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Isolation of developmental mutants of the ascidian *Ciona savignyi*

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Abstract Ascidians have been used extensively as model animals for experimental embryology. We report here the results of a pilot study with the aim of developing genetic methods for the ascidian *Ciona savignyi*. The chemical mutagen N-ethyl-N-nitrosourea (ENU) was used to induce point mutations. F1 animals, produced by using sperm from ENU-treated animals to fertilize untreated eggs, were grown to reproductive age. Sperm and eggs collected from the hermaphrodite F1 adults were used to generate self-fertilized F2 broods, which were then screened for recessive, zygotically acting mutations. Animals carrying potential mutations were outcrossed to wild type to test for the heritability of the phenotypes. We report on a number of mutants isolated using this method, including several with abnormalities in tail and notochord development.

Key words Ascidian · Mutagenesis · Development · Ethylnitrosourea (ENU)

Introduction

Genetics has provided powerful tools for the study of animal development, especially for the invertebrate model systems *Caenorhabditis elegans* and *Drosophila melanogaster*. Genetic techniques are now being applied to vertebrates, particularly zebrafish (Driever et al. 1996; Haffter et al. 1996). However, genetics in vertebrates poses many challenges. Feeding and housing large numbers of vertebrates can be costly and cumbersome. The longer generation time of vertebrates compared to the invertebrate models can severely slow the progress of studies. Finally, the larger genomes of vertebrates make identifying genes an even more difficult task.

The development of the non-vertebrate members of the chordate phylum (the cephalochordates and urochordates) has been studied extensively, and while sharing many features with vertebrates, these animals have unique properties that may facilitate genetic analysis. The ascidians (members of the urochordate sub-phylum), in particular, are well suited to genetic analysis. Many ascidian species have generation times as short as 1 month (Yamaguchi 1975). In addition, ascidians are hermaphrodites, and many species are capable of self-fertilization. Solitary ascidian species typically produce large numbers of gametes on a regular basis. Ascidian embryos develop quickly, are morphologically simple, and are relatively transparent.

A particularly important aspect of ascidians for genetic studies is the size and evolutionary history of the genome. The haploid genome of ascidians has been estimated to comprise approximately 1.8×10^8 bp – much smaller than that of vertebrates, and similar in size to the genomes of the invertebrate model animals *C. elegans* and *Drosophila* (Satoh 1994). The smaller genome of ascidians in comparison to that of vertebrates reflects a correspondingly smaller number of genes (Simmen et al. 1998). The history of the vertebrate genome appears to include two rounds of genome-wide gene duplication, which occurred following the divergence of the three chordate sub-phyla (Holland et al. 1994). Extensive additional gene duplications appear to have occurred in many amphibian species and in teleost fish (Ohno 1993; Amores et al. 1998). Because of these gene duplications in vertebrates, the ascidian genome may more closely reflect the "basic set" of genes needed for the development of the common chordate ancestor. The expansion of the vertebrate genome not only gave rise to the increased overall complexity of vertebrates, but also to extensive redundant and partially redundant gene function. Thus, mutagenesis screens in ascidians may allow the isolation of mutants which would be masked by redundant gene function in vertebrates.

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We report here the results of a pilot study with the goal of testing the feasibility of genetic screening in the ascidian *Ciona savignyi*. Our study involved first developing methods for rapid and reliable culture of large numbers of *C. savignyi*. We also report protocols for chemical mutatgenesis and screening for developmental abnormalities. Finally, the results of a screen with eighty mutagenized *C. savignyi* broods will be reported. In the pilot screen we have identified a number of developmental mutants with a range of phenotypes. The ability to analyze *C. savignyi* development genetically has the potential to contribute significantly to our understanding of chordate development in general.

Materials and methods

Fertilization and metamorphosis of *C. savignyi*

Gametes were collected from *C. savignyi* either by dissection or by light-induced spawning. For either procedure, adult *C. savignyi* were first placed in constant light for 2–4 days in running sea water to accumulate gametes (Lambert and Brandt 1967). The genital ducts of adults were then exposed by dissection. The ducts were torn open with fine forceps to release the gametes, which were then collected with a Pasteur pipet and transferred to plastic petri dishes containing filtered sea water supplemented with kanamycin and streptomycin (each at 50 μg/ml). For self-fertilization, the eggs and sperm from one individual were mixed, while for cross-fertilizations the gametes from two or more individuals were mixed.

Light-induced spawning was used when adults needed to be kept alive for repeated embryo collection. Spawning was induced by first placing the animals in the dark for a minimum of 3 h. Subsequent exposure of the animals to light induced shedding of eggs and sperm within 15 min in the majority of cases (>75%). For self-fertilizations, the animals were allowed to spawn individually in 300-ml beakers. For cross fertilizations, 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 containing filtered sea water and antibiotics. Adult *C. savignyi* could be induced to spawn repeatedly when allowed to recover for 1–2 weeks between spawnings.

