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A forkhead gene related to *HNF-3β* is required for gastrulation and axis formation in the ascidian embryo

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SUMMARY

We have isolated a member of the *HNF-3/forkhead* gene family in ascidians as a means to determine the role of winged-helix genes in chordate development. The *MocuFH1* gene, isolated from a *Molgula oculata* cDNA library, exhibits a forkhead DNA-binding domain most similar to zebrafish axial and rodent HNF-3β. *MocuFH1* is a single copy gene but there is at least one other related forkhead gene in the *M. oculata* genome. The *MocuFH1* gene is expressed in the presumptive endoderm, mesenchyme and notochord cells beginning during the late cleavage stages. During gastrulation, *MocuFH1* expression occurs in the prospective endoderm cells, which invaginate at the vegetal pole, and in the presumptive notochord and mesenchyme cells, which involute over the anterior and lateral lips of the blastopore, respectively. However, this gene is not expressed in the presumptive muscle cells, which involute over the posterior lip of the blastopore. *MocuFH1* expression continues in the same cell lineages during neurulation and axis formation, however, during the tailbud stage, *MocuFH1* is also expressed in ventral cells of the brain and spinal cord. The functional role of the *MocuFH1* gene was studied using antisense oligodeoxynucleotides (ODNs), which transiently reduce *MocuFH1* transcript levels during gastrulation. Embryos treated with antisense ODNs cleave normally and initiate gastrulation. However, gastrulation is incomplete, some of the endoderm and notochord cells do not enter the embryo and undergo subsequent movements, and axis formation is abnormal. In contrast, the prospective muscle cells, which do not express *MocuFH1*, undergo involution and later express muscle actin and acetylcholinesterase, markers of muscle cell differentiation. The results suggest that *MocuFH1* is required for morphogenetic movements of the endoderm and notochord precursor cells during gastrulation and axis formation. The effects of inhibiting *MocuFH1* expression on embryonic axis formation in ascidians are similar to those reported for knockout mutations of *HNF-3β* in the mouse, suggesting that *HNF-3/forkhead* genes have an ancient and fundamental role in organizing the body plan in chordates.

Key words: ascidian, forkhead gene, gastrulation, notochord formation, axis development, *HNF-3β*, *MocuFH1*

INTRODUCTION

Our understanding of the molecular mechanisms of embryonic development has increased dramatically with recent discoveries of developmentally important multigene families such as those encoding homeobox, helix-loop-helix, POU domain, and paired domain proteins (reviewed by Kenyon, 1994; Garrell and Campuzano, 1991; Herr et al., 1988; Burri et al., 1989). The proteins encoded by these gene families possess highly conserved DNA-binding domains and have been shown to function as transcription factors. Within these multigene families, similarity in amino acid sequences across species has made possible the cloning of related genes, many of which are involved in embryonic patterning and control of development in a variety of phyla (e.g., McGinnis and Krumlauf, 1992).

Over the past several years, a new family of transcription factors has emerged: the HNF-3/forkhead or winged-helix family. Winged-helix genes have been described in organisms from yeast to humans and share a highly conserved DNA-binding domain of about 110 amino acids (reviewed by Kaufmann and Knöchel, 1996). Since the identification of the first member of the family, the *Drosophila* gene *forkhead*, whose mutant phenotype is homeotic transformation of the terminal regions of the embryo (Weigel et al., 1989), a number of *HNF-3/forkhead* genes have been identified, many of them developmentally expressed. Vertebrate *HNF-3/forkhead* genes, such as mouse *HNF-3β* (Sasaki and Hogan, 1994), *Xenopus XFKH1* (Dirksen and Jamrich, 1992) and zebrafish *axial* (Strahle et al., 1993), display expression patterns implying a role in axial patterning and dorsoventral development of the notochord, central nervous system (CNS) and gut. *HNF-3β*, for example, is first expressed in the mouse node, an area corresponding to the organizer region of *Xenopus*, which is important in initial patterning of the body axes (Nieuwkoop et al., 1985). Later it is expressed in the notochord and floor plate of the neural tube, where it functions in dorsoventral pattern-

ing of the CNS, and in the endoderm, where it is important for liver differentiation. Mouse embryos homozygous for a targeted mutation in the *HNF-3β* gene show defects in organization of the node, paraxial mesoderm and neural tube, and fail to develop a gut tube (Weinstein et al., 1994; Ang and Rossant, 1994). The role of *HNF-3/forkhead* genes in cellular processes during gastrulation and axis formation is poorly understood, due to gene multiplicity and the complexity of gastrulation in the vertebrate embryo.

We have used ascidians to investigate the role of forkhead genes in embryogenesis. Ascidians have the smallest genome of any known chordate (Lambert and Laird, 1971), and exhibit only one or two copies of some of the regulatory genes that are present in multiple copies in vertebrates (Holland et al., 1994; di Gregorio et al., 1995; Ma et al., 1996). The ascidian tadpole larva has a simple body plan consisting of six different tissues and about 2500 cells and is considered a prototype of the ancestral chordate (Garstang, 1928; Satoh and Jeffery, 1995). There are 110 cells at the beginning of gastrulation in the ascidian embryo, whereas there are 10,000 cells in the *Xenopus* embryo. Therefore, the simplicity of the ascidian embryo permits complicated processes, such as cell movements during gastrulation, neurulation and axis formation, to be examined during embryonic development. Despite this reduced complexity, the ascidian tadpole larva exhibits the hallmarks of a chordate: a dorsal CNS, a notochord and a ventral gut.

Here we describe *MocuFH1*, a member of the *HNF-3/forkhead* gene family in the ascidian *Molgula oculata*. The single copy *MocuFH1* gene contains a winged-helix domain most closely related to that of axial and HNF-3β, is expressed in mid-line organizer tissues during gastrulation and axis formation, and is necessary for endoderm and notochord cell movements during gastrulation and axis formation.

MATERIALS AND METHODS

Biological materials

The ascidian *Molgula oculata* was collected and maintained at Station Biologique, Roscoff, France. The procedures used to prepare gametes for insemination and embryo culture have been described by Swalla and Jeffery (1990).

RNA isolation, cDNA synthesis and PCR

Total RNA from *M. oculata* gastrulae was isolated using the guanidinium isothiocyanate method (March et al., 1985). Then, cDNA was synthesized using the First-Strand cDNA Synthesis Kit according to the directions supplied by the manufacturer (Pharmacia Biosystems, Inc., Piscataway, NJ), and polymerase chain reaction (PCR) was performed on this cDNA at an annealing temperature of 47°C. Degenerate primers were designed against the amino acid sequences ITMAIQ (5′-ATHACNATGGCNCAG-3′) and GNMFEN (5′ CCRTTRTCRAACATRTTNCC-3′), which are highly conserved in the DNA-binding domains of various forkhead genes (Dirksen and Jamrich, 1992). An amplified product of 211 base-pairs (bp) was subcloned into the pCRII vector (TA Cloning Kit, Invitrogen, San Diego, CA) and sequenced using the dideoxy chain termination method (Sanger et al., 1977) with Sequenase (USB; United States Biochemical Corp., Cleveland, OH).

Library screening

A ^{32}P-labeled, random-primed PCR product was used as a probe to screen an *M. oculata* gastrula cDNA library prepared in the Uni-Zap vector (Stratagene, La Jolla, CA). The probe was hybridized to phage lifts on Biodyne A nylon filters (Pall BioSupport, East Hills, NY) at high stringency. Positive clones were in vivo excised to obtain the pBluescript phagemid with the cDNA insert (ExAssist helper phage kit, Stratagene). One phagemid, designated af3, contained a 2.2-kb insert, which was sequenced and shown to be a full-length ascidian forkhead cDNA. The af3 cDNA clone was designated *MocuFH1* (*M. oculata ForkHead 1*).

Southern and northern blots

Genomic DNA isolated from *M. oculata* sperm (Davis et al., 1986) was digested with *Eco*RI and *Hin*dIII, subjected to electrophoresis through agarose gels and transferred to nylon membranes (MSI; Fisher Scientific, San Francisco, CA). Blots were probed at high and low stringencies with the random-primed ^{32}P-labeled *MocuFH1* cDNA insert, prepared from af3 DNA by *Eco*RI and *Xho*I digestion. Blots were also probed at low stringency with the random-primed, ^{32}P-labeled 211-base-pair PCR product described above. The conditions for high and low stringency were as described by Swalla et al. (1993).

