Int. J. Dev. Biol. 42: 1085-1092 (1998)

Original Article

Cytoskeletal actin genes function downstream of HNF-3β in ascidian notochord development

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ABSTRACT We have examined the expression and regulation of cytoskeletal actin genes in ascidians with tailed *(Molgula oculata)* and tailless larvae *(Molgula occulta)*. Four cDNA clones were isolated representing two pairs of orthologous cytoskeletal actin genes *(CA1* and *CA2)*, which encode proteins differing by five amino acids in the tailed and tailless species. The *CA1* and *CA2* genes are present in one or two copies, although several related genes may also be present in both species. Maternal *CA1* and *CA2* mRNA is present in small oocytes but transcript levels later decline, suggesting a role in early oogenesis. In the tailed species, embryonic *CA1* and *CA2* mRNAs first appear in the presumptive mesenchyme and muscle cells during gastrulation, subsequently accumulate in the presumptive notochord cells, and can be detected in these tissues through the tadpole stage. *CA1* mRNAs accumulate initially in the same tissues in the tailless species but subsequently disappear, in concert with the arrest of notochord and tail development. In contrast, *CA2* mRNAs were not detected in embryos of the tailless species. Fertilization of eggs of the tailless species with sperm of the tailed species, which restores the notochord and the tail, also results in the upregulation of *CA1* and *CA2* gene expression in hybrid embryos. Antisense oligodeoxynucleotide experiments suggest that *CA1* and *CA2* expression in the notochord, but not in the muscle cells, is dependent on prior expression of Mocc FHI, an ascidian *HNF-3β*-like gene. The expression of the *CA1* and *CA2* genes in the notochord in the tailed species, downregulation in the tailless species, upregulation in interspecific hybrids, and dependence on *HNF-3β* activity is consistent with a role of these genes in development of the ascidian notochord.

KEY WORDS: *cytoskeletal actin, notochord development, forkhead genes, regressive evolution, ascidian tadpole larva*

Introduction

The origin of chordates has been the subject of speculation and debate for more than a century (Berrill, 1955; Jefferies, 1986). The chordates consist of the urochordates (or tunicates), the cephalochordates (e.g., amphioxus), and the vertebrates, which are unified in exhibiting a notochord, a dorsal hollow nerve cord, and pharyngeal gill slits. Molecular phylogenetic analysis suggests that the urochordates are the most ancient group of extant chordates, splitting from a common ancestor before the divergence of the cephalochordates and vertebrates (Turbeville *et al.*, 1994; Wada and Satoh, 1994). The ascidian urochordates have received the most attention in developmental biology because of their determinate mode of development and simple larval body plan (Satoh, 1994; Jeffery and Swalla, 1997). The ascidian tadpole larva has all the hallmarks of a chordate yet consists of only 2500 cells and six different tissues, and is considered a prototype for the ancestral chordate (Satoh and Jeffery, 1995). Therefore, studies of ascidian tadpole development may shed light on the origin of the chordate body plan.

We have developed a system consisting of two closely-related ascidian species with different modes of development to study the chordate body plan (Swalla, 1996; Jeffery, 1997). *Molgula oculata* (the tailed species) exhibits a swimming tadpole larva containing a notochord, a dorsal neural sensory organ (otolith), and striated tail muscle cells. In contrast, *Molgula occulta* (the tailless species) has

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0214-6282/98/$10.00

a derived immotile larva lacking these chordate features. Gastrulation and neurulation occur normally in the tailless species, but the notochord, otolith and muscle cells fail to differentiate (Swalla and Jeffery, 1990). The arrest in notochord development appears to play a central role in the loss of chordate features in the tailless species (Swalla, 1993; Jeffery and Swalla, 1997). Most pertinent to studies on the evolutionary origin of chordate features is that the notochord, otolith, and tail are restored in hybrids produced by fertilizing eggs of the tailless species with sperm of the tailed species (Swalla and Jeffery, 1990; Jeffery and Swalla, 1992). The restoration of these chordate features in hybrids suggests that their disappearance during the evolution of tailless larvae was caused in part by recessive mutations in genes regulating notochord development.

The genes involved in the loss of chordate features in tailless ascidians are intriguing in light of the possibility that they may also have been used to generate these structures during the evolution of the ascidian larval body plan. Candidates for some of these genes have been identified. The *Cymric (uro-1), lynx (uro-2)* and *Manx (uro-11)* genes, which were isolated from the tailed species using a subtractive screen, encode putative signal transducing molecules or transcription factors (Swalla *et al.*, 1993; Swalla, 1996). Direct evidence that *Manx* is required for tail restoration in hybrid embryos has been provided by antisense experiments (Swalla and Jeffery, 1996). The RNA helicase gene *bobcat*, identified by its close linkage to *Manx*, also appears to be involved in notochord and tail restoration (Swalla, Just, Pederson and Jeffery, unpublished). Finally, a putative ascidian ortholog of the winged-helix *(forkhead)* transcription factor *HNF3β* has been shown to be required for notochord and tail development in the tailed species (Olsen and Jeffery, 1997). Although some of the regulatory genes involved in notochord and tail development (Swalla *et al.*, 1993; Yasuo and Satoh, 1994; Corbo *et al.*, 1997; Shimauchi *et al.*, 1997; Erives *et al.*, 1998) have been characterized, the downstream structural genes involved in these morphogenetic processes remain to be determined.

