THE ROYAL SOCIETY

doi 10.1098/rstb.2001.0919

Developmental genetics in primitive chordates

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Recent advances in the study of the genetics and genomics of urochordates testify to a renewed interest in this chordate subphylum, believed to be the most primitive extant chordate relatives of the vertebrates. In addition to their primitive nature, many features of their reproduction and early development make the urochordates ideal model chordates for developmental genetics. Many urochordates spawn large numbers of transparent and externally developing embryos on a daily basis. Additionally, the embryos have a defined and well-characterized cell lineage until the end of gastrulation. Furthermore, the genomes of the urochordates have been estimated to be only 5–10% of the size of the vertebrates and to have fewer genes and less genetic redundancy than vertebrates. Genetic screens, which are powerful tools for investigating developmental mechanisms, have recently become feasible due to new culturing techniques in ascidians. Because hermaphrodite ascidians are able to self-fertilize, recessive mutations can be detected in a single generation. Several recent studies have demonstrated the feasibility of applying modern genetic techniques to the study of ascidian biology.

Keywords: ascidians; chordates; development; genetic screens; genomic tools

1. ASCIDIAN BIOLOGY AND DEVELOPMENT: AN OVERVIEW OF THE ASCIDIANS

(a) *Ascidians are among the oldest model animals of experimental embryology*

Adult ascidians, or sea squirts (subphylum Urochordata, class Ascidiacea), are sessile filter-feeders that show no obvious evolutionary similarity to their vertebrate cousins. The phylogenetic position of the ascidians as chordates was not proposed until 1866 by Kovalewsky, and was only obvious upon observation of the larvae, which possess the defining chordate characteristics of a dorsal, hollow nerve cord, notochord and tail muscles. By the turn of that century, ascidians were one of the primary model systems in the growing field of embryology. In one of the first studies of ascidian embryology, the teratologist L. Chabry (1887) showed that the disruption of specific blastomeres resulted in the loss of tissue types in the larva. In 1905, the seminal work of Conklin elegantly described the cell lineage of the ascidian embryo in a paper that remains a valuable reference to current researchers (Conklin 1905). Building on Conklin's pioneering work, researchers in the ensuing decades have greatly expanded our understanding of ascidian embryology at the cellular and molecular levels (Di Gregorio & Levine 1998; Nishida 1997).

The ascidians have been used as model animals in the study of determinative development, and the ascidian egg also undergoes extensive cytoplasmic reorganization following fertilization, leading to the segregation of maternal determinants. A recent analysis of cytoplasmic reorganizations in the ascidian *Phallusia mammillata* between fertilization and first division hints at the complexity of this process, and demonstrates distinct microfilament and microtubule-driven phases to the movement of cytoplasm (Roegiers *et al.* 1999). Probably the most studied of the ascidian cytoplasmic determinants has been the myoplasm, which promotes the autonomous differentiation of the primary muscle lineage (Satoh *et al.* 1996). In addition, maternal cytoplasmic determinants have been characterized in ascidians for endoderm (Marikawa & Satoh 1995) and epidermis specification (Ishida & Satoh 1998), as well as for gastrulation movements (Nishida 1996) and asymmetric cleavage (Nishida *et al.* 1999). A number of maternal mRNAs, including the posterior end mark (PEM) genes and *wnt5*, are actively localized to the posterior pole following fertilization and may provide clues to the molecular mechanisms of ooplasmic segregation (Sasakura *et al.* 1998; Satou & Satoh 1997; Yoshida *et al.* 1996). While the ascidians were thought to differ fundamentally from the vertebrates in their reliance on determinative mechanisms, the demonstration of the importance of polarized maternal determinants in *Xenopus* development (King *et al.* 1999), and the characterization of inductive interactions required for the development of notochord, nervous system, trunk lateral cells and mesenchyme in ascidians (Nishida 1997), reinforces the similarities between the chordate subphyla.

(b) *Urochordate genomes are unique among the chordates*

While the field of ascidian genomics is in its infancy, the urochordates are uniquely positioned to provide tremendous insight into chordate genome evolution and organization. Studies of the evolutionary history of chordate genomes indicate that the vertebrate subphylum has

