Development 126, 3293-3301 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV6405

Mutations affecting tail and notochord development in the ascidian *Ciona savignyi*

Yuki Nakatani, Robert Moody and William C. Smith*

Department of Molecular, Cellular and Developmental Biology, and Neuroscience Research Institute, University of California, Santa Barbara, CA 93106, USA
*Author for correspondence (e-mail: w_smith@lifesci.lscf.ucsb.edu)

Accepted 17 May; published on WWW 5 July 1999

SUMMARY

Ascidians are among the most distant chordate relatives of the vertebrates. However, ascidians share many features with vertebrates including a notochord and hollow dorsal nerve cord. A screen for N-ethyl-N-nitrosourea (ENU)-induced mutations affecting early development in the ascidian *Ciona savignyi* resulted in the isolation of a number of mutants including the complementing notochord mutants *chongmague* and *chobi*. In *chongmague* embryos the notochord fails to develop, and the notochord cells instead adopt a mesenchyme-like fate. The failure of notochord development in *chongmague* embryos results in a severe truncation of tail, although development of the tail muscles and caudal nerve tracts appears largely normal. *Chobi* embryos also have a truncation of the tail stemming from a disruption of the notochord. However, in *chobi* embryos the early development of the notochord appears normal and defects occur later as the notochord attempts to extend and direct elongation of the tail. We find in *chobi* tailbud embryos that the notochord is often bent, with cells clumped together, rather than extended as a column. These results provide new information on the function and development of the ascidian notochord. In addition, the results demonstrate how the unique features of ascidians can be used in genetic analysis of morphogenesis.

Key words: Ascidian, Notochord, Morphogenesis, *chongmague*, *chobi*, *Ciona savignyi*

INTRODUCTION

The notochord is one of the defining characteristics of chordates, and in vertebrates is well characterized as having both structural and inductive/patterning roles in embryogenesis. Morphogenic activity by the notochord contributes significantly to extension of the body along the anterior/posterior axis. For example, zebrafish with the *ntl* (*brachyury*) mutation fail to form a notochord, and as a result the tail does not elongate properly (Melby et al., 1996; Schulte-Merker et al., 1994). Once tailbud extension is complete, the notochord provides rigidity to the early body allowing for swimming motions in fish and amphibian larvae. However in comparison to fish and amphibians, the size of the avian and mammalian notochord is greatly reduced in relation to the cross-sectional area of the trunk or tail. In mammalian and avian embryos the structural function of the notochord may be diminished in relative importance to the other major role of the vertebrate notochord, that of providing patterning information to surrounding tissues. The vertebrate notochord, through its release of secreted inducing molecules such as *sonic hedgehog* and *noggin* (McMahon et al., 1998; Roelink et al., 1994), is essential for the proper development and patterning of numerous tissues including the neural tube (Placzek et al., 1993; Yamada et al., 1993), somites (Fan and Tessier-Lavigne, 1994; Pourquie et al., 1993), heart (Goldstein and Fishman, 1998) and pancreas (Hebrok et al., 1998).

In the non-vertebrate members of the chordate phylum, the cephalochordates and the urochordates, the notochord is a prominent feature of the body axis, and unlike in higher vertebrates is retained in the adults of many of these animals. The conserved expression patterns of a number of genes, including *brachyury* and *HNF-3β*, in the notochords of all chordates suggests that the common ancestor of the chordates had a well formed notochord (Corbo et al., 1997a,b; Holland et al., 1995; Olsen and Jeffery, 1997; Shimeld, 1997; Yasuo and Satoh, 1994). Consistent with data indicating a more recent divergence of the vertebrate and cephalochordate subphyla (Wada and Satoh, 1994), there appear to be unique aspects to the pattern of *brachyury* expression in ascidians, a class of the urochordates. In contrast to vertebrates and cephalochordates where *brachyury* is transiently expressed both in posterior mesoderm and notochord (Holland et al., 1995; Smith et al., 1991), the ascidian *brachyury* gene is only expressed in the notochord while a related T-box gene is expressed in the muscle lineage (Yasuo et al., 1996; Yasuo and Satoh, 1994).

In order to better understand chordate morphogenesis we have screened for N-ethyl-N-nitrosourea (ENU)-induced mutations that disrupt early development in the ascidian *Ciona savignyi* (Moody et al., 1999). Ascidians have many features

desirable of a genetic model system. First, ascidians are hermaphrodites with the capacity for self-fertilization. In addition, despite sharing many developmental features with vertebrates, ascidians have a much simpler morphology. The ascidian larval tadpole has approximately 2,500 cells, and a very limited number of cell types (Satoh, 1994). The cell lineage of the ascidians is fixed and well characterized up through gastrulation (Nishida, 1987). Equally important, the haploid genome of ascidians is approximately 1.8×10^8 base pairs (Lambert and Laird, 1971; Satoh, 1994), which is only 5-10% the size of vertebrate genomes, and is estimated to have only 15,500 protein-coding genes, approximately one-quarter the number estimated in vertebrates (Simmen et al., 1998). Furthermore, numerous studies indicate that ascidians have only single members of many gene families that are known to have multiple members in vertebrates (Di Gregorio et al., 1995; Holland, 1991; Meedel et al., 1997). Thus, it is expected that ascidians will have lower genetic redundancy than vertebrates, a potentially valuable feature for isolating mutants and characterizing gene function.