RNA for northern blots was isolated from embryos at different developmental stages, subjected to electrophoresis through formaldehyde gels and transferred to nylon membranes (MSI; Fisher Scientific). The phagemid af3, which contained the *MocuFH1*cDNA insert, was linearized with *Eco*RI and served as a template for the synthesis of an antisense RNA probe using T7 polymerase (Stratagene, La Jolla, CA) and [32P]UTP (800 Ci/mmol; Amersham, Arlington Heights, IL). Probes were hybridized to the blots and washed at high stringency (Swalla et al., 1993).

In situ hybridizations

For whole-mount in situ hybridizations, *M. oculata* embryos were dechorionated by treatment with 0.09% Pronase E (Sigma Chemical Company, St Louis, MO), fixed in 4% paraformaldehyde in MOPS buffer for 12 hours at 4°C and stored in 80% ethanol at –20°C. Some embryos were dechorionated manually after fixation using tungsten needles. Whole-mount in situ hybridization was performed according to Ma et al. (1996). Digoxigenin-labeled *MocuFH1* antisense probes were synthesized using T7 RNA polymerase and DIG-labeled UTP (BMB; Boehringer Mannheim Biochemicals, Indianapolis, IN), with *Eco*RI-digested af3 as the template. *MocuFH1* sense probes were made using T3 RNA polymerase (Stratagene, La Jolla, CA), with *Xho*I-digested af3 as the template. The signal was detected using an alkaline phosphatase-coupled secondary antibody and BCIP/X-phosphate reaction (Boehringer-Mannheim), which was allowed to proceed for 1 to 3 hours. Embryos to be photographed were then transferred into 100% ethanol and cleared in a 1:2 mixture of benzyl alcohol: benzyl benzoate (Ma et al., 1996). Embryos to be sectioned were postfixed in 4% paraformaldehyde in PBS, fixed in methanol at –20°C for 20 minutes and then in ethanol at –20°C for 20 minutes, incubated in 1:1 ethanol: polyester wax at 42°C for 1 hour and incubated in polyester wax at 42°C for 1 hour (Swalla et al., 1991). They were then placed in an embedding mold, cooled and sectioned at 8 μm.

For section in situ hybridizations, the method described by Jeffery (1989) was followed using a muscle actin probe synthesized from the *MocuMA1* genomic clone (Kusakabe et al., 1996) or a cytoskeletal actin probe synthesized from the *MocuCA4* cDNA clone (Swalla et al., 1993).

Oligodeoxynucleotide treatment

The following 18-mer phosphorothiolate-substituted oligodeoxyribonucleotides (ODNs) were synthesized by Oligos Etc., Inc. (Wilsonville, OR). Antisense Forkhead 1 (5′-CATAGTGATGTGGACAAA-3′) corresponds to nucleotides 29 to 42 of the *MocuFH1* cDNA sequence, whereas antisense Forkhead 2 (5′-AGAAGGTGGCGACGAAAG-3′) and sense Forkhead 2 (5′-CTTTCGTCGCCAC-

CTTCT-3′) correspond to nucleotides 46 to 63 of the *MocuFH1* cDNA sequence (Fig. 1). The ODNs were stored lyophilized at −20°C and a 30 nmole/μl stock solution was prepared in water prior to use in the experiments. The ODN treatments were carried out as described by Swalla and Jeffery (1996). Briefly, *M. oculata* embryos (100-150 embryos/ml) were suspended in Millipore-filtered sea water containing 30 μM ODN beginning just after first cleavage (about 60 minutes after insemination) and incubated at 16-20°C until hatching (10-12 hours after insemination). The morphology of the ODN-treated and control embryos was determined by light microscopy (Swalla and Jeffery, 1990). Some of the ODN-treated embryos were fixed in 4% paraformaldehyde or Bouin's fixative, embedded in Paraplast and sectioned, and the sections were stained with hematoxylin-eosin (Jeffery, 1989). Others were fixed in 4% paraformaldehyde in MOPS buffer at 4°C, then transferred into 100% ethanol and stored at −20°C in preparation for whole-mount in situ hybridization.

Enzyme assays

Embryos were fixed with 5% formalin-sea water for 30 minutes at 4°C, washed in 0.1 M sodium phosphate buffer. Acetylcholinestase (AChE) activity was assayed as described by Jeffery and Swalla (1991). Alkaline phosphatase activity was assayed as described by Bates and Jeffery (1987a).

RESULTS

Isolation and characterization of *MocuFH1*

Sequence similarity among the DNA-binding domains of various forkhead genes was used to design degenerate primers to amplify a conserved region from the ascidian *M. oculata*. A 211 bp PCR product was amplified from gastrulae cDNA, sequenced and found to exhibit a high degree of similarity to the corresponding region of known forkhead genes. The PCR product was used as a probe to screen an *M. oculata* gastrula cDNA library. The longest cDNA clone obtained, designated *MocuFH1*, was sequenced and found to contain a single open reading frame (ORF) of 1699 nucleotides (nts) flanked by 5′ and 3′ untranslated regions (UTRs) of 110 and 405 nts, respectively. Of two consecutive ATG triplets at the beginning of the ORF, the first is in a context most similar to the consensus eukaryotic translation initiation site (Kozak, 1991). The 3′ UTR of the *MocuFH1* cDNA contains a putative polyadenylation signal followed 14 nts downstream by a poly(A) tail (Fig. 1). The length of the *MocuFH1* cDNA clone is consistent with the size of the single 2.3 kb transcript detected in northern blots (see Fig. 4A).

The *MocuFH1* nucleotide sequence predicts a protein of 567 amino acids containing a DNA-binding domain (Fig. 1) with a high degree of similarity to those of other forkhead proteins (Fig. 2). When the MocuFH1 sequence was used to search the protein databases by BLAST, the best matches were to class I