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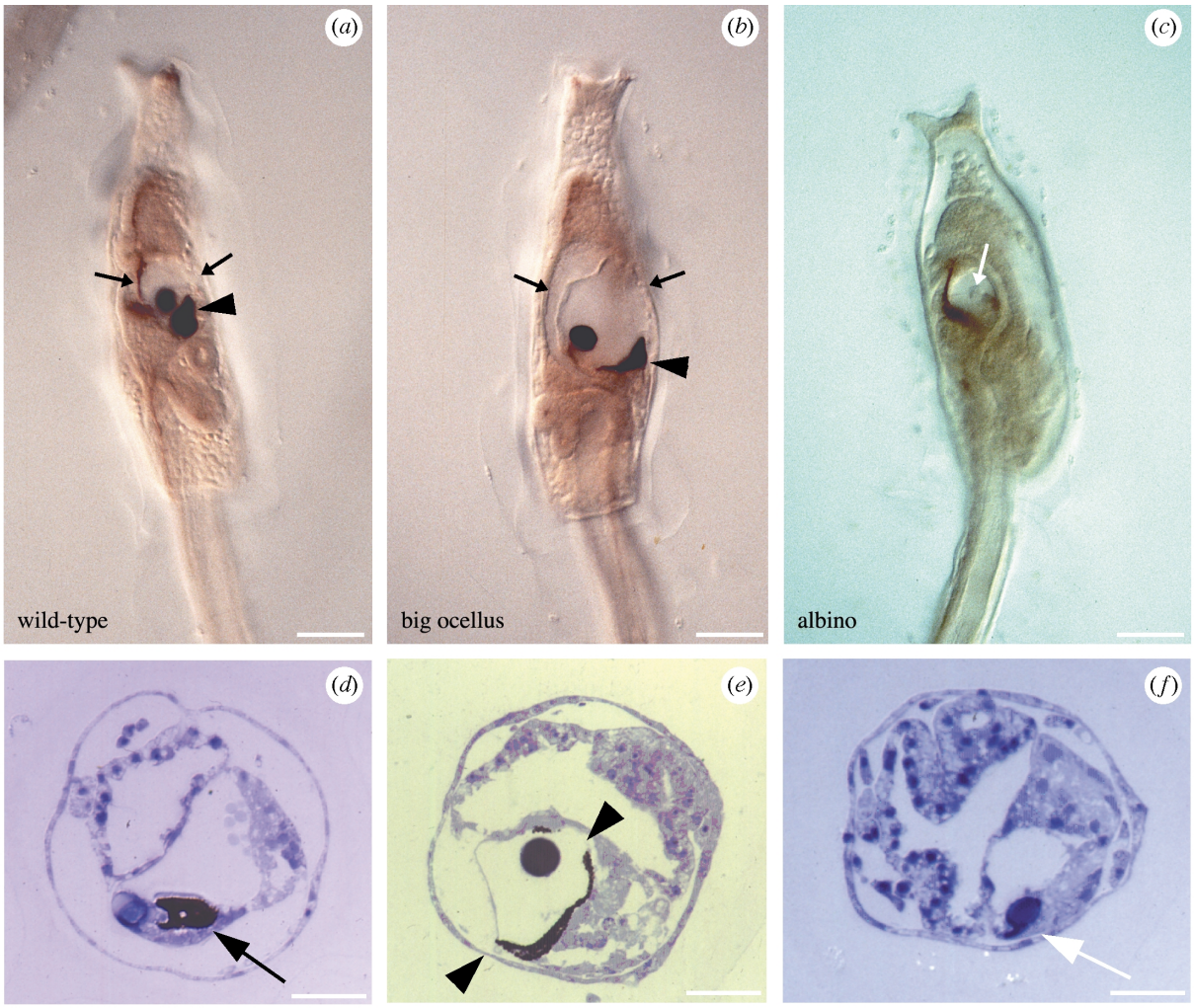

Figure 1. (*a*) Wild-type, (*b*) *bio* and (*c*) *alb C. intestinalis* larvae, which have been labelled with an anti-acetylated tubulin antibody, and (*d–f*) corresponding transversal histological sections across the sensory vesicle. Compared with wild-type larva (*a*), *bio* is characterized by an expanded sensory vesicle (black arrows in (*b*)) with an enlarged ocellus (arrowheads in (*b*) and (*e*)), while *alb* shows no sign of pigmentation in the sensory cells (white arrows in (*c*) and (*f*)). Bars = 50 µm.

undergone two genome-wide duplications since its divergence from the two invertebrate chordate subphyla (Meyer & Schart 1999; Sidow 1996). The consequences of the duplications are evident in the large gene families that are characteristic of the vertebrates. The recurring theme of four members in many gene families, such as in the mammalian *Hox* clusters, appears to be the result of these gene duplications. Following the genome-wide duplications, all possible variations of genomic reorganization appear to have occurred in the various vertebrate classes. These include loss of one or more duplicated genes, divergence of function and/or regulation of family members, and additional duplications both of smaller genomic regions and, as appears to have happened in teleost fishes and some amphibian species, larger-scale duplications, perhaps genome-wide. The genome-wide duplications in the vertebrates provided additional genetic material for natural selection and appear to help account for the tremendous complexity and diversity of the vertebrates. While models predict that duplicated genes should be quickly lost, except in those rare occasions when new divergent functions arise and are selected, examples of redundant and partially redundant gene function in vertebrates abound. Ascidians offer a significant advantage for genomic studies as they are not expected to have duplicated genes—thus having only a simple set of basic chordate genes.

(c) *The promise of ascidian developmental genetics*

Large-scale mutagenesis screens have been extremely useful for identifying developmentally important genes in *Drosophila* (Nüsslein-Volhard & Wieschaus 1980) and *Caenorhabditis elegans* (Brenner 1974), and more recently in vertebrates such as zebrafish (Mullins *et al.* 1994; Solnica-Krezel *et al.* 1994). Genetic screens are beginning to show promise in ascidians as well. Among the three ascidian species most extensively studied in developmental genetics, namely *Ciona intestinalis*, *Ciona savignyi* and *Halocynthia roretzi*, only the *Ciona* spp. are well suited for genetic screens. Large-scale genetic screens are impracticable in *H. roretzi* due to its limited geographical distribution, short spawning season (October to February), and long generation time. On the other hand, *C. intestinalis* and *C. savignyi* offer numerous advantages for mutant