We describe two mutants isolated from the ascidian *Ciona savignyi*, *chobi* (*chb*) and *chongmague* (*chm*), that disrupt notochord and tail development. In embryos homozygous for the *chm* mutation, the notochord fails to develop, and notochord cells instead take on a mesenchymal-like fate. The *chm* phenotype appears similar to that of zebrafish with the *no tail* (*ntl*) mutation, which results from a lesion in the *brachyury* gene (Schulte-Merker et al., 1994). In embryos homozygous for the *chb* mutation, notochord development appears to be disrupted later as the notochord attempts to elongate the body axis. The two notochord mutants described here provide insight into both the development and the function of the notochord in ascidians.

MATERIALS AND METHODS

Maintenance and fertilization of *C. savignyi*

C. savignyi were grown and maintained in running unfiltered sea water tanks as described by Moody et al. (1999). Gametes were collected from adults using a light-induced spawning procedure. The adults to be spawned were kept in constant light for 2 to 4 days in running filtered sea water to allow them to accumulate gametes (Lambert and Brandt, 1967). Spawning was induced by first placing the animals in the dark for a minimum of 3 hours. Reintroducing the animals to the light induced shedding of eggs and sperm in the majority of animals (>75%) within 15 minutes. For self-fertilizations, the animals were spawned individually in 300 ml beakers. For crossing individuals, two or more animals were placed in a beaker together and allowed to spawn. The spawned eggs were collected on 70 μm Nitex filters and then moved to plastic Petri dishes with filtered sea water with antibiotics (50 μg/ml each kanamycin and streptomycin).

Acetylcholinesterase staining

Muscle cells were stained using a chromogenic assay for acetylcholinesterase as described previously (Karnovsky and Roots, 1964), with the minor modification that embryos were fixed in 70% ethanol for 30 seconds prior to being placed in the chromogenic substrate solution.

Immunocytochemistry

Neural projections in *C. savignyi* tadpoles were visualized using an anti-acetylated tubulin antibody (Sigma; clone 6-11B-1). Embryos were fixed in cold methanol (–20°C) anthen ethanol (–20°C) for 10 minutes each. Immunofluorescent staining of whole-mounted specimens was carried out as described previously (Mita-Miyazawa et al., 1987). Localization of the primary antibody was visualized using fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibodies (Sigma).

DiI labeling

Fertilized eggs were dechorionated as described previously (Mita-Miyazawa et al., 1985), and cultured in agar-coated Petri dishes (0.9% agar in sea water) with filtered sea water containing 0.1% bovine serum albumin. DiI (1, 1′ dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyamine perchlorate; Sigma) was dissolved at 0.5 mg/ml in canola oil and sonicated. Blastomeres were labeled using glass capillary needles and a Picospritzer. A micromanipulator (Model MN-151, Narishige) was used to place an approximately 10 μm drop of the DiI solution on the surface of the blastomere and the capillary removed immediately. The labeled embryos were cultured separately in sea water plus 0.1% BSA until they developed to the desired stage.

In situ hybridization

In situ hybridization with muscle actin and CsEpi-2 antisense digoxigenin-labeled probes was performed as described previously (Wada et al., 1995) with minor modifications. Embryos were fixed for one-half hour at room temperature rather than overnight, and the hybridization was performed in 50% formamide, 6× SSC, 5× Denhardt's, 100 μg/ml salmon sperm DNA and 0.1% Tween-20 at 42°C.

Electroporation

The 3.5 kb *C. intestinalis brachyury* promoter-β-galactosidase fusion transgene construct was electroporated into one cell stage *C. savignyi* embryos as described previously for *C. intestinalis* (Corbo et al., 1997b).

RESULTS

Notochord and tail development can be genetically disrupted in *C. savigny*

The mutants characterized here, *chobi* (*chb*) and *chongmague* (*chm*) were isolated in a screen aimed at developing techniques for generating, identifying, and propagating N-ethyl-N-nitrosourea (ENU) induced mutations in *C. savignyi*. A complete description of the methods involved and a tabulation of results from the screen is published separately (Moody et al., 1999). In brief, the screen took advantage of the ability of *C. savignyi*, which are hermaphrodites, to self-fertilize, in order to screen for zygotically acting recessive mutations. First, the progeny of a cross between sperm from an ENU-treated adult and untreated eggs were raised to reproductive age (F_1s). Sperm and eggs were collected from each F_1 to produce a self-fertilized F_2 generation that was screened at tadpole stage for mutant phenotypes. Both *chb* and *chm* were identified in this screen due to their greatly truncated tails, and were present in the self fertilized F_2 brood at a frequency (approx. 25%) expected for recessive zygotic mutations. Sperm from the F_1 adult generating the mutant phenotypes was outcrossed to wild-type eggs to produce a family of F_2 adults. Individuals of this outcrossed family were crossed to test for transmission of the phenotype. For *chb* and *chm* the phenotypes of homozygous mutants from the self-fertilized F_2 generation and the outcrossed individuals were identical, indicating that both mutant phenotypes result from mutations at single