Larvae that were to be raised to adulthood were maintained in 10- or 15-cm petri dishes with sea water containing antibiotics at 16–18° C for several days, during which time the larvae attached to the sides and the bottom of the petri dishes and started metamorphosis. Once the majority of the larvae had attached, the petri dishes were moved to tanks containing flowing sea water (see below). A population of about 50 adults could be raised on a 15-cm plate. Dishes that were too crowded were culled after 3–4 weeks in culture. Once attached to the petri dishes, the animals remained strongly affixed until removed by hand. A number of procedures required that the adult *C. savignyi* be peeled from the dishes, spawned to identify the genotype, and then be returned to the flowing sea water tanks to accumulate new gametes for later spawning. We found that the adults did not readily reattach to petri dishes. Instead, adults that had been peeled from petri dishes were attached at their bases to squares of nylon window screen using nylon thread sewn through the tunic. The thread holds them against the screen allowing them to attach; attachment becomes firm only after several weeks.

Long-term culture of *C. savignyi*

Petri dishes with attached juvenile *C. savignyi* were suspended vertically in 15-l tanks supplied with unfiltered sea water at a flow rate of 2 l/min. The lids of the tanks were covered to inhibit algae growth. Up to six dishes, or approximately 200 animals, were grown per tank. Outflow from the tanks was passed through two 60 W UV sterilizers (Aquanetics). The bottoms of the tanks were vacuumed twice weekly to remove settled feces and silt.

ENU treatment

Two protocols for treating animals with the chemical mutagen N-ethyl-N-nitrosourea (ENU) were tested. The first was similar to one used successfully with zebrafish (Mullins et al. 1994). In this protocol (Protocol 1), the adult *C. savignyi* were soaked for 1 h in 3 mM ENU in sea water. The soaking was repeated two more times at 3-day intervals. In the second protocol (Protocol 2), the adults were injected in the base with 50 μl of 100 mM ENU in phosphate-buffered saline (PBS) and then soaked in 3 mM ENU for 3 h. This treatment was repeated 3 days later. Following the ENU treatments, the animals were rinsed extensively in sea water and kept isolated in closed aquariums for several days before being returned to the running sea water system.

The ENU-treated *C. savignyi* were maintained in the sea water tanks for 3 weeks after the end of the mutagenesis treatments. The animals were then moved to constant light for 3 days, following which sperm was collected by dissection and used to fertilize eggs from untreated animals. Sperm collected from a single ENU-treated animal was added to eggs from a single untreated animal in a 15-cm dish. Thus all of the F1 animals in any one dish were siblings, and are referred to as an F1 family. Once the larvae had settled, the dishes were transferred to the sea water tanks.

Cryostorage of *C. savignyi* sperm

Sperm was collected from dissected animals by first tearing the sperm duct with a pair of forceps. The sperm was collected with a Pasteur pipet, taking care to minimize the amounts of other fluid collected, and stored on ice until frozen. A 20-μl aliquot of the sperm was mixed in a 0.5 ml cryostorage vial with 80 μl of a solution containing 10% dimethylsulfoxide (v/v) and 10% (w/v) non-fat powdered milk in filter-sterilized sea water. The vial was then floated on a piece of styrofoam in liquid nitrogen in a standard laboratory ice bucket. After 20 min, the vials were transferred to a liquid nitrogen storage flask. *C. intestinalis* sperm frozen by this method was found to be viable after 9 months of storage, and presumably should be viable for much longer (Joe Corbo and Audrey Chang, personal communication). To thaw the sperm, the vials were warmed briefly in a 30° C water bath, filled with sea water, and then added directly to eggs.

Results

Long-term culture of *C. savignyi*

Ascidians feed by filtering sea water to collect plankton (Barrington 1965). We found that *Ciona* (both *C. intestinalis* and *C. savignyi*) could readily and reliably grow using only unfiltered (raw) sea water as a food source. Sea water for the marine laboratories at UC Santa Barbara is collected at an intake pipe located approximately 10 m deep and 500 m from shore. The incoming raw sea water is first delivered to a 40,000-l underground holding tank. Sea water is then pumped from the holding tank through a sand filter and then to laboratories as "filtered sea water". In order to have access to the raw sea water, we set up culturing tanks in the building that houses the 40,000-l holding tank. A pump was used to bring the sea water to a 15-l reservoir tank, from which it was then delivered to the culture

tanks by gravity feed (Fig. 1A). Effluent sea water from our culturing tanks is returned to the ocean (along with all of the sea water outflow from the marine laboratories). Because adult *Ciona* grown in the culturing tanks, including mutants generated in the mutagenesis screen (see below), were spawning daily, we wanted to ensure that no live gametes were released with the effluent water. In order to kill spawned gametes the effluent sea water was passed through two 60 W UV sterilizers. We found that when a suspension of the *C. savignyi* eggs and sperm was passed though the sterilizers and collected, no viable tadpoles were produced (not shown).