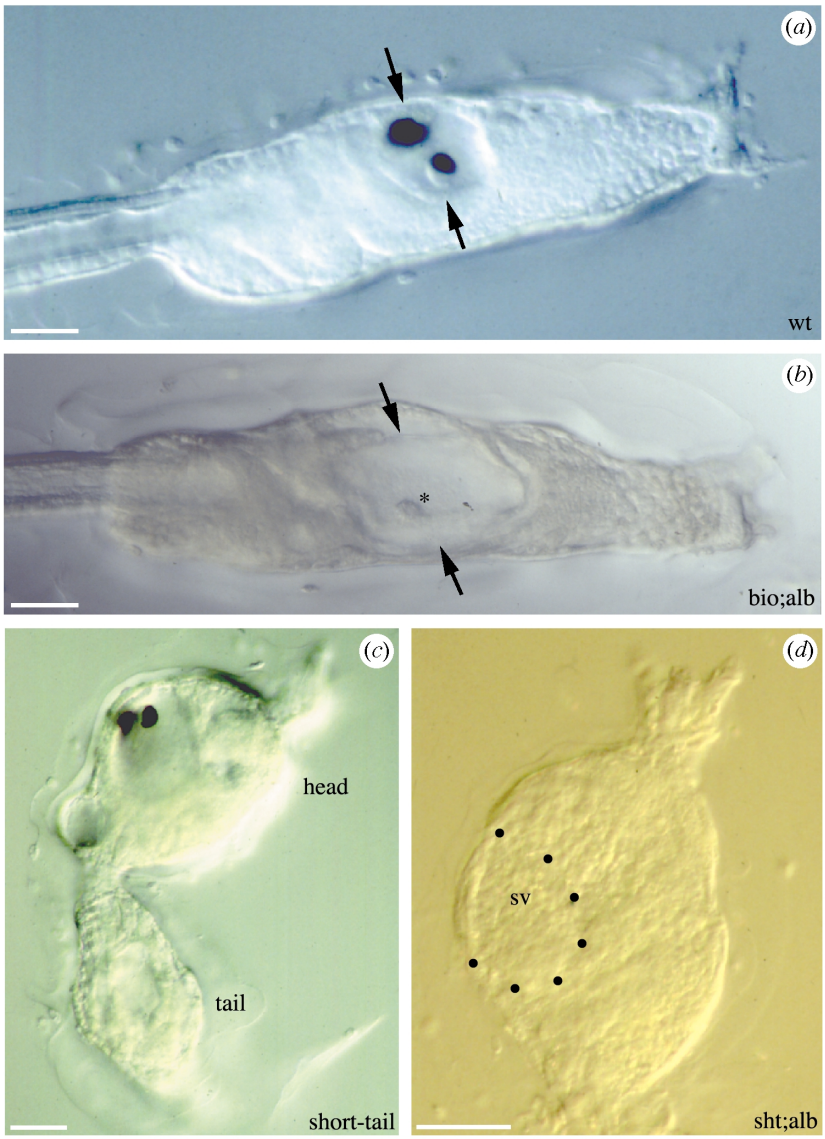

Figure 2. (*a*) Wild-type, (*c*) single and (*b*,*d*) double-mutant *C. intestinalis* larvae. (*a*) A strong expansion of the sensory vesicle (arrows in (*a*) and (*b*)) and lack of melanin are observed in *bio*;*alb*. (*b*) An asterisk indicates the unpigmented otolith. (*c*) *Short-tail* (*sht*) is an embryonic mutant phenotype showing severely affected morphogenetic movements. (*d*) A *sht* phenotype missing pigmentation in the sensory vesicle of the double homozygote *sht*;*alb* mutant (sv, outlined with dots). Bars = 50 µm.

analysis, including a wider distribution, two to three month generation times in captivity, and year-round breeding. Larvae from the solitary ascidian genus *Ciona* have a typical ascidian tadpole anatomy with six developed organ systems (adhesive organ, epidermis, tunic, tail musculature, notochord and nervous system) and four organ system rudiments (primordial pharynx, atrial primordia, gut and mesoderm). *Ciona* embryos develop quickly and are transparent, aiding in screens for mutant phenotypes. In addition to the desirable features of ascidians listed above (small genome, simple morphology, etc.), one more feature aids in mutagenesis screens: *C. intestinalis* and *C. savignyi* are hermaphrodites with the ability to self-fertilize. We have exploited this feature to generate homozygous mutant embryos in two generations, whereas either three-generation screens, or manipulations to generate haploid or gynogenetic embryos, are necessary for other chordate systems. Finally, the relatively small genome of ascidians (180 Mbp) compared with the higher chordates should greatly aid in efforts to map and isolate genes responsible for mutant phenotypes.

Progress has been made in developing genetic methods for both *C. savignyi* and *C. intestinalis*, and each species has its unique advantages. Pursuing research on both of these ascidians will be beneficial, particularly since efforts are being made to assign ancestral or basal functions to genes in these urochordates. In addition, recent advances in high-throughput genetic methods reduce the technological hurdles so that it is not necessary to concentrate all efforts on a single species. Finally, it has been reported that *C. savignyi* and *C. intestinalis* can cross-fertilize after removal of the chorion, although it is not known if the hybrids are viable (Byrd & Lambert 2000). If the hybrids do prove to be viable, the polymorphisms likely to exist between these two species could be valuable in future efforts to map mutations.

