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ORIGINAL ARTICLE

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Expression of the ctenophore Brain Factor 1 forkhead gene ortholog (*ctenoBF-1*) mRNA is restricted to the presumptive mouth and feeding apparatus: implications for axial organization in the Metazoa

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Abstract Ctenophores are thoroughly modern animals whose ancestors are derived from a separate evolutionary branch than that of other eumetazoans. Their major longitudinal body axis is the oral-aboral axis. An apical sense organ, called the apical organ, is located at the aboral pole and contains a highly innervated statocyst and photodetecting cells. The apical organ integrates sensory information and controls the locomotory apparatus of ctenophores, the eight longitudinal rows of ctene/comb plates. In an effort to understand the developmental and evolutionary organization of axial properties of ctenophores we have isolated a forkhead gene from the Brain Factor 1 (BF-1) family. This gene, *ctenoBF-1*, is the first full-length nuclear gene reported from ctenophores. This makes ctenophores the most basal metazoan (to date) known to express definitive forkhead class transcription factors. Orthologs of *BF-1* in vertebrates, *Drosophila*, and *Caenorhabditis elegans* are expressed in anterior neural structures. Surprisingly, in situ hybridizations with *ctenoBF-1* antisense riboprobes show that this gene is not expressed in the apical organ of ctenophores. *CtenoBF-1* is expressed prior to first cleavage. Transcripts become localized to the aboral pole by the 8-cell stage and are inherited by ectodermal micromeres generated from this region at the 16- and 32-cell stages. Expression in subsets of these cells persists and is seen around the edge of the blastopore (presumptive mouth) and in distinct ectodermal regions along the tentacular poles. Following gastrulation, stomodeal expression begins to fade and intense staining becomes restricted to two distinct domains in each tentacular feeding apparatus. We suggest that the apical organ is not homologous to the brain of bilaterians but that the oral pole of ctenophores corresponds to the anterior pole of bilaterian animals.

Keywords Axial patterning · Oral-aboral axis · Egg organization · Ctenophores · Forkhead gene

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Introduction

There is overwhelming evidence supporting the monophyly of the Metazoa (Christen et al. 1991; Wainright et al. 1993; Müller et al. 1994; Müller 1995, 1998; Borchiellini et al. 1998; Collins 1998; Medina et al. 2001). It should therefore be possible to reconstruct the changes leading to complex bilaterian body plans from "simpler" ancestral ones. There is growing evidence that the Ctenophora is the most basal extant eumetazoan taxon and that the cnidarians are the sister group of the Bilateria (Fig. 1). Ctenophores are a rather enigmatic

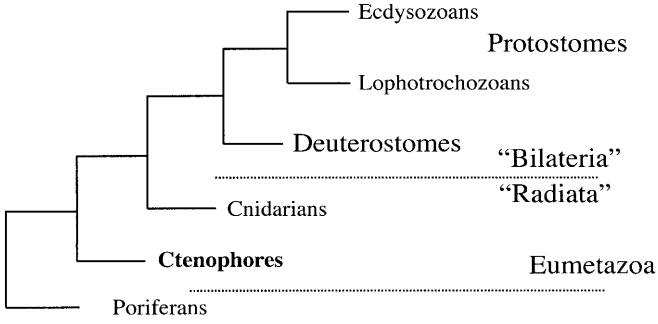


Fig. 1 Presumed phylogenetic position of the ctenophores based on molecular evidence (Medina et al. 2001; Peterson and Eernisse 2001; Podar et al. 2001). Ctenophores are the basal extant phylum of eumetazoans