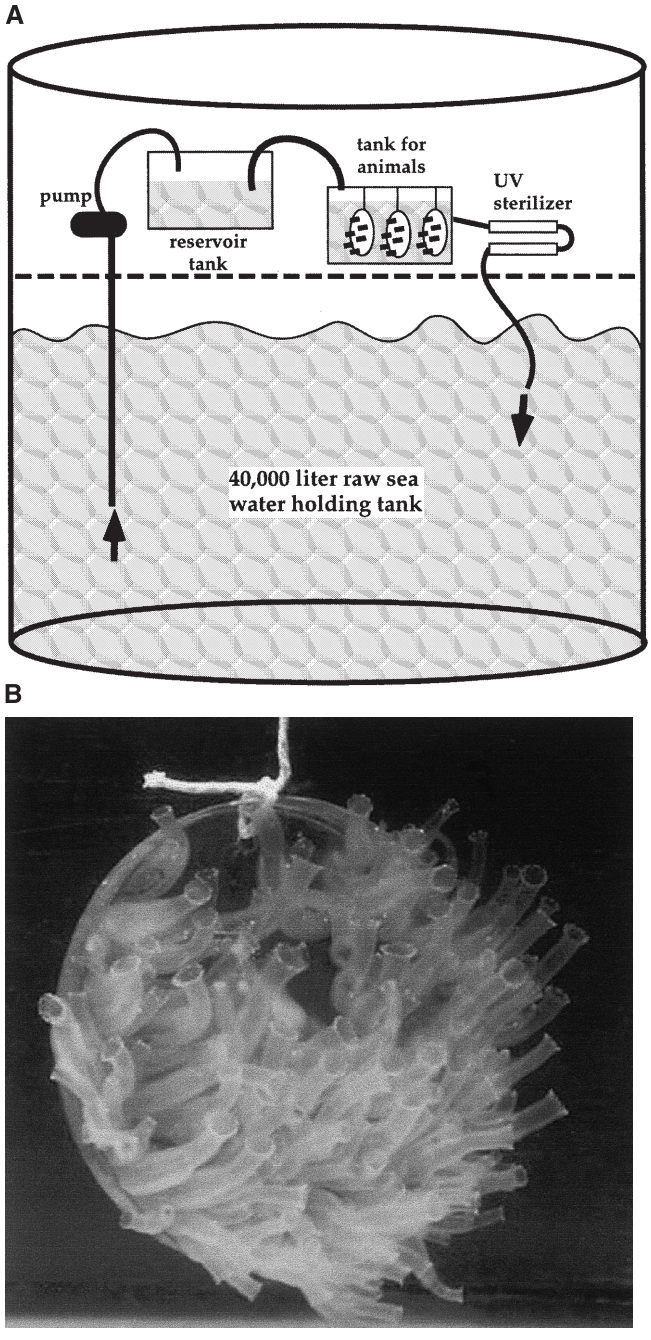

Fig. 1 **A** Schematic view of the arrangement used for culturing *C. savignyi*. Raw (unfiltered) sea water was drawn from a 40,000-liter underground holding tank and delivered to a 15-l reservoir tank. Gravity feed was then used to deliver the sea water at a rate of 2 l/min to 15-l tanks containing *C. savignyi* attached to petri dishes. Effluent water was passed through two 60 W UV sterilizers before being returned to the holding tank. **B** An F1 family of mutagenized *Ciona savignyi* growing on a 15 cm petri dish. Tadpole-stage *C. savignyi* were allowed to attach to the side and bottom of the petri dish. The dish was then suspended in a tank of flowing raw sea water until the animals reached reproductive age, shown here

The ease and economy of using raw sea water as a food source for *Ciona* is ideal for large-scale culturing. Labor involved in maintaining the animals is limited to twice-weekly vacuuming of the tanks. From April through December, the animals grew rapidly, and first produced gametes 7–8 weeks after fertilization, although we routinely allowed the animals to grow for at least 10 weeks in order to obtain more eggs. During the winter (January through March), the growth rate of juvenile *C. savignyi* was lower, although the mature adults continued to produce gametes throughout the winter. The reduced growth rate of the juveniles during the winter could be the result of a number of factors, including lower plankton concentrations and reduced water temperatures. Field studies have shown that the maturation time for *C. intestinalis* decreases with increase in sea water temperature, and can be as short as 1 month when water temperatures exceed 23° C (Yamaguchi 1975). The raw sea water provided to our animals ranged from a maximum of approximately 21° C in September and October to a minimum of approximately 13° C in March and April.