2. DIPLOID GENETIC SCREENS

(a) C. intestinalis

Located on the northern side of the Gulf of Naples, in southern Italy, the A. Dohrn Zoological Station offers several advantages for ascidian research, and *C. intestinalis* has been studied here for more than a century. A large ascidian culture facility will soon replace the small, successful pilot *Ciona* culture system developed by our colleagues, Alfonso Toscano and Paola Cirino. We have implemented reliable methods for raising continuous generations that have made our preliminary genetic screens possible. Both old and new facilities are based on an open system of filtered seawater, which continuously flows at a rate of one tank every 2 h.

Wild-type or heterozygote carrier animals that are raised in the facility produce consistently fewer oocytes than wild-caught individuals. Nonetheless, perhaps due to the stable conditions in the seawater system, or because of the constant and steady supply of food, the reproductive lifespan of the cultured *C. intestinalis* is longer than that of the wild animals. In terms of embryo supply, this feature compensates for the sudden declines that periodically occur in the natural populations of *C. intestinalis* in the Gulf of Naples, which often cause severe delays in experiments.

To identify genes that control developmental processes, we performed a set of small-scale zygotic mutagenesis screens, with the aim of generating mutant phenotypes. Adult wild-caught sea squirts were subjected to three 1 h incubations in a seawater solution containing different concentrations of *N*-ethyl-*N*-nitrosourea (ENU), with a 3 day recovery time between treatments. Mutagenesis caused by the alkylating agent ENU introduces random base-pair substitutions. In order to eliminate post-meiotically mutagenized sperm, which generate genetically mosaic progeny, treated animals were allowed to spawn repeatedly for three weeks, the approximate time required for spermatogonia to mature. Wild-type eggs were then fertilized with ENU-treated sperm, and the offspring grown to maturity.

Exploiting their natural ability to self-fertilize, F1 hermaphrodite animals are self-crossed, and the resulting F2 progeny scored for mutant phenotypes at various developmental stages (Sordino *et al.* 2000). To induce natural spawning and self-fertilization, wild-caught and laboratory-raised *C. intestinalis* adults are kept in continuous light for 3 days and then moved to dark boxes where simultaneous gamete spawning usually occurs within 1 h, with typical brood sizes of several hundred eggs. Before the next treatment, light-shocked animals return to a normal day–night cycle for at least one week. For outbreeding, gametes are collected by dissection in separate samples, and then larvae are allowed to settle and metamorphose in 15 cm Petri dishes, where young ascidiae remain until one week old. At this point, the Petri dishes are transferred to tanks where they are suspended upside down or vertically. All developmental stages are continuously supplied a mixture of dried microalgae and liquid food. In these conditions, an optimal number of about 500 *C. intestinalis* can be grown up to sexual maturity in three months in a 100 l tank (A. Toscano & P. Cirino, unpublished data).

During a pilot mutagenesis screen, we have analysed 62 potential F1 founders, which were derived from treatments with 2.7, 1.35 and 0.27 mM ENU. As expected, the frequency of induction of mutations is a sensitive function of ENU dosage. Indeed, the percentage of lethal early defects was directly related to ENU concentration while, conversely, the highest incidence of reproducible tissue-specific phenotypes was observed using the lowest concentration. The ENU-induced developmental phenotypes fell into several categories. Mutations affecting metamorphosis were mainly heterochronic, consisting of a prolonged larval stage, or characterized by reorganization and rotation of the endoderm that was not paralleled by tail resorption. Specific alterations of tail, head and nervous system formation, affecting tissue differentiation or morphogenetic processes, characterized other mutant phenotypes (P. Sordino, unpublished data). Two examples of recessive larval mutations are *big ocellus* (*bio*) (figure 1*b*,*e*) and *albino* (*alb*) (figure 1*c*,*f*). Both mutants were identified during the 0.27 mM ENU screening. They segregate in a Mendelian fashion and have now been transmitted through six generations. In *bio* mutant larvae, the most anterior part of the central nervous system, the cerebral vesicle, is greatly expanded (figure 1*b*,*e*). This phenotype does not seem to result from an increased proliferation or reduced apoptosis of neuroectodermal cells (data not shown), and it becomes morphologically evident at late tailbud stage. The ocellus, a larval sensory cell, increases in size, lining the dorsal part of the sensory vesicle. The *bio* mutant larvae undergo the initial steps of metamorphosis, with tail resorption, but die shortly thereafter. The *alb* mutant is characterized by the absence of pigment synthesis within the otherwise normally differentiating sensory organs, ocellus and otolith (figure 1*c*,*f*). Future experiments will address the cell autonomy of these defects.

In order to test ENU mutagenic rates for the *bio* locus, we crossed pre-meiotically mutagenized sperm with *bio* mutant eggs. Hit frequencies were 10% with 0.027 mM ENU and 20% with 0.135 and 0.27 mM ENU. These results are significantly higher than those obtained with similar treatments in zebrafish and mouse (Mullins & Nüsslein-Volhard 1993). One explanation could be that, unlike in other chordates, ascidian gonads are directly exposed to the mutagen. Compared with *C. intestinalis*, the species *C. savignyi* seems to be more resistant, as higher ENU concentrations are needed for efficient mutagenesis rates (Moody *et al.* 1999). However, more recessive alleles must be analysed in order to understand the mutagenic efficiency of ENU over the *C. intestinalis* genome.