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   1  GCA CGA GGA AAT TTC AGC CGC TTC ACT TTT TTC AAT TCT GTT GAT AAT AAA ATC TCA ACT   60
  61  GCT GCT GCA GTG CAA CAA ACA ATC GTC GAA TAC TTG TTT GTC CAC ATC ACT ATG ATG CTT  120
                                                                  M   M   L     3
 121  TCG TCG CCA CCT TCT AAG TAT CAG ACA TTT CAA CAA TCA TTT ACC AAC GGA ATG AAC GGT  180
   4   S   S   P   P   S   K   Y   Q   T   F   Q   Q   S   F   T   N   G   M   N   G   23
 181  TCT GTG CCA GGA TCT TAC TCG ATG AAT CCG ATG GCG ATC GGA GGA CCA TCA ACT CTT CAC  240
  24   S   V   P   G   S   Y   S   M   N   P   M   A   I   G   G   P   S   T   L   H   43
 241  TCC GGC ATG AAC GGG GGA TAC GGA AGC GGT ATG TTA AAC GGA ATG AAT GCT GCC GCC GGA  300
  44   S   G   M   N   G   G   Y   G   S   G   M   L   N   G   M   N   A   A   A   G   63
 301  ATG AAC TCG CAC CCA ACC CAC CAT TCT CAA ATG TCA GTC GGA GGT TCA GCC GCT TAC CCT  360
  64   M   N   S   H   P   T   H   H   S   Q   M   S   V   G   G   S   A   A   Y   P   83
 361  GGC ATA AAT CAA GGT GTT GGT CTC AGT CCA AAT ATG GCA TTA TCA ATG TGT ATT AAC CGT  420
  84   G   I   N   Q   G   V   G   L   S   P   N   M   A   L   S   M   C   I   N   R  103
 421  CGC ACA GAG AAG ACA TAT CGC AGG AAT TAC ACC CAT GCA AAA CCA CCA TAC AGC TAC ATC  480
 104   R   T   E   K   T   Y   R   R   N   Y   T   H   A   K   P   P   Y   S   Y   I  123
 481  TCA TTG ATC ACC ATG GCC TTG CAA TCC TCA CAA CAT AAG ATG ATG ACA CTT AGT GAA ATT  540
 124   S   L   I   T   M   A   L   Q   S   S   Q   H   K   M   M   T   L   S   E   I  143
 541  TAT CAA TGG ATT ATG GAC TTG TTT CCA TTC TAC AGA CAA AAT CAA CAG AGA TGG CAA AAC  600
 144   Y   Q   W   I   M   D   L   F   P   F   Y   R   Q   N   Q   Q   R   W   Q   N  163
 601  TCA ATC CGT CAT AGT TTG TCG TTC AAT GAC TGC TTT GTT AAA GTT CCG AGA TCT CCA GAT  660
 164   S   I   R   H   S   L   S   F   N   D   C   F   V   K   V   P   R   S   P   D  183
 661  AAG CCA GGG AAA GGA TCT TAT TGG TCA CTG CAC CCA GAT GCC GGA AAC ATG TTC GAG AAT  720
 184   K   P   G   K   G   S   Y   W   S   L   H   P   D   A   G   N   M   F   E   N  203
 721  GGT TGC TAC CTT CGT AGA CAA AAG CGA TTT AAG TGT AAA AAG ATG AAA TTT TCC GGT GAT  780
 204   G   C   Y   L   R   R   Q   K   R   F   K   C   K   K   M   K   F   S   G   D  223
 781  TCT ACT GAC ATG GAC AAC AAC GAC AAT TCT TCA AGC GAG GAA ATG CAC CAA CAA CAA TCA  840
 224   S   T   D   M   D   N   N   D   N   S   S   S   E   E   M   H   Q   Q   Q   S  243
 841  CCA TCT GGT TCT TTA TCA CCT TCC AAA GAA GTC ACT TCT CCA TCC AGT CCA CAC CCT CAC  900
 244   P   S   G   S   L   S   P   S   K   E   V   T   S   P   S   S   P   H   P   H  263
 901  ACC TCA TCG TAC AAT GAC ATA TCT GAC GTG ATG GAC GAC AAG GCT GCT CTG ACT CAA CAA  960
 264   T   S   S   Y   N   D   I   S   D   V   M   D   D   K   A   A   L   T   Q   Q  283
 961  CAA AGT TCA GTC GAG CAA AAC TCC CGT AAA GAA TTG GCA GAT CAA AGT TCA AAC GCT GAA 1020
 284   Q   S   S   V   E   Q   N   S   R   K   E   L   A   D   Q   S   S   N   A   E  303
1021  GCT TCG CCC AAT GAA AGG ATG CTG CAT CAT CAG CAG AAT ATC TAC TCA CAT TTG CAT CAA 1080
 304   A   S   P   N   E   R   M   L   H   H   Q   Q   N   I   Y   S   H   L   H   Q  323
1081  CAA AAT GCT GAC AGC AAC CTT CCT CAT CCA GAG CAA GGA AGA TTA TCT GCT GTT AAT AAT 1140
 324   Q   N   A   D   S   N   L   P   H   P   E   Q   G   R   L   S   A   V   N   N  343
1141  CAT CAT CAA AAC ACT GAA GTG GAA AAT ATC CAA CAT AGC AAT CAT GTT CGA ACA TCT TCA 1200
 344   H   H   Q   N   T   E   V   E   N   I   Q   H   S   N   H   V   R   T   S   S  363
1201  CCT GTC GAT GCA AAC CAA CAT TCA AAC AGC ATC ACA ACA AAC ACA AGA GAG AGA CAG AAT 1260
 364   P   V   D   A   N   Q   H   S   N   S   I   T   T   N   T   R   E   R   Q   N  383
1261  TAT TAT CAT GAA CCT TTG TTG GAA ACC AAA AGT GAT CCT CTG TCA TAT CCA TCC CAT CAT 1320
 384   Y   Y   H   E   P   L   L   E   T   K   S   D   P   L   S   Y   P   S   H   H  403
1321  TCA TTT TAC CTT TCC CAG TTG CAA GCT GCA GGA GCA CAT CAA GTT CAA CAT TAT CCT GGA 1380
 404   S   F   Y   L   S   Q   L   Q   A   A   G   A   H   Q   V   Q   H   Y   P   G  423
1381  CTT TCA CAC CAT GGA GCA TCT CAT CCT CTG GCA CAT TCC TTC ACC CAT CCC TTC TCC ATT 1440
 424   L   S   H   H   G   A   S   H   P   L   A   H   S   F   T   H   P   F   S   I  443
1441  TCA AGC TTG ATG AAT GCC GGT GGT GAG ATG CAA AGT TCG AAG GAG ATG AGG GCA TAT CAA 1500
 444   S   S   L   M   N   A   G   G   E   M   Q   S   S   K   E   M   R   A   Y   Q  463
1501  GAT GCC ATG CAA CAG TAC AGT TAT GGA ACA ACA GCA CAA GAT GTG CAT CAC GAC AAC ATC 1560
 464   D   A   M   Q   Q   Y   S   Y   G   T   T   A   Q   D   V   H   H   D   N   I  483
1561  TCA CCA CAA CAA ATA TCA ACA TTG GAA AAT GCA ACC GCA TCA ACT CCT GAC TCT GGT GAC 1620
 484   S   P   Q   Q   I   S   T   L   E   N   A   T   A   S   T   P   D   S   G   D  503
1621  GTT TCA ACA TCA ATA CCA TCG TCG AGT TCC AAC ACA CAC TCC CCA GAA AAT CTA CAA CAA 1680
 504   V   S   T   S   I   P   S   S   S   S   N   T   H   S   P   E   N   L   Q   Q  523
1681  CAA TAT TAT CAA ATG CAC TAC AAC ATG GAA TCA GCA AAT CCT GCA GTT TCA ACT CAC GAT 1740
 524   Q   Y   Y   Q   M   H   Y   N   M   E   S   A   N   P   A   V   S   T   H   D  543
1741  GGT TTG GGA AGT CTT GCT GAT GCA TAT TAT CAA GGG TGC GTA CAG CAG CAT AAT TCT AAT 1800
 544   G   L   G   S   L   A   D   A   Y   Y   Q   G   C   V   Q   Q   H   N   S   N  563
1801  GCA GCG ATT GCA TAA AAT ATT AAT ATC AAC AAA ATT AAT AAA TGT TTT GTG TGG GTT AGC 1860
 564   A   A   I   A   *
1861  AAT TCT TCC TTT GCT TGC GCC ATT ATC TTA TGG GTG GTG GCT TAA CGT TCC TGA TAA CAT 1920
1921  GGT ATG CAC TGA AAT GAT CAA ACC TCA TAG TAT TTA TTT CGG GGC ATG AAG CCA GAC TGA 1980
1981  AAT GCG GGT ATC TCT AAA CAT TCC ATG ACT TAA ATC GAA CAA TGA ACT TGA TAT GGA GTG 2040
2041  TCA TTT ACC GCC AGT AGT GTT AGG AAC AGT ATA AAT TAT GTC GTA ATA TCA TGT ATG TGT 2100
2101  ATA TAA ATG CAA TAA ATT TCG TAC TAA TTG CAA TTC TTA CTG TTG TTT AAC TGG CAT GAT 2160
2161  TAC TGC TTG CAA ACT TAA TAA ATC AAT GAA TGT TGA AAA AAA AAA AAA AAA AAA A       2215
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Fig. 1. The nucleotide and deduced amino acid sequences of *MocuFH1*. The positions of ODN-1 (nucleotides 97-114) and ODN-2 (nucleotides 118-135) are underlined in the 5′ region of the cDNA sequence. The putative forkhead DNA-binding domain is shaded and a putative polyadenylation signal is underlined. The GenBank accession number for *MocuFH1* is AF007905.

forkhead proteins (Sasaki and Hogan, 1993), such as zebrafish axial, *Xenopus* pintallavis/XFKH-1 and the rodent HNF-3 proteins. The alignments of MocuFH1 and some of these proteins are shown in Fig. 2. The forkhead domain of MocuFH1 is 92% identical to those of zebrafish axial and mouse HNF3β and 87% identical to those of *Drosophila* forkhead and *Xenopus* pintallavis/ XFKH-1. The forkhead domains of mouse HNF-3α and HNF-3γ (Lai et al., 1991) are only 82% and 84% identical to that of MocuFH1, respectively. The predicted MocuFH1 protein shows additional similarity to other forkhead proteins in two conserved regions near the C terminus (Fig. 2), which have been implicated in transcriptional activation (Pani et al., 1992). However, MocuFH1 shares no sequence similarity with other forkhead proteins outside the putative DNA-binding and transcriptional activation domains. The results suggest that the *MocuFH1* gene encodes a member of the class I subgroup of winged-helix proteins and is most closely related to the zebrafish *axial* and rodent *HNF3β* genes.