Our use of an open, free-flowing, raw sea water system for growing and maintaining animals presented several potential problems, including the possible introduction of predators. However, over the period of more than a year during which we have been using the system, this problem has not materialized. We have observed no unexpected loss of animals due to predation or other factors, such as changing sea water conditions. In fact, adult *C. savignyi* are hardy and can be maintained long term in our culture system. We do not know the maximum life span of animals in our tanks; however, animals as old as 9 months survive and continue to produce gametes.

A second concern we had was that wild *Ciona* tadpoles might be brought in with the unfiltered sea water and settle in our tanks. Although we occasionally did find colonial ascidians settling, contamination with wild *Ciona* has not been a problem. The *C. savignyi* that were intended for culture were grown attached to the inside surfaces of petri dishes (Fig. 1B), which were then suspended in the raw sea water tanks. We reasoned that if wild *Ciona* were coming in with the raw sea water and settling on our dishes we would expect to find them on both the inside and outside surfaces of the dishes; this has not been observed. In addition, we have not found *Ciona* growing on the dishes that were obviously of a different age from the animals that were intentionally settled. The fact that the water for the marine laboratories is collected

at an open ocean site, which is not a habitat for *Ciona*, may explain the lack of contamination.

ENU treatment

We tested two protocols for treating *C. savignyi* with the mutagen N-ethyl-N-nitrosourea (ENU). Both protocols were well tolerated by the animals. Neither protocol resulted in the death of more than 20% of the animals. The first protocol involved the simple soaking of *C. savignyi* in a solution of ENU in sea water. The animals appeared to find the ENU solution irritating and quickly closed and retracted their siphons. We were concerned that because the animals greatly reduced the amount of water siphoned during the treatment, they might not have been exposed to sufficiently high levels of ENU. To contend with this possibility, our second protocol involved injecting the animals with ENU followed by soaking for 3 h in ENU solution. Both treatments were used in our pilot screen, and both resulted in transmissible mutations, with neither being clearly superior to the other. The animals were maintained for 3 weeks following ENU treatment, with the intention of limiting mosaic transmission of point mutants. The time course of gametogenesis in *C. savignyi* has not been studied; however, we assumed that 3 weeks should be sufficient time for sperm that was derived from cells that were postmitotic at the time of ENU treatment to have been shed by spawning, and all new mature sperm would therefore have been derived from ENU-exposed premeiotic spermatogonia. Because we had no pre-existing *C. savignyi* mutants we were unable to easily measure how efficiently our ENU protocols induce point mutations. The two protocols represented our best guess, based on reports for other species, for treatments that would induce point mutations.

Design and results of the pilot screen

Figure 2 shows the design of the pilot screen for identifying and propagating *C. savignyi* individuals carrying mutations in zygotically acting genes. In brief, sperm from ENU-treated *C. savignyi* (G_0) was used to fertilize eggs from untreated animals to generate families of F1 larvae. Once settled and grown to reproductive age, the F1 adults were dissected to collect eggs and sperm. For each F1, a self-fertilized F2 generation (brood) was produced by mixing self sperm and eggs. F2 broods that successfully self-fertilized were screened at a single stage (early hatched tadpole) for obvious developmental ab-

