of oral structures inhibits *Cnox-2* expression.

Our results likewise suggest a down-regulation of *Cnox-2* in the presence of oral structures. First, the temporal progression documented in Figure 2 is one of development of oral structures and subsequent down-regulation of *Cnox-2* in oral regions. A second line of evidence derives from

our prior study of *Cnox-2* expression of polyp polymorphs of *Hydractinia* (Cartwright et al., '99). Dactylozooids are polyps of *Hydractinia* that lack the oral structures of the feeding polyps. Dactylozooids display high levels of *Cnox-2* expression throughout the entire polyp. Experimental transformation of dactylozooids into feeding polyps is accompanied by down-regulation of *Cnox-2* in the oral pole subsequent to the formation of oral structures (Cartwright et al., '99).

Recent studies on the gene *ks1* suggest that *Cnox-2* may act to directly suppress oral signals in the body column. *Ks1* is a molecular marker for oral pole specification in *Hydra* and is expressed in tentacle-specific epithelial cells (Weinziger et al., '94). Endl et al. ('99) found that *Cnox-2* binds to a promoter element of *ks1* in basal tissues, suggesting that *Cnox-2* acts to inhibit the oral-pole-specific gene, suppressing the development of oral structures in the body column. The temporal progression of continuous *Cnox-2* expression throughout the polyp followed by subsequent down-regulation in the oral region and continuous expression in the basal tissues in the mature polyp is consistent with our current understanding of polyp patterning as being regulated by a series of distinct processes dictating formation of the initial axis and subsequent maintenance of the axis in the adult polyp (Bode and Bode, '84).

***Cnox-2* expression is correlated with colony-wide patterning**

The development of a *Hydractinia* polyp is analogous to the development of a solitary organism in that a discrete period of morphogenesis is followed by a prolonged interval wherein a mature form is actively maintained. Morphogenesis of the colony itself is, in contrast, a continuous process, usually limited only by ecological factors. Colony form arises by the sequential application of a limited number of spatially restricted, morphogenetic processes (Braverman and Schrandt, '66). Three such processes are shared by virtually all colonial hydrozoans: the elongation of stolons, the branching of stolons to yield newly elongating tips, and the production of new polyps from stolons. The early ontogeny of a *Hydractinia* colony (Fig. 1B and C) is a paradigmatic example of colonial development.

Cnox-2 is differentially expressed in both of the morphogenetic zones that generate hydrozoan colonies. First, along the proximal-distal axis of stolons, with expression limited to the distal (tip)

region of both primary and laterally branched stolons (Fig. 3), and secondly in proximal regions of stolons only at locations where new polyps are being budded (Fig. 2A). Thus, there appears to be an association in differential *Cnox-2* expression with such seemingly disparate fates as tip elongation and polyp formation. As first noted by Saint-Hilaire ('30) and explored subsequently by many authors (Hale, '60, '68; Berrill, '61; Wyttenbach, '68; Belousov et al., '72, '89; Belousov, '73; Belousov and Dorfman, '74; Wyttenbach, '74), developing polyp bud rudiments and stolon tips are both regions where the stolon ectoderm possesses distinct behavioral and cytological specialization. Behaviorally, ectodermal cells in both regions adopt distinctive orientations relative to the growth axis and undergo a series of stereotypical changes in shape and position. Cytologically, this epithelia is distinguished by pronounced vacuoles in epitheliomuscular cells. While cytologically identical, stolon and polyp buds differ in the shape of the rudiment, a difference that Belousov ('73) argued governed the distribution of mechanical forces deforming the ectoderm in a fashion that leads either to elongation coupled with continual alteration in rudiment shape (polyps) or elongation with cyclic change in shape (stolon tips).

The correlation between *Cnox-2* expression and these two morphogenetic zones of the stolon nexus is strengthened by our demonstration that induction and elimination of growth zones results in concomitant changes in *Cnox-2* expression. By experimentally inducing tip formation, *Cnox-2* protein, which is undetectable in proximal regions of the unmanipulated stolons, becomes expressed at high levels in the developing tip at the time at which cytologically and behaviorally distinct tips appear (Fig. 4C). Stolon anastomosis, which results in the elimination of the cytological and behavioral specializations of the tip, is accompanied by a decline and eventually an elimination of *Cnox-2* expression (Fig. 4F).

***Cnox-2* expression is consistent with the evolution of polyp and colony form**

The patterns of *Cnox-2* expression documented here suggest quite different roles for this gene in the ontogeny of the polyp and the ontogeny of the colony as a whole. In polyp patterning, *Cnox-2* appears to be inhibited by the presence of oral structures and to negatively specify oral structures at the aboral pole following the establish-

ment of the mature axis. In colony patterning, *Cnox-2* appears to positively specify those regions of the stolon that form tip and polyp rudiments. Indeed, the sharp boundary of expression between the base of the polyp and the stolon from which it buds (Fig. 4B) suggests that *Cnox-2* is a marker for polyp–stolon boundaries. Our prior study of *Cnox-2* expression in stolonial mat in turn suggests that evolutionary changes in *Cnox-2* expression are correlated with changes in the aboral boundary of polyp tissue (Cartwright and Buss, '99).

These patterns of *Cnox-2* expression in polyps and stolons are temporally and spatially distinct and are associated with quite different patterns of epithelial cell movement and differentiation. This implies that a gene's involvement in distinct patterning systems may be under the control of different regulators.

In the absence of direct molecular evidence for cnidarians, comparative data are informative. If *Cnox-2* is subject to different regulators in the polyp and the colony patterning systems, then one would predict that changes in polyp morphology could arise without substantial modifications of colony form and vice versa. Indeed, a repeated trend within and between many hydrozoan clades is that of substantial evolutionary modification of the polyp (e.g., by expansion of the body column or the oral region, movement of the tentacles to various positions along the body column, changes in the placement of reproductive structures from positions in the body column to within the oral tentacle ring) without any coincident changes in colony form. The converse is also the case. Alterations in stolonial systems to generate elaborate tree-like morphologies or, in adherent forms, the production of substantial calcium carbonate skeletons have evolved without apparent changes in polyp morphology. Indeed, a fair epitome of the position of many comparative morphologists of the 19th century is that the cnidarian body plan is composed of a series of distinct regions, which may be homologized across taxa, and that axial changes in regions of polyp-, colony-, and medusa-forming modules underlie the remarkable diversity of forms that characterize the phylum Cnidaria (Agassiz, 1862, 1865; Metschnikoff, 1874; Brooks, 1886; Haeckel, 1888).

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