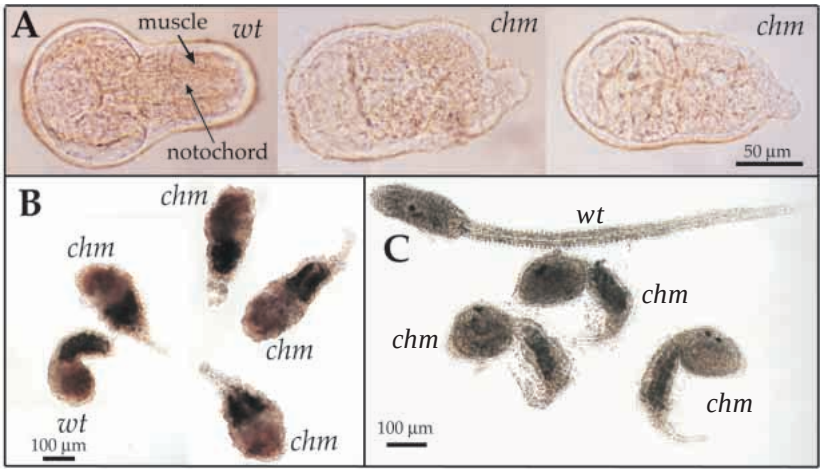

Fig. 1. *Chongmague* (*chm*) embryos and tadpoles. Representative embryos from outcrossed heterozygote *chm* adults are shown at early tailbud (A), mid tailbud (B), and tadpole (C) stages. For each of the three developmental stages shown, mutant (*chm*) as well as wild-type appearing siblings (*wt*) are shown.

chromosomal loci. Crosses between *chb* and *chm* heterozygotes produced all wild-type-appearing progeny. Thus it is mostly likely that the complimenting *chm* and *chb* mutations disrupt separate loci.

Chm embryos have a defect in notochord development

The *chm* phenotype was one of the most pronounced phenotypes observed in our screen. The hatched embryos had a shortened anterior/posterior axis and a narrow extension of tissue protruding from the posterior end. The *chm* phenotype was penetrant, with 25% of embryos from crossed heterozygotes showing an identical phenotype. We were unable to propagate *chm* as a homogzygote. In fact, *chm* tadpoles were never observed to start metamorphosis. For wild-type *C. savignyi* the tadpole stage persists only a few days, following which the larvae settle and undergo metamorphosis. The signal for the start of metamorphosis is adhesion of the larva by the anterior papilla to a substrate such as a rock or dock piling. Because the locomotion of *chm* embryos was so severely impaired (see below) they appeared to be unable to settle properly.

Fig. 1 shows outcrossed F_3 *chm* embryos/tadpoles and their phenotypically normal siblings at three stages of development. The embryos were observed from fertilization through swimming tadpole stages. *Chm* embryos did not appear to be grossly different from wild type until the early tailbud stage (Fig. 1A). There was no obvious phenotypic difference between *chm* and wild-type embryos at blastula, gastrula or neurula stages. We examined the expression of two genes, one for muscle actin and the other, CsEpi-2, which is an epidermis-specific gene (Chiba et al., 1998) by in situ hybridization in a large number of embryos from crossed *chm* heterozygotes at the late blastula stage (110 cell) and could identify no group with altered expression, indicating that the expression of these two genes in *chm* embryos at this stage is normal (not shown).

At early tailbud stages several consistent phenotypic differences from wild-type embryos were apparent in *chm* embryos. Two representative *chm* embryos are shown in Fig. 1A. The first characteristic of *chm* embryos at this stage is the reduced narrowing and extension of the tail in comparison to their wild-type-appearing siblings (*wt*). *C. savignyi* embryos are reasonably transparent, and under bright-field illumination the boundary between the notochord and the surrounding muscle can be seen at this stage in the wild-type-appearing sibling. In the *chm* early tail bud embryos no boundary or morphologically distinct notochord can be identified. The *chm* embryos did appear to have muscle cells properly located along the flanks of the forming tail (also see below), and a separate population of cells situated central to the muscle cells, probably made up of presumptive notochord cells, was also evident (Fig. 1A). Finally, at the posterior of the early tailbud *chm* embryos the beginnings of the narrow extension of tissue can be seen.

At the mid tailbud stage the morphology of the *chm* embryos is dramatically different from that of their wild-type-appearing siblings (Fig. 1B). In addition to the overall difference in shape of the embryos, the posterior extension has progressed further. In fact, if the extension of the cells from the posterior end is taken into account, *chm* embryos were often longer at this stage than wild-type embryos. The posterior extensions appear to be composed of epidermal cells. First, the cells of the extension are similar in size and appearance to the epidermal cells surrounding the tail and trunk, and they form a contiguous sheet with the tail epidermis (Fig. 2). In addition, cells of the extension expressed the gene *CsEpi-2* (not shown), but not muscle, neural, or notochord markers (Figs 3, 4 and 5).

At the hatched tadpole stage the failure of the tail to extend properly is most pronounced, and is probably due to the failure of the notochord to form properly (Fig. 1C, and see below). Most of the force for tailbud extension in ascidians is provided

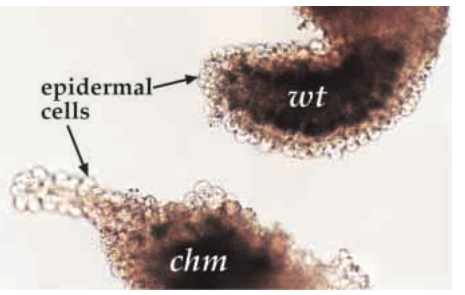

Fig. 2. High magnification view of tail from wild-type (*wt*) and *chongmague* (*chm*) mid tailbud-stage embryos. The figure show the round epidermal cells extending from the tail of the *chm* embryo.