Generation and description of multiple developmental phenotypes corresponding to combinations of heterozygosity and homozygosity represents an invaluable source of information regarding gene interactions. As shown in figure 2, classic diploid breeding schemes allowed us to obtain double homozygous mutants, demonstrating that the genome of *C. intestinalis* can carry more than one recessive allele (P. Sordino, C. P. Heisenberg, P. Cirino, R. Marino and R. De Santis, unpublished data).

Ciona spp. sperm can be readily collected and cryopreserved indefinitely as a backup for genetic stocks. Storage of frozen sperm allows us to preserve important lines and to save facility space. Sperm are collected from

the sperm duct with a tip-flamed Pasteur pipette into a microfuge tube on ice. Twenty microlitres of sperm are added to Millipore-filtered seawater (22 µm) containing 10% dimethylsulphoxide. The sample is frozen stepwise and has to be gradually thawed immediately before use.

(b) C. savignyi

An essential first step in developing genetic methods in ascidians was establishing conditions that would allow for the continuous culturing of animals through multiple generations. We tested a number of ascidian species including *C. intestinalis*, *Styela plicata* and *Clavelina huntsmani*, and found that *C. savignyi* was ideal in terms of hardiness, reliability and ease of culturing. The culturing procedure that we have developed, which has worked successfully and reliably for several years now, uses unfiltered seawater as a food source for the animals. The University of California at Santa Barbara is located on a promontory of the California coast and has extensive marine biology facilities. Unfiltered seawater is delivered to laboratories via an off-shore intake pipe. We have found that the intake seawater alone contains sufficient microalgae (the primary food source for ascidians) to support rapid and year-round growth. Animals are grown attached to 15 cm Petri dishes that are suspended in 260 l tanks (figure 3). Seawater is passed through the tank at *ca.* 8 l min^{-1} (or one tank volume every half hour). This high flow rate has allowed us to culture *C. savignyi* at densities as high as 600 adults per tank. We have reasoned that a high flow rate would be beneficial both for maximizing growth rate by ensuring that water was not depleted of algae, and for maintaining the health of the animals by rapidly flushing wastes and parasites and pathogens.

In a pilot study we screened for induced point mutations that generate identifiable zygotically acting recessive phenotypes. In brief, sperm collected from ENU-treated adults were crossed with wild-type eggs to generate heterozygous F1 individuals. At maturity, the F1s were self-fertilized to generate F2 broods that were screened for visible mutations at early-hatched tadpole stage (*ca.* 20 h post-fertilization). Sperm from each F1 was kept frozen, and if the corresponding F2 brood contained a potential mutation of interest, the F1 sperm was used to fertilize wild-type eggs to generate an outcrossed line.

Our ENU mutagenesis pilot screen consisted of examining the self-fertilized progeny (F2 broods) from 80 heterozygous F1s for developmental defects. Of the F2 broods, we categorized 25 as having potential mutations. From this initial collection of mutant lines, three showed penetrant defects in axial structures and were particularly interesting to us. Two of these mutants, 6.9 (also known as *chobi* (*chb*)) and 14.5 (also known as *chongmague* (*chm*)) have already been partially characterized and shown to have defects in notochord development (Nakatani *et al.* 1999). The other mutant, 17.3, has been shown to breed true when outcrossed, although it has not been extensively characterized. Although all three mutants have truncated tails, their phenotypes are distinct, and all three fall into different complementation groups. Of the three mutants, *chm* appears to disrupt notochord development the earliest. The presumptive notochord cells fail to take on the characteristic cuboidal shape and do not converge towards the midline (Miyamoto & Crowther 1985). In some respects, *chm* resembles the zebrafish *no tail* (*ntl*) mutation, which is in the *brachyury*/*T* gene (Halpern *et al.* 1993). Neither *chm* nor *ntl* causes a distinct fate change in notochord cells, such as is seen in the notochord-to-muscle fate change in the *floating head* mutation of zebrafish. Instead, the cells become mesenchyme-like in both *chm* and *ntl*. In addition, the defects in both *chm* and *ntl* are restricted largely to the notochord, and both mutants initiate *brachyury* expression, indicating that the earliest steps in notochord induction are not disrupted. We have speculated that *chm* may result from a mutation in the *brachyury* gene itself, or some other gene that acts in parallel with, or is downstream of *brachyury*. Ongoing experiments will determine if the *chm* mutation can be rescued by various candidate genes, including *brachyury*.

The other *C. savignyi* notochord mutant that has been partially characterized is *chb*. *Chb* embryos appear indistinguishable from wild-type until mid-tailbud stages, and early stages of notochord development such as the initial induction, *brachyury* expression and intercalation appear to occur normally. However, by late tailbud stages the tails of *chb* embryos are much shorter than wild-type embryos. The tail truncation, as with *chm*, appears to result from a problem with notochord morphogenesis. We have examined notochord structure in *chb* embryos both by following expression from a *brachyury* promoter-lacZ construct, and by labelling specific notochord lineages at the 64-cell stage with the lipophilic dye DiI. Both approaches show that the notochord of *chb* embryos is kinked and that the cells are often piled together, rather than forming a column. We speculate that the defect in *chb* embryos affects the ability of the notochord to form a rigid column. The cell movements of the notochord generate the primary force that elongates the ascidian body axis. A portion of the force is generated when the cells intercalate, but the majority of the force that extends the tail during tailbud stages is generated as the cells produce the matrix-filled vacuoles. We speculate that it is this later stage in notochord morphogenesis that is disrupted in *chb* embryos. Either the notochord in the mutant embryos does not generate sufficient force to elongate, and instead becomes folded and kinked, or the notochord may generate sufficient force, but a defect reduces its rigidity. A defect in the notochordal sheath, which surrounds and constrains the diameter of the notochord, may result in the notochord bending and crumpling rather than extending properly. These later stages in notochord morphogenesis are poorly understood, and thus we are particularly interested in understanding better the nature of this mutation.