Here we describe two pairs of orthologous cytoskeletal actin genes that are expressed in notochord, mesenchyme, and muscle cells of the tailed species. Downregulation of the *CA1* and *CA2* genes during embryogenesis in the tailless species, upregulation in hybrids, and dependence on the *HNF-3β*-like gene are consistent with a role for these genes in the normal development and evolutionary modification of the ascidian notochord.

Results

Isolation of cytoskeletal actin cDNA clones

Cytoskeletal actin cDNA clones were isolated from the tailed and tailless species. The *MoccCA1* clone was isolated from a

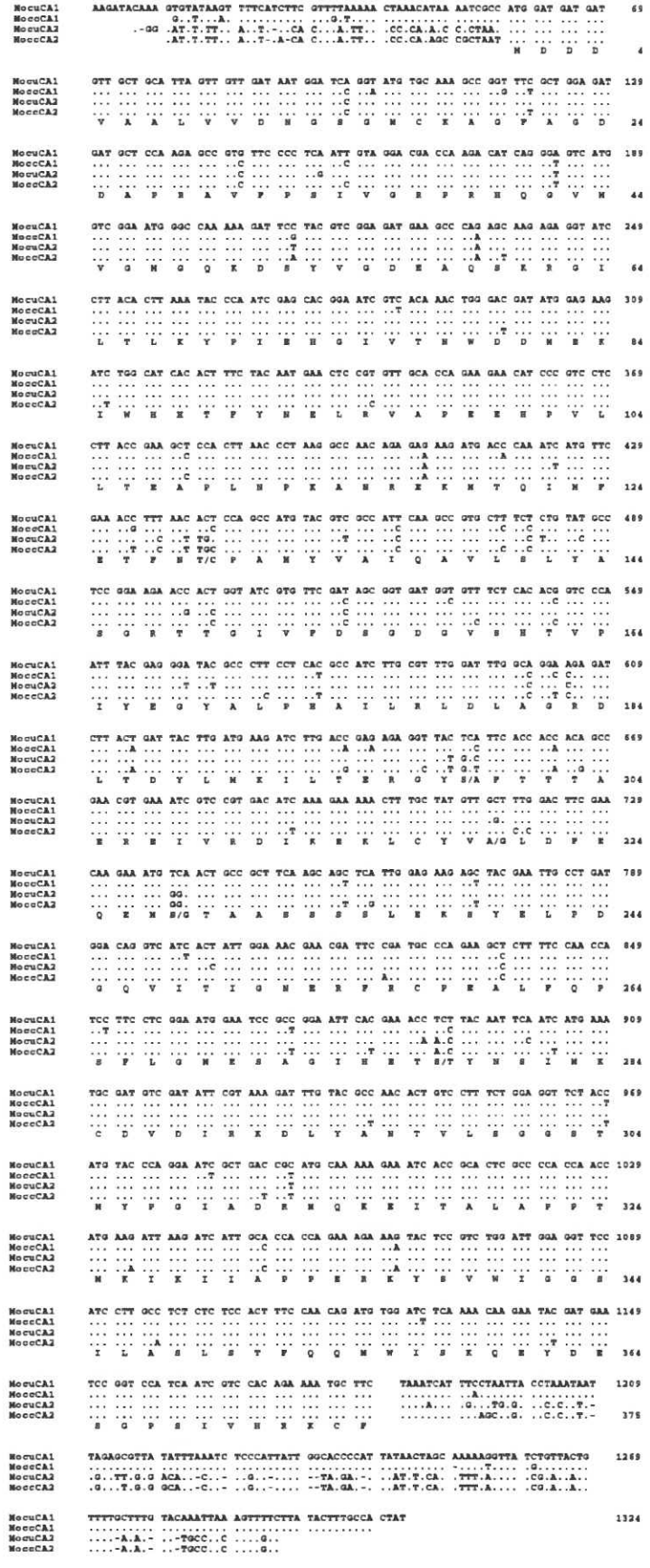

Fig. 1. Nucleotide and deduced amino acid sequences of the cytoskeletal actin cDNA clones. *The aligned sequences from top to bottom rows shows the MocuCA1, MoccCA1, MocuCA2, and MoccCA2 cDNA clones respectively. Dots indicate identical nucleotides and dashes represent gaps. The deduced amino acid sequence is shown at the bottom with differences in Mocu/MoccCA1 and Mocu/MoccCA2 indicated. The GenBank accession numbers of MocuCA1, MoccCA1, MocuCA2, and MoccCA2 are AF076516, AF076517, AF076518, and AF076519, respectively.*

cDNA library prepared from gonads of the tailless species and sequenced (Fig. 1). According to diagnostic amino acid positions, the deduced *MoccCA1* protein is a cytoskeletal actin (Table 1). The *MoccCA1* insert was then used to identify related actin clones by screening a gonad cDNA library of the tailed species. Two classes of cDNA clones encoding cytoskeletal actin mRNAs with different 5' and 3' UTRs were identified and sequenced (Table 1). The *MocuCA1* clone contains 5' and 3' UTRs similar to *MoccCA1*, whereas the *MocuCA2* clone contains different 5' and 3' UTRs. The cDNA library of the tailless species was then screened with the *MocuCA2* insert, and the nearly full length clone *MoccCA2*, which encodes a cytoskeletal actin mRNA similar to *MocuCA2*, was obtained and sequenced. The aligned nucleotide and predicted amino acid sequences of the *MocuCA1*, *MoccCA1*, *MocuCA2*, and *MoccCA2* clones are shown in Figure 1. The *MocuCA1* and *MoccCA1* clones are 96% similar at the nucleotide level, encode identical cytoskeletal actin isoforms (Table 1), and appear to represent orthologous genes (*CA1* genes). Likewise, the *MocuCA2* and *MoccCA2* clones are 95% similar at the nucleotide level, encode identical cytoskeletal actins (Table 1), and appear to represent another pair of orthologous genes (*CA2* genes). The *CA1* and *CA2* genes show about 96% conservation at the nucleotide level, with substitutions mostly in synonymous positions, and the corresponding proteins differ in five amino acid residues (Fig. 1). Thus, the cDNA clones represent orthologous genes encoding two different cytoskeletal actins in the tailed and tailless species.