MocuFH1 is a single copy gene

The forkhead genes are members of a multigene family (Kaufmann and Knöchel, 1996). Southern blots of *M. oculata* genomic DNA, hybridized with the full-length *MocuFH1* cDNA probe and washed at high stringency, showed only one or two bands (Fig. 3A), suggesting that *MocuFH1* is a single-copy gene. Another band appeared in the *Hin*dIII digests when the blot was washed at low stringency (Fig. 3B). When the same Southern blot was hybridized with a probe made from the conserved *MocuFH1* DNA-binding domain, however, additional bands were observed (Fig. 3C). These bands did not include the additional band seen in *Hin*dIII digests when *MocuFH1* was used as a probe, suggesting the existence of another gene in *M. oculata* with homology to the non-DNA-binding region of *MocuFH1*. The results suggest that *MocuFH1* is a single copy gene but that other related genes are present in the *M. oculata* genome.

MocuFH1 is expressed during gastrulation and axis formation

The temporal and spatial expression of *MocuFH1* during *M. oculata* embryogenesis was examined by northern blots and in

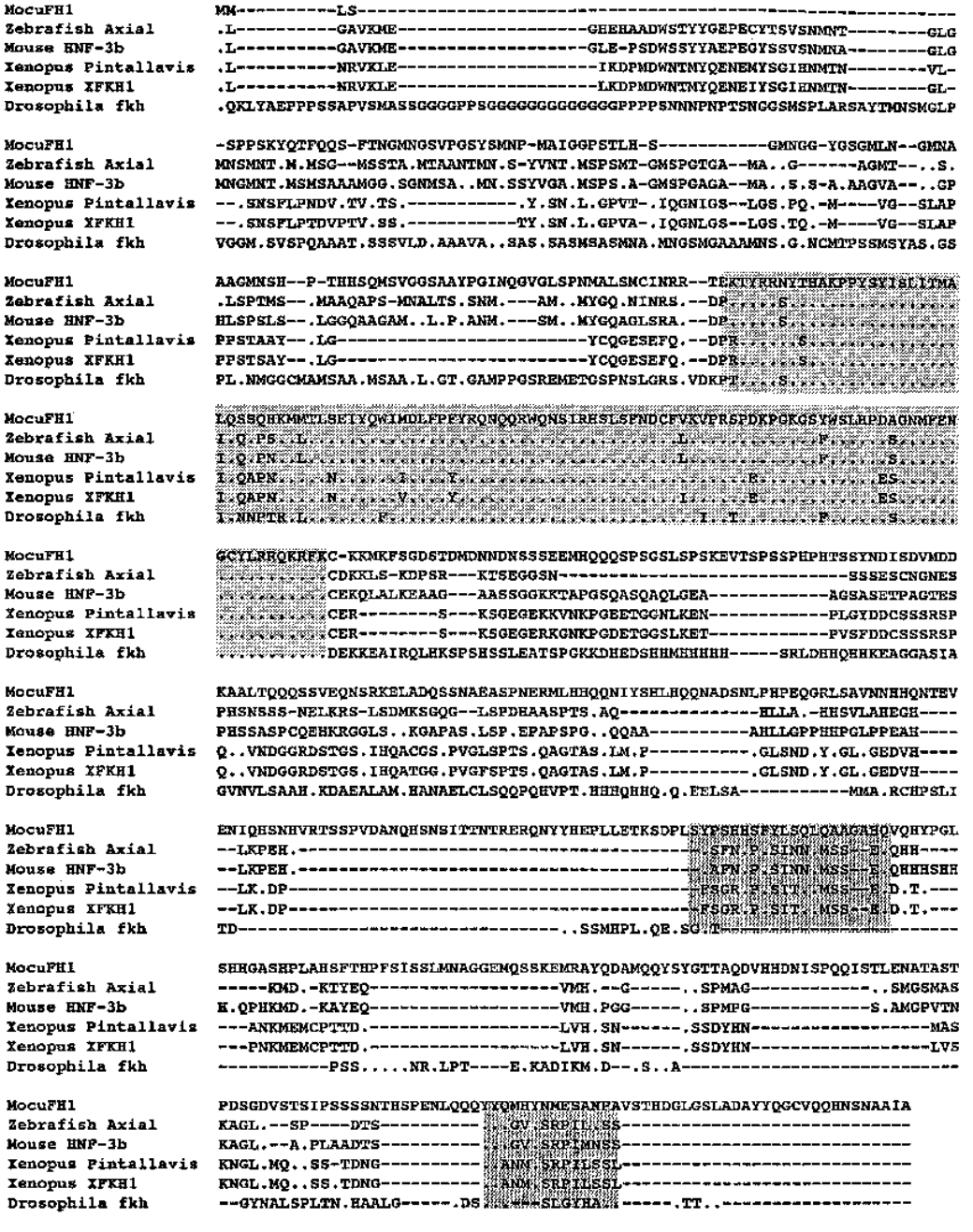

Fig. 2. Alignment of MocuFH1 with other class I forkhead proteins. MocuFH1 is aligned with zebrafish axial (Strahle et al., 1993), mouse HNF3β (Sasaki and Hogan, 1993), *Xenopus* pintallavis (Ruiz I Altaba and Jessell, 1992, *Xenopus* XFKH1 (Dirksen and Jamrich, 1992) and *Drosophila* forkhead (*fkh*) (Weigel et al., 1989). The dots represent identical amino acids and the dashes indicate gaps with respect to the MocuFH1 sequence. Light shading indicates the putative forkhead DNA-binding domains and dark shading indicates the putative transcription activation domains.

situ hybridization. Northern blots containing gonad and embryo RNA showed a single 2.3 kb transcript, which was first detected during gastrulation, peaked during neurulation and decreased in amount during the tailbud stages (Fig. 4A). *MocuFH1* transcripts could not be detected by northern hybridization in gonads, 8- to 16- cell embryos, or 64-cell embryos (although they were detected at the 44-64-cell stage by in situ hybridization; see below). The results suggest that *MocuFH1* does not have a maternal expression period and is expressed zygotically.

M. oculata embryos were fixed for whole-mount in situ hybridization at various stages of embryogenesis and some of these embryos were sectioned after hybridization to verify the pattern of *MocuFH1* expression (Fig. 4B-H). In 44-64 cell embryos, *MocuFH1* transcripts were confined to presumptive endoderm and notochord cells in the vegetal (future dorsal) hemisphere (Fig. 4B). Staining was more concentrated in the notochord than the endoderm cells at this stage. No staining was detected in embryos hybridized with a sense *MocuFH1* probe. In gastrulae, *MocuFH1* transcripts are present in presumptive endoderm cells, including the large vegetal cells, which initiate invagination (Conklin, 1905), in presumptive notochord cells, which involute over the anterior lip of the blastopore, and in mesenchyme cells, which involute over the lateral lips of the blastopore (Fig. 4C; also see Fig. 7A). *MocuFH1* transcripts were not detected in the presumptive muscle cells, which involute over posterior lip of the blastopore, or in the ectodermal cells, which spread over the vegetal hemisphere by epiboly. In neurulae, staining was restricted to the notochord, mesenchyme and endoderm cells (Fig. 4D,E). In tailbud stage embryos, *MocuFH1* transcripts were present in endoderm and mesenchyme cells, which enter the developing trunk (head) and presumptive notochord cells, which undergo convergence and extension to form the notochord and larval tail (Fig. 4F-H). *MocuFH1* transcripts were also detected in a row of cells on the basal side of the brain (Fig. 4H) and in the spinal cord (Fig. 4G). No transcripts were detected in the endodermal strand or muscle precursor cells at the tailbud stage (Fig. 4D-G). *MocuFH1* transcripts persisted in the endoderm and notochord cells through the late tailbud stage, although the staining in the notochord was gradually reduced. The results suggest that the *MocuFH1* gene is expressed in endoderm, mesenchyme and notochord cells during gastrulation and in these cells, as well as a restricted number of neural cells, during larval axis formation.

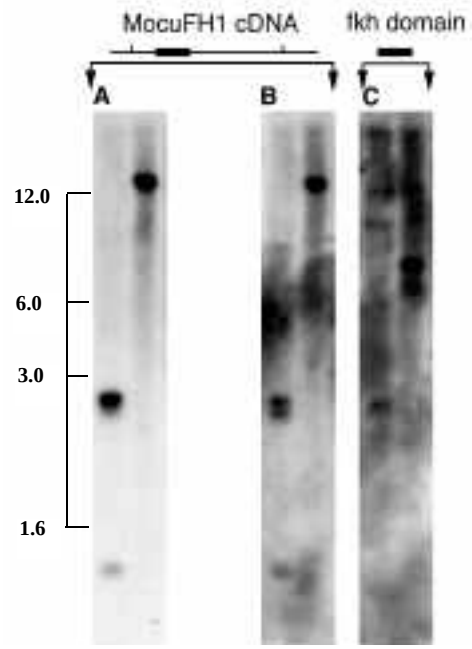

Fig. 3. Southern blot hybridization of *MocuFH1* full length and forkhead domain probes to *M. oculata* genomic DNA. (A,B) A blot hybridized to the full length *MocuFH1* probe and washed at high (A) or (B) low stringency. (C) The same blot as in A,B hybridized to the forkhead domain probe and washed at low stringency. Left lane, *Hin*d III digest; Right lane, *Eco*R1 digest.