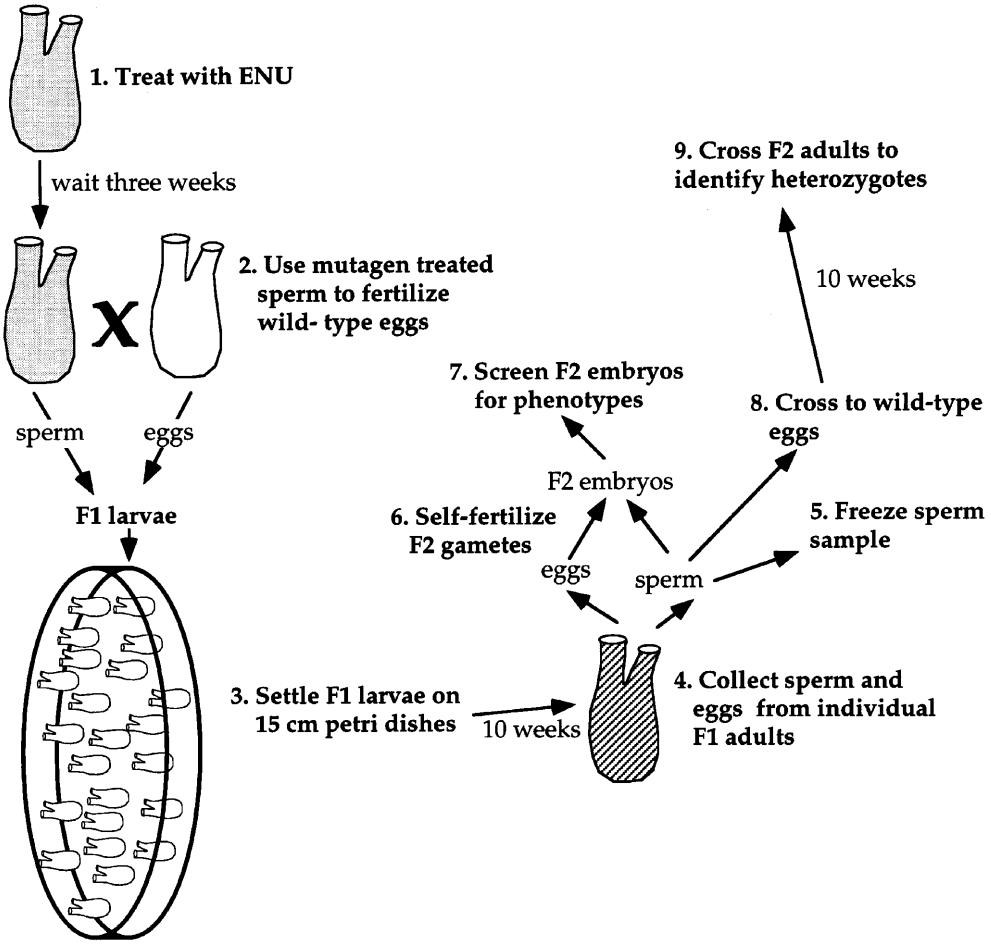

Fig. 2 Strategy for isolating zygotic mutants in *C. savignyi*. Adults were treated with the chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) (1). Sperm from ENU-treated adults was used to fertilize wild-type eggs (2), and the resulting larvae (F1s) were allowed to settle in 15 cm petri dishes (3). Once F1 animals reached reproductive age, sperm and eggs were collected from each individual (4), and a sample of the sperm was frozen (5). Sperm and eggs from each individual was used to produce a self-fertilized F2 generation (6), that was screened for abnormal phenotypes (7). Sperm from individuals that gave potential mutants in the F2 screen were crossed to wild type eggs to raise an F2 family (8). Members of the F2 family were crossed to identify heterozygotes carrying the particular mutation

normalities. For recessive mutations, 25% of the tadpoles in the F2 broods were expected to show a similar phenotype. Features examined in F2 broods included (but were not limited to) the overall shape of the tail and trunk, the ability to hatch, evidence of arrested development, the presence of the pigmented cells of the otolith and ocellus, contracting muscle, the presence/appearance of the sensory vesicle, notochord, and mesenchymal pouches of the trunk. Table 1 summarizes the results of the pilot screen. A total of 205 F1 adults from 13 F1 families were tested. Of these, 80 (39%) were self-fertile, and thus produced F2 progeny for phenotypic screening.

Table 1 Summary of the results of the pilot screen

Family[a]	Number of individuals screened[b]	Mutant/phenotype of F2 embryos[c]	Transmission to F3
3 (1)	5 (12)	3.1 Kinked axis	n.d.
		3.2 Kinked axis	No
5 (1)	9 (20)	5.2 Short tail	Yes
6 (1)	8 (12)	6.2 Short, thick tail	Yes
		6.5 Rounded, with pigment, muscles	No
		6.9 Short tail	Yes
7 (2)	5 (11)	7.6 No pigment cells, sensory vesicle	No
		7.11 Rounded; with pigment, muscles	Yes
8 (2)	2 (4)	8.1 Rounded; with pigment, muscles	No
10 (1)	1 (1)	None	
11 (2)	12 (24)	11.3 Rounded; with pigment, muscles	n.d.
		11.5 Rounded; with pigment, muscles	n.d.
		11.6 Rounded; with pigment, muscles	n.d.
		11.8 Rounded; with pigment, muscles	No
		11.11 Early arrest, no obvious pigment cells	n.d
		11.18 Rounded; with pigment, muscles	n.d
13 (2)	8 (32)	13.1 Short, kinked axis	n.d
		13.30 Early arrest, no obvious pigment cells	n.d
14 (2)	10 (16)	14.2 Short, kinked axis	n.d.
		14.5 Short axis, malformed head	Yes
		14.14 No pigment in otolith or ocellus	No
15 (2)	10 (24)	None	
16 (1)	3 (14)	16.1 Rounded, with pigment, muscles	n.d.
17 (1)	3 (11)	17.1 Short; kinked axis	No
		17.3 Short tail	Yes
18 (1)	4 (24)	18.16 Rounded, with pigment, muscles	Yes
		18.20 Rounded, with pigment, muscles	n.d.