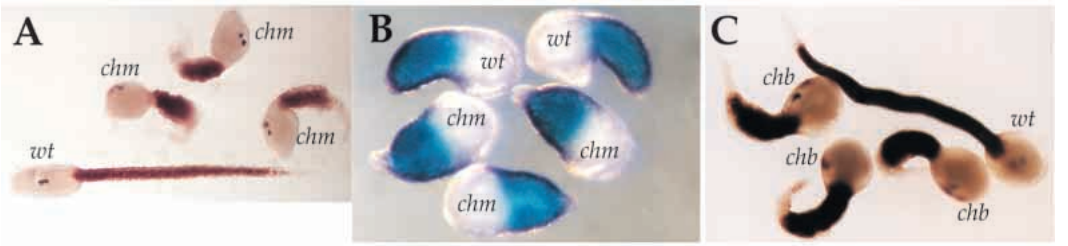

Fig. 3. Tail muscle in *chongmague* (*chm*) and *chobi* (*chb*) embryos and tadpoles. (A) *Chongmague* (*chm*) and wild-type (*wt*) tadpoles stained for muscle using a chromogenic reaction for acetylcholinesterase (brown). (B) In situ hybridization for muscle actin (blue) in early tailbud stage *chm* and wild-type (*wt*) embryos. (C) *Chb* late tailbud embryos stained for muscle as in A.

by the notochord; partial ascidian embryos lacking notochord cells have tails that show little elongation (Miyamoto and Crowther, 1985; Reverberi et al., 1960). The limited elongation seen in *chm* embryos may be due to the rearrangement and cell shape changes of the muscle cells following gastrulation (Cavey and Cloney, 1972).

The *chm* embryos hatched at approximately the same time as their wild-type appearing siblings, and although unable to swim, they occasionally twitched. Staining for the muscle product acetylcholinesterase shows that *chm* tadpoles have a short block of muscle cells in their truncated tail (Fig. 3A). As stated above, in situ hybridization for muscle actin in *chm* embryos at the 110 cell stage did not reveal any differences from the wild-type expression pattern. Likewise, at early tailbud stage the amount and overall morphology of the tail muscles appears largely normal, as determined by in situ hybridization for muscle actin (Fig. 3B). In ascidians, muscle actin gene expression starts as early as the 32 cell stage, and is expressed in blastomeres even before their developmental fate is restricted (Satou et al., 1995). Because the muscle lineage is restricted so early, and appears normal at the 110 cell stage, as well as the early tailbud stage, it would appear that muscle specification and induction is normal in *chm* embryos, and that the shortening of the muscle block seen in the tadpole (Fig. 2A) is secondary to the failure of the notochord to develop properly.

We found that a monoclonal antibody to acetylated tubulin strongly stained neural projections in *C. savignyi* tadpoles (Fig. 4). The majority of nerve cell bodies in *C. savignyi* are located in two ganglia, the sensory vesicle and the visceral ganglia (Nicol and Meinertzhagen, 1991). Although the anti-acetylated tubulin antibodies do not stain cell bodies, the projections from both of these centers are stained. The caudal nerve cord ('spinal cord') of the ascidian consists of four rows of glial cells (ependymal cells), and two parallel nerve tracts that arise from cell bodies located in the visceral ganglia. The anti-acetylated tubulin antibody does not react with the caudal ependymal cells, and the staining observed in the tail of wild-type and *chm* embryos most likely corresponds to motor neuron projections, as described previously (Nicol and Meinertzhagen, 1991; Takamura, 1998). Although the caudal nerve cord in *chm* embryos clearly is shorter than in wild-type embryos, its development appears normal at a gross level, although some fraying of the nerve tracts can be seen, probably due to the failure of the tail to elongate (Fig. 4B). In summary, the development and differentiation of the tail musculature and nerve tracts do not appear to be directly affected by the *chm* mutation, and abnormalities in the morphology of these tissues are probably secondary to earlier action of the *chm* mutation, probably on notochord development.

We have examined the development of the notochord in *chm* embryos in greater detail by examining the expression of *brachyury*. To examine *brachyury* expression in *chm* and wild-type embryos an expression construct containing 3.5 kb of the 5′ promoter region from the *C. intestinalis brachyury* gene driving the expression of β-galactosidase was electroporated into 1-cell stage embryos (Corbo et al., 1997b). In ascidians the expression of the notochord-specific *brachyury* gene is high during gastrulation, and then rapidly declines during neurula and tailbud stages, with higher expression persisting in the posterior eight cells through tailbud stages. In *chm* and wild-type embryos, we examined the expression of the reporter construct at mid tailbud stage, when most of the staining is due to residual β-galactosidase activity from gastrula stage expression. The expression pattern seen with this plasmid construct in wild-type *C. savignyi* was similar to that seen in *C. intestinalis* (Fig. 5A). Mosaic expression is observed in notochord cells and mesenchymal cells of the trunk of the mid tailbud embryo. At this stage, the column of notochord cells, some staining blue and others not, can clearly be distinguished in wild-type embryos. The morphogenesis of the notochord in *C. intestinalis* has been studied with the aid of time-lapse recording (Miyamoto and Crowther, 1985), and follows a similar sequence of morphogenic movements to those that have been well characterized in amphibians (Keller, 1991). In *C.*

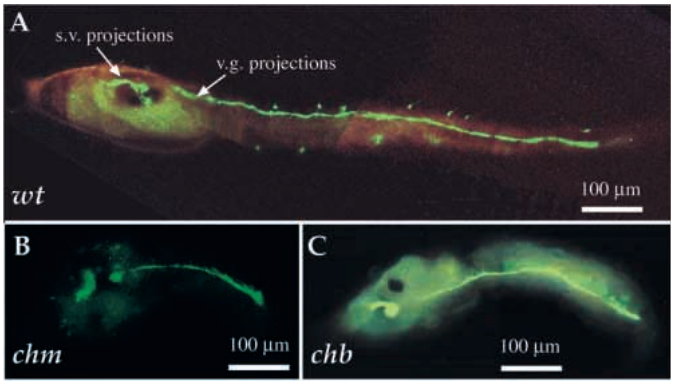

Fig. 4. Immunostaining of neural projections in wild-type (A), *chongmague* (*chm*) (B), and *chobi* (*chb*) (C) tadpoles with anti-acetylated tubulin antibody. The antibody stains projections from the two primary brain ganglia, the sensory vesicle (s.v.) and the visceral ganglion (v.g.).