Multiple cytoskeletal actin genes

The number of *CA1* and *CA2* genes was examined by hybridizing Southern blots with gene-specific 3' UTR probes (Fig. 2). In the tailed species, three bands (in some lanes one major and two or more minor bands) were usually seen in blots hybridized with the *CA1* probe (Fig. 2B). These bands were not recognized by the *CA2* probe (Fig. 2D). Thus, one or two copies of the *CA1* gene and/or some *CA1* related genes may be present in the tailed species. The number of bands recognized by the *CA1* probe was increased in the tailless species (Fig. 2A), suggesting that additional *CA1* and/

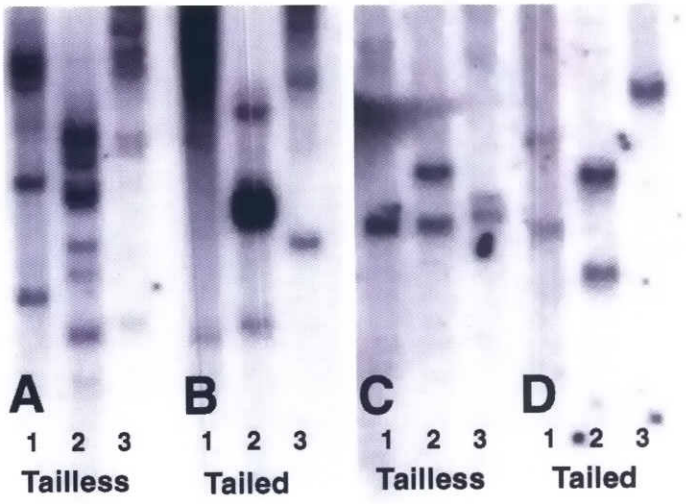

Fig. 2. Southern blot analysis of the *CA1* and *CA2* genes in the tailed and tailless species. (A,C) *Tailless species genomic DNA.* **(B,D)** *Tailed species genomic DNA. A-B. CA1 3' UTR probe. C-D. CA2 3' UTR probe. Lanes 1, 2, and 3 represent DNA digested with BamH1, EcoR1, and HindIII respectively. Hybridization and washing conditions are described in Materials and Methods.*

TABLE 1

COMPARISON OF DEDUCED AMINO ACID POSITIONS THAT DISTINGUISH CYTOSKELETAL AND MUSCLE ACTINS

Position(s)	Mocu/MoccCA1	Mocu/MoccCA2	SpCA8	HrCA1	MocuMA1	HrMA2/4
5-6	V-A	V-A	V-A	V-A	Q-T	T-T
8	V	V	V	V	C	C
16-17	M-C	M-C	M-C	M-C	L-V	L-V
76	V	V	V	V	I	I
103	V	V	V	V	T	T
129	T	T	T	T	V	V
153	F	F	M	F	L	L
162	T	T	T	T	N	N
176	L	L	F	L	A	A
201	T	T	T	T	V	V
225	Q	Q	S	T	Q	Q
260	A	A	A	A	T	T
267	L	L	L	L	I	I
272	A	A	A	A	A	A
279	Y	Y	Y	Y	Y	Y
287	V	V	V	V	I	I
297	T	T	T	T	N	N
365	S	S	S	S	A	A

The amino acid positions used to distinguish cytoskeletal and muscle actins are shown according to Vanderchove and Weber (1978). Deduced amino acid sequences were obtained as follows. SpCA8: Kovilur *et al.* (1993). HrCA1: Araki *et al.* (1996). MocuMA1: Kusakabe *et al.* (1996). HrMA1: Kusakabe *et al.* (1995). HrMA2/4: Kusakabe *et al.*(1992). The amino acid positions of MocuMA1 and HrMA2/4, which contain additional residues at their N-termini, were designated from alignments of the cytoskeletal actins.