[a] The results for each of the 18 mutagenized families are given. The mutagenesis protocol used is indicated in *parentheses*

[b] The number of successful self fertilizations for each family. The number given in *parentheses* is the total number of animals from each family that were tested (both self-fertile and self-sterile)

[c] See text for further details

Of the 80 self-fertile broods screened, twenty-five (31%) were judged to contain tadpoles with abnormal phenotypes at approximately the expected frequency for recessive mutations. In contrast, 50 self-fertilized broods from untreated *C. savignyi* were examined, and only one (2%) met our criteria for the presence of a potential mutation. The phenotypes observed in the F2 tadpoles are listed in Table 1. No obvious dominant mutations were found in this screen. We also indicate in Table 1 is whether the observed phenotypes were transmitted to F3 progeny when sperm from F1s was crossed to wild-type eggs (see below). Figure 3 shows F2 embryos representing the mutants that were isolated. Only those mutants that successfully transmitted the mutations when outcrossed are shown.

Tadpoles with the most common phenotype – which was observed in 11 of the 80 broods examined – were round and showed little extension of the anterior/posterior axis. They contained blocks of twitching muscle and pigment cells, indicating that they had not arrested early in development, although the majority of them failed to hatch. In Table 1 this phenotype is described as "rounded; with pigment, muscles". This phenotype is characterized by representatives of F2 broods 7.11 and 18.16 (Fig. 3).

Embryos displaying a different phenotype observed in two clutches also did not hatch. However, this class did not have any obvious pigment cells or twitching muscle, and appeared to have arrested early in development. Several clutches were observed to include tadpoles with normal trunks but with bent, and often shortened, tails. This phenotype is represented by clutches 5.2 and 6.2. A much more dramatic truncation of the tail was observed in three of the clutches, 6.9, 14.5 and 17.3 (Fig. 3). A number of other phenotypes were observed, including absence of pigment cells and the sensory vesicle, although these phenotypes were not transmitted.

The F2 screen identified 25 F1s carrying potential mutations. Fourteen of these were chosen for further study. To test whether the phenotypes resulted from heritable genetic changes at single loci, an aliquot of the F1 sperm was crossed to wild-type eggs to produce an outbred F2 family (Fig. 2). The F2 larvae were grown to reproductive age as described previously. Because the F1 animals would be heterozygous for any potential mutation, the F2 families were expected to contain a mixture of individuals either heterozygous for the mutation or homozygous wild-type. As an initial test for the transmission of the phenotypes, the F2 adults on the dish (typically 25–50) were spawned together using the light-induced spawning method (see Materials and methods). These progeny (F3s) were then examined for the occurrence of the mutant phenotype at an approximate frequency of 1:16. Of the 14 potential mutants that were chosen for study, seven tested positive in this assay (Table 1). F2s were then crossed pair-wise to identify