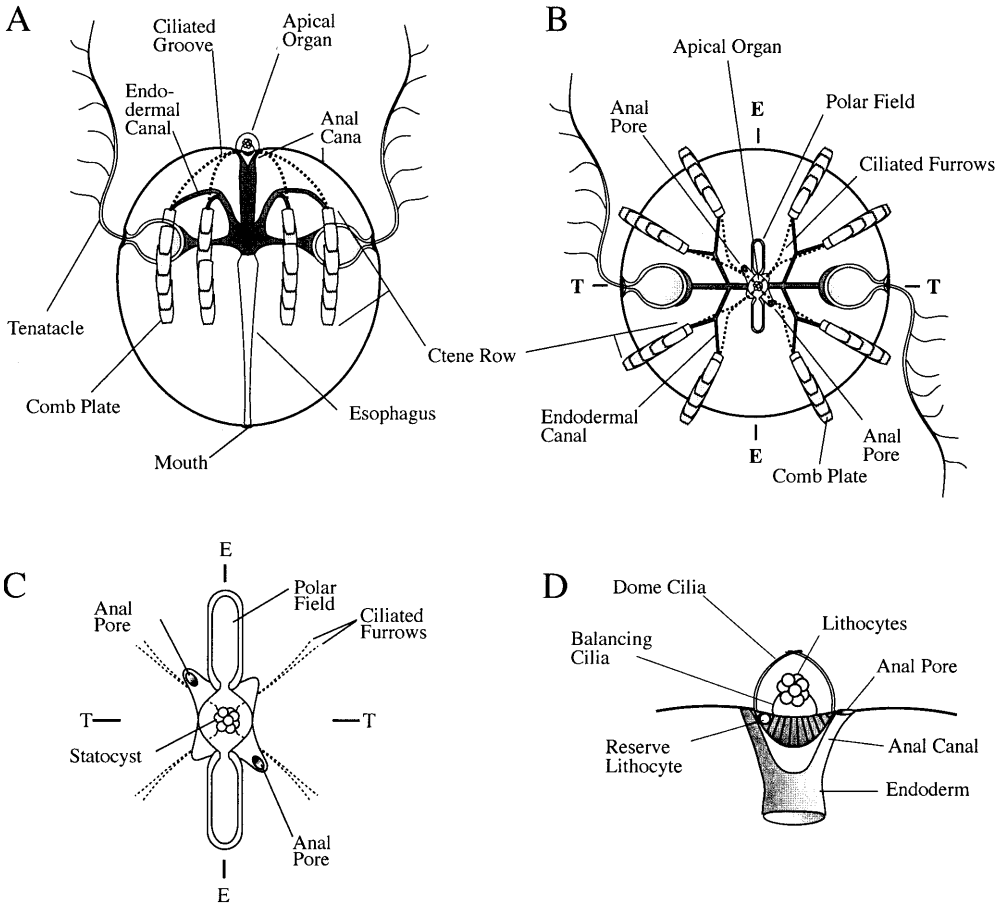


Fig. 2A–D Body plan of cydippid stage ctenophores (modified from Martindale and Henry 1999). **A** Lateral view showing the oral-aboral axis. The apical organ (statocyst) is located on the aboral surface. Endodermal canals (*heavy shading*) project out from the gut into the periphery and pass underneath the eight comb rows, to the two tentacle bulbs, and open to the external media in two diametrically opposed quadrants via the anal canals. **B** Aboral view showing the four nearly identical quadrants defined by the two planes of symmetry characteristic of all members of this phylum. One plane runs through the tentacles (*T*) and the other runs through the plane of the esophagus (*E*) and the polar fields that lie on the aboral surface. The tentacles are generated by stem cells at the base of the tentacle bulb throughout life and exit through the tentacle sheaths. Endodermal derivatives are *darkly shaded*. **C** Aboral view of the apical organ. Impulses generated by the gravity-sensing statocyst are transmitted (in part) to the eight comb rows by the ciliated furrows. **D** Lateral view of the apical organ. Mineral-containing lithocytes sit on top of four groups of balancing cilia. Replacement lithocytes are born from the floor of the apical organ. Large numbers of synapses reside in the floor of the apical organ. The statocyst resides under a group of non-motile dome cilia

group of marine carnivores. Virtually all species are holopelagic and feed with the aid of a pair of tentacles located on opposite sides of the mouth, although one derived group (the beroids) has lost their tentacles and another (platyctenes) has lost their comb plates and become benthic (Podar et al. 2001). The major longitudinal axis of all ctenophores is called the oral-aboral axis, along which run two planes of symmetry, the tentacular and the esophageal axes (Fig. 2A, B).

Most of the sensory and locomotory structures of ctenophores are cilia-derived. For example, ctenophores have eight longitudinal arrays of ectodermally derived ctene (comb) plates (Ctenophora = comb-bearers). Each comb plate is composed of thousands of cilia in longitudinal arrays that form “paddles”. The metachronal beating of these comb plates along each comb row accounts for the movements of these animals through the water column (Fig. 2A, B). The control of comb plate beating in individual comb rows is accomplished in large part by a central sensory structure called the apical organ located at the aboral pole of the animal. The apical organ is a richly innervated statocyst or “gravity amplifier” (Fig. 2C, D). The statocyst is housed under a cupola of non-motile dome cilia and is composed of a group of mineral-containing lithocytes perched upon four groups of balancing cilia. Each tuft of balancing cilia is functionally connected to a pair of ctene rows by the ciliated grooves (Fig. 2C) in each of the four quadrants. The position of the lithocytes atop the balancing cilia regulates the beating of the rows of comb plates. The apical organ also has neural connections to both tentacles (Hernandez-Nicaise 1973). The cells thought to mediate detection of light and regulate spawning are modified ciliated cells in the floor of the apical organ that contain lamellate bodies and synapse onto adjacent cells in the apical organ (Horridge 1964). Thus, the apical organ, with its high concentration of synapses (Hernandez-Nicaise 1973, 1991;

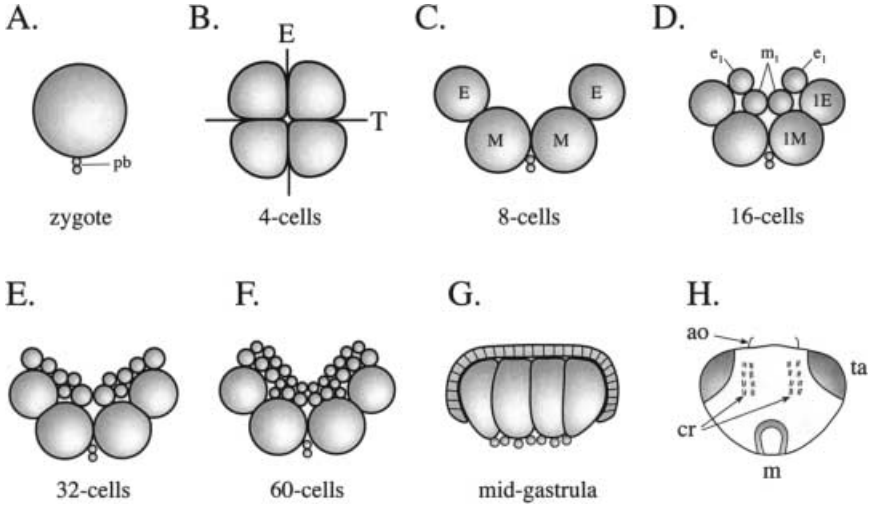


Fig. 3A–H Diagram of several stages of ctenophore development seen in lateral view (except **B**). The aboral pole is *up*, and the oral is *down* (except **B**). **A** The polar bodies (*pb*) mark the future oral pole. **B** Four-cell stage seen along the oral-aboral axis. The first cleavage plane defines the esophageal axis (*E*) and the second cleavage the tentacular axis (*T*). **C**–**F** Highly characteristic patterns of cleavages generate a cap of ectodermal micromeres at the aboral pole which move by epiboly to the future oral pole (**G**). The mesodermal cells are derived from micromeres born at the oral pole late in development (**G**). **H** Lateral view of a young cydippid. The mouth (*M*) and each tentacle apparatus (*ta*) form by invagination. The apical organ (*ao*) forms by convergence of components from each quadrant toward the aboral pole. The comb plate cilia for each comb row (*cr*) are just forming

Horridge 1974; Tamm 1982) integrates sensory information and initiates the appropriate motor/behavioral output. One interpretation of these data is that the centralized function of the apical organ is homologous to the bilaterian brain.

The aboral location of the apical organ in ctenophores raises an interesting question regarding the relationships of the axial properties of ctenophores to other metazoan animals. Various scenarios can be proposed. For example, the oral pole of ctenophores could be homologous to either the anterior or posterior end of bilaterians, or perhaps, the aboral pole (apical organ/"brain") of ctenophores is homologous with the anterior end of bilaterians. The oral pole of ctenophores could also be homologous to the ventral surface of bilaterians, or perhaps the axial properties of ctenophores bear no relationship to any other animal (see Beklemishev 1969; Willmer 1990 for review).