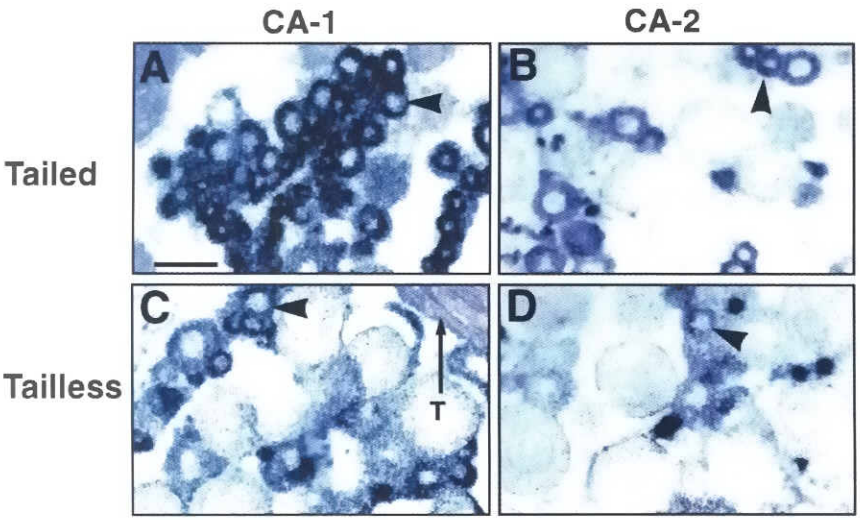

Fig. 3. Spatial distribution of *CA1* and *CA2* mRNA in gonads of the tailed (A,B) and tailless (C,D) species determined by *in situ* hybridization with *CA1* (A,C) and *CA2* (B,D) probes. *Arrowheads: small oocytes containing high concentrations of mRNA. T, testes. Bar; 100 μm; magnification is the same in each frame.*

or *CA1* related genes are present. One or two major bands were detected in blots containing tailed (Fig. 2D) or tailless (Fig. 2C) species DNA hybridized with the *CA2* probe, suggesting that *CA2* is present in one or two copies in both species. When the washing stringency was decreased, numerous bands appeared in blots hybridized with either of the probes (data not shown), suggesting that both species contain multiple *CA1*- and *CA2*-like cytoskeletal actin genes with similar 3' UTRs.

Maternal and embryonic expression of cytoskeletal actin genes

The spatial expression of the *CA1* and *CA2* genes in gonads of the tailed and tailless species was determined by *in situ* hybridization (Fig. 3). Ascidians are hermaphrodites with gonads containing sperm and oocytes at all stages of development. In the tailed species, *CA1* and *CA2* transcripts were distributed throughout the cytoplasm of small oocytes, gradually declined as oocytes increased in size during oogenesis, and were present at very low levels in large oocytes (Fig. 3A,B). No transcripts were detected in the testes. A similar expression pattern was observed in gonads of the tailless species (Fig. 3C,D). The results suggest that the *CA1* and *CA2* genes are active primarily during early oogenesis in the tailed and tailless species.

The expression of the *CA1* and *CA2* genes during embryonic development was determined by *in situ* hybridization (Figs. 4 and 5). The short *CA1* and *CA2* 3' UTRs required the use of RNA probes containing the protein coding regions of the genes to obtain sufficient signal in these experiments. However, hybridization conditions were refined to maximize the detection of gene specific transcripts, as shown by our ability to recognize different *CA1* and *CA2* expression patterns in the tailless species (see below). The low levels of *CA1* and *CA2* transcripts characteristic of mature oocytes (Fig. 3) persisted in fertilized eggs and cleaving embryos (data not shown). However, transcript levels increased during gastrulation, indicating that the *CA1* and *CA2* genes are expressed zygotically as well as maternally.

The expression of *CA1* is shown in Figure 4. In the tailed species, *CA1* transcripts were first detected in the presumptive muscle and mesenchyme cells at the mid-gastrula stage (Fig. 4A). By the early tailbud stage (Fig. 4B), *CA1* mRNA also appeared in the notochord cells (Fig. 4B). Thereafter, *CA1* transcripts persisted in the notochord, mesenchyme, and muscle cells through the tadpole larva stage (Fig. 4C). In the tailless species, *CA1* transcripts showed a distribution similar to that of tailed species between gastrulation and the 6 h stage (Fig. 4D-E). Subsequently, however, *CA1* transcript levels declined, in concert with arrested tail development, and were absent or detectable at very low levels through larval hatching (Fig. 4F). The tailed phenotype can be restored by fertilizing eggs of tailless species with sperm of the tailed species (Swalla and Jeffery, 1990). In these hybrid embryos, *CA1* transcripts were detected in the presumptive muscle cells during gastrulation, appeared in the notochord cells in 6 h. embryos, and in contrast to their behavior in the tailless species, persisted in these tissues through the hatching stage (Fig. 4G). The decline in *CA1* mRNA levels in the tailless species relative to the tailed species and an increase in hybrids during gastrulation was confirmed by northern blotting experiments (Fig. 4H). Thus, the *CA1* expression pattern of the tailed species was restored in hybrid embryos.

Figure 5 shows *CA2* expression in tailed species, tailless species, and hybrid embryos. In the tailed species, *CA2* expression was similar temporally and spatially to that of *CA1*, with mRNA accumulation restricted to the mesenchyme, muscle, and notochord cells (Fig. 5A-C). In contrast to *CA1*, however, *CA2* transcripts were undetectable, or detected at very low levels, during embryogenesis in the tailless species (Fig. 5D-F), implying that the *CA2* gene is downregulated. In hybrid embryos, *CA2* transcripts accumulate in the muscle and notochord cells at levels comparable to the tailed species (Fig. 5G), indicating that the expression pattern of the tailed species was restored.

The results show that embryonic expression of the *CA1* and *CA2* genes is restricted to the notochord and other mesodermal tissues beginning at gastrulation of the tailed species, downregulated in the tailless species, and restored in hybrid embryos. The patterns of *CA1* and *CA2* gene downregulation are different in the tailless species: the *CA1* genes are actively expressed during and

for a few hours after gastrulation but not at later stages of development, whereas the *CA2* genes are not expressed at all during embryogenesis.