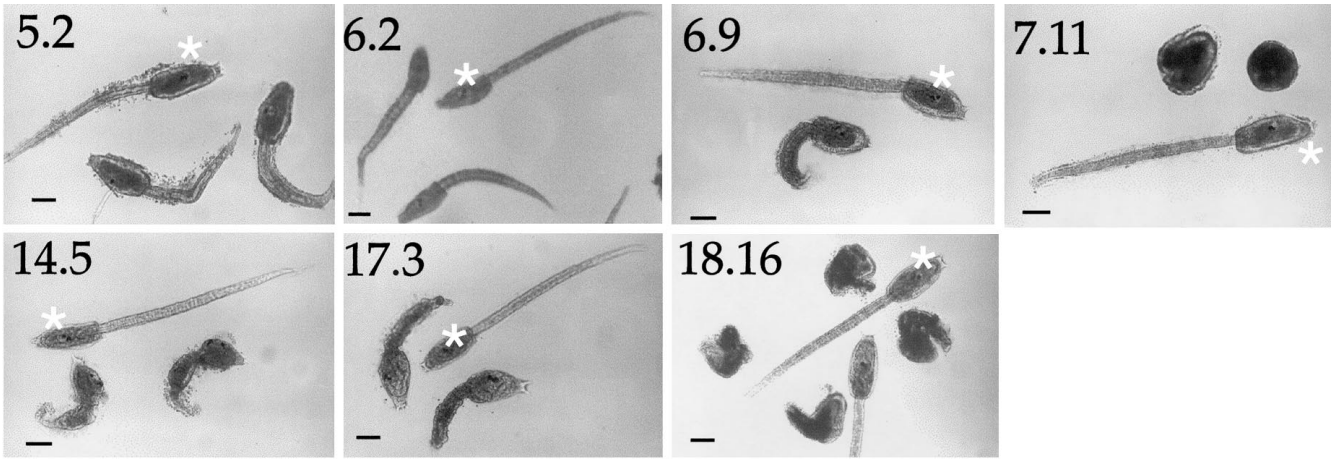

Fig. 3 Mutants isolated in the pilot screen. The mutant tadpoles and their apparently wild-type siblings (*asterisks*) are shown. In the numbering system used, the first number refers to the mutagenized F1 family and the second number is unique to the individual. Tadpoles with mutations 5.2 and 6.2 were observed to have short, thick tails. Tadpoles with the 6.9 mutation have severely truncated tails. Embryos homozygous for mutation 7.11 did not hatch, but had differentiated tissues such as muscle and pigment cells. Tadpoles with the 14.5 mutation had both a shortened tail and a misshapen trunk/head. These embryos did not have an identifiable notochord. Tadpoles with the 17.3 mutation had a truncated tail – although the tail appeared to be longer than that of mutant 6.9 – and they had a kinked notochord phenotype. Finally, embryos with the 18.16 mutation were similar to those with the 7.11 mutation. The tadpole-stage mutants shown for 18.16 were removed from the chorion, unlike the 7.11 mutants shown (*Bars* 100 μm)

heterozygous mutant and homozygous wild-type individuals.

The identified F2 heterozygotes were attached to pieces of nylon screen with thread (see Materials and Methods) and were returned to the raw sea water tanks. Gametes were collected from crossed F2 heterozygotes at approximately 2-week intervals using the light-induced spawning technique, and used to generate F3 embryos for analysis. Additional experiments have focused on mutants 6.9 and 14.5, which were found to have defects in notochord development, along with other defects. Complementation analysis suggests that the two mutations are due to lesions in different loci. A complete report on the development of these two mutants will be published separately (Nakatani et al. 1999).

One concern we had about screening for recessive mutations by self-fertilization was the possibility of uncovering pre-existing mutations. Because the wild population of *C. savignyi* presumably reproduces to some degree by self-fertilization, there is likely to be selective pressure for such self-fertilizations to produce viable offspring. As was stated above, examination of 50 self-fertilized broods from untreated *C. savignyi* uncovered only one potential mutation (not shown). In addition, to control for pre-existing mutations, the parentage of each mutagenized F1 screened was carefully recorded. We reasoned that a mutant phenotype resulting from a pre-existing mutation should appear frequently within a family. Among the 13 families we screened, one family (number 11) was observed to have a consistent and recurring phenotype in the progeny of 5 of the 12 self-fertilized F1s tested (Table 1). It is possible that this phenotype resulted from a preexisting recessive mutation, although it is also possible that a large clonal population derived from an ENU-mutated spermatogonium was responsible. We are in the process of developing an inbred line of *C. savignyi* that will be used for future mutagenesis screens, which should help us to distinguish more readily between induced and pre-existing mutations.

Discussion

C. savignyi can be cultured through multiple generations

Ascidians have many desirable features for genetic analysis. In addition to their close relationship to the vertebrates, ascidians have a small genome that is likely to encode fewer redundant gene functions than do vertebrate genomes; they have fixed cell lineages in early development, and are self-fertile hermaphrodites (Satoh 1994). Over 2,000 species of ascidians have been identified. Members of the genus *Ciona* are particularly attractive because of their short generation time (Berrill 1947; Dybern 1965; Yamaguchi 1975) and the ease with which large numbers of gametes can be collected. A necessary first step in establishing genetic methods for *C. savignyi* was the development of a system for culturing large numbers of animals through multiple generations. Few reports on laboratory culturing of ascidians are available in the literature. Success has been reported in raising *C. intestinalis* (Berrill 1947) and the colonial ascidian *Botryllus schlosseri* (Boyd et al. 1986) in closed aquariums. Although we found that we were able to grow *C. intestinalis* in aquariums, feeding them a mixture of cultured *Nannochloropsis* and *Chaetocerous gracilis* (unpublished), the procedure was laborious and did not seem ideal for culturing large numbers of animals. Instead, we turned to an open culture system to

raise *Ciona*, taking advantage of the raw sea water delivered into the marine laboratories at UC Santa Barbara. This system has proven to be reliable, convenient and economical. By running the sea water through the tanks at a high flow rate (2 l/min) we were able to grow the animals at densities as high as 200 animals in a 15-l tank.