An embryonic fate map for the first 60 cells has been generated using intracellular markers for the ctenophore *Mnemiopsis leidyi* (Martindale and Henry 1999). The mesendoderm is generated from the animal pole, which also becomes the mouth. Most of the components of the apical organ (dome cilia, balancing cilia, ciliated grooves, and the floor of the apical organ) are derived from the progeny of ectodermal micromeres generated from the aboral pole from both the E and M macromeres, although the lithocytes are generated by endodermal precursors from the 3E lineage (Fig. 3). The aboral micromeres proliferate and spread over the mesendodermal macromeres by epiboly during gastrulation, with the blastopore becoming the adult mouth. The tentacles form from two ectodermal patches that invaginate and fuse with muscle precursors born from the oral end of the E and M lineages (Martindale and Henry 1999). The tentacles are generated continuously from the central region of the tentacle bulbs and exit the animal through the ectodermal tentacle sheathes. The apical organ arises from the coalescence of components from all four quadrants toward the aboral pole as the eight ctene rows have begun to form following gastrulation. Unfortunately, previous studies investigating the axial properties of ctenophores have shed little light on the relationship of axial properties of ctenophores to bilaterian representatives (Martindale and Henry 1997).

In order to understand the relationship of axial properties of ctenophores to other metazoans, we have begun to examine the expression of ctenophore orthologs of several developmental regulatory genes with known regional expression patterns in other animals. Here we report the cloning and expression of the first full-length nuclear gene in any ctenophore (*ctenoBF-1*), a winged helix domain transcription factor of the class 7 (subclass G), "Brain Factor 1" (BF-1). This is the most basal metazoan described to date to possess any forkhead class gene, thus adding insight into the depth of the eumetazoan "genetic toolkit". Orthologs of this gene are expressed in the anterior region of *Drosophila* and *Caenorhabditis elegans*, and the developing forebrain of chordates. *CtenoBF-1* is expressed transiently around the mouth and in discreet regions of the tentacular feeding apparatus but not in the apical organ. Implications of these results are discussed with respect to body plan organization in the Metazoa.

Materials and methods

Animals

Adult specimens of the lobate ctenophore *M. leidyi* were collected in Eel Pond or off the rock jetty at NOAA in Woods Hole, Massa-

chusetts during the months of July and August. Self-fertile adult animals were placed in the dark at night and naturally fertilized embryos collected approximately 8 hours later. Embryos were raised in 0.22-µm filtered seawater (FSW) at approximately 20°C until the desired stage.

Isolation of *ctenoBF-1*, a ctenophore forkhead family gene

Degenerate primers were designed against the amino acid sequences ITMAIQ and MFENGC that are highly conserved in the DNA-binding domains of most forkhead family genes. The sequences of the primers are: upstream primer 5′-AYNACNAT-GGCNATHCA-3′ and, downstream primer 5′-CANCCRTTYT-CRAACAT-3′. A 240-base pair (bp) fragment was obtained by PCR (annealing temperature of 46°C) from a *M. leidyi* newly hatched juvenile cDNA library. Amplified products were blunt-end cloned (Novagen) and sequenced on both strands. A ^{32}P-labeled, random-primed PCR product was used as a probe to screen replica lifts from the *M. leidyi* juvenile cDNA library at high stringency (hybridization: 6× SSC, 1% SDS, 5× Denhardt's solution at 65°C for 16 h; washing: 0.5× SSC, 1% SDS at 65°C). Nine positive clones were obtained by screening 3.2×10^5 pfu of the library and subcloned into pBluescript SK. All nine clones contained a 1.5-kb insert. Six of them were sequenced on both strands and shown to be the same cDNA.

Phylogenetic analysis

A comparison of forkhead superfamily genes based on the amino-acid sequence of the forkhead domain was made in order to establish gene orthology. Deduced protein sequences were aligned and gaps were introduced to obtain alignment with maximal similarity. Molecular phylogenetic relationships of the forkhead superfamily were analyzed by means of neighbor-joining using the PHYLIP (version 3.5).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using a method based on the protocol of Seaver et al. (2001). Embryos and juveniles were fixed in 4% formaldehyde in FSW at 4°C overnight, rinsed three times in PBS and stored in methanol at –20°C. Juveniles were relaxed for several minutes with a 1:1 mixture of FSW and a 7% $MgCl_2$ solution before fixation. Prior to hybridization, specimens were washed for 5 min each in 60% methanol-40% PBT (PBS + 0.1% Tween-20), and 30% methanol-70% PBT, and four times in PBT. The vitelline membrane was removed with sharpened tungsten needles under a dissecting microscope and devitellinated embryos were incubated in 2 µg/ml proteinase K for 20 min at 37°C. Proteinase K digestion was stopped by washing three times with PBT and specimens were refixed with 4% formaldehyde in FSW for 1 h. After washing five times for 5 min in PBT, samples were heat-treated at 80°C for 20 min to reduce endogenous alkaline phosphatase activity.

Specimens were washed for 10 min in hybridization buffer (50% formamide, 5× SSC, pH 4.5, 0.05% Tween-20, 1% SDS, 50 µg/ml heparin, 50 µg/ml yeast total RNA) and then prehybridized in fresh hybridization buffer for 3 h at 60°C. Hybridization was performed overnight at 60°C in hybridization buffer containing 0.1 ng/µl 1.5 kb digoxygenin-labeled probe synthesized with the MegaScript transcription system (Ambion, Austin, Tex.). Following hybridization, embryos were successively washed twice for 20 min with 100% hybridization buffer at 60°C, 75% hybridization buffer-25% PBT at 55°C, 50% hybridization buffer-50% PBT at RT, and 25% hybridization buffer-75% PBT at RT. After two 20- min washes with PBT, some specimens were treated with RNase A (1 µg/ml) for 20 min at 37°C and then washed five times with PBT for 5 min each. Specimens were blocked with Boehringer-Mannheim Blocking buffer for 1 h at RT, and then incubated with 1:2,000 alkaline phosphatase conjugated anti-digoxygenin Fab fragments (Roche) in the blocking buffer (overnight at 4°C). Specimens were washed with PBT five times for 10 min each and alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.5, 0.1% Tween-20) three times for 5 min. Signal detection was carried out in the alkaline phosphatase buffer with NBT and BCIP.