Cytoskeletal actin expression in the notochord is dependent on forkhead gene activity

The ascidian forkhead gene *MocuFH1*, a putative homolog of vertebrate *HNF3β*, is expressed in the prospective endoderm and notochord cells, where it is required for morphogenetic cell movements (Olsen and Jeffery, 1997). Since *CA1* and *CA2* are expressed in the notochord, the possibility that these genes are functionally dependent on *MocuFH1* activity was examined using antisense procedures (Swalla and Jeffery, 1996). Previous studies have shown that the antisense ODNs used in these experiments are effective in suppressing the accumulation of *MocuFH1* mRNA but not other mRNAs during ascidian embryogenesis (Olsen and Jeffery, 1997). In antisense ODN-treated embryos, the presumptive notochord cells involute over the anterior lip of the blastopore, but they are subsequently unable to undergo cell movements resulting in notochord formation and fail to develop into tadpole larvae. By contrast, the tail muscle cells develop normally in these antisense ODN-treated embryos (Olsen and Jeffery, 1997). When embryos of the tailed species were treated with sense *MocuFH1* ODNs they formed normal tadpole larvae, and expressed the *CA1* and *CA2* genes in the tail muscle and notochord cells (Fig. 6A,B,E,F). Treatment with antisense *MocuFH1* ODNs resulted in arrested embryos lacking brain sensory organs and tails and undifferentiated notochord cells (see Olsen and Jeffery, 1997). In the antisense ODN-treated embryos, *CA1* or *CA2* transcripts were not detected in the notochord cells, although mRNA was expressed strongly in the presumptive muscle cells at the posterior lip of the blastopore (Fig. 6C,D,G,H). The results show that *CA1* and *CA2* expression in the notochord but not the muscle cells is dependent on *MocuFH1* gene activity.

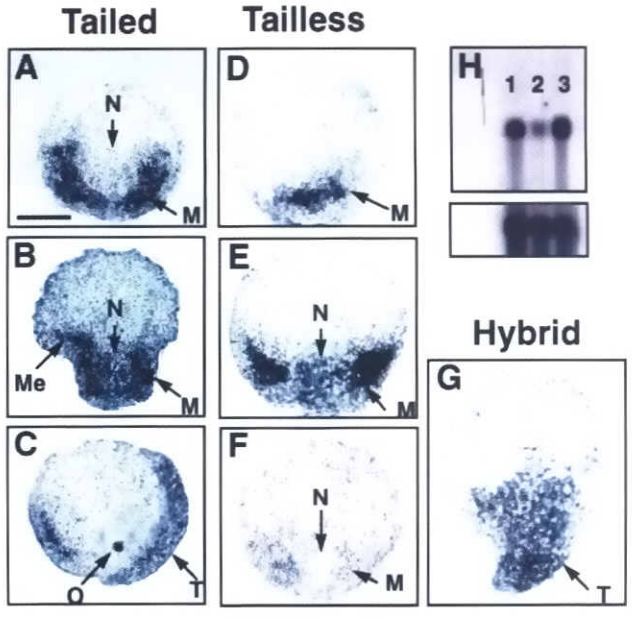

Fig. 4. Spatial distribution of *CA1* mRNA in tailed species (A-C), tailless species (D-F) and hybrid (G) embryos determined by *in situ* hybridization with the *CA1* (A-F) probe. (A-C) *Tailed species. Frontal (A,B) or sagittal (C) sections through the anteroposterior axis of mid-gastrulae* **(A)**, *early tailbud* **(B)**, *and late tailbud* **(C)** *embryos.* **(D-F)** *Tailless species. Frontal sections through the anteroposterior axis of mid-gastrulae* **(D)**, *6 h (equivalent to early tailbud) embryos of the tailless species* **(E)**, *and 11 h (equivalent to late tailbud) embryos.* **(G)** *Hybrid (11hr). Sagittal section through the anteroposterior axis. M, muscle cells; Me, mesenchyme cells; N, notochord cells; T, tail; O, otolith sensory cell. Bar, 20 μm; magnification is the same in each frame.* **(H)** *Upper frame. Accumulation of CA1 mRNA in gastrulae of (1) the tailed species, (2) the tailless species, and (3) hybrids determined by northern blot hybridization. Lower frame. rRNA loading control for upper frame.*

Discussion

The evolutionary regression of chordate features in tailless ascidian larvae is caused in part by an arrest in notochord development (see Swalla, 1993; Jeffery, 1997). Here we describe two cytoskeletal actin genes whose expression patterns in the tailed and tailless species and dependence on an *HNF-3β*-like gene suggest a role in development of the ascidian notochord. Changes in the expression of these genes in the tailless species suggest that they may also be involved in regression of the chordate body plan in ascidians.

The four cDNA clones we have identified encode orthologous pairs of cytoskeletal actin genes, based on a high degree of nucleotide conservation in their protein coding regions and 5' and 3' UTRs. The *CA1* and *CA2* genes are present in one or two copies in each species, although both species appear to exhibit related genes with similar 3' UTRs. Multiple muscle actin genes have been identified previously in ascidians (Kusakabe *et al.*, 1991,1992,1995; Beach and Jeffery, 1992). The adaptive significance of these reiterated muscle actin genes may be to mediate rapid differentiation of tail muscle cells, and thereby enhance dispersal of the tadpole larva (Beach and Jeffery, 1992). Our results suggest that the ascidian genome also contains multiple cytoskeletal actin genes. Similar to the muscle actin genes, the expression of multiple *CA1* and *CA2* genes in the notochord and tail muscle cells may have evolved to enhance larval development and dispersal. Alternatively, multiple *CA1* and *CA2* genes may be employed to mediate tissue specific expression during embryogenesis.

