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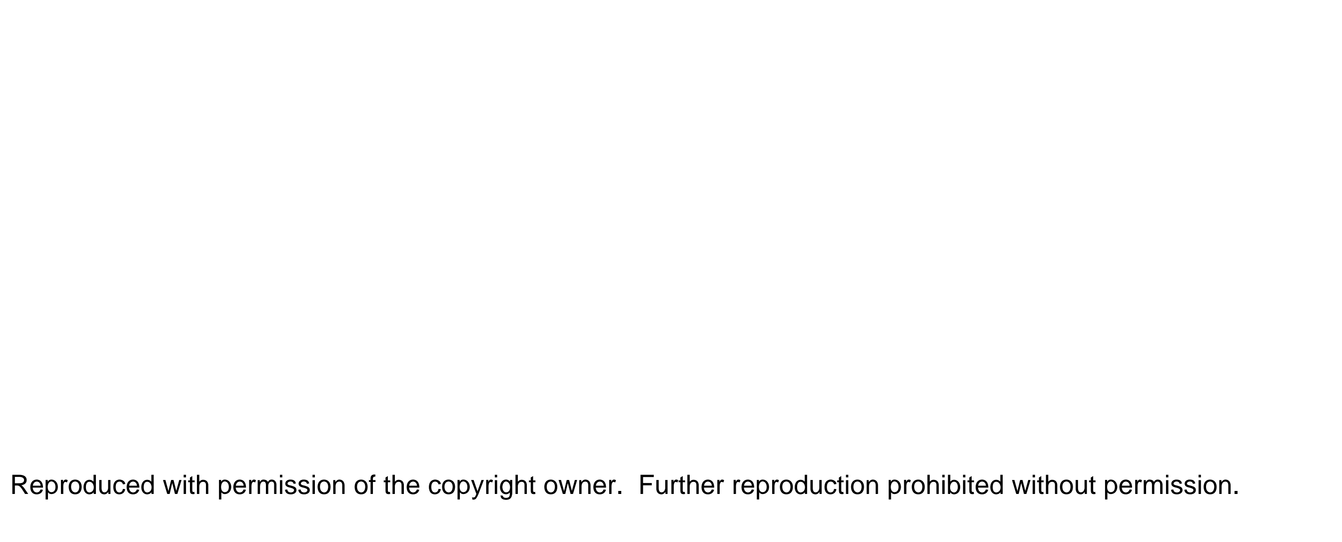

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The Role of Forkhead Genes in Ascidian Development

By

CATHERINE LEIGH OLSEN
B. S. (Duke University) 1991

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in

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in the

GRADUATE DIVISION

of the

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Approved:

Committee in Charge

1998

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Catherine Leigh Olsen
Cell and Developmental Biology
November, 1998

The role of forkhead genes in ascidian development

Abstract

Two modes of development have been observed in ascidians: tailed species produce a larva featuring a tail with notochord, spinal cord, and rows of muscle cells, as well as a pigmented otolith sensory organ in the brain, while tailless species produce a larva lacking the tail and otolith. Investigation of the closely related ascidian species *Molgula oculata* (tailed) and *Molgula occulta* (tailless), as well as hybrids produced by their cross-fertilization and exhibiting a tail and otolith, has proven useful in identifying genes whose differential expression is responsible for a switch in developmental mode during evolution. The genes *MocuFH1* (tailed) and *MoccFH1* (tailless), members of the *HNF-3/forkhead* gene family, have been identified as key components in the modification of the chordate body plan which has occurred in the tailless species.

In the tailed species, *MocuFH1* was expressed early in notochord, endoderm, and mesenchyme precursors and later in the trunk endoderm, in notochord, and in ventral cells of the brain and spinal cord. Similar *MoccFH1* expression patterns were observed in the tailless species through the neurula stage, but subsequently transcript levels decrease relative to *MocuFH1* levels in the tailed species. Disruption of the *MocuFH1* mRNA using antisense oligodeoxynucleotides (ODNs) prevents normal gastrulation and leads to axially deficient embryos lacking a tail and otolith. Expression of *FH1* in hybrids is very similar to that in the tailed species, and levels remain high after neurulation as in the tailed embryos. When hybrid embryos are treated with antisense ODNs, they fail to develop an archenteron and are unable to form a tail and otolith as they normally would, indicating that *FH1* is required for the restoration of tailed features to hybrid embryos. However,

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when tailless embryos are treated with antisense ODNs, they are able to form morphologically normal larvae featuring an archenteron, suggesting that they do not require *MoccFH1* to pattern their larvae. In summary, the data implicate the *MocuFH1* and *MoccFH1* genes in the processes of gastrulation, anterior movements of endoderm to form the archenteron, and posterior movements of notochord cells to form the tail.

Approved: ______________________________

Chairperson of the Dissertation Committee

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INTRODUCTION

A key approach to understanding how embryonic development evolves is the investigation of closely-related species with different modes of development (Swalla et al., 1993; Swalla and Jeffery, 1996). The rationale behind the study of such species is that, because they are closely related, and therefore recently diverged, they are likely to differ in the expression or presence of only a few genes. A simple switching mechanism may alter the expression of these genes, leading to a dramatically different phenotype. The identification of the genes involved can yield information on the mechanisms causing a change in developmental mode.

Two modes of development have been observed in ascidians: tailed species produce a larva featuring a tail with notochord, spinal cord, and rows of muscle cells, as well as a pigmented otolith sensory organ in the brain, while tailless species produce a larva lacking the tail and otolith (Jeffery and Swalla, 1990). The closely related ascidian species *Molgula oculata* (tailed) and *Molgula occulta* (tailless) have proven useful in the search for genes whose differential expression is responsible for the switch in developmental mode. In hybrid embryos made by fertilizing *M. occulta* eggs with *M. oculata* sperm, tailed features including a neural sensory cell and/or short tail with extended notochord cells are restored (Jeffery and Swalla, 1992). A subtractive hybridization screen revealed that only a few genes are expressed differently between the two species; these genes, known as the *uro* genes, are expressed at higher levels in the tailed species and at least one of them, *Manx*, has been shown to be responsible for the restoration of tailed features to hybrid embryos (Swalla and Jeffery, 1996).

The search for other genes involved in the switch from tailed to tailless development led to genes known to be involved in axis formation in other species, which are candidates for interacting with the ascidian *uro* genes. Foremost among these axis-forming genes are those encoding members of the forkhead family. Also known as winged-helix or *HNF-3* genes, these putative transcription factors share a conserved 110-

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amino acid DNA binding domain and are expressed embryonically in many species (Kaufmann and Knochel, 1996). In vertebrate embryos, forkhead genes are expressed in the organizer region and later in the notochord and floor plate of the neural tube, where they are responsible for dorsoventral patterning. *HNF-3β* in mouse is required for notochord and gut formation, as well as for development of a normally patterned neural tube (Weinstein et al., 1994; Ang and Rossant, 1994). The forkhead genes have been implicated in notochord and neural development. Therefore, they present themselves as strong candidates for genes that might be expressed in a tailless ascidian species, which does not form a notochord or pigmented otolith in its brain. The relative simplicity of the ascidian larva also makes it an attractive choice of model system for determining the role of forkhead genes in patterning the chordate body plan.

The overall goal of this research was to identify forkhead genes expressed embryonically in the tailed ascidian *M. oculata* and the tailless *M. occulta* and to ascertain whether differences in expression of these genes could be responsible for the change from tailed to tailless development. Our approach was as follows: (1) to clone and characterized the expression of forkhead genes in the tailed species and to determine the function of these genes using antisense oligodeoxynucleotides (ODNs); (2) to clone and characterize the expression of orthologous forkhead genes in the tailless species and hybrids, to study their function using antisense ODNs, and to compare them in the tailed species and hybrids; and (3) to use the cloned forkhead genes for the production of a polyclonal antibody to be used as a tool for discovering the presence and expression patterns of the forkhead protein in tailed, tailless, and hybrid embryos, as well as in embryos of other tailless species.

The key questions to be answered in this dissertation were (1) whether forkhead genes are required for development of tailed features, including a tail with extended notochord and a brain with pigmented otolith sensory cell; (2) whether forkhead genes are differentially expressed and/or required in the tailless species, which does not form the

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notochord or otolith; and (3) whether forkhead proteins are present in both the tailed and tailless species and in the same temporal and spatial patterns. The third question was an important one to address because the expression of mRNA alone does not guarantee that proteins are present.

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CHAPTER ONE

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 and characterized 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-3ß. *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 oligodexoynucletides (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 acethycholinesterase, 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 the *HNF-3/fork head* genes have an ancient and fundamental role in organizing the body plan in chordates.

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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 Knochel, 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 the 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 patterning of the CNS, and in the endoderm, where it is important for liver differentiation. Mouse embryos

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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; diGregorio 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.

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MATERIALS AND METHODS

Biological materials

The ascidian *Molgula oculata* was collected and maintained at Station Biologique, Roscoff, France. The procedures used to prepare gametes and 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^{0}C. Degenerate primers were designed against the amino acid sequences ITMAIQ (5'-AT(A/C/T)AC(A/C/G/T)ATGGC(A/C/G/T)CAG-3') and GNMFEN (5'-CC(A/G)TT(C/T)TC(A/G)AACAT(A/G)TT(A/C/G/T)CC-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 release 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

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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 at 4^{0}C and stored in 80% ethanol at -20^{0}C. Some embryos were dechorionated manually after fixation using tungsten needles. Whole-mount in situ hybridization was performed according to Ma et al. (1996). Digoxygenin-labelled *MocuFH1* antisense probes were synthesized using T7 RNA polymerase and DIG-labelled UTP (BMB; Boehringer Mannheim Biochemicals, Indianapolis, IN), with *Eco*RI-digested af3 as the template. *MocuFH1* sense probes were made using T3 RNA polymerase

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(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^{0}C for 20 minutes and then in ethanol at -20^{0}C for 20 minutes, incubated in 1:1 ethanol: polyester wax at 42^{0}C for one hour, and incubated in polyester wax at 42^{0}C for one 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'-CTTTCGTCGCCACCTTCT-3') correspond to nucleotides 46 to 63 of the *MocuFH1* cDNA sequence (Fig.1). The ODNs were stored lyophilized at -20^{0}C and a 30 nmole/μg 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 min after insemination) and incubated at 16-20^{0}C until hatching (10-12 hrs 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%

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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 4^0C, then transferred into 100% ethanol and stored at -20^0C in preparation for whole-mount in situ hybridization.

Enzyme assays

Embryos were fixed with 5% formalin-sea water for 30 min at 4^0C and washed in 0.1 M sodium phosphate buffer. Acetylcholinesterase (AChE) activity was assayed as described by Jeffery and Swalla (1991). Alkaline phosphatase activity was assayed as described by Bates and Jeffery (1987a).

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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 of 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 nucleotides, 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 that was 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 data bases by BLAST, the best matches were to class I 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 Figure 2. The forkhead domain of MocuFH1 is 92% identical to those of zebrafish axial and mouse HNF-3β 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.,

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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 *HNF-3β* 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 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, 8-16 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

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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 the endoderm, mesenchyme, and notochord cells during gastrulation and in these cells, as well as a restricted number of neural cells, during larval axis formation.

***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*

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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 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 if 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 embryo 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 muscle cells but not in notochord cells of ODN-2-treated embryos (Fig. 6D). In

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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 embryo, 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 a 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). Embryos that completed gastrulation also expressed *MocuCA4* in notochord cells, suggesting that loss of expression of *MocuCA4* correlates with the developmental defects resulting from a loss of *MocuFH1* function. 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

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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 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 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 embryo 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 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*

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expression is required for morphogenetic movements of the notochord and endoderm cells during gastrulation and axis formation.

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DISCUSSION

The *HNF-3/forkhead* genes are members of a family of winged-helix transcription factors expressed 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 zebrafish axial and rodent HNF-3ß 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 *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; diGregorio et al., 1995; 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 the zebrafish axial and the rodent HNF-3ß 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 *HNF-3ß* 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

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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 of 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 vertebrate CNS. During vertebrate body axis formation, *HNF-3ß* 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 Kaufmann 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.

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Although definitive transplantation experiments have not been done in ascidians, fate mapping studies have shown that 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.

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 (Weinstien 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 embryos could also be caused by the absence of a notochord.

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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 subsequent cleavages are normal and gastrulation 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 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 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 eveluated 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.

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The downstream targets of the winged-helix transcription factors have not been elucidated in great detail, although some evidence suggestes that *Brachyury* (*T*) may act downstream of *HNF-3β* 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 *MocuFH1* and *T* genes in the notochord becaue 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 post-gastrulation morphogentic 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 with 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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Table 1. Effect of *MocuFH1* ODNs on development of tadpole larvae

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

A total of 14 experiments was done. The % normal otolith and tail development is indicated relative to development in untreated controls.

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Figure 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.

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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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Figure 2. Alignment of MocuFH1 with other class I forkhead proteins. MocuFH1 is aligned with zebrafish axial (Strahle et al., 1993), mouse HNF-3β (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.