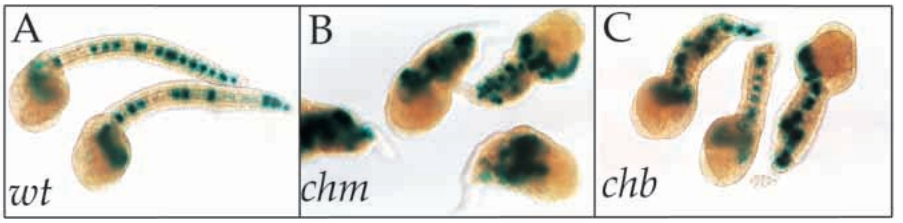

Fig. 5. Expression of *Ciona intestinalis brachyury* promoter-β-galactosidase transgene construct in wild-type (A), *chongmague* (*chm*) (B), and *chobi* (*chb*) (C) tailbud stage embryos. Mosaic expression of the transgene (blue) is seen both in notochord cells of the tail and mesenchymal cells in the trunk.

intestinalis, cells of the notochord rudiment progress from a loosely packed group of isodiametric cells to an intercalated single row of columnar cells at early tailbud stage. In *chm* embryos the *brachyury*-expressing cells appear loosely packed and appear to have never undergone medial intercalation (Fig. 5B). In addition, the *brachyury*-expressing cells in *chm* tailbuds were more round than notochord cells. Because the 3.5 kb *C. intestinalis brachyury* promoter construct gives reporter gene expression both in the notochord and mesenchyme cells, it is possible that in *chm* embryos presumptive notochord cells in the tail have become mesenchyme-like. The shape and loose arrangement of the *brachyury*-expressing cells in *chm* embryos is consistent with their becoming mesenchymal, and may be similar to the fate of notochord cells in the zebrafish *ntl* mutant (Halpern et al., 1993; Melby et al., 1996).

We used DiI labeling to look more closely at the notochord lineage in *chm* embryos, and to distinguish cells of the notochord lineage from the mesenchyme lineage. Specifically we wanted to determine if the presumptive notochord cells in *chm* mutants were changing fate to a identifiable differentiated cell type. In *C. elegans*, for example, loss of determination factors in one cell type often results in that cell adopting the fate of its sister cell (Schnabel and Priess, 1997). The fixed cell lineage of ascidians allows us to directly address questions of cell fate transformation. The development and lineage of the 40 cells that make up the ascidian notochord is well characterized (Nishida, 1987). The A7.3 and A7.7 blastomere pairs, which become restricted to notochord fate at the 64-cell stage, each contribute 16 cells, while the B8.6 blastomere pairs, which become restricted at the 110-cell stage, contribute the posterior eight cells (see Corbo et al. 1997b for a diagram of the notochord lineages). We focused on the A7.3 blastomere, which is directly descendant from the A6.2 blastomere. The sister of the A7.3 blastomere is the A7.4 blastomere, which is fated to make nervous system. *Chm* and wild-type embryos in which one of the A7.3 blastomeres had been labeled with DiI were observed at mid- and late-tailbud stages (Fig. 6). In wild-type embryos, the eight notochord cells derived from this blastomere could be clearly distinguished. In *chm* embryos the A7.3 descendants remained grouped together in the trunk of the embryo and single cells could not be distinguished. Consistent with the results of the *brachyury* promoter expression, the presumptive notochord cells in *chm* embryos had not undergone medial intercalation. At the 32-cell stage we also labeled the A6.2 blastomere, which gives rise to the A7.3

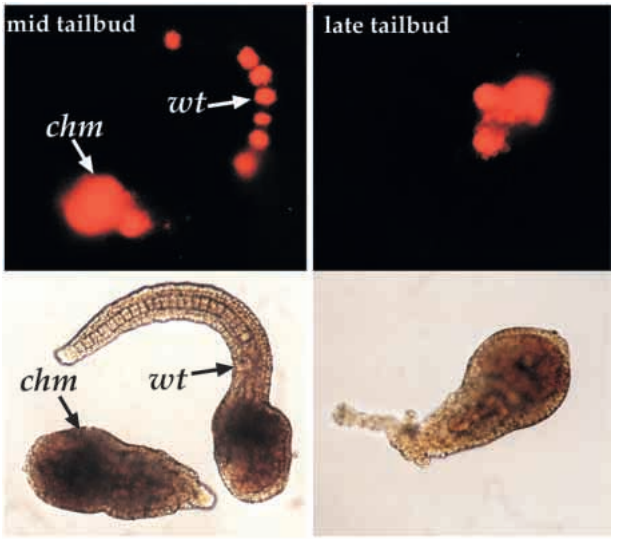

Fig. 6. DiI labeling of the A7.3 lineage in *chongmague* (*chm*) embryos. One of the A7.3 blastomere pair was labeled at the 64-cell stage. In wild-type embryos (wt) this blastomere gives rise to eight notochord cells, but in the mutant these cells remain grouped together in the trunk of the embryo, as seen in the top two panels. Embryos are show both at the mid and late tailbud stages.