MocuFH1 is necessary for gastrulation and patterning of the tadpole larva

The absence of maternal expression suggested the use of antisense procedures to study the role of the *MocuFH1* gene in embryogenesis. Recent experiments with phosphorothiolate-substituted oligodeoxynucleotides (ODNs) have shown that the *Manx* gene is required for development of the tadpole larva (Swalla and Jeffery, 1996). Therefore, antisense ODNs were used to examine the developmental role of *MocuFH1*.

Embryos were treated with ODNs beginning at first cleavage and incubated until hatching, about 10-12 hours after fertilization. Antisense ODN-1 had no effect on embryogenesis and was not used in subsequent experiments. Antisense ODN-2, however, showed reproducible effects on gastrulation and axis formation. The phenotypes of ODN-2-treated and control embryos are shown in Fig. 5. Sense and antisense ODN-2-treated embryos were able to cleave and initiate gastrulation normally and showed no morphological differences with respect to the controls. Patterning defects were first observed in antisense ODN-2-treated embryos at the mid-gastrula stage. Ascidian gastrulation is initiated by the invagination of large endoderm precursor cells at the vegetal pole (Conklin, 1905). After the archenteron is formed, additional endoderm cells involute over the lips of the blastopore, and later the presumptive notochord, mesenchyme and muscle cells involute over the anterior, lateral and posterior lips of the blastopore, respectively (see Satoh, 1978; Jeffery, 1992; Swalla, 1993 for descriptions of ascidian gastrulation). Control (Fig. 5A,D) and sense (Fig. 5C,F) ODN-2-treated embryos exhibited normal invagination and involution, but gastrulation was defective in most of the antisense ODN-2-treated embryos (Fig. 5B,E). The effects of antisense ODN-2 varied in severity between different clutches of embryos. However, three classes of embryos could be distinguished in every experiment (Fig. 5M-O). In severely affected embryos, the internalization of the presumptive endoderm cells was incomplete, leaving a large endodermal mass outside the embryo and the presumptive notochord cells were arrested as a cluster anterior to the endodermal mass (Fig. 5E,O). The endodermal mass and internalized endoderm cells stained positively for alkaline phosphatase (Fig. 6E), an endoderm-specific marker in ascidians (Bates and Jeffery, 1987b). However, the cytoskeletal actin gene *MocuCA4* (Swalla et al. 1993), which is normally expressed in notochord and muscle cells of *M. oculata* embryos (Fig. 6C), was expressed in muscle cells but not in notochord cells of ODN-2-treated embryos (Fig. 6D). In moderately affected embryos,

an increased number of endodermal cells were able to invaginate, reducing the size of the endodermal mass and some of the notochord cells appeared to involute, but did not undergo further movements inside the embryo (Fig. 5N). The moderately affected embryos also did not express *MocuCA4* in notochord cells (data not shown). Even in the most severely affected embryos, however, the presumptive muscle cells were able to involute (Figs 5E, 6A,B), although subsequent movements of the myoblasts were affected and these cells remained as stationary ring around the posterior base of the endodermal mass (see Fig. 6A). Finally, a few embryos in each antisense ODN-2 experiment were able to complete an apparently normal gastrulation (Fig. 5M). The results suggest that antisense ODN-2 inhibits cell movements that begin during mid-gastrulation.

Some of the ODN-2-treated and control embryos were fixed at the gastrula and early tailbud stages and subjected to in situ hybridization to monitor the accumulation of *MocuFH1* mRNA. The gastrulae that developed from controls and sense ODN-2-treated embryos showed normal distributions of *MocuFH1*mRNA (Fig. 7A,C). In contrast, most moderately and severely affected gastrulae that developed from embryos treated with antisense ODN-2 showed lower levels of staining in the endodermal mass (Fig. 7B). When early tailbud embryos were examined, however, the level of staining was similar in the control and ODN-2-treated embryos (Fig. 7D-F), although the embryos treated with antisense ODN-2 showed an endodermal mass and presumptive notochord. Thus, the initial reduction in *MocuFH1*mRNA at the gastrula stage in antisense ODN-2-treated embryos appears to be temporary. A transient reduction of mRNA in chick embryos treated with antisense ODNs corresponding to the *Slug* gene has also been reported (Nieto et al., 1994). The normal gastrulae that developed from embryos treated with antisense ODN-2 stained more intensely for *MocuFH1* mRNA after in situ hybridization than those with defective gastrulation (data not shown), suggesting that variations in the effects of antisense ODN-2 are based on differential suppression of target mRNA. Similar variability in experiments with *Manx* antisense ODNs was shown to be related to ODN penetration (Swalla and Jeffery, 1996). The results suggest that antisense ODN-2 reduces the level of *MocuFH1* transcripts early during gastrulation.

The inability of most antisense ODN-2-treated embryos to gastrulate also affected subsequent axis formation during larval development (Table 1). After gastrulation, most of the endoderm cells move toward the anterior pole of the embryo to form the larval head, and the notochord, muscle and some endoderm cells (the endodermal strand) move posteriorly to form the larval tail. The CNS is formed during neurulation by