3. MATERNAL FACTORS REGULATE MULTIPLE EARLY EVENTS IN ANIMAL DEVELOPMENT

Screens for maternal-effect genes in the invertebrate model animals *Drosophila* and *C. elegans* have demonstrated the key roles played by maternal factors in a myriad of early developmental events ranging from body-axis polarity and asymmetric cell divisions to establishment of the germ line and the *C. elegans* 'founder cells' (Bowerman 1998; St Johnston & Nüsslein-Volhard 1992). The role of maternal factors in vertebrate development has been most

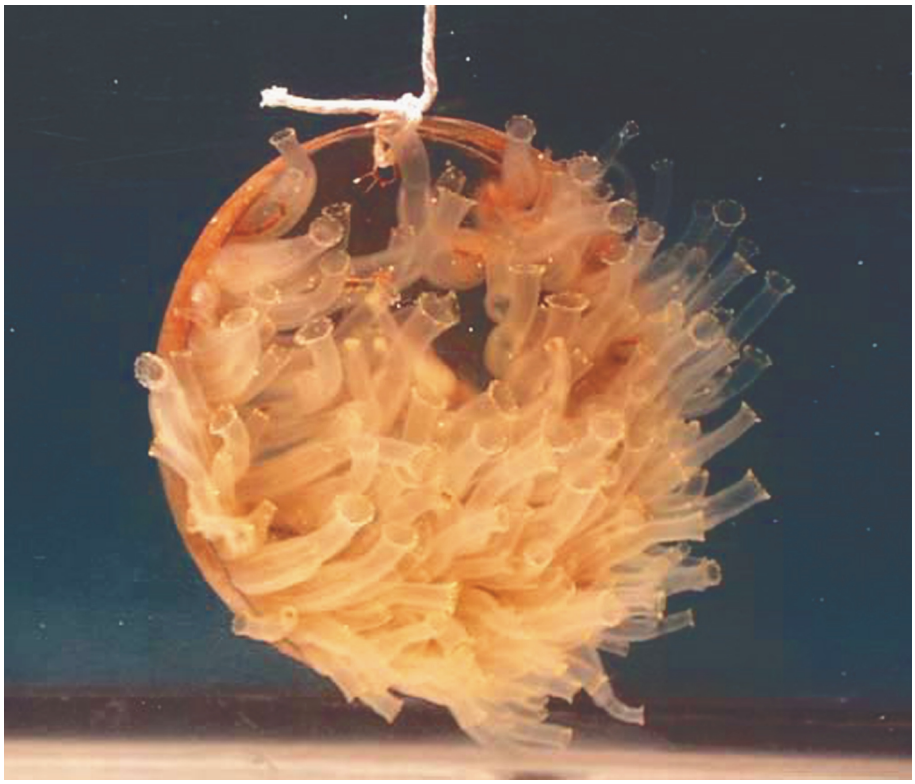

Figure 3. Adult *C. savignyi* cultured on 15 cm Petri dishes. *C. savignyi* larvae are placed in Petri dishes and allowed to attach to the bottom and sides. The dishes are then suspended in 260 l tanks with running seawater. The animals reach reproductive age in eight to ten weeks.

extensively studied in *Xenopus*. A number of maternal mRNAs are known to be localized to distinct regions of the *Xenopus* egg (King *et al.* 1999). Efforts are ongoing to isolate and characterize the cytoplasmic determinants in ascidians using biochemical and molecular cloning techniques. The development of forward genetic methodologies offers new possibilities for characterization of maternal-effect genes in ascidians. The difficulties of generating and propagating mutants in chordates are amplified when considering maternal-effect genes. However, several of the unique properties of ascidians, particularly their abilities to generate both eggs and sperm and to self-fertilize, facilitate procedures for screening and recovering maternal-effect mutants.

Large-scale screens for maternal-effect mutations are underway in zebrafish, and the results of a pilot screen have been reported (Pelegri *et al.* 1999; Pelegri & Schulte-Merker 1999). This screen has used artificial gynogenesis to produce homozygous mutagenized females. The early pressure procedure used to induce gynogenesis suffers from a number of shortcomings, including low viability of the F2s, and variable degrees of homozygosity. The early pressure blocks cytokinesis at meiosis II, resulting in diploid oocytes that are then activated with UV-irradiated sperm. Because the oocytes have completed meiosis I, recombination between homologous chromosomes introduces the variability in homozygosity such that loci proximal to the centromere show the highest probability of being homozygous in the gynogenic F2s (approaching 50%), while those distal to the centromere show the lowest probability (approaching 0%). Thus, the screen is biased towards loci that are most tightly linked to the centromere. Our maternal screen strategy does not suffer from this bias (§3a) and, theoretically, all loci should show the same probability (25%) of being homozygous in the F2 adults. The second difficulty with the published zebrafish protocol is the difficulty in recovering mutations, since the F3 embryos are likely to be inviable. The current strategies require both recovery of the mutation through either parental F1 females, or F2 siblings and require maintenance of large stocks of animals and extensive crosses to recover the mutation. Because *C. savignyi* are hermaphrodites, we will be able to recover the mutation by outcrossing the sperm from the F2 to wild-type eggs. The only potential difficulties in recovery could be due either to dominant mutations, which are unlikely since the F1s would be expected to be inviable, or paternal lethals, which are expected to be extremely rare.