Results

Isolation and characterization of the ctenophore BF-1 gene (*ctenoBF-1*)

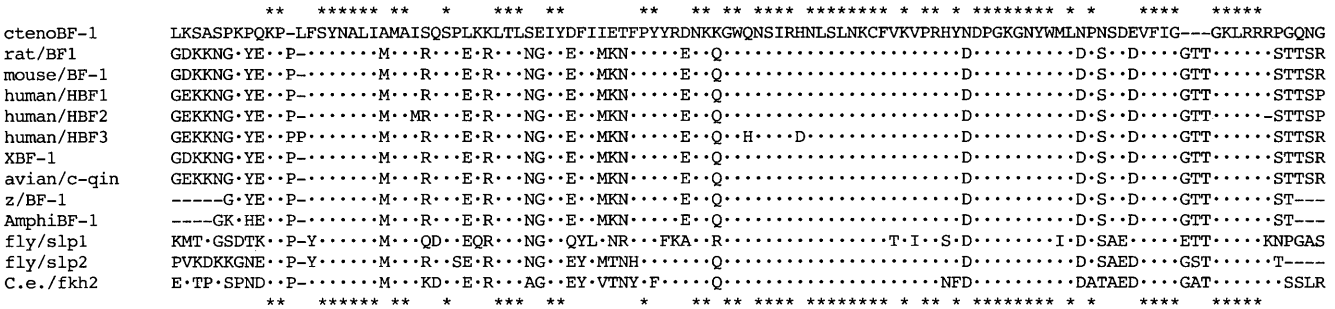
PCR and replica lift screening of a ctenophore juvenile cDNA library resulted in the isolation of what might be the first complete cDNA ever characterized in a ctenophore, *ctenoBF-1*. The 1,540-bp clone contains a single open reading frame of 318 amino acids (DDBJ/EMBL/GenBank accession number AF477500). The ATG at the position 372–374 corresponds to the Kozak (1986) consensus sequence, ACCATGG, and is likely to be the translation initiation codon of the *ctenoBF-1*-encoded protein. Several termination codons were found upstream of the codon for methionine and downstream of an in-frame termination codon on the 3′ end. No consensus eukaryotic polyadenylation signal was detected in the 3′ UTR although a poly-A like stretch was found. We do not know if this clone represents a truncated cDNA or whether this is characteristic of other ctenophore cDNAs. The overall G-C content of this transcript is 49%; 52% in the coding region; 51% for the 5′ UTR and 35% in the 3′ UTR (not including the polyA tail).

BLAST searches of the deduced polypeptide of *ctenoBF-1* reveal that it contains a class 7 (Kaufmann and Knöchel 1996) or subclass G (Kaestner et al. 2000) forkhead domain (Fig. 4A). This class is represented by amphioxus "Brain factor" *BF-1*, rat *BF-1*, mouse *BF-1*, the two *Drosophila* orthologs *slp1* and *slp2*, and a nematode gene *fkh2*. The forkhead domain of *ctenoBF-1* is 70% identical at the amino acid level to the forkhead domain of mouse, rat, and amphioxus *BF-1* genes and 59% and 64% to *Drosophila slp1* and *slp2*, respectively.

In addition to the forkhead domain there is another conserved region in forkhead family genes (Weigel and Jäckle 1990; Pani et al. 1992). The second encodes the amino acids HPFSI and is thought to be a transactivation domain. It is present in the C terminus of class 1 genes but is found in the N terminus of the class 7 genes in both chordates and *Drosophila* (Grossniklaus et al. 1992; Bourguignon et al. 1998). Interestingly, this HPFSI domain is located in the N terminus in *ctenoBF-1* (Fig. 4B) supporting the assignment of *ctenoBF-1* to class 7 forkhead genes.

To confirm that *ctenoBF-1* belongs to the BF-1 gene class, a phylogenetic analysis encompassing a large number of forkhead genes was performed grouping the genes into 15 different subclasses according to Kaestner et al. (2000). Neighbor-joining analysis (Fig. 5) shows

A



B

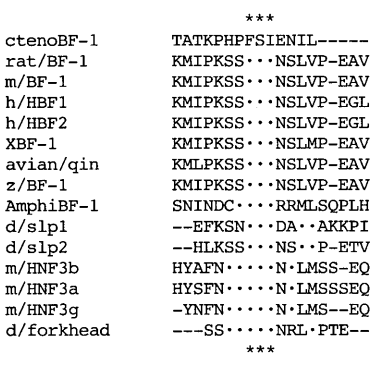


Fig. 4 Comparison of the amino acid sequence of **A** the fork head domain and **B** another conserved region of ctenoBF-1 with those of rat BF-1 (Tao and Lai 1992), mouse BF-1 (Li et al. 1996), amphioxus BF-1 (Toresson et al. 1998), human HBF1, HBF2 and HBF3 (Murphy et al. 1994), *Xenopus* XBF-1 (Bourguignon et al. 1998), avian qin (Chang et al. 1995), zebrafish BF-1 (Toresson et al. 1998), *Drosophila* slp1 and slp2 (Grossniklaus et al. 1992), *C. elegans* fkh-2 (Molin et al. 2000), mouse HNF-3α, HNF-3β and HNF-3γ (Lai et al. 1991), and *Drosophila* forkhead (Weigel et al. 1989) Identical residues to ctenoBF-1 protein are indicated by *dots* and *dashes* represent gaps. Amino acid positions that are identical in all genes are marked with *asterisks*

that *ctenoBF-1* is the basal gene member in the subclass G (class 7) represented by *Amphioxus*, rat, mouse, amphibian, and zebrafish *BF-1*, *Drosophila slp1* and *slp2*, *C. elegans fkh2*and planaria *Djfkh8*. The grouping of *ctenoBF-1* with other class 7 genes suggests that *ctenoBF-1* is a ctenophore ortholog of the class 7/subclass G, forkhead superfamily.