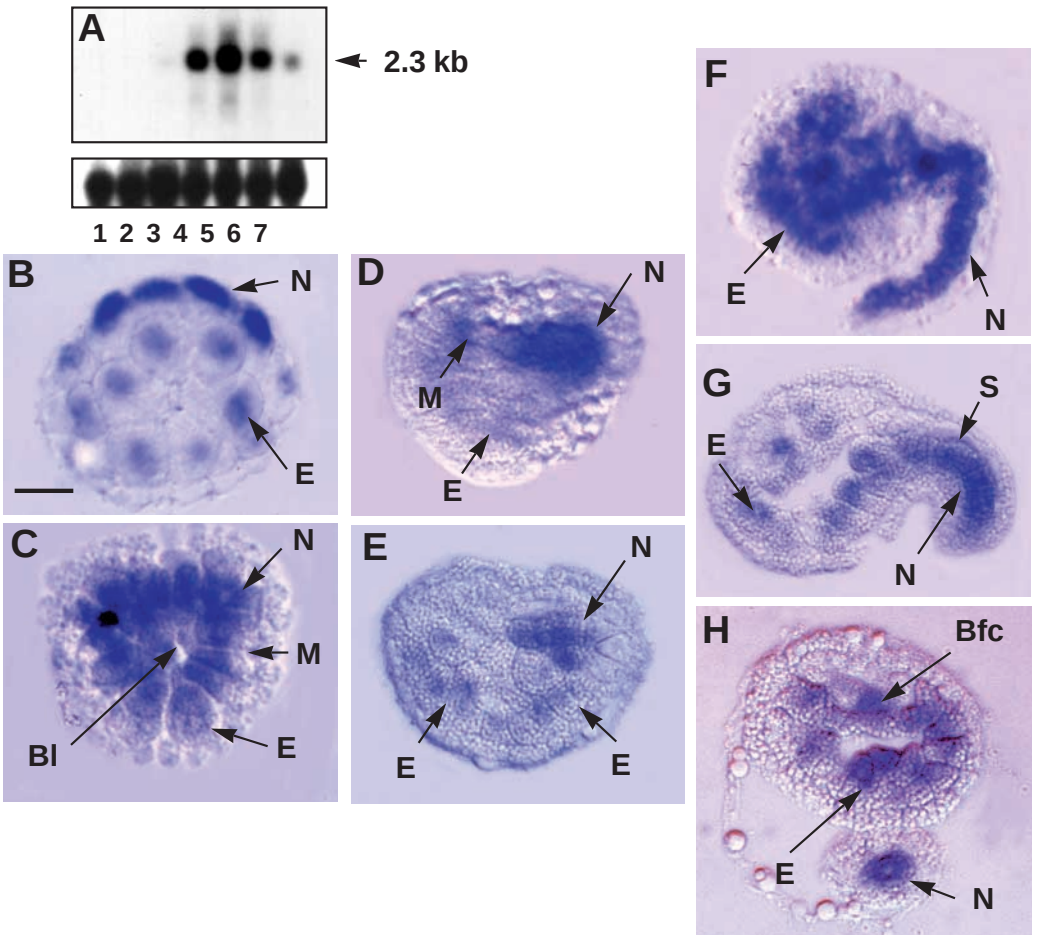

Fig. 4. Temporal and spatial accumulation of *MocuFH1* mRNA during *M. oculata* development. (A) A northern blot containing RNA from (1) gonads, (2) 8- to 16-cell embryos, (3) 32- to 64-cell embryos, (4) mid-gastrulae, (5) neurulae, (6) mid-tailbud embryos and (7) late tailbud embryos. Top: *MocuFH1* probe. The single 2.3 kb *MocuFH1* transcript is indicated by the arrow. Bottom: 18S rRNA loading control. (B-H) Embryos subjected to whole-mount in situ hybridization with *MocuFH1* antisense RNA probe. (B-D,F) Whole mounts. (E,G,H) Sections of whole mounts. (B) A 44-/64-cell embryo showing staining in presumptive notochord (N) and endoderm (E) cells. (C) An early gastrula showing staining of presumptive notochord (N) cells at the anterior lip of the blastopore (Bl), the mesenchyme cells (M) at the lateral lips of the blastopore and endoderm cells (E) that have entered the blastopore. (D,E) Neurulae showing staining in the notochord (N), mesenchyme (M) and endoderm (E) cells. (F-H) Mid-tailbud stage embryos showing staining in the notochord (N) and spinal cord (S) cells in the tail and in the endoderm (E) and brain floor (Bfc) cells in the head. (B,C,H) Embryos are shown with anterior poles at the top; (D-G) embryos are shown with their anterior poles at the left. Scale bars in B,E, 20 μm; magnification is the same in B,C,G, and in D,E,F, respectively.

folding of ectodermal cells at the dorsal midline of the embryo to form the neural tube. Controls and embryos treated with sense ODN-2 developed into normal tadpole larvae (Fig. 5G,I,J,L). In contrast, embryos treated with antisense ODN-2 failed to develop an embryonic axis at frequencies comparable to the controls (Table 1). The severely affected embryos formed mushroom-shaped structures with a bulging endodermal mass, but lacking a definitive head, a CNS with an otolith and a tail (Fig. 5H,K). Sections through these embryos showed that they lacked a neural tube and that the endodermal mass contained endoderm and notochord cells (Fig. 5K), but the latter were not arranged in single file, as they appear in the tail of tadpole larvae. The moderately affected embryos did not form mushroom-shaped bodies, but appeared as permanent gastrulae, not extending a notochord or developing an apparent anteroposterior axis. The appearance of these embryos and the

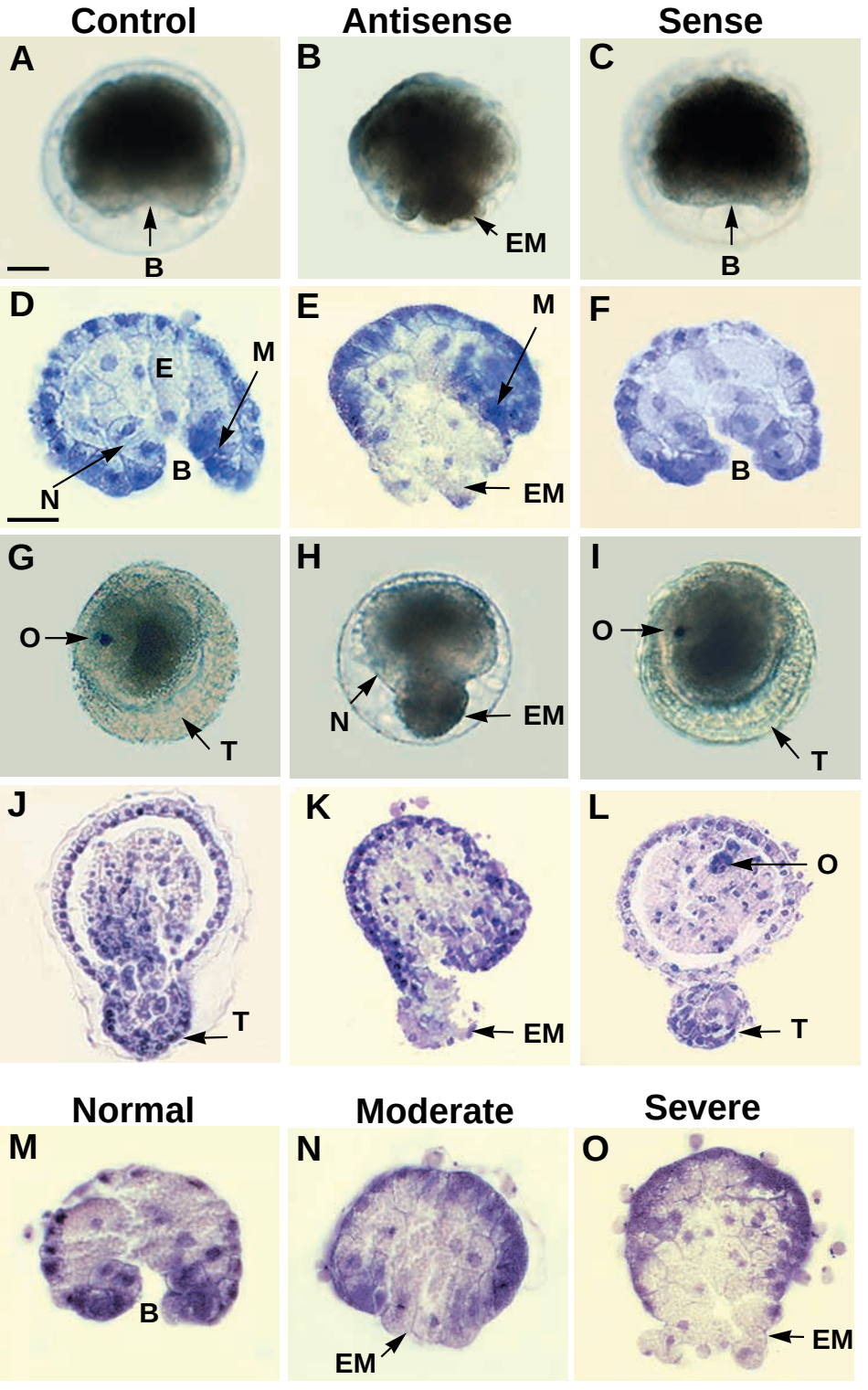

Fig. 5. The effect of antisense ODNs on *M. oculata* embryogenesis. (A,D,G,J) Untreated embryos; (B,E,H,K,M-O) embryos treated with antisense ODN-2; (C,F,I,L) embryos treated with sense ODN-2. (A-C) Mid-gastrulae viewed from a lateral side. (D-F) Sagittal sections of mid-gastrulae treated as in A-C. The gastrulae shown in A,D,C,F have normal morphology with endoderm cells (E) invaginated into the archenteron, notochord cells (N) involuted at the anterior lip and muscle cells (M) involuted at the posterior lip of the blastopore (B). The endodermal mass (EM) is shown protruding from the vegetal pole region in embryos treated with antisense ODN-2 (B) but not in untreated embryos (A) or embryos treated with sense ODN-2 (C). The muscle cells involute in untreated and ODN-treated embryos. (G-I) Mid-tail bud stage (11 hour) embryos viewed from a lateral side; (J-L) sections through the head and tail of mid-tailbud (11 hour) stage embryos treated as in G-I. The mid-tailbud embryos shown in G,J and I,L have normal heads containing an otolith (O) and tails (T). Heads and tails are not distinguishable in 11 hour embryos treated with antisense ODN-2 (H,K), which still contain a protruding endodermal mass (EM). (M-O) Sections of mid-gastrulae with normal (M), moderately affected (N) and severely affected morphology after treatment with antisense ODN-2. Scale bars in A,D, 20 μm; magnification is the same in A-C, G-I, D-F, J-L and M-O, respectively.