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```
MocuFH1                MM----------LS------------------------------------------------------------
Zebrafish Axial        .L----------GAVKME-------------------GHEHAADWSTYYGEPECYTSVSNMNT--------GLG
Mouse HNF-3-beta       .L----------GAVKME-------------------GLE-PSDWSSYYAEPEGYSSVSNMNA--------GLG
Xenopus Pintallavis    .L----------NRVKLE--------------------IKDPMDWNTMYQENEMYSGIHNMTN--------VL-
Xenopus XFKH1          .L----------NRVKLE--------------------LKDPMDWNTMYQENEIYSGIHNMTN--------GL-
Drosophila fkh         .QKLYAEPPPSSAPVSMASSGGGGPPSGGGGGGGGGGGGPPPPSNNNPNPTSNGGSMSPLARSAYTMNSMGLP

MocuFH1                -SPPSKYQTFQQS-FTNGMNGSVPGSYSMNP-MAIGGPSTLH-S-----------GMNGG-YGSGMLN--GMNA
Zebrafish Axial        MNSMNT.M.MSG--MSSTA.MTAANTMN.S-YVNT.MSPSMT-GMSPGTGA--MA..G------AGMT--..S.
Mouse HNF-3-beta       MNGMNT.MSMSAAAMGG.SGNMSA..MN.SSYVGA.MSPS.A-GMSPGAGA--MA..S.S-A.AAGVA--..GP
Xenopus Pintallavis    --.SNSFLPNDV.TV.TS.-----------.Y.SN.L.GPVT-.IQGNIGS--LGS.PQ.-M----VG--SLAP
Xenopus XFKH1          --.SNSFLPTDVPTV.SS.-----------TY.SN.L.GPVA-.IQGNLGS--LGS.TQ.-M----VG--SLAP
Drosophila fkh         VGGM.SVSPQAAAT.SSSVLD.AAAVA..SAS.SASMSASMNA.MNGSMGAAAMNS.G.NCMTPSSMSYAS.GS

MocuFH1                AAGMNSH--P-THHSQMSVGGSAAYPGINQGVGLSPNMALSMCINRR--TEKTYRRNYTHAKPPYSYISLITMA
Zebrafish Axial        .LSPTMS--.MAAQAPS-MNALTS.SNM.---AM..MYGQ.NINRS.--DP.....S.................
Mouse HNF-3-beta        HLSPSLS--.LGGQAAGAM..L.P.ANM.---SM..MYGQAGLSRA.--DP.....S.................
Xenopus Pintallavis    PPSTAAY--.LG-------------------------YCQGESEFQ.--DPR......S...............
Xenopus XFKH1          PPSTSAY--.LG-------------------------YCQGESEFQ.--DPR......S...............
Drosophila fkh         PL.NMGGCMAMSAA.MSAA.L.GT.GAMPPGSREMETGSPNSLGRS.VDKPT....S.................

MocuFH1                LQSSQHKMMTLSEIYQWIMDLFPFYRQNQQRWQNSIRHSLSFNDCFVKVPRSPDKPGKGSYWSLHPDAGNMFEN
Zebrafish Axial        I.Q.PS..L..................................L............F.....S......
Mouse HNF-3-beta       I.Q.PN..L..................................L............F.....S......
Xenopus Pintallavis    I.QAPN.....N......I....Y..........................E............ES......
Xenopus XFKH1          I.QAPN.....N......V....Y...................I......E............ES......
Drosophila fkh         I.NNPTR.L.......F..........................I..T........F.....S......

MocuFH1                GCYLRRQKRFKC-KKMKFSGDSTDMDNNDNSSSEEMHQQQSPSGSLSPSKEVTSPSSPHPHTSSYNDISDVMDD
Zebrafish Axial        ...........CDKKLS-KDPSR---KTSEGGSN----------------------------SSSESCNGNES
Mouse HNF-3-beta       ...........CEKQLALKEAAG---AASSGGKKTAPGSQASQAQLGEA------------AGSASETPAGTES
Xenopus Pintallavis    ...........CER--------S---KSGEGEKKVNKPGEETGGNLKEN------------PLGYDDCSSSRSP
Xenopus XFKH1          ...........CER--------S---KSGEGERKGNKPGDETGGSLKET------------PVSFDDCSSSRSP
Drosophila fkh         ...........DEKKEAIRQLHKSPSHSSLEATSPGKKDHEDSHHMHHHHH-----SRLDHHQHHKEAGGASIA

MocuFH1                KAALTQQQSSVEQNSRKELADQSSNAEASPNERMLHHQQNIYSHLHQQNADSNLPHPEQGRLSAVNNHHQNTEV
Zebrafish Axial        PHSNSSS-NELKRS-LSDMKSGQG--LSPDHAASPTS.AQ--------------HLLA.-HHSVLAHEGH----
Mouse HNF-3-beta       PHSSASPCQEHKRGGLS..KGAPAS.LSP.EPAPSPG..QQAA----------AHLLGPPHHPGLPPEAH----
Xenopus Pintallavis    Q..VNDGGRDSTGS.IHQACGS.PVGLSPTS.QAGTAS.LM.P----------.GLSND.Y.GL.GEDVH----
Xenopus XFKH1          Q..VNDGGRDSTGS.IHQATGG.PVGFSPTS.QAGTAS.LM.P----------.GLSND.Y.GL.GEDVH----
Drosophila fkh         GVNVLSAAH.KDAEALAM.HANAELCLSQQPQHVPT.HHHQHHQ.Q.EELSA-----------MMA.RCHPSLI

MocuFH1                ENIQHSNHVRTSSPVDANQHSNSITTNTRERQNYYHEPLLETKSDPLSYPSHHSFYLSQLQAAGAHQVQHYPGL
Zebrafish Axial        --LKPEH.----------------------------------------.SFN.P.SINN.MSS--E.QHH----
Mouse HNF-3-beta       --LKPEH.----------------------------------------.AFN.P.SINN.MSS--E.QHHHSHH
Xenopus Pintallavis    --LK.DP-----------------------------------------FSGR.P.SIT..MSS--E.D.T.---
Xenopus XFKH1          --LK.DP-----------------------------------------FSGR.P.SIT..MSS--E.D.T.---
Drosophila fkh         TD-------------------------------..SSMHPL.QE.SG.T-------------------------

MocuFH1                SHHGASHPLAHSFTHPFSISSLMNAGGEMQSSKEMRAYQDAMQQYSYGTTAQDVHHDNISPQQISTLENATAST
Zebrafish Axial        -----KMD.-KTYEQ-------------------VMH.--G-----..SPMAG------------..SMGSMAS
Mouse HNF-3-beta       H.QPHKMD.-KAYEQ-------------------VMH.PGG-----..SPMPG------------S.AMGPVTN
Xenopus Pintallavis    ---ANKMEMCPTTD.-------------------LVH.SN------.SSDYHN------------------MAS
Xenopus XFKH1          ---PNKMEMCPTTD.-------------------LVH.SN------.SSDYHN------------------LVS
Drosophila fkh         -----------PSS.....NR.LPT----E.KADIKM.D--.S..A---------------------------