The expression pattern of *ctenoBF-1*

The spatial localization of *ctenoBF-1* transcripts in embryos at different developmental stages was revealed by means of whole-mount hybridization (Fig. 6). Control embryos hybridized with digoxygenin-labeled sense probes did not show signals above background (data not shown). Expression of *ctenoBF-1* is initially detected at low levels in uncleaved zygotes. There is weak staining in the thin ectoplasmic layer surrounding the entire embryo (Fig. 6A, B). In some embryos the staining was more pronounced on one side of the embryo and several clear spots were seen that are probably male pronuclei (Fig. 6A). Ctenophore embryos, including *M. leidyi*, are known to undergo physiological polyspermy with as many as 5–20 sperm entering the egg (Carré and Sardet 1984).

By first cleavage (Fig. 6B) the distribution of transcripts has begun to change. Transcripts appear to accumulate at the site of first cleavage (presumptive oral pole) and the aboral pole, rather than in equatorial ectoplasm. At the 2-cell stage, transcripts are concentrated along the cleavage plane and towards one end of the embryo (data not shown). Staining increases in intensity during the early cleavage stages and by the 8-cell stage *ctenoBF-1* transcripts are localized to the aboral pole of the embryo (Fig. 6C). At the 16- to 32-cell stages expression is confined to the aboral region of the embryo where it is inherited by ectodermal micromeres of both E and M lineages (Fig. 6D). The intensity of expression increases in descendants of aboral ectodermal micromeres during epibolic gastrulation movements. From mid to late gastrulation heavy staining is seen in ectodermal cells surrounding the blastopore and in cells along the tentacular axis from the blastopore towards the aboral surface (Fig. 6E, F). Staining persists in cells associated with the mouth and ectodermal portions of the esophagus (Fig. 6G, I) during late gastrulation and early cydippid formation and then disappears (Fig. 6J, L). There is a noted absence of signal at the aboral pole where the apical organ will form, and along the esophageal axis (Fig. 6E–L). No evidence of staining in mesodermal or endodermal derivatives was detected, nor was any asymmetric expression detected along the anal axis (Martindale and Henry 1997).

A relatively uniform pattern of *ctenoBF-1* expression in the presumptive tentacle buds during gastrulation was followed by a gradual condensation into distinct oral and aboral regions. Each quadrant expresses *ctenoBF-1* in a small region on the aboral surface (Fig. 6, arrows). The aboral domain, initially diffuse (Fig. 6F), becomes focused into a discreet spot (Fig. 6H). As the tentacle bud begins to differentiate, but prior to invagination, the aboral tentacular domain becomes crescent-shaped and is

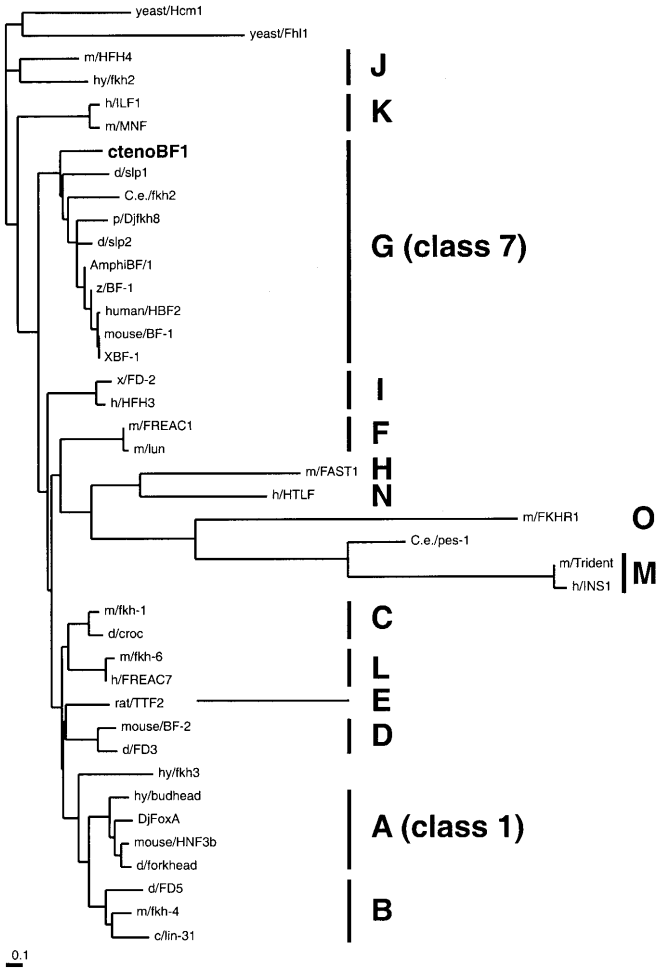


Fig. 5 Phylogenetic tree constructed by the Neighbor-Joining method. The sequences of the forkhead domains were used as characters. The tree was rooted using the yeast HCM1 and FHL1. Members for each subclass (*A–O*; Kaestner et al. 2000), and *class 1* and *7* (Kaufmann and Knöchel 1996) were included. The *branch lengths* indicate the evolutionary distance between the different sequences. This tree classifies ctenoBF-1 into the "subclass G (class 7)'' genes. The references for the sequences are as follows: yeast HCM1 (Zhu et al. 1993), yeast FHL1 (Hermann-Le Denmat et al. 1994), human HFH3 and mouse HFH4 (Clevidence et al. 1993), hydra fkh2, fkh3 and budhead (Martinez et al. 1997), human ILF1 (Li et al. 1992a), mouse MNF (Bassel-Duby et al. 1994), *Xenopus* XFD-2 (Lef et al. 1994), mouse FREAC1 (Hellqvist et al. 1996), mouse lun (Miura et al. 1998), mouse FAST1 (Weisberg et al. 1998), human HTLF (Li et al. 1992b), mouse FKHR1 (Biggs and Cavenee 2001), *C. elegans* pes-1 (Molin et al. 2000), mouse Trident (Korver et al. 1997), human INS1 (Yao et al. 1997), mouse fkh-1, fkh-4 and fkh-6 (Kaestner et al. 1993), *Drosophila* crocodile (Häcker et al. 1995), human FREAC7 (Pierrou et al. 1994), rat TTF2 (Zannini et al. 1997), mouse BF-2 (Hatini et al. 1994), *Drosophila* FD3 and FD5 (Häcker et al. 1992), planaria DjFoxA (Koinuma et al. 2000), *C. elegans* lin-31 (Miller et al. 1993), and planaria Djfkh8 (Koinuma and Agata, personal communication)

positioned along the boundary between the tentacle bud and the adjacent aboral epidermis (Fig. 6H). Following tentacle bud invagination, the two spots on either side of a tentacle bud converge towards the midline, and fuse to form a single domain of expression (Fig. 6J, K).