Table 1. Effect of *MocuFH1* ODNs on development of the tadpole larva

	Otolith		Tail	
ODN-2	Embryos	% normal	Embryos	% normal
Sense	848	85	847	93
Antisense	840	24	840	18

The pooled results of a total of 14 experiments are shown. The % normal otolith and tail development is indicated relative to development in untreated controls. The proportion of affected embryos was approximately the same at the gastrula stage.

expression of AChE in non-migratory myocytes resemble the phenotype of *Molgula occulta*, an ascidian species closely related to *M. oculata* that does not undergo notochord and tail formation during normal larval development (Swalla and Jeffery, 1990; Jeffery and Swalla, 1991). The defects in larval development did not include the muscle cells, which do not express the *MocuFH1* gene (Fig. 4), and were able to express muscle actin mRNA and the muscle-specific enzyme AChE (Fig. 6A,B). The results suggest that *MocuFH1* expression is required for morphogenetic movements of the notochord and endoderm cells during gastrulation and axis formation.

DISCUSSION

The *HNF-3*/*forkhead* genes are members of a family of winged-helix transcription factors expressed in dorsal mid-line organizing centers in vertebrate embryos. We describe here the ascidian gene *MocuFH1*, which encodes a winged-helix protein with a DNA-binding domain most closely related to zebrafish axial and rodent HNF-3β proteins. The *MocuFH1* gene is expressed in mid-line tissues of the ascidian embryo, including the presumptive notochord, endoderm and ventral cells of the CNS. Functional studies show that *MocuFH1* is required for morphogenetic movements of the notochord and endoderm cells during gastrulation and axis formation, indicating that the *HNF-3 /forkhead* genes have a fundamental role in establishing the chordate body plan.

Ascidians have the smallest genome of any chordate (Lambert and Laird, 1971). Accordingly, genes that exist in multiple copies in vertebrates, such as the forkhead genes (Lai et al., 1991; Sasaki and Hogan, 1993), may be fewer and function in a less complicated fashion in ascidians (Holland et al., 1994; di Gregorio et al., 1995; 1994; Ma et al., 1996). Southern blot analysis showed that *MocuFH1* is a single copy gene. Nonetheless, hybridization with a probe restricted to the *MocuFH1* DNA-binding domain suggests that there are other related winged-helix genes in the *M. oculata* genome. The conserved DNA-binding domain of MocuFH1 is most similar to the forkhead domain of zebrafish axial and the rodent HNF3β proteins. Except for this region and two putative transcription activation domains, MocuFH1 is divergent from all known winged-helix proteins. Thus, until additional ascidian winged-helix genes are identified, the evolutionary relationship between the *MocuFH1* and *HNF3β* genes cannot be determined with certainty. Considering the low number of bands in Southern blots, however, it is likely that ascidians have fewer winged-helix genes than higher vertebrates.

Despite its divergence from other winged-helix proteins, the embryonic expression pattern of *MocuFH1* is remarkably similar to that of the vertebrate *HNF-3* genes. In *Xenopus* and the mouse, one or more *HNF-3* genes are first expressed in the organizer/node region, and later in the notochord, endoderm and floor plate (Lai et al., 1991; Dirksen and Jamrich, 1992; Sasaki and Hogan, 1993; Ruiz i Altaba et al., 1993). The expression of *MocuFH1* in vegetal blastomeres of 44-/64-cell embryos, in the presumptive endoderm, mesenchyme and notochord cells during gastrulation and axis formation, and in ventral mid-line cells in the CNS of ascidian embryos resembles the expression patterns seen in vertebrate embryos. The similarity of *MocuFH1* expression in ascidians and vertebrates, members of different chordate subphyla, suggests that their embryonic midline tissues may deploy the same genes to organize the body plan.

The possible homology of the ascidian and vertebrate notochord has been appreciated for some time, and is supported by expression of *Brachyury* (*T*) (Yasuo and Satoh, 1994; Corbo et al., 1997) and the *HNF-3*/ *forkhead* -related gene in the ascidian notochord. The expression pattern of the *MocuFH1* gene described here also provides evidence that ventral cells of the ascidian CNS, including both the brain and spinal cord, have a common evolutionary origin with the floor plate cells of the vertebrate CNS. During vertebrate body axis formation, *HNF-3β* seems to induce the floor plate by activating the expression of *sonic hedgehog* in the notochord and floor plate cells (Echelard et al., 1993; Sasaki and Hogan, 1994). Thus, it will be interesting to determine whether a similar relationship exists between *MocuFH1* and *sonic hedgehog* in the notochord and CNS during axis formation in the ascidian embryo. There are also similarities between the *MocuFH1* and *HNF-3* genes in the endoderm and its derivatives. The *HNF-3* genes are expressed in the liver, lungs, intestine and stomach of adult mice (reviewed by Kaufman and Knöchel, 1996). Likewise, the *MocuFH1* gene is expressed in the differentiating endoderm throughout the development of the tadpole larva. Although the ascidian larva does not exhibit tissues resembling the endodermally derived organs of vertebrates, a pharynx with gill slits, stomach and intestine are prominent features of the adult, which differentiate from *MocuFH1*-expressing cells in the head endoderm during metamorphosis.

Although definitive transplantation experiments have not been done in ascidians, fate-mapping studies have shown that the presumptive notochord cells reside at the anterior lip of the blastopore (Conklin, 1905; Nishida, 1987), suggesting that this region may be the functional equivalent to the vertebrate organizer (Nieuwkoop et al., 1985). Consistent with this idea, the presumptive notochord cells exhibit relatively high levels of *MocuFH1* expression in the ascidian embryo. The expression domain of *MocuFH1* also includes prospective endoderm at the vegetal pole, the first cells to invaginate during gastrulation (Conklin, 1905). The sensitivity of the vegetal pole region to ultraviolet irradiation during ooplasmic segregation (Jeffery, 1990) suggests that this region may function similarly to the Nieuwkoop Center in *Xenopus* embryos. Endoderm cells that arise from the vegetal pole region of the ascidian embryo contain cytoplasmic factors responsible for initiating gastrulation (Bates and Jeffery, 1987b; Nishida, 1996). These factors are candidates for direct or indirect regulators of *MocuFH1* expression in the invaginating endoderm.

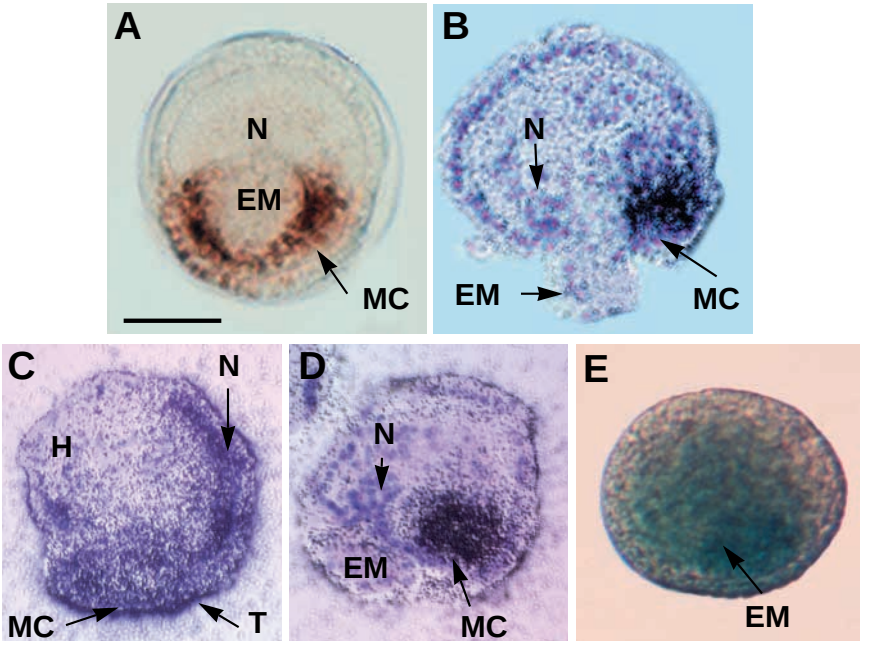

Fig. 6. Expression of muscle, notochord, endoderm markers in *M. oculata* embryos treated with antisense ODN-2. (A) A whole-mount 11 hour embryo showing AChE activity (red-brown stain) in a ring of muscle cells (MC) around the endodermal mass (EM). The anterior pole of the embryo is at the top. (B) A section of an 11 hour embryo subjected to in situ hybridization with a muscle actin probe showing muscle actin mRNA in muscle cells. The anterior pole of the embryo is at the left. The scale bar in A is 50 μm; magnification is the same in A-E. (C,D) Sections of (C) an sense ODN-2-treated mid-tailbud embryo and (D) an antisense ODN-2-treated embryo subjected to in situ hybridization with a cytoskeletal actin probe. (E) A whole-mount 11 hour embryo showing AP activity (green) in the internal endoderm and endodermal mass. N, notochord cells; H, larval head; T, larval tail.