MocuFH1                PDSGDVSTSIPSSSSNTHSPENLQQQYYQMHYNMESANPAVSTHDGLGSLADAYYQGCVQQHNSNAAIA
Zebrafish Axial        KAGL.--SP----DTS----------...GV.SRPIL.SS-----------------------------
Mouse HNF-3-beta       KAGL.--A.PLAADTS----------...GV.SRPIMNSS-----------------------------
Xenopus Pintallavis    KNGL.MQ..SS-TDNG----------..ANM.SRPILSSL-----------------------------
Xenopus XFKH1          KNGL.MQ..SS.TDNG----------..ANM.SRPILSSL-----------------------------
Drosophila fkh         --GYNALSPLTN.HAALG-----.DS...---SLGYHA..-----.TT..-------------------
```

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Figure 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 low (B) 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.

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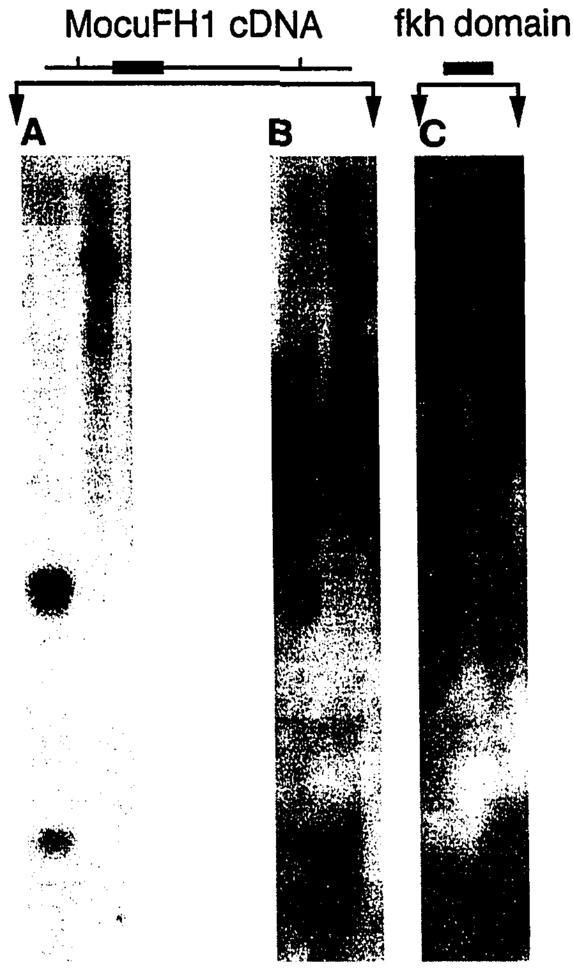

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Figure 4. Temporal and spatial accumulation of *MocuFH1* mRNA during *M. oculata* development. (A) A northern blot containing RNA from (1) gonads, (2) 8- to 16-cell embryos, (3) 32- to 64-cell embryos, (4) mid-gastrulae, (5) neurulae, (6) mid-tailbud 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 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.

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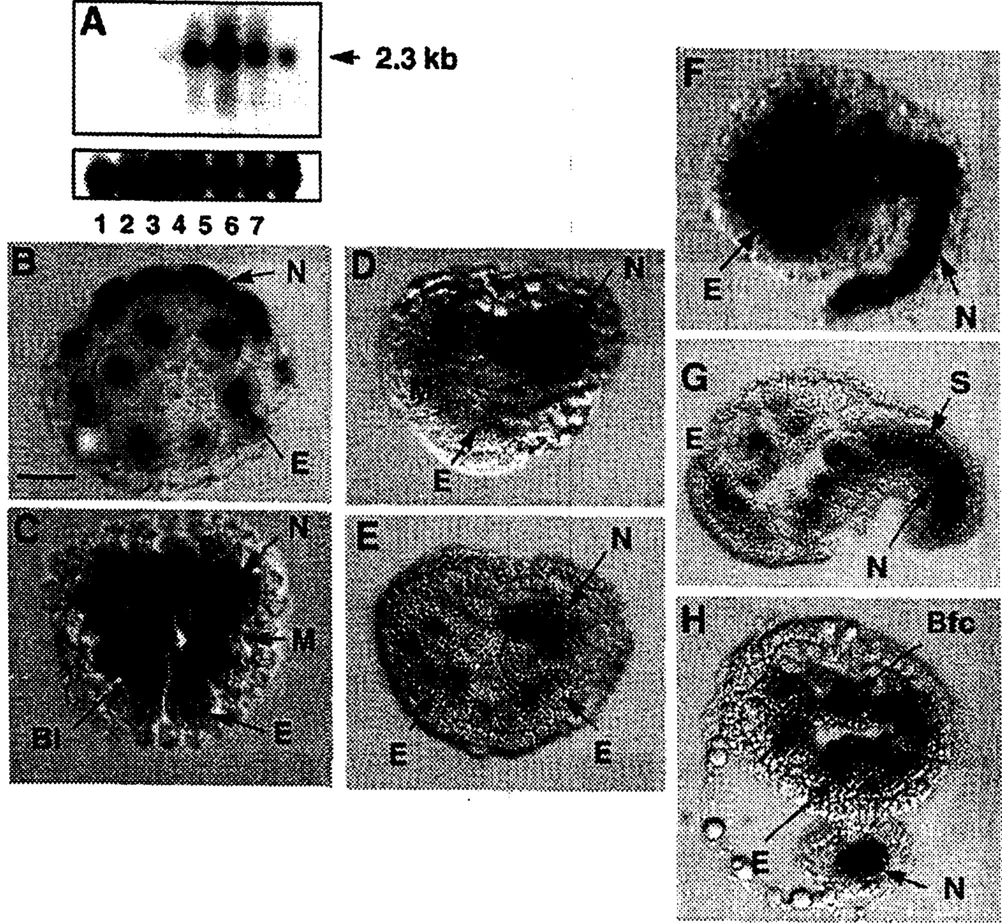

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Figure 5. The effect of antisense ODNs on *M. oculata* embryogenesis. (A,D,G,J) Untreated embryos; (B,E,H,K,M-O) embryos treated with antisense ODN-2; (C,F,I,L) embryos treated with sense ODN-2. (A-C) Mid-gastrulae viewed from a lateral side. (D-F) 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-tailbud stage (11 hour) embryos viewed from a lateral side; (J-L) sections through the head and tail of mid-tailbud stage (11 hour) 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.

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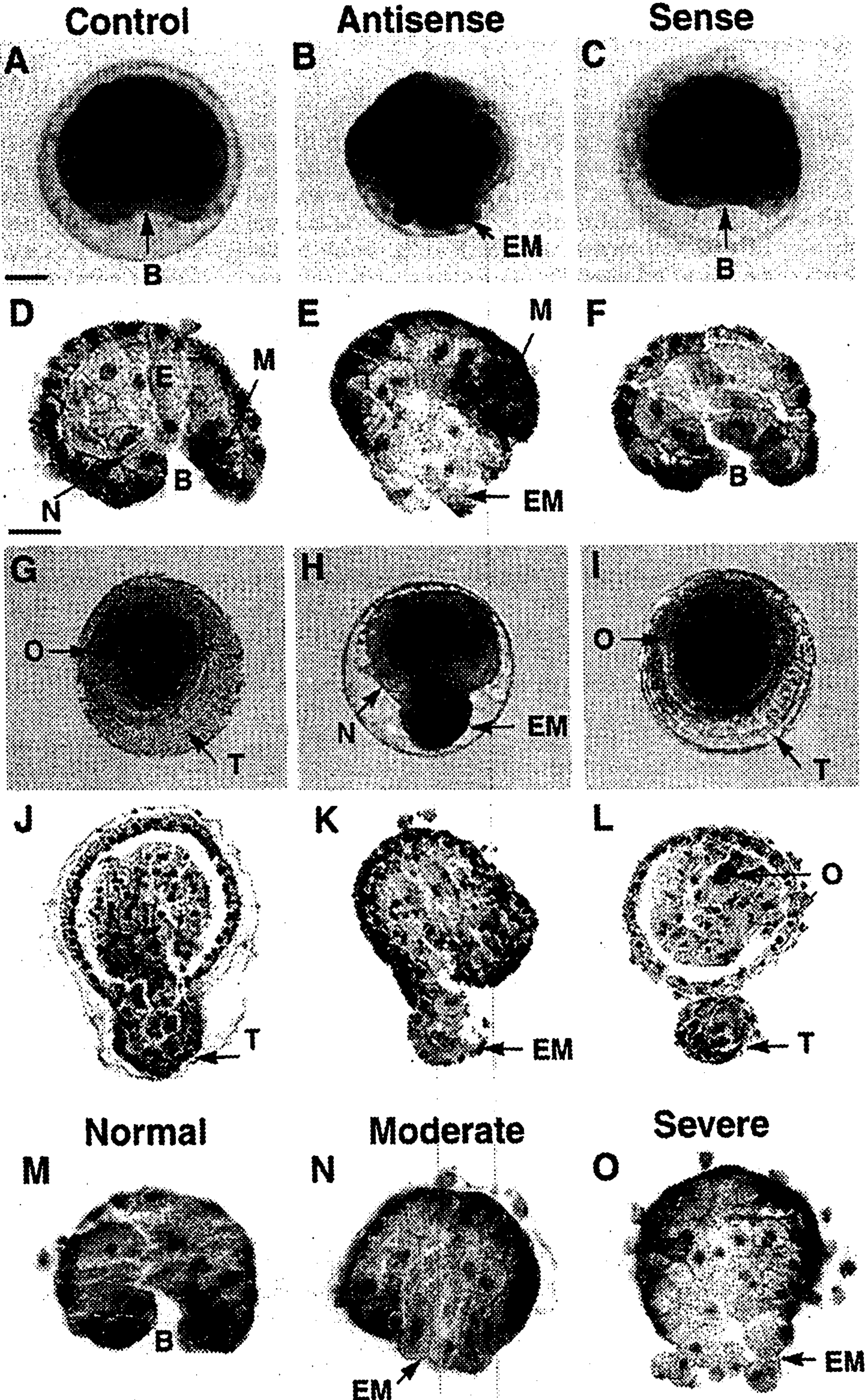

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Figure 6. Expression of muscle, notochord, endoderm markers in *M. oculata* embryos treated with antisense ODN-2. (A) A whole-mount 11 hour embryo showing AChE activity (red-brown stain) in a ring of muscle cells (MC) around the endodermal mass (EM). 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) a 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.

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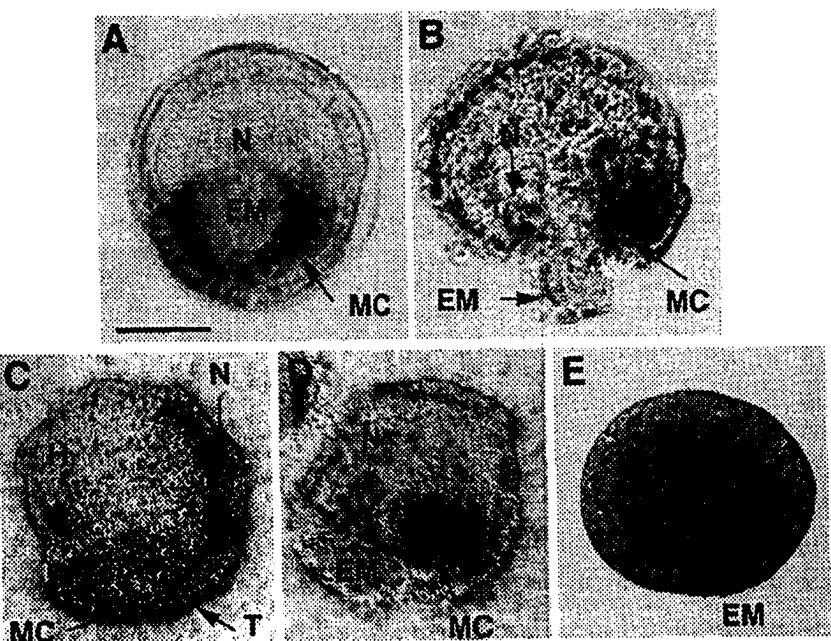

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Figure 7. Expression of *MocuFH1* mRNA in antisense ODN-2 treated and control *M. oculata* embryos. Whole mount *in situ* hybridization was done with an *MocuFH1* antisense RNA probe. (A-C) Mid-gastrulae that developed from untreated embryos (A) or embryos treated with antisense (B) or sense (C) ODNs. (D-F) Early tailbud embryos that developed from 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.

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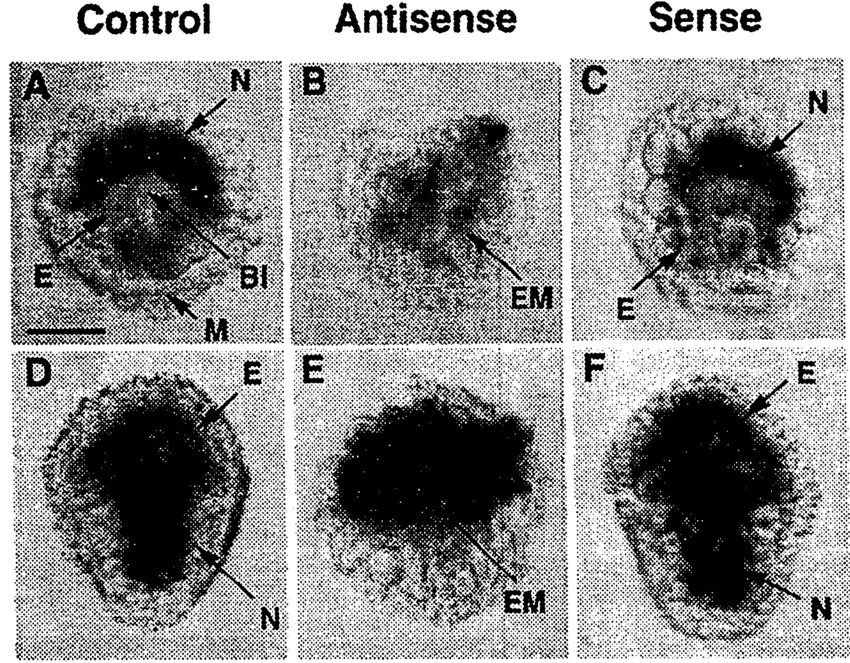

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CHAPTER TWO

Role of an *HNF-3β*-related forkhead gene in evolutionary reorganization of the chordate body plan in ascidians

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SUMMARY

To determine the role of *HNF-3*/forkhead genes in the evolution of ascidian development, we have studied the *MoccFH1* gene in *Molgula occulta* (the tailless species), a species lacking a conventional tailed larva. The *MoccFH1* cDNA sequence suggests that the encoded gene is orthologous to the *MocuFH1* forkhead gene in *Molgula oculata* (the tailed species), a species with a tailed larva, and is most closely related to the vertebrate *HNF-3β* gene. Here we compare the expression and developmental role of *MoccFH1* in the tailless species relative to the orthologous gene in the tailed species. We also investigate the function of the gene in hybrid embryos in which tailed larval features (otolith sensory organ, notochord, and tail) lost during evolution in the tailless species are restored. In hybrids, the gene will be referred to as *FH1*. Similar to *MocuFH1* in the tailed species, *MoccFH1* is expressed in presumptive notochord, mesenchyme, and endoderm cells during gastrulation and neurulation in the tailless species. However, in contrast to *MocuFH1*, *MoccFH1* is downregulated during later stages of development in the tailless species. The pattern and timing of gene expression in hybrids is the same as in the tailed species, indicating upregulation occurs in concert with restoration of tailed larval features. Antisense oligodeoxynucleotides (ODNs) were used to disrupt *MoccFH1/FH1* function in tailless species and hybrid embryos. In response to treatment with antisense ODNs, tailless species embryos showed a defect in the ability of endodermal cells to invaginate and/or involute into the archenteron; however, they were able to complete subsequent development and form tailless larvae. In contrast, most hybrid embryos treated with antisense ODNs failed to restore tailed larval features. The difference in response of hybrid and tailless species embryos to antisense ODNs shows that the forkhead gene is required for otolith, notochord, and tail formation in hybrid larvae, but not for the development of tailless larvae, which normally lack these structures. The results suggest that changes in forkhead gene expression are involved in evolutionary reorganization of the larval body plan in ascidians.

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INTRODUCTION

Our understanding of the evolution of developmental mechanisms has been revolutionized by the discovery of gene families with conserved developmental functions in diverse phyla (Stein et al., 1996; Littlewood and Evan, 1995; Jun and Desplan, 1996; Ryan and Rosenfeld, 1997). Genes such as those in the homeobox family have provided insights into the way that the same genes may be employed in embryonic patterning by different organisms (McGinnis and Krumlauf, 1992). Conversely, the study of closely related species whose developmental processes differ radically has led to the identification of novel genes that function in changing pattern-generating events during evolution (Swalla et al., 1993; Swalla and Jeffery, 1996).

The urochordates (or tunicates), inferred by phylogenetic analysis to be the most primitive extant chordates (Turbeville et al., 1994; Wada and Satoh, 1994), have been popular subjects for embryological research since the turn of the century (reviewed by Satoh, 1994). More recently, the ascidian urochordates also have proved useful in the study of the evolution of developmental mechanisms (reviewed by Jeffery, 1997). Most ascidians exhibit a tadpole larva, which has a simple body plan but is recognizable as a chordate by the presence of a notochord and a dorsal central nervous system (CNS). The mode of ascidian development culminating in the tadpole larva is known as urodele or tailed development. In contrast, a small number of ascidian species, mostly in the family Molgulidae, have modified the conventional mode of development and exhibit a tailless larva (Berrill, 1931; Jeffery and Swalla, 1990). These species, referred to anural or tailless developers, have fewer notochord precursor cells, which fail to undergo the normal morphogenetic movements leading to tail formation. In addition, the tailless larvae lack a neural sensory organ (otolith) and tail muscle cells, which are required for swimming and dispersal of the tadpole larva. According to phylogenetic analysis, urodele development is the ancestral condition, whereas anural development has arisen secondarily (Hadfield et al., 1995). Tailless species have been derived independently at

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least four times within the Molgulidae, and once or twice in the family Styelidae, suggesting that a simple switching mechanism may regulate this developmental change.

Molgula oculata, a tailed species, and *Molgula occulta*, a closely-related tailless species, have been used as a model system to identify genes whose differential expression may be responsible for the evolution of tailless development (reveiwed by Jeffery, 1997). Because the two species are closely related, differences in gene expression between them are presumably few and specific to their different developmental modes. These two species have the additional advantage of being capable of interspecific fertilization generating viable hybrid larvae (Swalla and Jeffery, 1990). When *M. occulta* (the tailless species) eggs are fertilized with *M. oculata* (the tailed species) sperm, an otolith and a short tail (reflecting the reduced number of notochord cells) are restored in the hybrid larvae. Since otolith and tail restoration are due to the expression of zygotic genes in hybrid embryos (Jeffery and Swalla, 1992), antisense oligodeoxynucleotides (ODNs) have been used to test whether candidate genes identified by subtractive procedures are required for the restoration of chordate features (Swalla and Jeffery, 1996). The *Manx* gene, which encodes a putative zinc-finger protein, is expressed in precursors of posterior embryonic tissues, including the epidermis, muscle, notochord, and neural tube in the tailed species (Swalla et al, 1993). This gene is downregulated zygotically in the tailless species, and expression is restored in the hybrid embryo. Disruption of zygotic *Manx* expression by antisense ODNs prevents restoration of the otolith and tail in hybrid larvae, demonstrating that *Manx* is required for development of these larval features (Swalla and Jeffery, 1996).

Regulatory genes known to be involved in axial patterning in other species could also play a role in modifying the ascidian tadpole larva. The *HNF-3*/forkhead genes are prime candidates for such genes due to their involvement in axis, notochord and neural tube formation in vertebrate embryos (Sasaki and Hogan, 1994; Dirksen and Jamrich, 1992). The putative transcription factors encoded by the forkhead genes share the

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winged-helix motif, a 110-amino acid DNA-binding domain, and are expressed in organizer tissues during embryogenesis (Kaufmann and Knochel, 1996). For example, *HNF-3β* is first expressed in the node in the mouse embryo and the dorsal lip of the blastopore in the *Xenopus* embryo, regions involved in initial patterning of the body axes (Tam et al., 1997; Nieuwkoop et al., 1985). Later this gene is expressed in the notochord and floor plate of the neural tube, where it functions in dorsoventral patterning of the CNS, and in the endoderm, where it may be involved in 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. They also fail to form a notochord or a gut tube, although endoderm is present (Weinstein et al., 1994; Ang and Rossant, 1994).

We identified a forkhead gene (*MocuFH1*) with a winged-helix motif similar to *HNF-3β* in the tailed species (Olsen and Jeffery, 1997). The disruption of the *MocuFH1* gene by antisense ODNs showed that it is required for gastrulation, axial patterning, and tail formation during larval development. Here we show that the *MoccFH1* gene, which is orthologous to the *MocuFH1* gene, is downregulated during later embryogenesis in the tailless species. The pattern of *MoccFH1* expression characteristic of the tailed species is restored in hybrid embryos. Treatment of tailless species embryos with antisense ODNs causes a defect in gastrulation but does not prevent the development of a tailless larva. In striking contrast, antisense ODNs prevent the restoration of the otolith and tail in hybrid embryos. Together, these data imply that *MoccFH1* is involved in the evolutionary reorganization of the chordate body plan in the ascidian larva. Because *MocuFH1* and *MoccFH1* are so closely related, the differences between the tailed and tailless species are probably due to the differences in timing of gene expression rather than the nature of the translated protein.