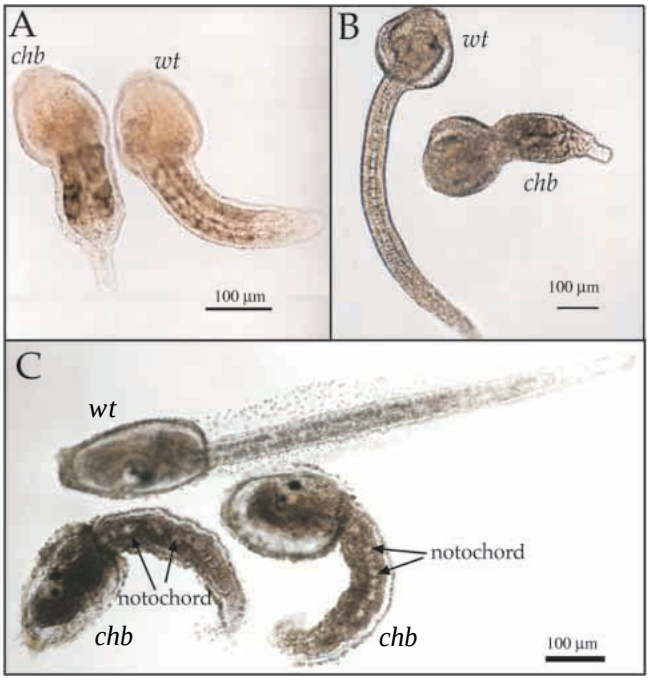

Fig. 7. *Chobi* (*chb*) embryos and tadpoles. Representative embryos from outcrossed heterozygote *chb* adults are shown at early tailbud (A), mid tailbud (B), and tadpole (C) stages. For each of the three developmental stages shown, mutant (*chb*) as well as wild-type appearing siblings (*wt*) are shown.

blastomere and cells fated to the central nervous system, the A6.4 blastomere, which gives rise to the A7.7 blastomere, spinal cord and muscle, and the B6.2 blastomere, which gives rise to the B8.6 blastomere as well as mesenchyme and muscle. In all of these lineage tracings from the 32-cell stage the presumptive notochord cells did not show the intercalation behavior. In summary, the results of the *brachyury* expression plasmid data and the lineage tracing show that the development of the notochord cells is disrupted very early in *chm* embryos. Although the presumptive notochord cells had expressed *brachyury*, they do not assume the morphology or behavior of notochord cells. While it is not clear how far down the pathway to notochord development these cells progressed, they did not appear to have adopted the fate of their sister cells. It is unclear if the mesenchyme morphology of the presumptive notochord cells is indicative of a cell fate change, since other processes, such as arrested development, may result in a similar cellular phenotype.

chobi embryos show a defect in tail elongation

Although both *chb* and *chm* tadpoles have tail truncations, the appearance of the two mutants is different (compare Figs 1C and 7C), and a complementation test suggests that the phenotypes result from lesions at different loci. At a gross level, *chb* embryos appear phenotypically wild type up through early tail bud stages. Only at the mid tailbud stage could *chb* embryos be readily distinguished from wild-type embryos (Fig. 7A). At this stage, the tail of *chb* embryos did not display the sweeping curve found in wild-type tails, and is tapered to a finer point at the posterior end. The extent of elongation of the tail was already less than wild type. A few hours later when tail extension in wild-type embryos has progressed considerably, the tail of *chb* embryos is much shorter and thicker (Fig. 7B). In addition, *chb* embryos have a posterior extension of ectoderm cells that appear similar, but much shorter, to those seen in *chm* embryos. *Chb* tadpoles (Fig. 7C) hatch normally and have twitching muscle. The trunk of *chb* tadpoles appears similar to that of wild-type embryos, unlike *chm* tadpoles which have a more rounded trunk (Fig. 1C).

Unlike *Chm* embryos, *Chb* embryos had identifiable notochords, as can be seen in hatched tadpoles (Fig. 7C, arrows). Despite the tail truncation, the development of the muscle and nervous system in *chb* embryos appears essentially normal, as determine by acetylcholinesterase and anti-acetylated tubulin immunostaining (Figs 3C, 4C). We observed a great variability in the morphology of the notochords between *chb* embryos, although in no cases was the extension of the notochord as great as seen in wild-type embryos. The notochords in *chb* embryos were visualized with the *C. intestinalis brachyury* promoter construct, and with DiI labeling, as described above. From observations of hatched *chb* tadpoles under Nomarski optics it appeared initially as though *chb* embryos had fewer notochord cells than wild type (Fig. 7C). However upon closer examination, the notochord cells in *chb* embryos are often kinked or folded back upon each other making them difficult to count. This kinking of the notochord can be seen in *chb* embryos electroporated with the *brachyury* promoter construct (Fig. 5C). In order to determine if *chb* embryos were making all 40 notochord cells found in wild-type embryos, we followed the development of cells in each of