Maternal expression of the *CA1* and *CA2* genes is restricted to small oocytes in the hermaphroditic gonads of both species. Thus, the *CA1* and *CA2* genes are distinct in their expression patterns from the cytoskeletal actin gene *SpCA15*, which is expressed in both developing sperm and oocytes in the ascidian *Styela plicata* (Beach and Jeffery, 1990). The *SpCA15* gene is thought to function in cell proliferation during the embryonic and adult phases of the life cycle (Beach and Jeffery, 1990). In contrast, the restricted expression of the *CA1* and *CA2* genes implies a more specific role in early oogenesis, possibly in elaborating the actin cytoskeleton in the oocyte cortex (Swalla *et al.*, 1991; Jeffery, 1995).

Embryonic expression of the *CA1* and *CA2* genes is restricted to the mesenchyme, notochord, and muscle cells in the tailed species. Thus, these genes are also distinct in their embryonic expression patterns from *SpCA15*, which is active in the rapidly-dividing ectodermal and neural cells of *Styela* embryos (Beach and Jeffery, 1990). In addition, *CA1* and *CA2* expression is more extensive in the notochord and muscle cells than the cytoskeletal

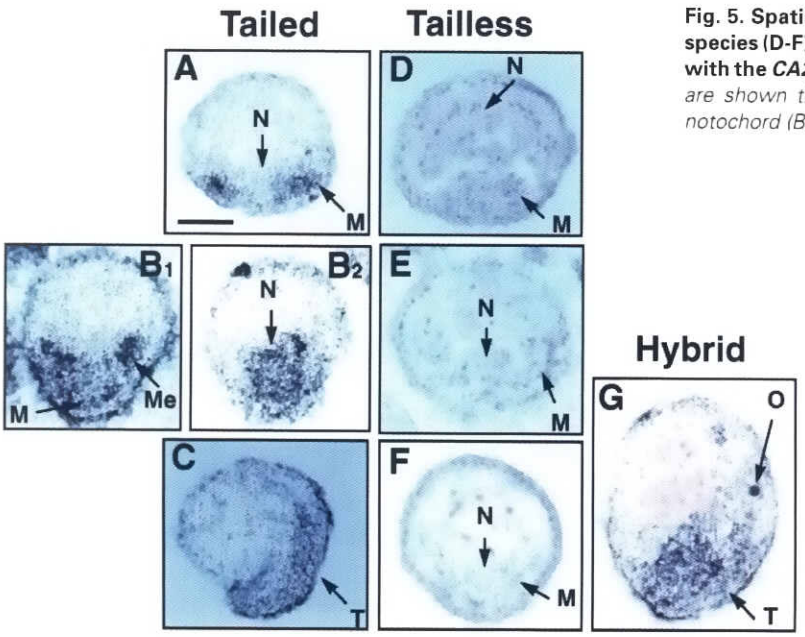

Fig. 5. Spatial distribution of *CA2* mRNA in tailed species (A-C), tailless species (D-F), and hybrid (G) embryos determined by *in situ* hybridization with the *CA2* probes. *In B, frontal sections of the same early tailbud embryos are shown through the planes of the mesenchyme and muscle (B1) and notochord (B2) cells. Other details are the same as indicated in Figure 4.*

actin gene *HrCA1*, which operates primarily in mesenchyme cells of the ascidian *Halocynthia roretzi* (Araki *et al.*, 1996). Thus, in contrast to the cytoskeletal actin genes identified previously in ascidians, the *CA1* and *CA2* genes may be involved in more general aspects of larval mesoderm development.

Contrasting expression patterns in the tailless species and hybrids suggest that the *CA1* and *CA2* genes have different roles in the development and regression of the tadpole larva. The mesoderm cells involute over the lips of the blastopore at the midgastrula stage and subsequently move to their final positions along the anteroposterior axis during neurulation and the tailbud stages (Jeffery and Swalla, 1997). The involution movements of mesodermal cells are not changed in the tailless species, although the succeeding posterior movements of the prospective notochord and muscle cells that establish the tail are suppressed (Swalla and Jeffery, 1990). We have shown that *CA1* transcripts accumulate in the mesoderm cells during gastrulation and neurulation in both species (albeit less extensively in the tailless species), then disappear in concert with arrested tail development in the tailless species. On this basis, we suggest that the *CA1* gene is required for embryogenesis. The *CA1* gene is likely to be involved in mesoderm involution during gastrulation, thus accounting for its expression in both species. In contrast, the *CA2* gene, which is downregulated throughout development of the tailless species and upregulated in hybrids, may be involved in post-gastrulation morphogenetic events leading to tail formation in the tailed species, and therefore nonessential in the tailless species embryos. The *CA1* gene could also have a secondary role in tail formation because expression is restored in the notochord and muscle cells coincident with the reappearance of chordate features in hybrid embryos. It will be interesting to determine the molecular basis for changes in *CA1* and *CA2* gene expression in the tailless species.