A second domain of tentacle-associated expression is apparent at the oral boundary between the tentacle bud and oral epidermis (Fig. 6 arrowheads). Like the aboral domain, it begins as a larger more diffuse area on either side of the tentacular pole (Fig. 6E) and converges towards the tentacular midline (Fig. 6E–I). Before invagination this oral domain becomes more intense as the tentacle bud enlarges (Fig. 6H, I), and subsequent to invagination becomes reduced to a single line of expression along the oral edge of the tentacle apparatus (Fig. 6J, K). *CtenoBF-1* expression wanes shortly after hatching. Only small areas of staining can still seen over the background pigmentation of the tentacles in both oral and aboral domains (Fig. 6L). Thus, *ctenoBF-1* is expressed embryonically in ectodermal cells around the mouth and in the tentacle bud/apparatus, but not in the apical organ, mesendoderm, or esophageal ectoderm.

Because this is the first report of in situ hybridizations in embryonic ctenophore tissue, we utilized RNase A, which degrades single stranded RNA, to determine the fidelity of our hybridization conditions. RNase A treatment following antisense riboprobe hybridization reduced background and in some cases increased the time it took for definitive alkaline phosphatase signal to become apparent. This was particularly true for early stages in which transcript levels were probably lower than at later developmental stages. These treatments did not significantly change the pattern of hybridizations, however, and the sense controls were blank.

Discussion

We have used PCR with degenerate forkhead primers to isolate a gene from an early juvenile-stage cDNA library of the lobate ctenophore *M. leidyi*. To our knowledge, this is the first full-length nuclear gene ever characterized from the phylum Ctenophora. It has a typical eukaryotic mRNA structure with a 5′ and 3′ UTR, methionine start site, and a 318-base pair open reading frame. A polyadenylation signal was not detected; however this could be due to truncation of the 3′ UTR during library construction.

BLAST searches of GenBank and phylogenetic reconstruction analyses indicate that this gene is a class 7 (Fox G) forkhead transcription factor with orthology to the "Brain Factor 1" genes of chordates, including *Amphioxus*; (Tao and Lai 1992; Murphy et al. 1994; Li et al. 1996; Toresson et al. 1998), *Drosophila* (Grossniklaus et al. 1992) and nematodes (Molin et al. 2000). As many as 10–15 distinct families of forkhead class genes have been described in bilaterians (Kaufmann and Knöchel 1996; Kaestner et al. 2000), but none in any poriferans, making *M. leidyi* the most basal metazoan expressing forkhead class genes to date. Winged helix family transcription factors have been described in yeast (Zhu et al. 1993; Hermann-Le Denmat et al. 1994) so it is likely that they will be eventually found in sponges as well. Cnidarians, the likely sister group to higher bilaterians,