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MATERIALS AND METHODS

Biological materials

The ascidians *Molgula oculata* and *Molgula occulta* were collected and maintained at Station Biologique, Roscoff, France. Hybrids were made by fertilizing *M. occulta* eggs with *M. oculata* sperm. Methods of embryo culture and preparation of gametes for insemination have been described by Swalla and Jeffery (1990).

Library screening

The full-length *MocuFH1* cDNA previously described by Olsen and Jeffery (1997) was labeled with ^{32}P by random priming and used to screen an *M. occulta* 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 AF-1, contained a 2.2-kb insert, which was sequenced and found to encode a cDNA similar to *MocuFH1*. The AF-1 cDNA was named *MoccFH1* (*M. occulta* ForkHead 1).

Southern and Northern blots

Genomic DNA isolated from *M. occulta* sperm (Davis et al., 1996) was digested with *Eco* RI and *Hin*d III, electrophoresed 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 *MoccFH1* cDNA insert, which was prepared from AF-1 DNA by *Eco* RI digestion. High- and low-stringency washes were conducted as described by Swalla et al. (1993).

RNA for Northern blots was isolated from embryos at different stages as described previously (Swalla et al., 1993), electrophoresed through formaldehyde gels, and transferred to nylon membranes (MSI; Fisher Scientific, San Francisco, CA). A phagemid previously designated as af-3, containing the *MocuFH1* cDNA insert, was linearized with *Eco* RI to serve as the template for synthesis of an antisense RNA probe

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using T7 RNA polymerase (Stratagene, La Jolla, CA) and [^{32}P]UTP (800 Ci/mmol; Amersham, Arlington Heights, IL). Probes were hybridized to blots and washed at high stringency (Swalla et al., 1993).

In situ hybridizations

The whole-mount in situ hybridization method described by Olsen and Jeffery (1997) was followed using antisense and sense *MocuFH1* riboprobes synthesized from af-3 DNA.

Oligodeoxynucleotide treatment

The following 18-mer phosphorothiolate-substituted oligodeoxynucleotides (ODNs) were synthesized by Oligos, Etc., Inc. (Wilsonville, OR). As described previously (Olsen and Jeffery, 1997), antisense ODN-2 (5'-AGAAGGTGGCGACGAAAG-3') and sense ODN-2 (5'-CTTTCGTCGCCACCTTCT-3') span nucleotides 46 to 63 of the *MocuFH1* cDNA sequence. Antisense ODN-3 (5'-AGAAGGTGGCGACGATAA-3') and sense ODN-3 (5'-TTATCGTCGCCACCTTCT-3') span nucleotides 115 to 132 of the *MoccFH1* cDNA sequence, which corresponds to the same region of *MocuFH1* spanned by ODN-2. ODNs were stored lyophilized at -20°C, and a 30 nmole/ml stock solution was prepared in water just prior to use.

ODN treatment of embryos was performed as described by Swalla and Jeffery (1996) and Olsen and Jeffery (1997). Briefly, embryos (100-150/ml) were suspended in Millipore-filtered seawater containing 30 mM ODN beginning just after first cleavage (about 60 minutes post-fertilization) and incubated at 16-20°C until hatching (10-12 hours post-fertilization). The morphology of ODN-treated embryos was examined by light microscopy (Swalla and Jeffery, 1990). A subset of the ODN-treated embryos was fixed in 4% paraformaldehyde or Bouin's fixative, embedded in Paraplast, and sectioned, and the sections were stained with hematoxylin-eosin (Jeffery, 1989).

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RESULTS

Isolation and characterization of *MoccFH1*

The cDNA clone *MocuFH1* (Olsen and Jeffery, 1997) was used to screen a tailless species gastrula cDNA library. A 2.2-kb cDNA was isolated, sequenced, and found to contain an open reading frame (ORF) of 1703 nucleotides flanked by 5' and 3' untranslated regions (UTRs) of 109 and 387 nucleotides respectively (Fig. 1). The first of two consecutive ATG triplets at the beginning of the ORF is in a context most similar to the consensus eukaryotic translation initiation site (Kozak, 1991). The 3' UTR of the *MoccFH1* cDNA contains a putative polyadenylation signal beginning 17 nucleotides before the end of the cDNA, but no poly (A) tail is present. Because the size of the *MoccFH1* clone resembles the 2.3-kb transcript observed on Northern blots (see Fig. 3A), *MoccFH1* is presumed to be a full-length clone.

The *MoccFH1* and *MocuFH1* sequences are 94% similar at the nucleotide level and exhibit highly conserved 5' and 3' UTRs. There is a substitution of two nucleotides in the region of the *MoccFH1* and *MocuFH1* clones used to design antisense ODNs (Fig. 1; and see below). Comparison of deduced amino acid sequences revealed that the MoccFH1 and MocuFH1 proteins are 96% similar and exhibit 100% identity in the putative winged helix DNA-binding domain (Fig. 1). The MoccFH1 and MocuFH1 protein sequences are more similar to each other than they are to the two other HNF-3-like forkhead genes recently identified in ascidians (Corbo et al., 1997; Shimauchi et al., 1997). A BLAST search indicated that MoccFH1 is a member of the HNF-3/forkhead gene family and is most similar to *Ciona* Ci-fkh (Corbo et al., 1997), amphioxus AmHNF-3-1 (Shimeld, 1997), and the vertebrate HNF-3ßs respectively. When a Southern blot containing tailless species genomic DNA was probed with *MoccFH1* and washed at high or low stringency, only one or two bands were detected (Fig. 2). The results indicate that *MoccFH1* is a single copy gene that is orthologous to the *MoccFH1* gene and most closely related to the vertebrate *HNF-3ß* genes.

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MoccFH1 is downregulated after neurulation in the tailless species

The temporal and spatial expression of *MoccFH1* was determined by northern blots and in situ hybridization. Northern blots showed that *MoccFH1* transcripts were absent from eggs and early cleaving embryos, but began to accumulate by the gastrula stage, persisted at the same levels through the neurula stage, and then decreased in amount at later developmental stages in the tailless species (Fig. 3A). The spatial distribution of *FH1* transcripts was compared in the tailed and tailless species by in situ hybridization (Fig. 3B-M). After hybridization, some of the embryos were sectioned to examine the expression patterns in more detail (Fig. 3E, I, M). Confirming previous results (Olsen and Jeffery, 1997), tailed species embryos showed transcripts in the presumptive endoderm, notochord, and mesenchyme cells at the gastrula stage, in the same cell types at the neurula stage, and in the endoderm and notochord cells at the tailbud stages (Fig. 3B-E).

The expression pattern in the tailless species corresponded to the tailed species through the neurula stage (Fig. 3B-C, G-H). In tailless species gastrulae (Fig. 3F), *MoccFH1* mRNA was present in the presumptive endoderm cells, including the large vegetal cells that initiate invagination (Conklin, 1905), the presumptive notochord cells, which involute over the anterior lip of the blastopore, and the presumptive mesenchyme cells, which involute over the lateral lips of the blastopore. Northern blots confirmed that transcript levels are approximately the same at the gastrula stage in the two species (data not shown). In tailless species neurulae (Fig. 3G), mRNA was detected in the endoderm and notochord cells, and possibly in the mesenchyme cells, which are difficult to identify at this stage. As in the tailed species, no expression was detected in the presumptive muscle or ectodermal cells. At the later stages of development, which correspond to the period of tail formation in the tailed species, *MoccFH1* staining was downregulated in the tailless species (Fig. 3E, I). Although there was variability in staining intensity of tailless species embryos within a single experiment (Fig 3H shows

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one of the most intensely-stained embryos, and Fig. 3I a weakly stained embryo), in no case did staining match the higher levels observed in the tailed species. Decreased staining was evident in the notochord and endoderm cells; however, the endoderm cells showed the most dramatic decline in *MoccFH1* transcripts in the tailless species (Fig. 3E, I). The results suggest that *MoccFH1* is expressed normally during gastrulation and neurulation but downregulated at later developmental stages in the tailless species.

***MoccFH1* is necessary for normal gastrulation but not larval development in the tailless species**

Antisense ODNs have been used to demonstrate that the *MocuFH1* gene is required for gastrulation, axial patterning, and tail formation in the tailed species (Olsen and Jeffery, 1997), and also to show that the *Manx* gene is necessary for restoration of chordate features in hybrid larvae (Swalla and Jeffery, 1996). Since *MoccFH1* is not expressed maternally (Fig. 3A), the antisense approach was employed here to determine whether this gene is necessary for development in the tailless species. Two distinct antisense ODNs were used for the different species in these experiments because *MoccFH1* differs from the corresponding region in *MoccFH1* (Fig. 1). Antisense ODN-2 corresponds to the region beginning four nucleotides downstream of the translation start site in *MocuFH1*, and was previously shown to inhibit mRNA accumulation and development in the tailed species (Olsen and Jeffery, 1997), whereas ODN-3 corresponds to the same region in *MoccFH1*.

Embryos were treated with antisense and control ODNs beginning at first cleavage and incubated until hatching, about 12 hours post-fertilization. Similar results were obtained when embryos of both species were treated with ODN-2 or ODN-3, indicating that the two-nucleotide difference did not affect the phenotypes. Typical ODN-2 treated embryos are shown in Figure 4. Embryos of both species were able to undergo cleavage and to initiate gastrulation normally after treatment with antisense ODNs. By the mid-gastrula stage, however, morphological changes were evident in these

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embryos (Fig. 4A-F). Normally, gastrulation is initiated by invagination of large endoderm precursor cells at the vegetal pole (Conklin, 1905). An archenteron then forms, after which more endoderm precursors involute over the blastopore lips (Fig. 4A, D). The endoderm cells are followed by presumptive notochord, mesenchyme, and muscle cells, which involute over the anterior, lateral, and posterior lips of the blastopore, respectively. We found that tailed species embryos treated with antisense ODNs were unable to complete normal gastrulation (Fig. 4C) or to form axial structures, including a head, a CNS with an otolith, and a tail (see below; Olsen and Jeffery, 1997). Instead, most of these embryos showed a large mass of endodermal cells at the normal position of the blastopore, lacked an archenteron, and did not exhibit posterior movements of the presumptive notochord cells after involution (Fig. 4C, M).