We were able to raise both *C. savignyi* and *C. intestinalis* in our raw sea water system. However, *C. savignyi* were chosen for our mutagenesis studies for a number of reasons. *C. savignyi* were found to be hardier overall, and we were able to maintain adults throughout the year. In addition, adult *C. savignyi* are significantly smaller than adult *C. intestinalis*. The smaller size of the *C. savignyi* was particularly advantageous in establishing conditions that would allow the growth of large numbers of *C. savignyi* at high density.

C. savignyi is amenable to genetic analysis

It proved to be relatively straightforward to adapt the available techniques for mutational analysis to *C. savignyi*. The animals withstood ENU treatment well, and this mutagen produces visible mutant phenotypes that breed true. Controlled self- and cross-fertilizations of *C. savignyi* were easy to perform, either by natural spawning or by in vitro fertilization. The fact that the adults remain attached to petri dishes, or other substrates, makes managing a colony and keeping track of individuals easy. Our ability to self-fertilize *C. savignyi* is a great benefit in screening for recessive mutations. Self-fertilization allowed us to skip an entire generation that would have to be raised in sexually dimorphic animals in order to produce animals homozygous for an induced mutation (Mullins et al. 1994). We found that approximately 40% of *C. savignyi* were capable of self-fertilization (thus, 60% of mutagenized animals were not screened). The block to self-fertilization in *C. intestinalis* has been shown to be at the chorion, and we have found, in agreement with published results, that chemical treatment to remove the chorions renders all eggs self-fertile (Rosati and de Santis 1978). However, we found that the procedure itself produces developmental abnormalities at a sufficiently high frequency as to prevent its use in our screens. We have attempted to produce lines of *C. savignyi* that were completely self-fertile by raising the progeny of a self-fertilization. So far this strategy has not been successful, and the self-fertilized progeny are no more self-fertile than the wild population (not shown).

Our pilot screen of 80 mutagenized F1s resulted in seven mutant lines [although only 14 of 25 potential mutants were tested for transmission when outcrossed (Table 1)]. Five of the seven mutations isolated caused defects that were largely restricted to the development of the tail. We anticipate that a larger screen would result in a broader range of phenotypes. Our pilot screen was too small to provide an accurate estimate of the incidence of zygotic mutations produced by our mutagenesis protocols. As discussed below, our observed rate of about 7–12 mutations per 100 mutagenized genomes probably should be considered to lie at the low end of the range for the true frequency of induced zygotic mutations. This frequency of mutant recovery is lower than has been reported for similar screens in zebrafish (Driever et al. 1996; Haffter et al. 1996). Part of the difference may be due to the fact that we screened for mutations at a single stage (hatched tadpole). Because the ascidian tadpole is so simple, and does not form structures such as the digestive system, heart, and blood until after metamorphosis, it is likely that many mutations were missed in our screen. Fortunately, *C. savignyi* are able to complete metamorphosis in petri dishes within a few days of fertilization, which should allow us to perform more extensive screens in the future. In addition, our mutagenesis protocol, which was based on those used for zebrafish, may not be optimal for *C. savignyi*. Now that we have mutants in *C. savignyi*, it will be feasible to test the efficiency of other mutagenesis procedures, including different doses and times of exposure to ENU, as well as using different chemical mutagens, ionizing radiation, and insertional mutagenesis.

Half of the abnormal phenotypes identified in self-fertilized F2 embryos were found to be heritable. There are various factors that probably account for the failure to transmit many of the phenotypes, the most obvious being that some were not due to genetic changes, but rather resulted from factors such as the age or condition of the eggs. However some of the phenotypes, such as an ocellus/otolith pigmentation phenotype that was seen in exactly 25% of brood 14.14 (Table 1), are likely to have resulted from a genetic change. Perhaps this and other phenotypes depend upon the appropriate genetic background, which might have been present only in self-fertilized F2s, but not in outbred F3s.

Prospects for ascidians in the genetic analysis of chordate development

Ascidians were one of the earliest experimental models used in the study of animal development (Conklin 1905), and through the decades have proven to be a valuable experimental system. The development of genetic methods provides a new tool that can draw from the already extensive knowledge of ascidian development. The goals of the study reported here were to determine the feasibility of genetic screens in ascidians. While the procedures developed here will certainly be refined in the future, we have been able to establish protocols that allow for reliable, large-scale culture of *C. savignyi*, chemical mutagenesis, and identification and propagation of mutants. The unique features of ascidians, such as hermaphroditism, self-fertility, a sessile adult form, and rapid development have facilitated this project. Our long-term goals include large-scale screens for developmental mutants and the identification of mutated genes

responsible for the phenotypes. Again, the special features of *C. savignyi*, such as their relatively small genome, should aid in these projects.

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