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).

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. 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.

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; 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; Olsen and Jeffery, 1997; Shimeld, 1997; Yasuo and Satoh, 1994), 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 $1.8 \times 10^8$ 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 (>75%) within 15 minutes. For self-fertilizations, the animals were 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).

**Dil 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. Dil (1, 1’ dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine 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 Dil 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, 6x SSC, 5x 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. savignyi

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 (F1s). Sperm and eggs were collected from each F1 and F2 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 F2 brood at a frequency (approx. 25%) expected for recessive zygotic mutations. Sperm from the F1 adult generating the mutant phenotypes was outcrossed to wild-type eggs to produce a family of F2 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 F2 generation and the outcrossed individuals were identical, indicating that both mutant phenotypes result from mutations at single chromosomal locations.
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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 homozygote. 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 F3 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. 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 tailbud 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. 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.](image)

![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.](image)
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 morphogenetic movements to those that have been well characterized in amphibians (Keller, 1991). In C. savignyi, the formation and maintenance of the notochord is driven by the expression of the brachyury gene. To examine

![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.](image-url)
Ascidian notochord mutants

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 asidian 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
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 Cm 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 determined 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.](image-url)
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 indicates that the presumptive notochord cells in chm mutants 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/foxkhead 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/foxkhead 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. oculata. However, the phenotype of M. oculata, as well as M. oculata/M. oculata hybrids treated with antisense Manx or bobcat oligonucleotides, 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 (fh) 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 strand 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 fh (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-3 β, 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. Chelm embryos thus may provide an tool for uncovering what, if any, roles the ascidian notochord has in patterning the embryo.

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REFERENCES


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