Antisense ODNs also disrupted gastrulation in the tailless species, although the effects appeared less dramatic than in the tailed species and were manifest by a slight endodermal bulge on the vegetal surface (Fig. 4F). This small bulge appears to be the equivalent of the large endodermal mass observed in tailed species embryos treated with an antisense ODNs. No effects on gastrulation were observed in sense ODN-treated embryos of either species (Fig. 4B, E). Figure 4G-L shows antisense ODN-treated embryos of the tailed species at the larval stage. Compared to untreated and sense ODN-treated controls (Fig. 4G, H), larvae that developed from antisense ODN-treated tailless species embryos appeared normal (Fig. 4I). They showed a distinct archenteron surrounded by large endodermal cells in the anterior region, and a notochord placode, muscle precursor cells, and neural cells in the posterior region (Fig. 4), although the larvae did not hatch. Thus, although gastrulation was somewhat disrupted, tailless species embryos were able to complete development and form morphologically normal tailless larvae. This result is in contrast to tailed species and hybrid embryos treated with antisense ODNs, which do not develop normal posterior larval features (Olsen and

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Jeffery, 1997). The results suggest that *MoccFH1* is necessary for normal gastrulation but not for larval development in the tailless species.

***FH1* expression is restored and required for otolith and tail development in hybrid embryos**

The spatial expression of *FH1* was determined by in situ hybridization in hybrid embryos. Staining in hybrid embryos was similar to the tailed and tailless species at the gastrula and neurula stages (Fig. 3B-C, F-G, J-K). In addition, northern blots showed that transcript levels in hybrid gastrulae were comparable to those seen in the tailed and tailless species (data not shown). After neurulation, however, about 70% of the hybrid embryos expressed *FH1* in the notochord and endoderm at the high levels seen in the tailed species (Fig. 3D-E, L, M) rather than the lower levels usually observed in the tailless species (Fig. 3I). The observation that not all embryos regained a high level of expression correlates with the fact that chordate characteristics are not restored uniformly in hybrids (Swalla and Jeffery, 1990; Jeffery and Swalla, 1992). Thus, hybrid embryos can restore the prolonged expression of the *FH1* message observed during the later stages of larval development in the tailed species, but not in the tailless species.

In order to determine whether the development of the otolith and tail requires *FH1*, hybrid and tailed species embryos were treated with antisense and sense ODNs. The results were similar for antisense ODN-2 and ODN-3 (Table 1). Both antisense ODNs reduced the number of embryos with otoliths and tail to low levels relative to untreated controls, and sense ODNs showed lesser phenotypic effects. The phenotypes of typical antisense ODN-treated and control hybrids are shown in Figure 4. Antisense ODN-treated hybrids were abnormal (Fig. 4L). At gastrulation, they showed a larger endodermal mass than tailless species embryos (data not shown, but see Fig. 4N) and at later stages of development they lacked a distinct archenteron, had disorganized endoderm cells, which protruded from the embryo (Fig. 4N), and failed to form otoliths and tails (Fig. 4N). In contrast, sense ODN-treated hybrids were normal, possessing a

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short tail and/or an otolith, as well as a distinct archenteron (Fig. 4J, K). Figure 4M-O compares the phenotypes of antisense ODN-treated tailed species, tailless species, and hybrid embryos observed after the untreated controls had hatched. The tailed species and hybrid embryos exhibit highly abnormal phenotypes, showing protruding endodermal masses instead of tails. In contrast, embryos of tailless species at the same stage of development were normal (see Swalla and Jeffery, 1990). The results suggest that the enhanced or prolonged expression of the *FH1* gene is necessary for restoration of chordate features in hybrid embryos.

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DISCUSSION

Members of the forkhead gene family are involved in axial patterning in vertebrates (Kaufmann and Knochel, 1996). *HNF-3β*, a developmentally expressed forkhead gene, has been implicated in early gastrulation, formation of the notochord, neural tube, and gut (Sasaki and Hogan, 1994; Weinstein et al., 1994; Ang et al., 1994), as well as in cell differentiation and metabolism (Duncan et al., 1998). Analysis of the expression and effects of disrupting *MocuFH1*, a forkhead gene related to *HNF-3β* in the tailed ascidian *Molgula oculata*, has revealed an ancient and conserved role for these genes in the processes of gastrulation, notochord formation, and CNS development in a primitive chordate (Olsen and Jeffery, 1997). Here we demonstrate that the forkhead gene *MoccFH1* has a role in the evolutionary regression and restoration of the chordate body plan in the tailless ascidian *Molgula occulta*. Our results provide the first evidence that forkhead genes are involved in axial reorganization during chordate evolution.

We have isolated and sequenced a cDNA clone encoding the *MocuFH1* gene in the tailless species. The *MoccFH1* clone encodes a single copy gene that is 93% and 96% similar to *MocuFH1* at the nucleotide and deduced amino acid levels respectively, exhibits highly conserved 5' and 3' UTRs, and has a winged-helix DNA binding domain identical to *MocuFH1*. Thus, the *MoccFH1* gene in the tailless species is the orthologue of the *MocuFH1* gene in the tailed species. Among the vertebrate forkhead genes, *MoccFH1* and *MocuFH1* are most similar to the *HNF-3β* genes and have conserved expression patterns along the axial midline.

The transcript levels and spatial expression patterns of *MoccFH1* in the tailless species are similar to that of the tailed species throughout the early embryonic stages, when development appears nearly identical to the tailed species (Swalla and Jeffery, 1990). Downregulation of the *MoccFH1* gene after neurulation in the tailless species, however, suggests that it may not be required for later embryogenesis, when morphogenetic movements appear to cease. The additional observation that *MoccFH1*

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expression was restored in hybrid embryos implies that this gene may be involved in the development of tailed larval features (otolith and tail), possibly by promoting the differentiation and posterior morphogenetic movements of notochord cells.

Treatment of tailed species embryos with antisense ODNs previously demonstrated that *MocuFH1* is required for gastrulation and axis formation during embryogenesis of the tailed species (Olsen and Jeffery, 1997). Significantly, the phenotype of the antisense-ODN treated embryos, which failed to undergo the morphogenetic movements necessary for tail formation, was reminiscent of normal development in tailless species embryos, which do not undergo these cell rearrangements (reviewed by Jeffery, 1997). When treated with antisense ODNs, embryos of the tailless species gastrulate somewhat abnormally but appear to recover in later embryonic stages, and eventually develop into normal tailless larvae. Perhaps this is because the cell movements following gastrulation in the tailless species are limited, compared to the more posterior extensive movements required to form a tadpole larva (reviewed by Jeffery, 1997). The results are consistent with the possibility that *MoccFH1* is required only during the early stages of embryogenesis in the tailless species, when it is necessary for normal gastrulation, but not at later stages, when embryos lack the extensive notochord cell movements seen in the tailed species and must compensate for the defect in ability of endodermal cells to completely internalize to continue development.

Evidence that *MoccFH1* is required for the development of the otolith and tail is provided by experiments in which hybrid embryos were treated with antisense ODNs. Hybrid embryos produced by fertilization of tailless species eggs with tailed species sperm develop an otolith, notochord, and tail, based on the expression of zygotic genes (Swalla and Jeffery, 1990; Jeffery and Swalla, 1992). The failure of antisense ODN-treated hybrids to develop the tail and otolith at the frequency of control embryos in our studies supports the hypothesis that the *FH1* gene is necessary for the restoration of these chordate features. Hybrid embryos contain the same number of notochord precursor cells

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as tailless species embryos, yet these cells are able to rearrange themselves to form an extended tail, albeit a short one compared to that of tailed species embryos, which possess about four times as many notochord cells (reviewed by Jeffery, 1997). Formation of the otolith in hybrids suggests that this neural feature may develop as a result of enhanced differentiation of presumptive notochord, endoderm, or spinal cord cells, which are thought to induce the CNS in ascidians (Reverberi et al., 1960; Nishida, 1991). In addition, the phenotypes of antisense ODN-treated hybrids suggest that there are defects in the organization of endodermal cells and the formation of the archenteron. However, these defects are apparently not extensive enough to affect subsequent posterior development of the tailless embryo. Taken together, these data implicate the *MoccFH1* and *MocuFH1* genes in three processes involving cellular rearrangements in ascidian embryos: (1) gastrulation, (2) anterior movements of endoderm cells to form the archenteron, and (3) posterior movements of notochord cells to form the larval tail. In addition to these processes, the development of inductive potential in the prospective notochord cells is likely to require the function of these genes.

These results provide suggestions as to the nature of changes in gene expression which correlate with a switch in the mode of larval development. It is conceivable that the reduction or truncation of *MoccFH1* gene expression during later development in the tailless species is one change that, along with others such as a difference in *Manx* gene expression (Swalla and Jeffery, 1996), led directly to anural development. Indeed, preliminary experiments (Olsen and Jeffery, unpublished) suggest that *Manx* functions upstream of *MocuFH1* in a gene cascade leading to tail development. Alternatively, changes in *MoccFH1* gene expression may be a consequence rather than the cause of anural development; perhaps selection for the conventional expression pattern was relaxed in the absence of tail development, and was subsequently lost. In this case, mutations in genes that function upstream of or in parallel to *MoccFH1* may be responsible for triggering anural development. Investigation of forkhead gene expression

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in other, independently-derived tailless species (Hadfield et al., 1995) may shed more light on the role of these genes in the evolution of anural development.

Future studies on forkhead genes in ascidians will focus on identifying their interactions with other genes implicated in notochord development and axis formation, such as *T* (Yasuo and Satoh, 1994) and *Manx* (Swalla et al., 1993). Antisense ODN inhibition of *Manx* (Swalla and Jeffery, 1996) results in the same phenotype as inhibition of *MoccFH1* in hybrid embryos, suggesting that these genes may function in the same pathway leading to tail restoration. Weinstein et al. (1994) proposed that *HNF-3ß* is located upstream of *T* in a hierarchy of transcription factors involved in notochord development in the mouse. The ascidian *T* gene is also expressed in the notochord (Yasuo and Satoh, 1994; Corbo et al., 1997). Therefore, antisense ODN experiments may be insightful in understanding genetic interactions between *FH1*, *T*, and *Manx*. Further investigations of the genetic pathways underlying the switch from urodele to anural development in ascidians will provide insights into the ways that chordates have evolved different modes of development. The pursuit of genes expressed differentially between embryos developing via one developmental mode versus the other, along with antisense ODN studies, should help reveal the pathways by which the body plan is produced and may have originally appeared in the chordate ancestor.