(a) *Pilot screen for maternal-effect mutations*

A pilot screen has been undertaken by the laboratory of W.C.S. to assess the feasibility of generating maternal-effect mutations in *C. savignyi*. Our experimental approach involves generating homozygous F2 hermaphrodites that are screened for maternal-effect mutations, and so far this method appears promising. The protocol used in this screen is shown diagrammatically in figure 4. As with the zygotic screen in *C. savignyi*, ENU was used to mutagenize adults. Sperm from ENU-treated animals were crossed with wild-type eggs. In brief, the screen takes advantage of the fact that *C. savignyi* has the ability to self-fertilize to generate homozygous adults in only two generations. Because *C. savignyi* is hermaphrodite, we are able use a single animal both to screen for maternal effects using the

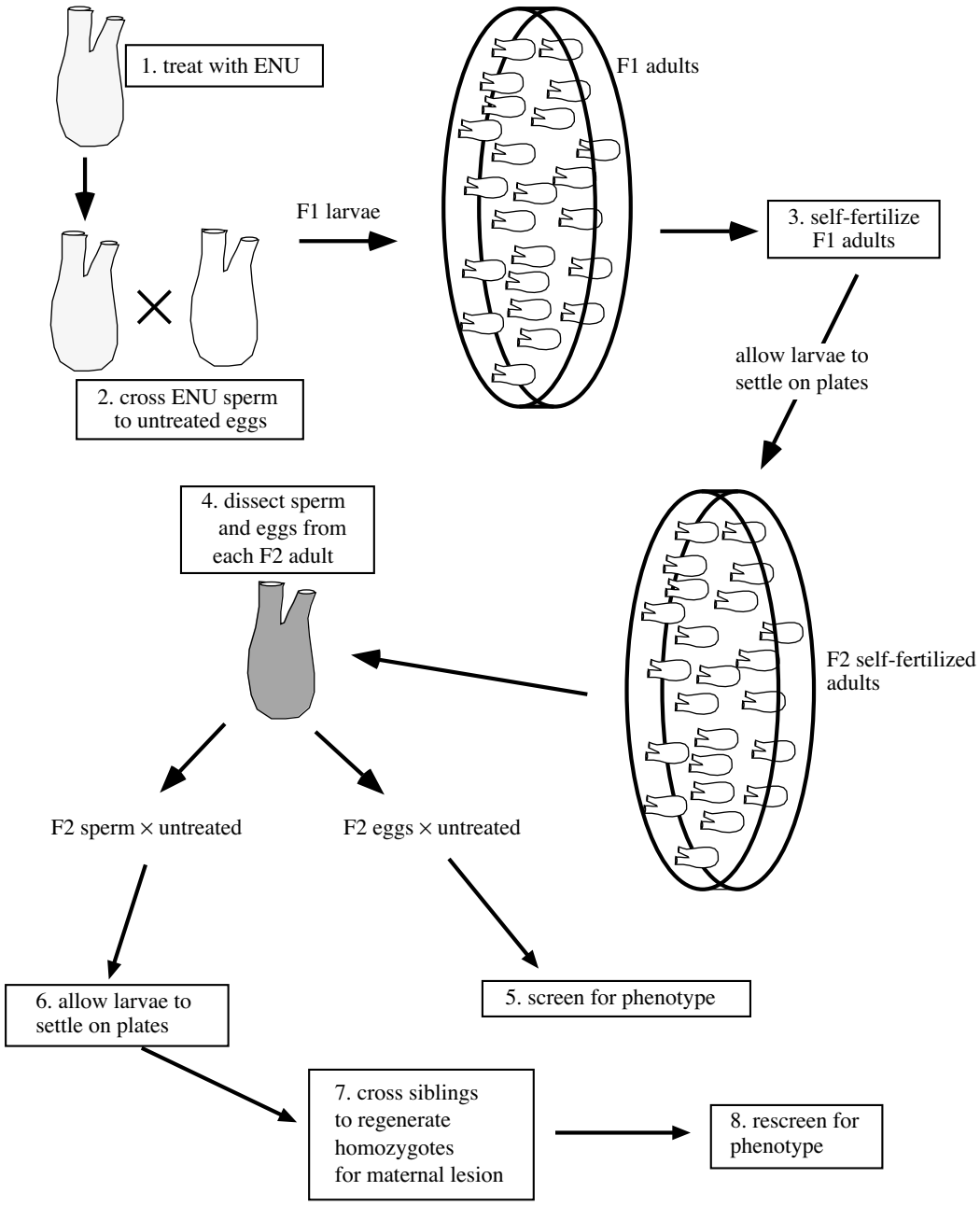

Figure 4. Strategy for isolation of maternal-effect mutations.