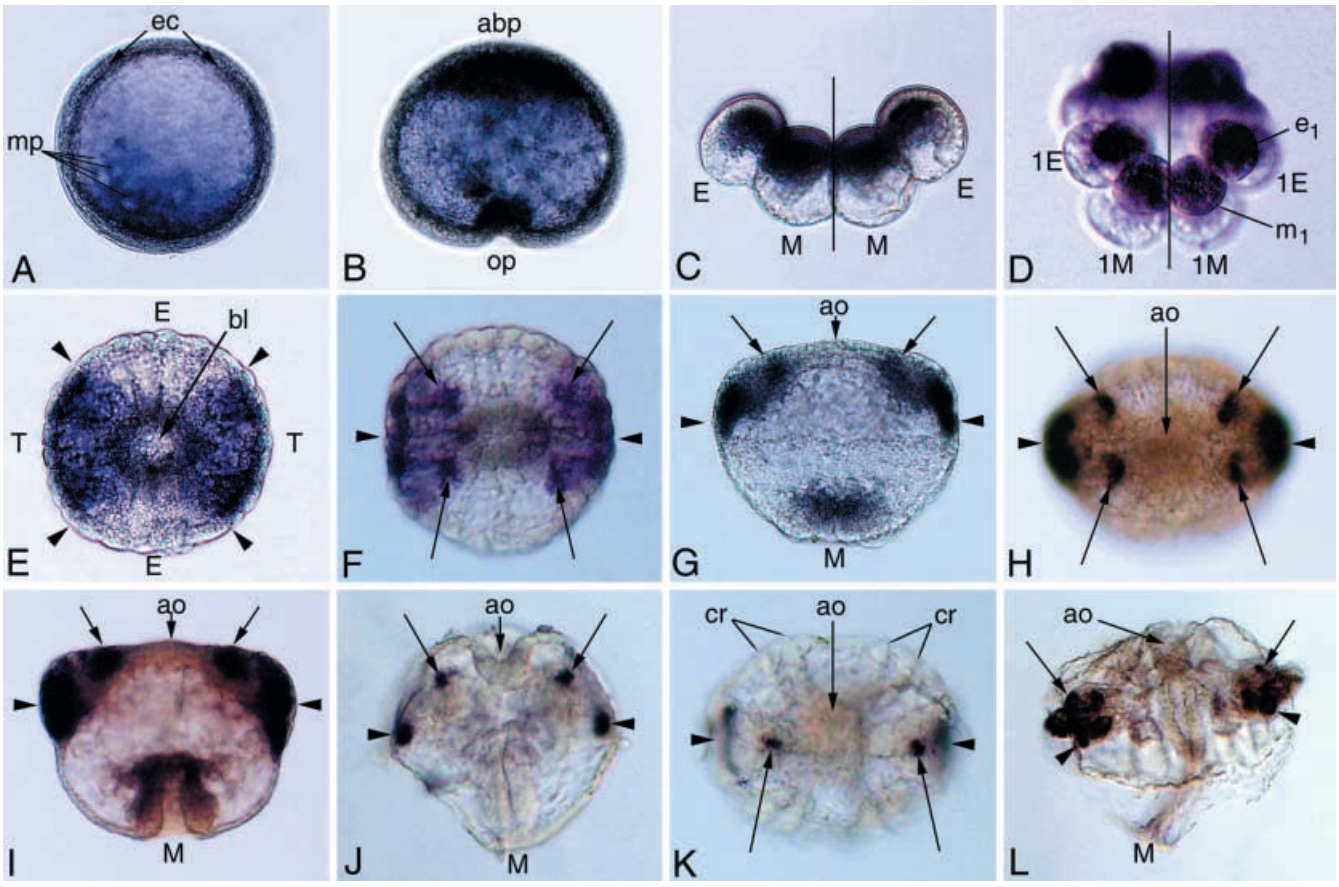


Fig. 6A–L Spatial expression of *ctenoBF-1* during embryogenesis as revealed by whole-mount in situ hybridization with a 1.5-kb digoxygenin-labeled antisense riboprobe. Some embryos at the early cleavage stages were not treated with RNase A after the hybridization (**A**–**D**). All views are from the esophageal pole (*vertical lines* in **C** and **D**) and the tentacular axis runs *left-right* on the page. **A** Uncleaved zygote showing ectoplasmic expression (*ec*). The light areas are probably male pronuclei (*mp*) that exclude cytoplasmic staining prior to their disappearance. **B** Early first cleavage. Note staining around site of first cleavage. **C** An 8-cell stage showing staining in the ectoplasm at the aboral pole. **D** A 16-cell embryo seen in an oblique lateral-aboral view. Staining is seen predominantly in the e_1 and m_1 micromeres but not the oral pole of the macromeres, *1E* and *1M* (esophageal plan indicated by *line*). **E** Oral view of a mid-late gastrula showing the circular blastopore (*bl*). *CtenoBF-1* transcripts are present in the ectoderm surrounding the blastopore (presumptive mouth) and along the ectoderm (*between arrowheads*) of the tentacular axis (*T*). Note the absence of staining along the esophageal axis (*E*). **F** Aboral view of a mid-late gastrula, showing that the entire tentacle apparatus expresses transcripts, but that each quadrant generates a small aboral patch (*arrows*) and tentacular midline domain (*arrowheads*) of high-expression cells. As gastrulation proceeds, these two domains make the aboral and oral domains, respectively. **G** Lateral view of a late gastrula showing oral labeling (*M*) and strong expression in subregions of the presumptive tentacle apparatus (*arrows* and *arrowheads*). Note the absence of staining at the aboral pole (*ao*) and along the esophageal axis. **H** Aboral view of a late gastrula showing the consolidation of oral (*arrowheads*) and aboral (*arrows*) domains within the larger tentacle bud. The four aboral domains become crescent-shaped and delimit the boundary between aboral ectoderm and presumptive tentacle apparatus. **I** Lateral view of a late gastrula just before the internalization of the tentacle buds. A small amount of staining persists around the mouth, with most of the highly expressing cells being located in the two tentacular domains. **J** Lateral and **K** aboral views showing that after involution of the tentacle apparatus and before hatching, the staining regions from two adtentacular quadrants fuse to form a single aboral (*arrows*) and oral (*arrowhead*) domains along the tentacular midline. During these stages the mesoglea swells and the body wall can shrink during hybridization conditions (*cr* ctene rows). **L** Following hatching, *ctenoBF-1* expression wanes and can be seen in two small patches within the tentacle apparatus (*arrows* and *arrowheads*). Note the brown staining of the tentacle proper occurs in sense controls and is not an authentic hybridization signal

possess at least three forkhead genes (no class 7 genes reported), but only one (class 1) member has been characterized in detail (Martinez et al. 1997). We do not know how many forkhead class genes exist in ctenophores, but is likely that the early eumetazoan "genetic toolkit" possessed at least several members of the forkhead family of transcription factors prior to the diversification of the Bilateria.

Development of ctenophores is rapid, and it is therefore difficult to say for certain whether the expression of *ctenoBF-1* seen in freshly spawned zygotes is maternally expressed. Staining intensity increases in the first few hours following fertilization so it is therefore likely that transcription of this gene is also activated zygotically. We did not attempt to quantify the amount of *ctenoBF-1* transcript by a northern hybridization or RT-PCR meth-

ods. Unfortunately, no information is currently known about the timing of the activation of zygotic gene expression in ctenophores. Expression of *ctenoBF-1* disappears soon after hatching which is interesting as juvenile and adult ctenophores are highly regenerative (see Martindale and Henry 1997 for review). It would appear that individual cell types do not require the permanent or prolonged expression of this transcription factor in order to maintain their differentiated state. It will be interesting to see if *ctenoBF-1* is expressed during adult regeneration in patterns similar to those seen during embryogenesis.

Virtually all ctenophores are self-fertile hermaphrodites and eggs are fertilized by multiple (5–20) sperm at the time of spawning (Carré and Sardet 1984; personal observation). Although sperm can enter the egg at any position (Carré et al. 1991), many eggs tend to get fertilized in one hemisphere because they are exposed to sperm as they exit the female gonopore, particularly during in vitro spawnings when sperm concentrations are high. Sperm asters are known to concentrate the yolk-free ectoplasm into characteristic "plaques" before their dispersal, which occurs by microtubule-dependent waves of cytoplasmic reorganization starting from the site of pronuclear fusion and moving to the opposite pole at a rate of 10–20 μm/minute (Houliston et al. 1993). Cytoplasmic reorganization continues throughout the next few cleavage stages as the ectoplasm, and the developmental potential to form discreet structures, become localized to the aboral pole (Yatsu 1912; Freeman 1976, 1977; Houliston et al. 1993).

The spatial localization of *ctenoBF-1* transcripts is dynamic and mirrors many of the cytoplasmic reorganizations of the zygote. Initially the expression is present uniformly in the ectoplasm in the 1-cell zygote. Transcripts then appear to become localized to one end of the zygote, possibly organized by sperm asters (Fig. 6A). The distribution of *ctenoBF-1* transcripts begins to change as the first cleavage furrow forms (Fig. 6B) and they appear around the site of first cleavage and the future aboral pole, with fewer transcripts in equatorial regions. Transcript localization is concentrated to the aboral pole by the 8-cell stage when *ctenoBF-1* expression becomes inherited by the ectodermal micromeres at the aboral pole (Fig. 6C, D). It will be interesting to see if transcripts of other genes follow the same changes in distribution as seen here.