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Table 1. The effect of ODNs on development of hybrid and tailed species embryos.

	Hybrid		Tailed Species	
	otolith (% control)	tail (% control)	otolith (% control)	tail (% control)
ODN-2				
Sense	53	62	96	98
Antisense	12	37	2	0
ODN-3				
Sense	78	78	86	90
Antisense	18	8	26	25

Pooled results of one representative experiment for each ODN are shown. The percent otolith and tail development are indicated relative to development in untreated controls. Each ODN treatment involved from 100 to 200 embryos.

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Figure 1. The nucleotide and deduced amino acid sequences of *MoccFH1*, compared to *MocuFH1*. Amino acids which differ between the two cDNA clones are listed side by side with the *MoccFH1* amino acid listed first (e.g., S/G). The putative forkhead DNA-binding domain is shaded, and the sequence used to design sense and antisense ODNs has a line above it. The GenBank accession number for *MoccFH1* is AF082992.

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```
MoccFH1     1  --CACGAGGAAATTTCAGCCGCTTCATTTTTTTGAGTTCTGTTAATAATAAAATCTCTACC  59
MocuFH1     1  GG........................C......C.A.......G.............A..T  61

MoccFH1    60  GCTGCTGCAGTGCAATAAACTACCGTCGAATATTTGTTTGTCCGC-TCACTATGATGTTA  118
MocuFH1    62  ...............C....A.T.........C..........A.A...........C.T  121
            1                                                    M  M  L    3

MoccFH1   120  TCGTCGCCACCTTCTAAATATCAGACATTTCAACAATCATTTACCAACGGAATGAACGGT  178
MocuFH1   122  .................G..........................................  181
            4   S  S  P  P  S  K  Y  Q  T  F  Q  Q  S  F  T  N  G  M  N  G   23

MoccFH1   179  TCTGTGCCAGGATCCTACTCAATGAACCCGATGGCAATCGGAGGACCATCAACTCTTCAC  238
MocuFH1   182  ..............T.....G.....T........G........................  241
           24   S  V  P  G  S  Y  S  M  N  P  M  A  I  G  G  P  S  T  L  H   43

MoccFH1   239  TCCGGCATGAACGGAGGTTATGGAAGCAGCATGTTAAACGGAATGAATGCTGCCGCTGGT  298
MocuFH1   242  ..............G..A..C......G.T..........................C..A  301
           44   S  G  M  N  G  G  Y  G  S S/G M  L  N  G  M  N  A  A  A  G   63

MoccFH1   299  ATGAACTCGCATCCAACACATCATTCTCAAATGTCAGTCGGAGGTTCACCTGCTTACCCT  358
MocuFH1   302  ...........C........C...........................G.C.........  361
           64   M  N  S  H  P  T  H  H  S  Q  M  S  V  G  G  S P/A A  Y  P   83

MoccFH1   359  GGCATAAATCAAGGTGTTGGTCTCAGTCCAAATATGGCATTATCAATGTGTATCAACCGT  418
MocuFH1   362  .....................................................T......  421
           84   G  I  N  Q  G  V  G  L  S  P  N  M  A  L  S  M  C  I  N  R  103

MoccFH1   419  CGCACAGAGAAGACATACCGCAGAAATTACACGCATGCCAAACCCCCATATAGCTACATC  478
MocuFH1   422  .................T.....G........C.....A.....A.....C.........  481
          104   R  T  E  K  T  Y  R  R  N  Y  T  H  A  K  P  P  Y  S  Y  I  123

MoccFH1   479  TCACTGATTACCATGGCCTTACAATCATCTCAGCACAAGATGATGACTCTTAGTGAAATT  538
MocuFH1   482  ...T....C...........G.....C..A..A..T...........A............  541
          124   S  L  I  T  M  A  L  Q  S  S  Q  H  K  M  M  T  L  S  E  I  143

MoccFH1   539  TATCAATGGATTATGGACTTGTTCCCATTCTACAGACAAAACCAACAAAGATGGCAAAAC  598
MocuFH1   542  .......................T.................T.....G............  601
          144   Y  Q  W  I  M  D  L  F  P  F  Y  R  Q  N  Q  Q  R  W  Q  N  163

MoccFH1   599  TCAATCCGTCATAGTTTGTCGTTCAATGACTGCTTTGTTAAAGTTCCGAGATCTCCAGAT  658
MocuFH1   602  ............................................................  661
          164   S  I  R  H  S  L  S  F  N  D  C  F  V  K  V  P  R  S  P  D  183

MoccFH1   659  AAGCCAGGGAAAGGATCTTATTGGTCACTACACCCAGATGCCGGAAACATGTTTGAGAAC  718
MocuFH1   662  .............................G.......................C.....T  721
          184   K  P  G  K  G  S  Y  W  S  L  H  P  D  A  G  N  M  F  E  N  203

MoccFH1   719  GGTTGTTATCTTCGACGTCAAAAGCGATTTAAGTGTAAAAAGATGAAATTTTCTGGTGAT  778
MocuFH1   722  .....C..C.....TA.A...................................C......  781
          204   G  C  Y  L  R  R  Q  K  R  F  K  C  K  K  M  K  F  S  G  D  223

MoccFH1   779  TCTGGTGACACGGACAACAACGACAATTCTTCAAGCGAAGAAATGCACCAACAACAATCG  838
MocuFH1   782  ...AC.....T...........................G....................A  841
          224   S G/T D T/M D  N  N  D  N  S  S  S  E  E  M  H  Q  Q  Q  S  243

MoccFH1   839  CCATCTGGTTCTTTATCGCCCTCCAAAGAAGTCACTTCTCCATCCAGTCCACACCCTCAC  898
MocuFH1   842  .................A..........................................  901
          244   P  S  G  S  L  S  P  S  K  E  V  T  S  P  S  S  P  H  P  H  263

MoccFH1   899  ACCGCATCGTACAATGACATATCTGACGTGATGGACGACAAGGCTGCTGTGACTCAACAA  958
MocuFH1   902  ...T............................................C...........  961
          264   T A/S S  Y  N  D  I  S  D  V  M  D  D  K  A  A V/L T  Q  Q  283

MoccFH1   959  CAAAGCTCAGTCGAGCAAAACTCCCATAAAGAATTGACAGATCAAAGTTCAACCGCTGAT  1018
MocuFH1   962  .....T...................G..........................A......A  1021
          284   Q  S  S  V  E  Q  N  S H/R K  E  L T/A D  Q  S  S T/N A D/E 303

MoccFH1  1019  GCTTCACCCAATGAAAGGATGCTGCACCATCAGCAGAATATCTACTCACATTTGCATCAA  1078
MocuFH1  1022  .....G....................T.................................  1081
          304   A  S  P  N  E  R  M  L  H  H  Q  Q  N  I  Y  S  H  L  H  Q  323

MoccFH1  1079  CAAACTGCTGACAGCAACCTCCCTCATCAAGAACAAGGCAGATTATCTGCTGTTAATAAT  1138
MocuFH1  1082  ....A...............T.......C...G.....A.....................  1141
          324   Q T/N A  D  S  N  L  P  H Q/P E  Q  G  R  L  S  A  V  N  N  343

MoccFH1  1139  CATCATCAAAACACAGAAGTGGAAAACATCCAACACAGCAATCATGTCCGAACATCTTCA  1198
MocuFH1  1142  ..............T...........T........T...........T............  1201
          344   H  H  Q  N  T  E  V  E  N  I  Q  H  S  N  H  V  R  T  S  S  363

MoccFH1  1199  CCTGTCGATGCAAACCAACATTCAAACAGCATCACAACAAGCACAAGAGAGAGACAGAAT  1258
MocuFH1  1202  ........................................A...................  1261
          364   P  V  D  A  N  Q  H  S  N  S  I  T  T S/N T  R  E  R  Q  N  383

MoccFH1  1259  TACTACCATGAACCTTTGCTGGAAAGCAAAAGTGATCCGCTCTCATATCCGTCGCATCAT  1318
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MocuFH1  1262  ..T..T............T......C............T..G........A..C......  1321
          384   Y  Y  H  E  P  L  L  E S/T K  S  D  P  L  S  Y  P  S  H  H   403

MoccFH1  1319  TCATTTTACCTTTCGCAGTTGCAAGCTGCAGGAGCTCATCAAGTTCAACATTATCCAGGA  1378
MocuFH1  1322  ..............C....................A....................T...  1381
          404   S  F  Y  L  S  Q  L  Q  A  A  G  A  H  Q  V  Q  H  Y  P  G   423

MoccFH1  1379  CTTTCTCATCATGCTGGAGCAACTCATCCTCTGGCTCACTCCTTCAGCCATCCCTTCTCC  1438
MocuFH1  1382  .....A..C..---.......T.............A..........C.............  1438
          424   L  S  H  H A/- G  A T/S H  P  L  A  H  S  F S/T H  P  F  S   442

MoccFH1  1439  ATCTCAAGTCTGATGAATGCTGGTGGTGAGATGCAGAGTTCAAAGGAGATGAGAGCTTAC  1498
MocuFH1  1439  ..T.....CT..........C..............A.....G...........G..A..T  1498
          443   I  S  S  L  M  N  A  G  G  E  M  Q  S  S  K  E  M  R  A  Y   462

MoccFH1  1499  TAGGATGCCATGCAACAGTATAGTTATGGCACAACAACACAAGAAGTACATCATGACAAC  1558
MocuFH1  1499  C...................C........A......G.......T..G.....C......  1558
          463   Q  D  A  M  Q  Q  Y  S  Y  G  T  T T/A Q E/D V  H  H  D  N   482

MoccFH1  1559  ATCTCACCGCAGCAAATATCAACACTGGAAAATGCAACCGCTTCAACTCCTGATTCCGGC  1618
MocuFH1  1559  ........A..A............T................A...........C..T..T  1618
          483   I  S  P  Q  Q  I  S  T  L  E  N  A  T  A  S  T  P  D  S  G   502

MoccFH1  1619  GACGTTTCAACATCAATACCATCGTCCAGTTCCAATGCACACTCACCAGAAAATCTCCAG  1678
MocuFH1  1619  ..........................G........CA.......C...........A..A  1678
          503   D  V  S  T  S  I  P  S  S  S  S  N A/T H  S  P  E  N  L  Q   522

MoccFH1  1679  CAACAATATTATCAAATGCATTACAACATGGAATCGTCAAATCCGGCAGTTTCAACTCAT  1738
MocuFH1  1679  ...................C..............AG.......T..............C  1738
          523   Q  Q  Y  Y  Q  M  H  Y  N  M  E  S S/A N  P  A  V  S  T  H   542

MoccFH1  1739  GATGGCTTGGGAAGTCTTGCTGATGCATATTATCAAGGGTGCGTACAGCAGCATAATTCT  1798
MocuFH1  1739  .....T......................................................  1798
          543   D  G  L  G  S  L  A  D  A  Y  Y  Q  G  C  V  Q  Q  H  N  S   562

MoccFH1  1799  AATGCAGCGATTGCATAAAATATTAATATCAACCAAATTAATAAATGTTTTGTGTGGGTT  1858
MocuFH1  1799  ...............................A............................  1858
          563   N  A  A  I  A  *                                             567

MoccFH1  1859  AGCAATTCTTATTTTGCTTGCGCCATTATGT-----GTGATGGCTTAACGTTCCTTTCCG  1913
MocuFH1  1859  ..........CC.................C.TATGG...G...............----.  1914

MoccFH1  1914  ATAACATGGTATGCACTGAAATGATCAAACCTCATAGTATTTATTTCGGGGC-TGAAGCC  1972
MocuFH1  1915  .....................................................A......  1974

MoccFH1  1973  AGATTGAAATGCGGGTATCTCTGAACATGAACATTCCATGACCATATATCGAACAATGAA  2032
MocuFH1  1975  ...C.................A.------............T-..A..............  2027

MoccFH1  2033  CTGATATTATGGAGTGTCATTTATGACCAGTAGTGTTAGGAACAGTATAAATTATGTCGT  2092
MocuFH1  2028  ..--.GA...............CCG...................................  2085

MoccFH1  2093  AATATCATGTATGTGTATATAAATGCAATAAATTTCGTACTAATTGCAATTCTTACTGTT  2152
MocuFH1  2086  ............................................................  2145

MoccFH1  2153  GTTTAACTGGCATGATTACTGCTTGCAAACTTAATAAATCCTCG--TGCCG---------  2201
MocuFH1  2146  .........................................AT.AA..TT.AAAAAAAAA  2205

MoccFH1        -----------
MocuFH1  2206  AAAAAAAAAAA                                                   2216
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Figure 2. Southern blot hybridization of tailless species genomic DNA using the *MoccFH1* probe. (A, B) The same blot hybridized with the full-length *MoccFH1* probe and washed at high (A) or low (B) stringency. Lane 1: *Hind* III digest. Lane 2: *Eco* RI digest.

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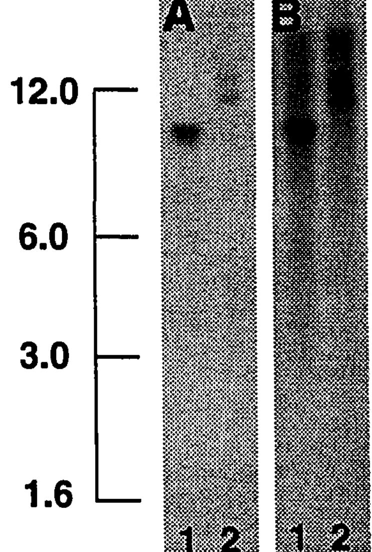

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Figure 3. Temporal and spatial accumulation of *Mocc/MocuFH1* mRNA during embryonic development of the tailed species, tailless species, and hybrids. (A) A northern blot containing RNA from (1) unfertilized eggs, (2) fertilized eggs, and (3) gastrulae (4 hours), (4) neurulae (5 hours), 6 hour embryos, (6) 8 hour embryos, 10 hour embryos of the tailless species. Top: *MocuFH1* probe. The 2.3-kb *MoccFH1* transcript is indicated by the arrow. Bottom: 18S rRNA loading control. (B-M) Embryos subjected to in situ hybridization with the *MocuFH1* antisense RNA probe. (B-E) Tailed species. (F-I) Tailless species. (J-M) Hybrids. (B-D, F-H, J-L) Whole mounts. (E,I,M) Sections of whole mounts. (B) A tailed species gastrula showing staining in presumptive notochord (N) cells at the anterior lip of the blastopore, mesenchyme (Me) cells at the lateral lips of the blastopore, and endoderm (E) cells inside the blastopore. (C-E) Tailed species neurula (C) and early tailbud stages (D-E; 6 hours) showing staining in the notochord (N) and endoderm (E) cells. (F) A tailless species gastrula showing staining in notochord (N), mesenchyme (Me), and endoderm (E) cells. (G-I) Tailless species neurula (G) and 6 hour embryos (H-I) showing staining in notochord (N) and endoderm (E) cells. (J-M) Hybrid gastrula (J), neurula (K), and 6 hour embryos (L,M) showing staining in notochord (N) and endoderm (E) cells. (E,I,M) Sections of 6 hour tailed species (E), tailless species (I), and hybrid (M) embryos, showing downregulation of *MoccFH1* transcripts in the tailless species and restoration in hybrids. Embryos are shown with their anterior poles at the top (B-C, F-G, J-K) or to the left (D-E, H-I, L-M). Scale bar, 20 mm; magnification is the same in B-M.