Another similarity between *MocuFH1* and *HNF-3β* genes is lack of expression in mesodermal derivatives responsible for muscle cell formation. In the ascidian embryo, the presumptive larval muscle cells involute over the posterior lip of the blastopore and are devoid of *MocuFH1* transcripts at this stage as well as later stages of development when myoblasts differentiate into larval tail muscle cells. Similarly, mammalian embryos do not express *HNF-3β* in paraxial mesoderm, although related winged-helix genes are expressed in the somites and other non-notochordal mesodermal derivatives (Sasaki and Hogan, 1993; Miura et al., 1993; Kaestner et al., 1993). Despite the lack of *HNF-3β* expression in paraxial mesoderm, knockout mice exhibit defects in somite organization during later development, presumably due to the absence of a notochord (Weinstein et al, 1994). Although ascidian embryos deficient in *MocuFH1* transcripts are able to express muscle cell markers, the myoblasts are not organized properly into muscle bands, as they are in the tail of normal larvae. The inability of myoblasts to differentiate into muscle cells in antisense-treated ascidian embryos could also be caused by the absence of a notochord.

In mouse embryos, loss of *HNF-3β* gene function through targeted mutagenesis leads to defective development of the node, notochord and floorplate (Ang and Rossant, 1994; Weinstein et al., 1994). Earlier effects on gastrulation have been more difficult to interpret, primarily because of the relative complexity of cell movements. In contrast, ascidian embryos begin gastrulation with only 110 cells (Conklin, 1905), the cell movements initiated at this stage of development are well known (Satoh, 1978; Jeffery, 1992; Swalla, 1993) and a fate map of the gastrula is available (Nishida, 1987). When *M. oculata* embryos are treated with antisense ODNs at first cleavage, the succeeding cleavages are normal and gastrulation

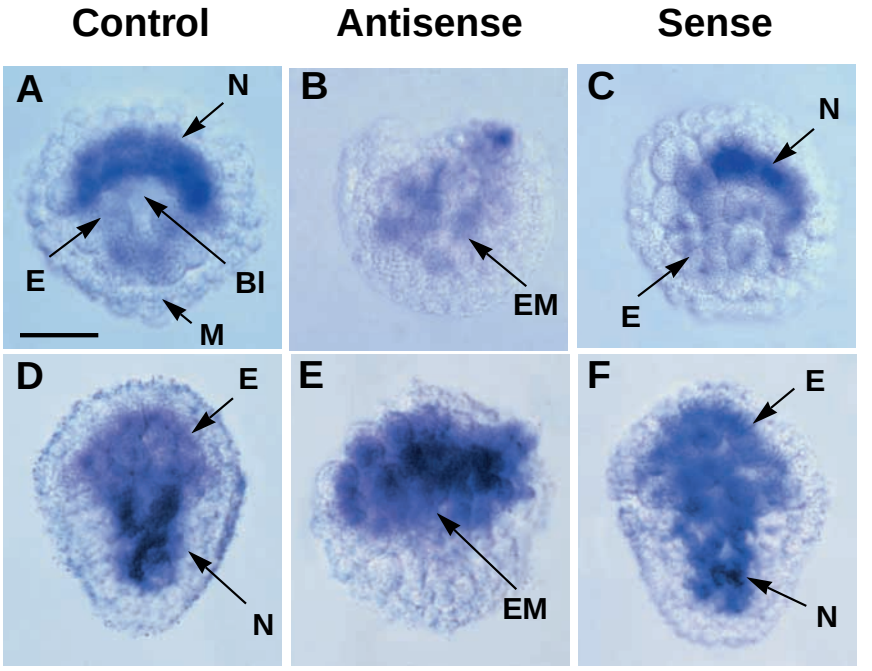

Fig. 7. Expression of *MocuFH1* mRNA in antisense ODN-2-treated and control *M. oculata* embryos. Whole-mount in situ hybridization was done with an *MocuFH1* antisense RNA probe. (A-C) Mid-gastrulae that developed from untreated embryos (A) or embryos treated with antisense (B) or sense (C) ODNs. (D-F) Early tailbud embryos that developed from embryos untreated embryos (D) or embryos treated with antisense (E) or sense (F) ODNs. N, notochord; E, endoderm; Bl, blastopore; EM, endodermal mass. Embryos are shown with their anterior poles at the top and photographed from the dorsal (vegetal) side. Scale bar in A is 50 μm; magnification is the same in each frame.

is initiated by invagination of the endoderm cells, but the ensuing movements of endoderm and notochord cells are inhibited. Later in development, the notochord, CNS and embryonic axis do not appear, resulting in a grossly abnormal embryo. The effects of antisense ODN-2 are thought to be specific for *MocuFH1* because (1) antisense ODN-2 suppresses the accumulation of *MocuFH1* transcripts during gastrulation, (2) sense ODN-2 and other antisense ODNs have no affect on development, (3) antisense ODN-2 does not affect other zygotic genes, such as the muscle actin and AChE genes, (4) there are no direct effects on gastrulating tissues that do not express the *MocuFH1* gene, such as the prospective larval muscle cells, and (5) the effects of antisense *MocuFH1* ODNs on embryonic development are different from those of antisense ODNs corresponding to other genes (Swalla and Jeffery, 1996). We conclude that *MocuFH1* is required for cell movements beginning at the midgastrula stage, including involution of endoderm and notochord cells during gastrulation, and interdigitation and posterior movements of notochord precursor cells during axis formation. The effects of inhibiting *MocuFH1* on the presumptive mesenchyme cells, which express *MocuFH1* and involute over the lateral lips of the blastopore, cannot be evaluated at present because of the lack of appropriate markers. Finally, it seems likely that failure of CNS development is due to the inability of the notochord/endoderm cells to signal to neuroectoderm and/or to attain the appropriate spatial organization to interact with the neuroectoderm.

The downstream targets of the winged-helix transcription factors have not been elucidated in great detail, although some evidence suggests that *Brachyury* (*T*) may act downstream of *HNF3β* in the mouse (Weinstein et al., 1994). The fact that alkaline phosphatase activity is present in ascidian embryos treated with forkhead antisense ODNs suggests that expression of this enzyme is not dependent on *MocuFH1* in the endoderm. Unfortunately, we were unable to examine the relationship between the *MouFH1* and *T* genes in the notochord because the ascidian *Brachyury* cDNA, cloned from *Halocynthia roretzi* (Yasuo and Satoh, 1994), does not react with *Molgula oculata*, which is phylogenetically distant from other ascidians (Hadfield et al., 1995). However, expression of the cytoskeletal actin gene *MocuCA4* in the notochord, but not the muscle cells, was inhibited by treatment with forkhead antisense ODNs. This suggests that *MocuCA4* is a direct or indirect downstream target of *MocuFH1* in the developing notochord.

It is significant that the effects of *MocuFH1* inhibition in some *M. oculata* embryos resemble the natural phenotype of *M. occulta*, a closely related species that lacks postgastrulation morphogenetic movements resulting in notochord and tail development (Swalla and Jeffery, 1990). Several *M. oculata* genes have been identified that are downregulated during *M. occulta* development (Swalla et al, 1993), including *Manx*, which is required for tail formation (Swalla and Jeffery, 1996). It will be interesting to investigate *MocuFH1* expression and function in *M. occulta* embryos, and to determine whether there are interactions with the *Manx* gene.

The ascidians (along with the salp and larvacean tunicates) are the sister group of a clade containing the cephalochordates (amphioxus) and the vertebrates (Wada and Satoh, 1994). As such, the ascidians represent a basal group within the Phylum Chordata. The deployment of *HNF-3*/ *forkhead* genes to organize cell movements during gastrulation and axis formation in ascidians and mammals suggest that these genes have an ancient and fundamental role in organizing the body plan during chordate development.

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Note added in proof

While this article was in proof, Corbo et al. (*Development* **124**, 2335-2344, 1997) reported the isolation of a *Ci-frch*, a forkhead gene in the ascidian *Ciona intestinalis*. The Ci-frch and MocuFH1 proteins show 96% conservation in their forkhead domains but no significant amino acid conservation outside this region, and are possibly encoded by different forkhead genes. The *Ci-frch* and *MocuFH1* genes also show slightly different expression patterns.