CtenoBF-1 expression increases during cleavage stages in the aboral micromeres during the 16- and 32 cell-stages. These micromeres give rise to the ectodermal tissues including the epidermis, apical organ, comb rows, and tentacles. During the subsequent epibolic gastrulation movements, *ctenoBF-1* is quickly down-regulated in sublineages of aboral micromeres that will form the apical organ and epidermal cells along the esophageal axis. Expression persists in cells surrounding the blastopore (future mouth) and in subsets of cells in the tentacular feeding apparatus. The exact identity of the *ctenoBF-1*-expressing cells in the oral/tentacular regions is unknown because cell-type-specific markers in ctenophores are not yet available. One possible link between the staining in the tentacle buds and the oral region is that they both undergo some sort of invagination during their development. The condensation of expression into discreet regions of the tentacle apparatus during later tentacle bud development might indicate multiple roles for *cteno-BF-1* in cell type determination and morphogenetic movements.

One of the fundamental issues in eumetazoan evolution is determining the relationship of the axial properties of ctenophores (and cnidarians) to those of bilaterians. *CtenoBF-1* was originally cloned to provide one of several possible positional markers to address this question. *Brain Factor-1* orthologs in vertebrates (mouse, rat, *Xenopus*, and zebrafish) as well as *Amphioxus* are an anterior forebrain marker involved in both the patterning and differentiation of anterior telencephalic neural structures (Tao and Lai 1992; Xuan et al. 1995; Li et al. 1996; Bourguignon et al. 1998; Toresson et al. 1998). It is somewhat difficult to generalize about the role of *BF-1* orthologs in protostomes. The two taxa in which class 7 forkhead genes have been investigated are *Drosophila* and two nematode species. The closest ortholog of *ctenoBF-1* in *Drosophila* is *slp1*. The earliest role of *slp1* is as an anterior gap gene and it is expressed in the most anterior 30% of the blastoderm. Mutations of *slp1* generate abnormal antennal, ocular, and mandibular head segments (Grossniklaus et al. 1992, 1994). Anterior *slp1* expression becomes down-regulated on the ventral surface of the embryo (Grossniklaus et al. 1992, 1994) and at later stages shows pair rule and segment polarity expression patterns in both ectoderm and mesoderm in the trunk (Cadigan et al. 1994). The closest *ctenoBF-1* ortholog in nematodes is *fkh-2* which is first expressed in the AB blastomere located at the anterior end of the nematode embryo (Molin et al. 2000). The AB cell gives rise to a variety of different anterior cell types in the head, 62% of which are neural (Sulston et al. 1983). Molin et al. (2000) suggest that these class 7 orthologs had a role in anterior patterning in the protostome/deuterostome ancestor. It will be interesting to determine whether the down-regulation of *ctenoBF-1* along the esophageal axis is related to the down-regulation seen at the ventral surface in *Drosophila*.

It is of some interest that we do not detect expression of *ctenoBF-1* in the developing apical organ. Although *ctenoBF-1* is initially expressed at high levels in cells born at the aboral pole, it is quickly down-regulated in these apical cells and persists in cells derived from more orally located lineages. The apical organ contains the highest density of neural synapses (Hernandez-Nicaise 1973, 1991), possesses cells implicated in photodetection (Horridge 1964) and its statocyst is clearly involved with the coordination of locomotory activity (Tamm 1982). Furthermore, the apical organ plays a role in controlling the global symmetry properties during regenerative patterning (Freeman 1967; Martindale 1986). The absence of expression of class 7 Brain Factor 1 genes in the apical organ is thus surprising since such a high per-

centage of genes are expressed in neural structures at some stage of development in most animals. This data, along with the fact that the apical organ of ctenophores fails to express serotonin (Hay-Schmidt 2000) suggests that the apical organ of ctenophores might not be homologous with the central nervous system of bilaterians. Alternatively, class 7 brain factor genes could have been co-opted for neural specification subsequent to the origin of the ctenophore lineage or lost in the apical organ in the ctenophore lineage.

If the absence of *ctenoBF-1* expression in the ctenophore apical organ means that the ancestral role of the *BF-1* gene was not in brain/neural cell type specification, perhaps its region-specific role in anterior development is the common theme seen in the animals thus examined. If this were true, it suggests that the oral staining around the blastopore (Fig. 6E, F) and stomodeum (Fig. 6G, I) in *M. leidyi* is homologous with the anterior expression seen on all other studied bilaterians. The power-stroke of comb plate beating in ctenophores propels the animal mouth-first (Tamm 1982) so that the preferred direction of locomotion supports the anterior placement of the mouth. The ancestral ctenophore is now thought to be tentaculate (Podar et al. 2001), but the tentacles of ctenophores do not appear to be homologous with the tentacles of cnidarians or other metazoan animals. One interpretation is that the tentacles evolved in association with the oral pole and *ctenoBF-1* was then co-opted for tentacle formation. The tentacles are generated by most of the same cell lineages as those surrounding the mouth and esophagous (Martindale and Henry 1999) and in adult *M. leidyi* the tentacles are positioned immediately adjacent to the mouth, suggesting their ontogenetic, functional, and potentially evolutionary, relationship to the oral pole.

One feature uniting both ctenophores and cnidarians is that the site of first cleavage (the “animal” pole) is causally involved with the establishment of the oral pole (Freeman 1977, 1980). The idea that the oral pole of “prebilaterians” (ctenophores and cnidarians) is related to the anterior end of other metazoans is supported by Hox gene expression in an anthozoan embryo. A posterior Hox gene (*Anthox1*) is expressed at the aboral pole and anterior Hox and ParaHox genes (*Anthox6* and *Anthox2*, respectively) at the oral pole of planula stage embryos (Finnerty, Paulson, and Martindale, in preparation). No authentic Hox class genes have been reported in ctenophores (or sponges) so it is currently not possible to use Hox gene expression to compare the axial properties of ctenophores with other eumetazoans. It is of course too early to make definitive conclusions about the relationships of metazoan symmetry properties based on the expression of one gene, but as basal members of the Eumetazoa, ctenophores provide a crucial link for understanding the origins of all bilaterian body plans.

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