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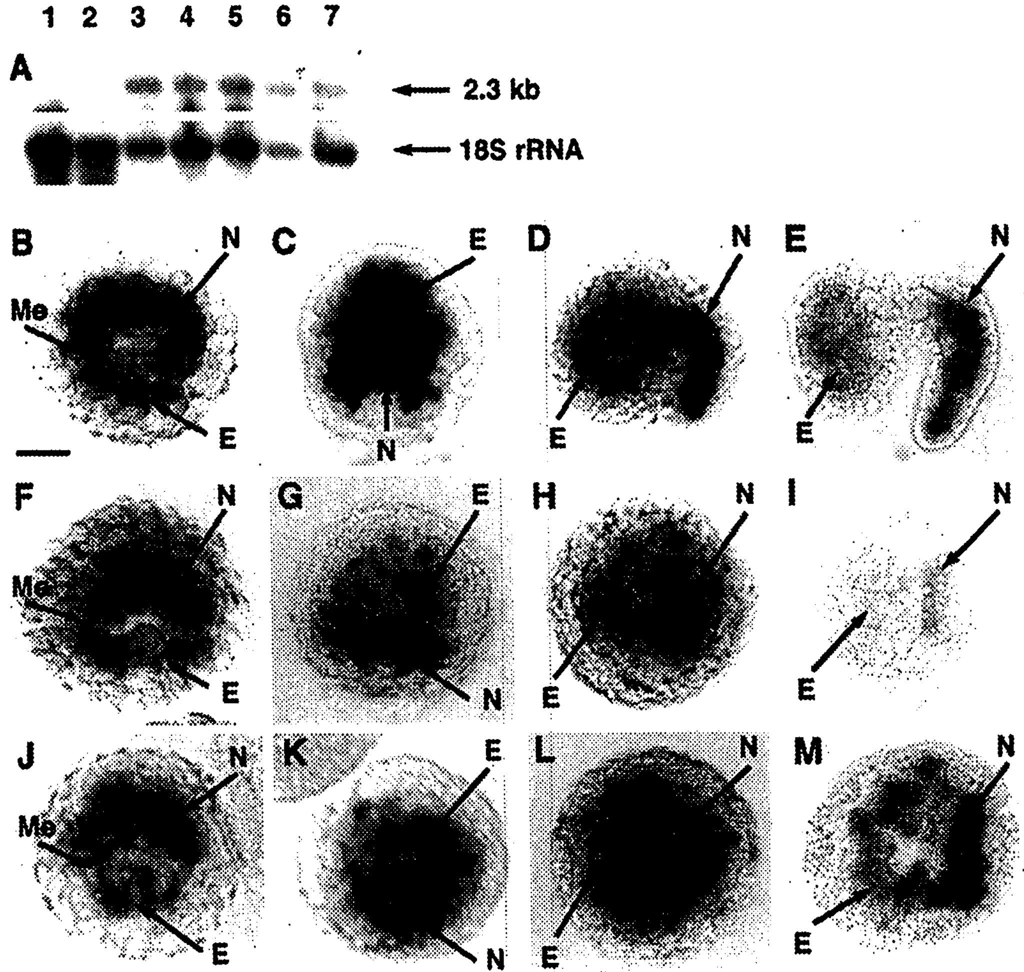

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Figure 4. The effect of antisense ODNs on development in tailed species, tailless species, and hybrid embryos. (A,D,G,J) Untreated control embryos. (B,E,H,K) Sense ODN-2 treated embryos. (C,F,I,L-O) Antisense ODN-2 treated embryos. (A-F) Sagittal sections of tailed (A-C) and tailless (D-F) species mid-gastrulae viewed laterally. Untreated and sense ODN-treated gastrulae in A,B,D,E show 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). (C,F) Antisense ODN-treated tailed (C) and tailless (F) species gastrulae showing the endodermal mass (EM) or bulge respectively at the vegetal pole. (G-L) Transverse sections of 11 hr tailless species (G-I) and hybrid (J-L) embryos. (G,H,J,K) Untreated (G,J) and sense ODN-treated (H,K) tailless species (G,H) and hybrid (J,K) embryos showing normal morphology, including an archenteron (Ar) surrounded by endoderm cells (E). Hybrid embryos also exhibit an otolith (O). (I,L) Antisense ODN-treated tailless species (I) and hybrid (L) embryos. Tailless species embryos exhibit normal morphology but hybrid embryos are missing the otolith and exhibit somewhat disorganized endoderm cells (E) and sometimes lack an archenteron. (M-O) Living antisense ODN-treated tailed species (M), tailless species (N), and hybrid (O) embryos. The tailed species and hybrid embryos exhibit a large endodermal mass (arrowheads), whereas the tailless species embryo shows normal morphology. Scale bar, 20 mm; magnification is the same in each frame.

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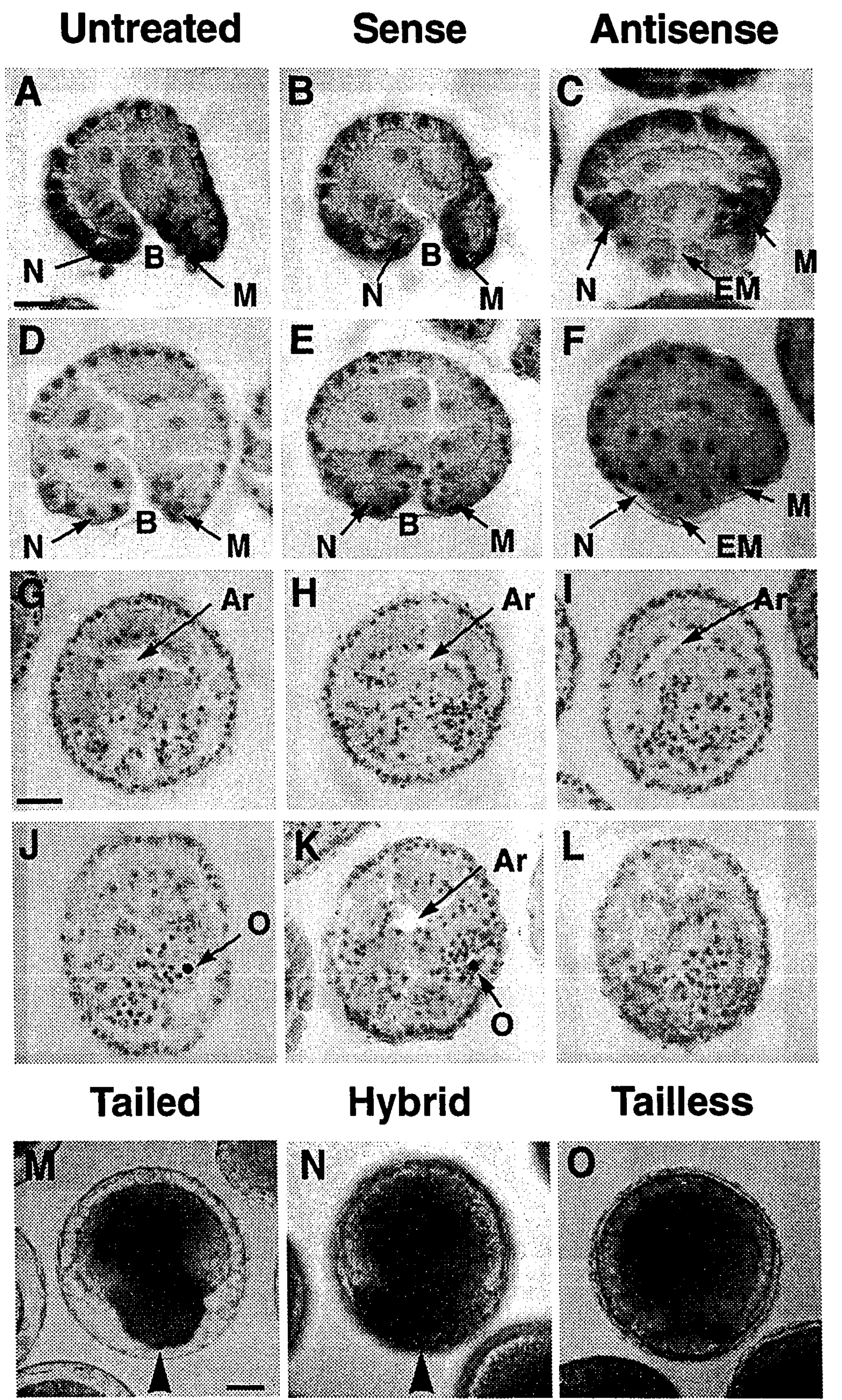

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CHAPTER THREE

A polyclonal antibody against the C-terminal portion of MocuFH1 recognizes a 57-kD protein in ascidian embryos

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INTRODUCTION

The investigation of different modes of development in closely related ascidian species has led to the discovery of several genes whose differential expression is involved in a switch in developmental mode. The *Manx* gene is expressed zygotically in embryos of *Molgula oculata* (the tailed species), a species whose larva has a tail, notochord, and otolith sensory organ, but not in embryos of a closely related species, *Molgula occulta* (the tailless species), whose larvae are tailless and lack a notochord and otolith (Swalla et al., 1993). *Manx* expression in hybrids made by crossing eggs of the tailless species with sperm of the tailed species correlates with the presence of a tail and otolith in these embryos, while treatment of these embryos with antisense oligodeoxynucleotides (ODNs) directed against *Manx* mRNA abolishes the development of these tailed larval features (Swalla and Jeffery, 1996). The forkhead gene *MocuFH1* is required for tail, notochord, and otolith formation in the tailed species (Olsen and Jeffery, 1997), but expression of its orthologue, *MoccFH1*, is truncated during later development, correlating with the lack of tail development in the tailless species. The two are referred to herein as *FH1* since their sequences are so similar. The pattern and amount of forkhead gene expression, as determined by in situ hybridization, is similar in the two species through the neurula stage, after which transcript levels decrease in the tailless species.

Although the presence and level of *FH1* transcripts have been examined in both the tailed and tailless species, it is unknown whether the protein product is translated and stable. It is critical to determine whether differences in protein levels may be responsible for the differential requirement for the gene in larval development of these two species. To this end, we have expressed a portion of the MocuFH1 protein in bacteria and have used this protein for the production of a polyclonal antibody. The antibody, designated FH-40, recognizes a 57-kilodalton (kD) protein in homogenates of tailed, tailless, and hybrid embryos.

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MATERIALS AND METHODS

Expression vector preparation

A part of the *MocuFH1* coding region lacking the forkhead DNA-binding domain was selected for preparation of a fusion protein to be used as an antigen in the production of a polyclonal antibody (Berkeley Antibody Company, Richmond, CA). Because the *MocuFH1* DNA-binding domain is highly conserved relative to its counterpart in mammalian *HNF-3β*, and to avoid cross reactivity of the antibody with other ascidian forkhead proteins, a region of the *MocuFH1* cDNA sequence outside of the forkhead domain was chosen (Figure 1). A 1020-nucleotide *Xho* I/*Xmn* I restriction fragment encoding the C-terminal 204 amino acids of the predicted MocuFH1 protein (designated fh-1020) was chosen according to several criteria. First, it does not include the forkhead DNA-binding domain. Second, examination of the predicted amino acid sequence of this fragment using the MacVector program (IBI-A Kodak Co., New Haven, CT) showed that its antigenicity and hydrophilicity were in a range acceptable for its use as an antigen. Third, the predicted size of 204 amino acids, or 40 kD, was large enough so that conjugation to a larger carrier protein was unnecessary.

The expression vector pRSET (Invitrogen, San Diego, CA) was used for preparation of an MocuFH1 fusion protein (Figure 2). This vector allows expression of a gene as an N-terminal peptide fusion, with the fusion peptide containing six tandem histidine residues. The fh-1020 insert was cloned into pRSET(C), the version of pRSET with the correct reading frame for the insert, using *Eco* RI linkers. The construct was sequenced to insure that the insert was present in the proper orientation and reading frame for translation.

Expression and purification of the fh-1020 fusion protein

BL21 bacteria (Novagen, Madison, WI) were transformed with the pRSET(C)/fh-1020 construct and incubated with IPTG (Fisher Scientific, Pittsburgh, PA) to induce expression of the fh-1020 fusion protein. Cell lysate prepared under denaturing

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conditions according to Qiagen protocols (Qiagen Inc., Valencia, CA) and subjected to the French pressure cell press (American Instrument Company, Silver Spring, MD) was applied to a column containing Ni-NTA Superflow resin (Qiagen Inc.) to purify the fusion protein. Column eluates were electrophoresed through 12% polyacrylamide gels, and the proteins were visualized by staining with Coomassie blue (Sigma Chemical Company, St. Louis, MO). Proteins on some gels were transferred electrophoretically to nitrocellulose paper (MSI; Fisher Scientific, San Francisco, CA) and probed with the T7.Tag antibody (Novagen, Madison, WI) to determine the presence of the fusion protein. The Pierce BCA protein assay was used to measure the amount of protein purified (Pierce, Rockford, IL). A total of 3 mg of protein, enough for antibody production in two rabbits, was obtained from a one-liter bacterial culture.

Polyclonal antibody production

Antibody production was performed by Berkeley Antibody Company (Richmond, CA) according to their standard protocol. Briefly, preimmune serum from four New Zealand white rabbits was collected and used to probe Western blots containing the fh-1020 protein. The two rabbits with the least reactive preimmune sera were chosen for antibody production. These two rabbits were initially injected with 500 mg of fh-1020 antigen each, followed 21 days later by a boost injection of 250 mg each. Ten days after the boost injection, serum samples from both rabbits were collected and assayed for antibody titer using indirect ELISA. Boost injections with fh-1020 antigen were repeated every 21 days and antibody titer assayed until the desired titer was obtained. At this time, antiserum was collected from the rabbit with the higher antibody titer and purified over a Protein A column. The resulting polyclonal antibody preparation was designated FH-40.

SDS-PAGE and Western blots

Homogenates of *M. oculata*, *M. occulta*, and hybrid embryos 7 hours post-fertilization were prepared in sodium dodecyl sulfate (SDS)-containing sample buffer (25% glycerol; .156 M Tris, pH 6.8; 5% SDS; 12.5% β-mercaptoethanol), and the

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proteins were separated by electrophoresis in 12% polyacrylamide gels. The fh-1020 protein was used as a control. Proteins were then transferred to nitrocellulose paper (MSI; Fisher Scientific), which was blocked using PBS containing 0.1% Tween 20 and 3% BSA (PBT/BSA). The blots were then cut into strips and incubated with a 1:1000 dilution of preimmune serum from the rabbit in which the FH-40 antibody was produced or a 1:100 dilution of the purified FH-40 antibody, each in PBT. Blots were rinsed three times with PBT and then incubated in a 1:5000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody (Promega, Madison, WI) in PBT. Blots were then rinsed three times in PBT and developed using H_2O_2, with diaminobenzidine (DAB) as a chromogenic substrate.

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RESULTS

FH-40 antibody recognizes a protein in ascidian embryos

At 7 hours post-fertilization (F+7), tailed species embryos are at the mid-tailbud stage, with notochord cells extended single file and muscle cells in rows on either side of the notochord. Most of the morphogenetic movements which produce the larva have been completed, while development of the central nervous system, indicated by the appearance of the otolith, is still in progress. Tailless species embryos at F+7 have apparently completed all morphogenetic movements necessary for larval development; there is no extension of the notochord precursor cells, and while the muscle precursor cells produce acetylcholinesterase, they do not extend into rows or twitch. In the tailed species and hybrids, Northern blots and in situ hybridization have shown that *MocuFH1* transcripts are present at high levels through the mid-tailbud stage and then begin to decrease in amount during later tailbud stages. In the tailless species, the decrease in transcript levels of *MoccFH1* is more dramatic, with levels much lower by F+6 than in the tailed species. Because the deduced MocuFH1 and MoccFH1 proteins are nearly identical, the difference in timing of expression, rather than the function of the proteins themselves, is likely to be responsible the tailless phenotype. The truncation of *MoccFH1* expression in the tailless species may be related to tail regression during evolution (Olsen and Jeffery, unpublished results).

Western blots containing embryonic extracts, and the fh-1020 fusion protein as a control, were probed with FH-40 antibody. The antibody recognized a band of about 35 kD in the lane containing the fh-1020 fusion protein (data not shown). This is consistent with the 40-kD weight predicted for the fusion protein. In lanes containing embryonic extracts, FH-40 recognized a band of 57 kD, as well as bands of 27 and 20 kD (Figure 3). The 57-kD band is consistent with a predicted molecular weight of 62 kD for the full-length FH1 protein, while the 27- and 20-kD bands may be degradation products of FH1.