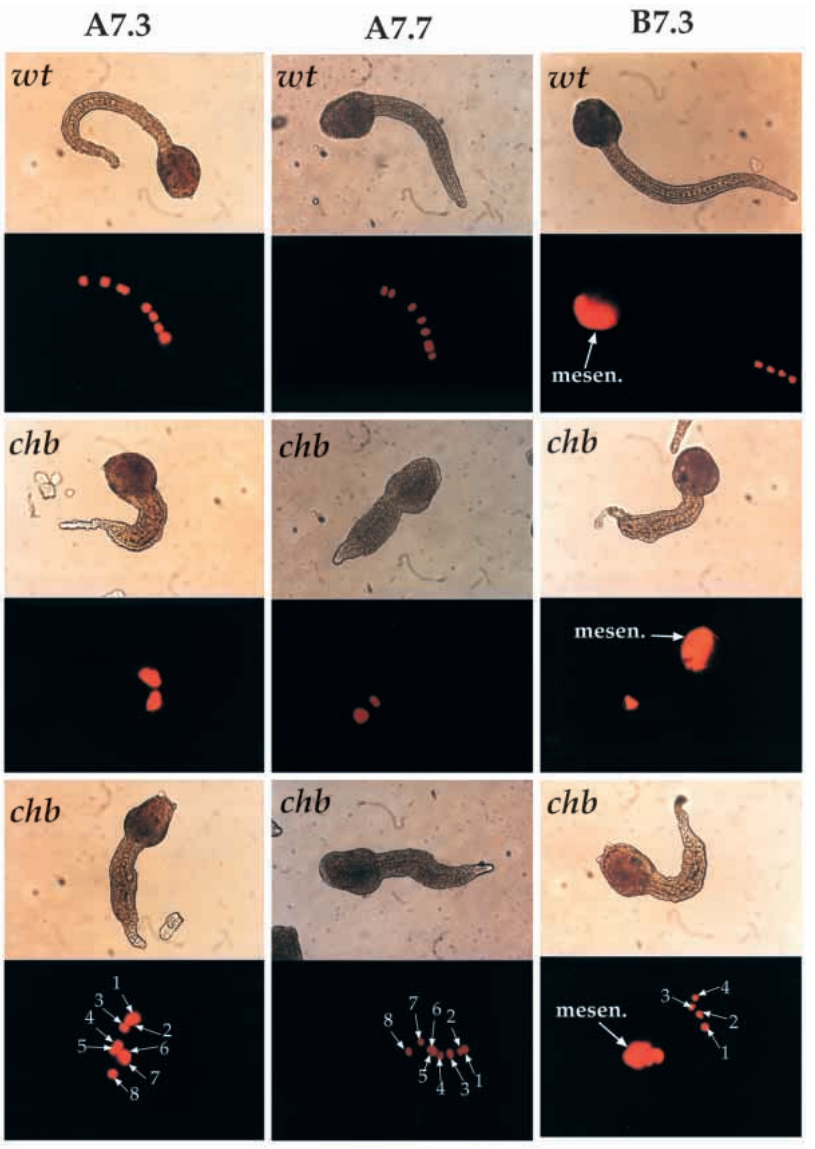

Fig. 8. DiI labeling of the notochord lineage in *chobi* (*chb*) embryos. *Chb* embryos and their wild-type siblings (*wt*) were labeled with DiI in one of either the A7.3, A7.7 or B7.3 blastomeres. *wt* embryos show the normal lineage for each blastomere. The A7.3 and A7.7 blastomere give rise to the anterior 32 of the 40 notochord cells found in *C. savignyi*. By labeling one each of the A7.3 and A7.7 blastomere pairs we expect to observe eight labeled notochord cells. The B7.3 blastomere pair give rise to the posterior eight notochord cells, and thus labeling one of the blastomeres should result in four labeled notochord cells. Two *chb* mutants are shown for each labeled blastomere and indicate the range of staining patterns observed. In cases where the individual cells can be counted, the number of cells is indicated.

the restricted notochord lineages (the descendants of the A7.3, A7.7 and B8.6 blastomeres, see above) by DiI labeling. We directly labeled one each of the A7.3 and A7.7 blastomere pairs (Fig. 8). To follow the B8.6 blastomere, we labeled the B7.3 blastomere which gives rise to the B8.6-derived notochord cells, as well as mesenchyme. Fig. 8 shows the range of DiI labeling patterns observed in tailbud *chb* embryos. In some labeled *chb* embryos we were unable to distinguish individual notochord cells, while in others they could be easily counted. However, we were able to identify enough embryos in which all of the expected cells could be identified to conclude that there is not a general loss of notochord cells in *chb* embryos. It is also clear that notochord cells in *chb* embryos had undergone medial intercalation. The intercalation process is responsible for only a portion of the elongation force for the notochord. Cell shape changes following intercalation contribute significantly to extending the notochord (Miyamoto and Crowther, 1985). The notochord is surrounded by a sheath of extracellular matrix that restrains the diameter of the notochord, and thus directs force towards elongation in the anterior/posterior axis. A direct or indirect effect of the *chb* mutation may be a weakening of the sheath, leading to a defect in the elongation of the entire tail.

DISCUSSION

It is the presence of the notochord that most clearly demonstrates the close evolutionary link between the ascidians and the vertebrates. The importance of the notochord as a structural element in the ascidian tadpole is consistent with the truncated phenotypes we have observed for *chm* and *chb*. In *chm* there appears to be an early disruption in the pathway to notochord formation leading to a failure of a morphologically distinct notochord to develop, while in *chb* there appears to be a defect in the structure of the notochord causing it to bend and buckle rather than form a rigid column of cells.