The *forkhead* gene *HNF-3β* is required for gastrulation, axis formation, and differentiation of axial tissues in vertebrate embryos (Ang and Rossant, 1994; Sasaki and Hogan, 1994; Weinstein *et al.*, 1994). Based on expression in axial organizing centers (Corbo *et al.*, 1997; Shimauchi *et al.*, 1997) and axis disrupting effects of *forkhead* antisense ODNs (Olsen and Jeffery, 1997), similar developmental roles have been proposed for putative *HNF-3* orthologs in ascidians. The downstream targets of *HNF-3β* have not been determined in chordates, although *Brachyury (T)* may be regulated by *HNF-3β* in the mouse (Ang and Rossant, 1994). Our antisense ODN results suggest that the *CA1* and *CA2* genes are among the direct or indirect targets of the *HNF-3β*-like gene in the ascidian notochord. We envision that the *CA1* and *CA2* genes function in an *HNF-3β*-regulated gene cascade(s) involved in notochord development. The lack of antisense ODN effects on *CA1* and *CA2* expression in the tail muscle cells verifies the specificity of the antisense ODNs and is consistent with the absence of *HNF-3β* gene expression in the tail muscle cells (Corbo *et al.*, 1997; Olsen and Jeffery, 1997; Shimauchi *et al.*, 1997). Therefore, other upstream regulatory factors must control *CA1* and *CA2* expression in the tail muscle cells during larval development. A possible candidate for such a regulatory factor is the ascidian *snail* gene, whose expression domain includes the tail muscle cell precursors (Erives *et al.*, 1998).

Downregulation of the *CA1* and *CA2* genes during embryogenesis of the tailless species suggests that these genes may be involved in the regressive evolution of the notochord and tail via changes in the regulation of upstream control genes, such as *HNF-3β*. If so, changes in the regulation of *HNF3β* would also be expected in the tailless species. *MoccFH1*, an *HNF3β*-like forkhead gene orthologous to the *HNF3β*-like forkhead gene *(MocuFH1)* in the tailed species (Olsen and Jeffery, 1997), has recently been isolated in the tailless species (Olsen and Jeffery, unpublished data). Although *MoccFH1* is expressed during gastrulation, it is downregulated after neurulation (Olsen and Jeffery, unpublished data), consistent with the possibility that it may be one of the upstream control genes responsible for changes in *CA1* and *CA2* expression during development of the tailless species.

It has been difficult to establish relationships between different regulatory genes in ascidians, which do not offer tractable genetic approaches to dissect developmental pathways. Our results suggest that antisense ODN procedures can be used to investigate gene cascades during ascidian development. Using these procedures, here we have identified for the first time some of the structural genes *(CA1* and *CA2)* that function downstream of the transcription factor HNF-3β during ascidian development.

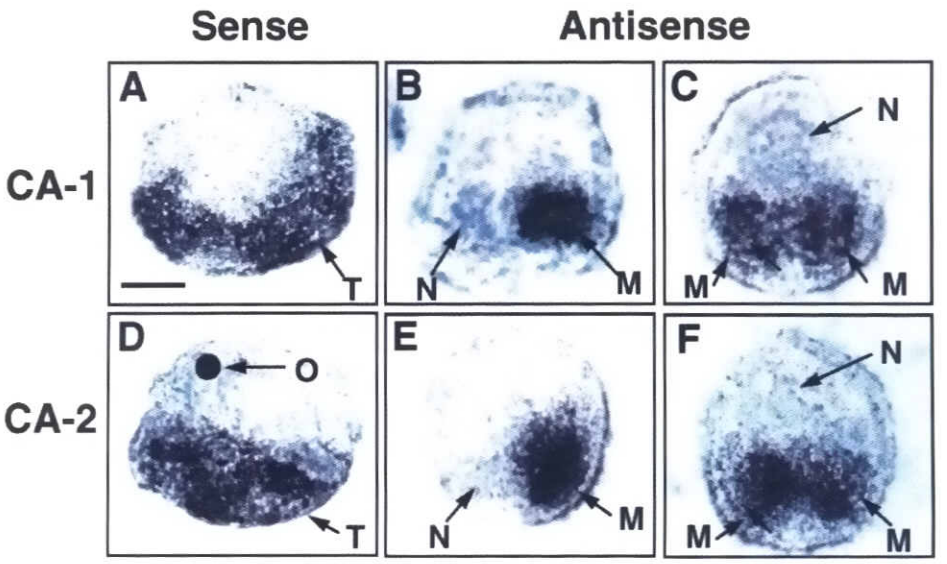

Fig. 6. Effect of *MocuFH1* ODNs on *CA1* (A-C) and *CA2* (D-F) mRNA accumulation in embryos of the tailed species determined by *in situ* hybridization with *CA1* and *CA2* probes. (A-D) *Sagittal* **(A,B,D,E)** *and frontal* **(C,F)** *sections through 11 h. embryos treated with sense* **(A,D)** *or antisense* **(B,C,E,F)** *MocuFH1 ODNs. M, muscle cells; N, notochord cells; O, otolith sensory organ; T, tail. Bar, 20 μm; magnification is the same in each frame.*

Materials and methods

Animals, gametes and embryos

The ascidians *M. oculata* and *M. occulta* were collected at Station Biologique, Roscoff, France. The procedures used to obtain gametes, culture embryos, and prepare interspecific hybrids have been described previously (Swalla and Jeffery, 1990).