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Although transcript levels in the tailless species decrease sooner than in tailed and hybrid embryos, immunoblots containing embryo homogenates and probed with FH-40 antibody reveal that the FH1 protein product is present in tailed, tailless, and hybrid embryos at F+7 . The amount of protein loaded in each lane of the blot is equal, but it is difficult to draw conclusions about the amount of FH1 protein present in different batches of embryos since the rate of fertilization and the number of normally developing embryos is variable among the three samples of developing embryos. However, it can be concluded that the antibody recognizes a protein that is likely to correspond to FH1.

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DISCUSSION

The expression of numerous forkhead genes has been analyzed with northern blots and in situ hybridization in various model systems, but in few instances has the presence and localization of the protein products been examined. Generally, it is assumed that the protein product is present in temporal levels and spatial patterns corresponding to those of the mRNA. In the case of forkhead genes expressed in tailed and tailless ascidian embryos, where disruption of the mRNA in the tailed species produces a severe axial patterning defect (Olsen and Jeffery, 1997) and disruption of mRNA in the tailless species has little effect on patterning (Olsen and Jeffery, unpublished results), it is very important to ascertain whether the protein products of the forkhead genes are present in both species. In producing the polyclonal antibody FH-40, raised against a portion of the MocuFH1 protein product, we have created a valuable tool to answer the question of whether *MocuFH1* and *MoccFH1* mRNA is actually translated, stable, and correctly localized in both species.

Western blots have revealed that a 57-kD protein recognized by the FH-40 antibody is present in tailed, tailless, and hybrid embryos at a stage equivalent to the mid-tailbud stage in the tailed species. This suggests that *MocuFH1* and *MoccFH1* are not only transcribed in both species and in hybrids, but that their transcripts are translated. These results provide evidence that the MoccFH1 gene product may have some role in embryonic development of the tailless species, even though its disruption produces less dramatic defects than in the tailed species and hybrids. Development may be compromised, as demonstrated by the failure of forkhead antisense ODN-treated tailless embryos to hatch at the same levels as controls (Olsen and Jeffery, unpublished results), if the MoccFH1 protein is not present at appropriate levels, is not properly localized, or even fails to undergo possible post-translational modification.

Western blots using embryo homogenates of different developmental stages (not available to us at the time this research was performed) should be done in the future to

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determine whether FH1 protein is present at different timepoints in tailed, tailless, and hybrid embryos. Future experiments employing immunocytochemistry of whole mount and/or sectioned embryos should address the question of where the FH1 protein is expressed in embryos.

Another potentially informative use for the FH-40 antibody is to examine expression of forkhead proteins in other tailless species whose embryo, unlike those of *M. occulta*, lack a placode of notochord precursor cells. The potential alteration of forkhead protein expression in later embryonic stages of such species may indicate that MoccFH1 is important in tailless development. These experiments depend on the ability of the FH-40 antibody to cross-react with the forkhead proteins of other ascidian species. The amino acid sequence of the 40-kD MocuFH1 fragment used as an antigen is 30% identical to the corresponding sequence in *HrHNF3-1*, a recently identified forkhead gene of the pyurid ascidian *Halocynthia roretzi* (Shimauchi et al., 1997), and 29% identical to the corresponding sequence in the more distantly related ascidian *Ciona intestinalis* (Corbo et al., 1997), a member of a different order (order Phlebobranchia) than Halocynthia and Molgula (order Stolidobranchia). Although this degree of identity is much lower than the 96% identity between the same region of the MocuFH1 and MoccFH1 amino acid sequences, it is still possible that FH-40 may recognize proteins in other species, especially among the Molgulids, in which most tailless species are found. It is also conceivable that the *Ciona* and *Halocynthia* forkhead genes identified may not be the orthologues of the *MocuFH1* and *MoccFH1* genes and that the true orthologues are present though as yet unidentified. Western blot analysis must be done using embryo homogenates from other species in order to determine whether FH-40 can serve as a useful tool for the exploration of axial patterning in other ascidian species exhibiting altered developmental modes.

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Figure 1. *MocuFH1* nucleotide and amino acid sequences. The underlined region is the fh-1020 fragment which was cloned into pRSET(C) and expressed as a fusion protein.

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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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Figure 2. Diagram of the pRSET expression vector (Invitrogen, San Diego, CA). The fh-1020 fragment of *MocuFH1* was cloned into the *Eco* RI site of the multiple cloning site (MCS). A six-histidine fusion peptide is located at the N-terminus of the encoded fusion protein [$(His)_6$].

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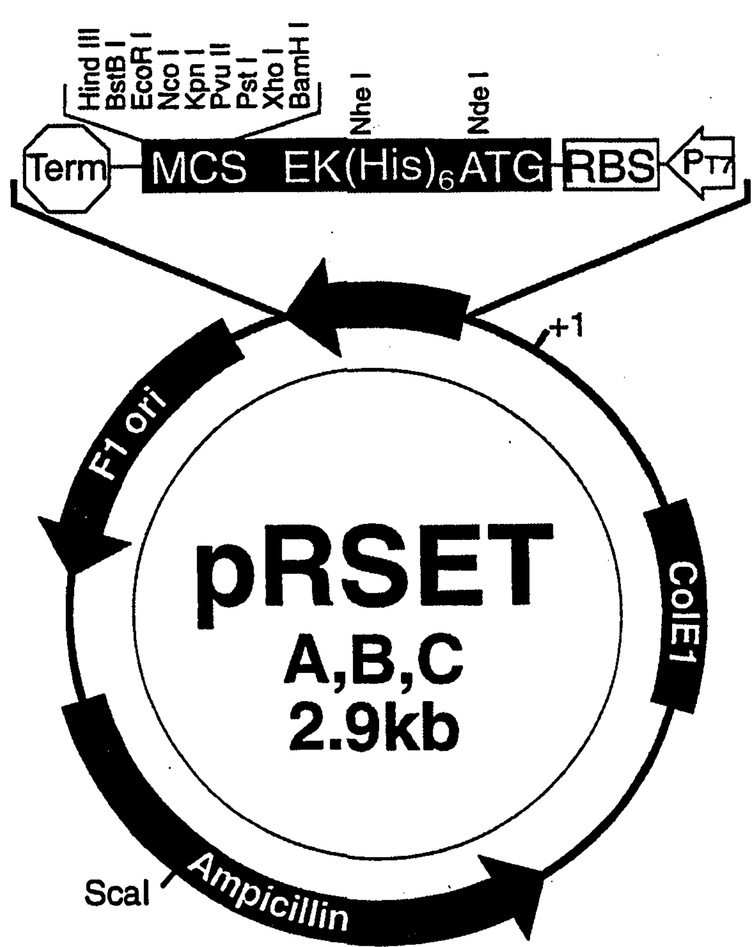

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Figure 3. Western blot containing homogenates of *M. oculata* (tailed; A,B), *M. occulta* (tailless; C,D), and hybrid (E,F) embryos and probed with preimmune serum (A,C,E) or purified FH-40 antibody (B,D,F). The FH-40 antibody recognizes a 57-kD band consistent with the predicted 62-kD size of the MocuFH1 protein. The 27- and 20-kD bands also recognized by FH-40 may be degradation products of MocuFH1.

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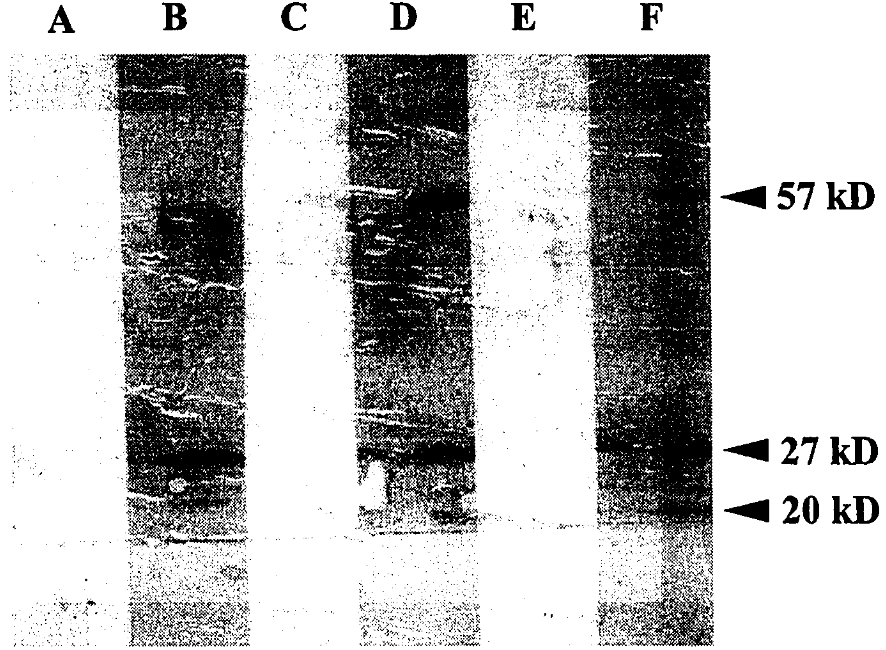

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CONCLUSIONS

The closely related ascidian species *Molgula oculata* (tailed) and *Molgula occulta* (tailless) have been proven useful tools for embryological studies, perhaps most importantly in the quest to identify the genes whose differential expression is responsible for a switch in developmental mode during evolution. The forkhead genes *MocuFH1* (tailed species) and *MoccFH1* (tailless species) have been identified as key players in the modification of the chordate body plan that has occurred in the tailless species. Presented here are the overall conclusions which can be drawn from the research presented above.

The *MocuFH1* gene isolated in this study was found to be most closely related to the *HNF-3β* gene in vertebrates. In the tailed species, *MocuFH1* expression was found in the vegetal cells of the 44/64-cell embryo, including those cells which initiate gastrulation, in the notochord, endoderm, and mesenchyme precursors which involute over the lips of the blastopore early in gastrulation, and later in the trunk endoderm, in notochord, and in ventral cells of the brain and spinal cord. Disruption of the *MocuFH1* mRNA using antisense oligodeoxynucleotides (ODNs) prevents gastrulation from occurring normally and leads to axially deficient embryos which lack a tail and otolith. From these results it can be concluded that *MocuFH1* is required for gastrulation and axis formation in tailed species embryos. Thus, the function of the *HNF-3β* gene in axis organization appears to be conserved in ascidian and vertebrate chordates.

In the tailless species, *MoccFH1*, the ortholog of *MocuFH1*, is expressed in patterns similar to those of *MocuFH1*-- in endoderm, notochord precursors, and mesenchyme-- through the neurula stage. Subsequently the transcript levels decrease relative to *MocuFH1* levels in the tailed species. Expression of *FH1* in hybrids is very similar to that in the tailed species, and levels remain high after neurulation as in the tailed embryos. When hybrid embryos are treated with antisense ODNs, they are unable to form a tail and otolith as they normally would, indicating that *FH1* is required for the restoration of the tailed features to hybrid embryos. However, when tailless embryos are

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treated with antisense ODNs, gastrulation appears somewhat abnormal but they are able to form apparently normal larvae with a distinct archenteron and posterior features, including the vestigial placode of notochord cells. These results suggest that *FH1* is necessary for the development of the tail, along with differentiated notochord, and otolith in hybrid embryos, but it is unnecessary for larval development in the tailless species.

A polyclonal antibody produced against a fusion protein representing the C-terminal 204 amino acids of MocuFH1 recognized a 57-kilodalton (kD) protein in tailed, tailless, and hybrid embryos 7 hours after fertilization, a late embryonic stage equivalent to a mid-tailbud embryo in the tailed species. It is concluded from this observation that FH1 protein is present in all three types of embryos, albeit at different concentrations, even though FH1 is not required for tailless larvae to develop. What cannot be concluded from the antibody results is whether the protein is functional in all three embryos. Whether it is functional or not, though, it is apparently not necessary for development to proceed in the tailless species.

The investigation of forkhead genes in ascidians has demonstrated a conserved role for these genes in patterning the chordate body plan. The tailed ascidian species *M. oculata* has been shown to use *MocuFH1* for the same processes-- gastrulation, axial patterning, notochord formation, central nervous system development-- as vertebrate embryos use other forkhead genes, such as *HNF-3β*. On the other hand, the tailless *M. occulta* possesses the ortholog *MoccFH1* but does not seem to require it for the patterning of its larva. The presence of MoccFH1 protein in embryos, along with the fact that the antisense ODN-treated tailless species embryos do not gastrulate normally and do not hatch at the same levels as controls, suggests that MoccFH1 may still have a function in these embryos. However, this function is arguably much less important to tailless development than it is to the ancestral tailed mode of development. Further studies of the expression of FH1 protein in tailed, tailless, and hybrid embryos should provide more insight into the role of this protein in a switch in developmental modes. An important

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goal of research on forkhead genes is the identification of other genes which interact with forkhead. Future experiments using antisense ODNs to disrupt the transcripts of other axial patterning genes, such as *Manx* (Swalla and Jeffery, 1996) and *T* (Yasuo and Satoh, 1994), will help to identify these interacting genes and elucidate the pathways by which the chordate body plan originally arose.

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