The screen that resulted in the isolation of *chb* and *chm* was small, and served primarily as a pilot to future, larger screens (Moody et al., 1999). Because of the small size of the screen, we do not know if mutations disrupting tail formation will be extremely common. While mutations in a number of genes might be expected to produce a phenotype similar to *chb*, the phenotype of *chm* embryos points to a more specific genetic lesion. While the molecular lesion responsible for the *chm* phenotype remains unknown, there are several candidate genes. In fact, the *chm* mutation has several features in common with the zebrafish *no tail* (*ntl*) mutation. The appearance of *chm* embryos is similar to that of zebrafish with the *ntl* mutation (compare our Fig. 1C with Fig. 1A in Halpern et al., 1993), although *ntl* embryos do not have the posterior extension of the epidermal cells seen in *chm* embryos. In both mutants the primary effects appear largely restricted to the notochord, and the gross development of the head and muscles is normal. The fixed lineage of ascidians allowed us to follow the precise fate of presumptive notochord cells. We observed that the presumptive notochord cells in *chm* mutants adopt a mesenchyme-like morphology, similar to the fate of notochord cells in the zebrafish *ntl* mutation. These results suggest that the *chm* mutation disrupts notochord development at approximately the same stage as the zebrafish *ntl* mutation, and raises the possibility that the *chm* mutation involves *brachyury*. Although we observe the expression of the *brachyury* promoter reporter gene (β-galactosidase) in *chm* mutants, this does not exclude the mutation being in the *brachyury* gene itself. In the zebrafish *ntl* mutant, the early expression of brachyury persists (Schulte-Merker et al., 1994). Other candidates for *chm* include *Hnf-3β/forkhead* which is known to be essential for axial development in both mice and ascidians (Ang and Rossant, 1994; Olsen and Jeffery, 1997; Weinstein et al., 1994), although the targeting of *Hnf-3β/forkhead* transcripts in the ascidian *Molgula oculata* with antisense oligonucleotides results in a severe gastrulation defect not seen in *chm* embryos.

Other genes that are known to be essential for notochord development, such as the *Manx* and *bobcat* genes isolated from the *Molgulid* ascidians (Swalla et al., 1993) appear to be less likely candidates. Down regulation of *Manx* and *bobcat* expression appears to contribute significantly to the tailless phenotype of *M. occulta*. However, the phenotype of *M. occulta*, as well as *M. oculta/M. occulta* hybrids treated with antisense *Manx* or *bobcat* oligonucletides, is much more severe than *chm* and includes disruption of the sensory organs and secondary muscle (Swalla and Jeffery, 1996; Swalla et al., 1999). Likewise the *Not* genes, whose loss leads to a notochord to muscle transformation in the zebrafish *floating head* (*flh*) mutation (Amacher and Kimmel, 1998) would also appear to be less likely candidates.

One of the most striking features of both *chm* and *chb* mutants is the posterior extension of the epidermal cells. While the extensions do not contain notochord, muscle or neural tissue, we cannot rule out that they contain the posterior endodermal strand. The ascidian endodermal strand is thought to be related to the vertebrate hypochord (Corbo et al., 1997a). Consistent with the possibility that the endodermal stand is directing the posterior elongation are several lines of evidence suggesting that the extensions do not result from autonomous behavior of the ectodermal cells. In vertebrates, cells of the neural ectoderm are known to undergo convergent extension causing elongation of the spinal cord in the anterior/posterior axis (Elul et al., 1997). However, the non-neural ectoderm is not thought to have autonomous extension activity. Furthermore, the epidermal cells in both the *chm* and wild-type embryos at this stage are round and loosely packed, very different than the flattened shape these cells take on in the tail of the tadpole (Katz, 1983), and not at all indicative of cells undergoing convergent extension. Because we see this phenomenon in both *chm* and to a lesser extent in *chb* embryos (see below), we assume it is a secondary result deriving from the failure of the axial mesoderm to extend properly, and may indicate a normal process that only became evident in these mutants. Whatever the mechanism that causes the posterior extension of the epidermal cells, it does not appear to be common to all chordates, since in zebrafish mutants having defects in axial mesoderm, such as *ntl* and *flh* (Schulte-Merker et al., 1994; Talbot et al., 1995), there is no similar epidermal phenotype.

While the structural importance of the notochord in ascidian development is well established, it is less clear whether the ascidian notochord has significant inducing/patterning activity on neighboring tissues. It is known, however, that ascidian notochord cells do signal to each other to induce notochord development (Nakatani and Nishida, 1994). If the notochord,

or notochord precursor cells, have an inductive role in patterning the ascidian embryo, a likely target would be the nervous system. Other roles of the vertebrate notochord such as patterning the somite into myotome, sclerotome, and dermatome (Fan and Tessier-Lavigne, 1994; Marcelle et al., 1997) obviously would not be found in ascidians. Some aspects of patterning the dorsal/ventral axis of the neural tube appear to be conserved between ascidians and vertebrates (Corbo et al., 1997a). It was observed that the ventral-most of the four ependymal cells of the ascidian spinal cord expressed the gene *HNF-3β*, while the lateral cells of the closing neural tube express the gene *snail*. This approximates the pattern found in the closing vertebrate neural tube, and suggests that much of the mechanisms for patterning the neural tube preceded the split of the chordate sub-phyla. In vertebrates, signals arising from the notochord, as well as the overlying ectoderm, are important in patterning dorsal/ventral aspect of the neural tube. *Chm* embryos thus may provide an tool for uncovering what, if any, roles the ascidian notochord has in patterning the embryo.

We thank Shota Chiba, Michael Levine, and Kazuhiro W. Makabe for providing us with plasmids. We also thank Toshio Hirano for teaching us blastomere labeling techniques. This work was supported by grants from the National Science Foundation (IBN-9724094) and the Beckman Young Investigator Program to W. C. S.; Y. N. is a recipient of a Human Frontier Science Project post-doctoral fellowship.

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