Preparation and screening of cDNA libraries

The cDNA libraries were prepared in the lambda ZAPII vector (Stratagene, La Jolla, CA) using poly(A)+RNA isolated from gonads of the tailed and tailless species (Swalla *et al.*, 1993). The *MoccCA1* (*Molgula occulta* cytoskeletal actin 1) cDNA clone was obtained during a screen for other cDNA clones in the tailless species gonad cDNA library. The insert of the *MoccCA1* clone was labeled with [32P] dCTP (3000 Ci/mmole, Amersham, Arlington Heights, IL) using the Random Primed DNA Labeling Kit (U.S. Biochemicals, Cleveland, OH) and used to screen the tailed species gonad cDNA library. The *MocuCA1* (*Molgula oculata* cytoskeletal actin 1; originally named in *MocuCA-4* in Swalla *et al.*, 1993) and *MocuCA2* cDNA clones were isolated. The tailless species cDNA library was screened with a probe generated by polymerase chain reaction (PCR) from the 3' untranslated region (UTR) of the *MocuCA2* clone to isolate the *MoccCA2* cDNA clone. The positive clones were purified by additional rounds of screening, and the lambda ZAPII *in vivo* excision protocol (Stratagene) was used to recover the cDNA clones in Bluescript SK(-).

DNA sequencing and computer analysis

The cDNA clones were sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) using [35S]-dATP (800 Ci/mmole; New England Nuclear, Boston, MA) and Sequenase (United States Biochemicals). Oligonucleotide primers were made on a Pharmacia LKB Gene Assembler Plus (Pharmacia Biosystems, Piscataway, NJ). Synthesized oligonucleotides were deprotected with NH4OH, purified with Oligoclean (U.S. Biochemical) and diluted to 25 ng/ml with distilled water.

Sequencing showed that *MoccCA1*, *MocuCA1*, and *MoccCA2* were apparently full-length clones, whereas *MocuCA2* lacked the 5' part of the coding region. A PCR based approach was utilized to obtain the complete *MocuCA2* sequence. As the tailed species cDNAs were directionally cloned, a primer (T3) was chosen located on the vector at the 5' end of the inserted cDNAs. The *MocuCA2* primer was located 250 base pairs (bp) from the 5' end of the cDNA so that the resulting PCR products would include sufficient overlap to allow their identification. The T3 and *MocuCA2* primers were used to PCR amplify the cDNA library. The resulting products were separated on an agarose gel, and a 500bp band was excised and ligated into the pCR1000 vector (InVitrogen, Carlsbad, CA). The sequence of the subcloned PCR product matched *MocuCA2* exactly in the overlapping region and included the unsequenced 5' part of the coding region and the 5' untranslated region (UTR).

The sequences were read and compared with the Mac Vector Program (IBI-A Kodak Co, New Haven, CT). Sequence alignments were done using Clustal V (Higgins and Sharp, 1988).

Filter hybridizations

Southern hybridizations were carried out with DNA isolated from gonads according to Davis *et al.* (1986). DNA was digested with 50 units of *Bam*H1, *Eco*R1, or *Hind*III and separated on 0.8% agarose gels containing ethidium bromide. The DNA was transferred to a nylon membrane, baked for two hours at 80°C under vacuum, and pre-hybridized with 10% dextran sulfate, 1% SDS, 1.0 M NaCl with 100 ng sheared and denatured salmon sperm DNA at 65°C. The DNA probes, which consisted of the 3' UTRs of the *MoccCA1* and *MoccCA2* cDNAs, were excised from the inserts with appropriate restriction enzymes and labeled with [^{32}]P using the Random Primed DNA Labeling Kit. Hybridizations were carried out at 65°C, and blots were washed in 5XSSC, 0.2% SDS and then in 2XSSC, 0.2% at 50°C prior to autoradiography.

Northern hybridizations were done according to Swalla *et al.* (1993) using total RNA isolated from gastrula stage embryos by the procedure of March *et al.* (1985) and a [32P]-labeled *MoccCA1* probe.

In situ hybridizations

Specimens were fixed in 3:1 ethanol: acetic acid or in 5% formalin in Millipore filtered sea water (MFSW) for 30 min at 4°C, embedded in paraplast, and sectioned. *In situ* hybridization was carried out using [35S]-labeled *MoccCA1* and *MoccCA2* riboprobes according to Swalla *et al.* (1993).

Antisense oligodeoxynucleotide procedures

The antisense (5'-AGAAGGTGGCGACGAAAG-3') and sense (5'-CTTTCGTCGCCACCTTCT-3') oligodeoxyribonucleotides correspond to nucleotide positions 46 to 63 of the *MocuFH1* cDNA sequence (Olsen and Jeffery, 1997). The 18-mer phosphorothiolate-substituted ODNs were synthesized by Oligos Etc., Inc. (Wilsonville, OR). The ODNs were stored

lyophilized at -20°C. A 30 nmole/μl stock solution was prepared in water prior to use in the experiments. Embryos (100-150 embryos/ml) were suspended in MFSW containing 30 μM ODN beginning just after first cleavage (about 60 min after insemination) and incubated until hatching (10-12 h after insemination) (Swalla and Jeffery, 1996).

Acknowledgments

We thank J. Reardon and K. Fletcher for technical assistance and the staff at Station Biologique de Roscoff for provision of experimental animals and laboratory space. This research was supported by NIH grant HD-13970 (W.R.J) and NSF grants IBN 94-17799 (W.R.J), IBN 98-07899 (W.R.J), and IBN 93-04958 (B.J.S).

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Received: July 1998
Accepted for publication: September 1998