egg and to recover and propagate any potential mutations using the sperm. In the pilot screen, we have examined the progeny from crosses between the eggs from 75 ENU-treated, self-fertilized F2s and wild-type sperm. The embryo broods were allowed to develop to an age equivalent to that of early-hatched tadpoles, at which point they were examined for obvious abnormalities. Of the 75 broods examined, 54 (72%) had over 90% normal-appearing tadpoles and were eliminated. The 21 remaining broods had varying percentages (20–100%) of tadpoles showing abnormalities. For penetrant mutations, we would expect 100% of the embryos in the brood to show a similar phenotype. Sixteen of the broods showed abnormalities in more than 90% of tadpoles. Of these, two showed zero fertilization. Eleven broods had what we referred to as 'early arrest' and showed no obvious signs of gastrulation or hatching, and three broods had 'axial defects' and showed either shortened or absent tails. Finally, among the remaining five broods that showed abnormalities in lower percentages, a similar set of phenotypes was observed. The difficulty with analysing these broods is determining which abnormalities were due to induced mutations, which might be pre-existing mutations, and which may be non-heritable or environmental abnormalities. As discussed in §4b, we are currently developing inbred lines of *C. savignyi* that have very low levels of background abnormalities for use in future screens and which should largely eliminate uncertainty about the origin of observed abnormalities.

The definitive test concerning whether these phenotypes are the products of maternal-effect mutations will come when we determine if these phenotypes can be recovered in successive generations. The sperm from each of the F2s carrying candidate maternal mutations was

used to fertilize wild-type eggs to generate an outcrossed, heterozygous family (figure 4). Members of this family were then crossed to regenerate homozygotes. At the time of writing, this generation had just reached maturity, and eggs are being tested for the recovery of mutations by crossing to wild-type sperm. This procedure has required four generations in order to test for recovery of the candidate mutations, and has been underway for over 10 months. It is important to stress that the *C. savignyi* lines that we have identified so far are only candidates, and as we have observed in a zygotic screen (Moody *et al.* 1999), many candidates may not breed true.

4. PROSPECTS FOR ASCIDIAN DEVELOPMENTAL GENETICS

If we consider the acceleration that has characterized genetics and genomics of ascidians in the last few years, it is tempting to predict that a wide range of genomic tools, such as those framed in this section, will soon be readily available.

(a) *Expressed sequence tags (EST) databases*

Although a complete and annotated genome sequence from an ascidian would be immensely valuable in the study of chordate genome evolution and for mapping mutant loci, an alternative approach that has proven very useful in other organisms has been to focus sequencing efforts on transcribed regions of the genome through the partial sequencing of cDNAs (i.e. ESTs). For ascidians, the potential returns from large-scale EST projects are particularly high. First, because the ascidian genome is expected to contain approximately one-quarter of the number of genes of vertebrate genomes, the number of cDNAs that need to be sequenced in order to obtain a good representation of transcribed regions is much smaller. Furthermore, it is expected that the reduced number of genes in ascidians relative to the vertebrates will be reflected primarily in a reduction in the number of members within gene families, and not in fewer overall gene families. Thus, one can expect from random sequencing of ascidian cDNAs to get a higher frequency of unique classes of genes, as opposed to new variants within gene families. Finally, data from ascidian EST projects will provide a wealth of information on chordate genome evolution. The genome-wide duplications that have occurred in vertebrates provided new starting material for evolution and have probably contributed to the large gene families of vertebrates. Although ascidians have not stopped evolving since their split with the vertebrates, the fact that they have not undergone the genome-wide duplications is likely to have put different selective pressure on their single copy of genes in comparison with the multicopy genes in vertebrates. Thus, both the sequence and function of ascidian genes may provide insights into the ancestral roles of these genes in the common chordate ancestor.

Research tools in the form of sequence information provided by ESTs will also help drive efforts in ascidian biology forward in many ways. While the list of known and sequenced ascidian genes isolated by homology, expression cloning, or differential expression techniques, is growing, the total number of genes in the databases is much smaller than for other chordate model organisms. EST projects promise to provide a flood of new sequence information. As experimental tools, the ESTs will provide probes to study expression levels of genes in both wild-type and mutant embryos either by conventional techniques, or by arrayed gene chips. When the EST projects are coupled with *in situ* hybridization screens, new molecular probes for investigating spatial regulation of gene expression and embryonic pattern formation will become available. Finally, the ESTs will be of great value for genetic studies. Polymorphisms in untranslated regions of the ESTs can be mapped through meiotic recombination to provide both genetic markers and candidate genes to test for mutation or rescue.

Several ascidian EST projects are currently underway. A project at Kyoto University led by Dr Kazuhiro W. Makabe is sequencing 5′ and 3′ ends from unfertilized eggs of *H. roretzi*, as well as performing whole-mount *in situ* hybridization to examine expression patterns. Several thousand clones have already been sequenced, and detailed information is available on the Internet (http://www.genome.ad.jp/magest). An analogous, intensive effort for *C. intestinalis* is led by Professor Noriyuki Satoh at Kyoto University (http://ghost.zool.kyoto-u.ac.jp/). Similarly, two more *C. intestinalis* EST projects are underway at the Marseilles Institute of Developmental Biology (IDBM, France), led by Dr Patrick Lemaire, and the University of Padova (Italy), led by Professor Giorgio Valle in collaboration with R.D.S. These projects aim to sequence cDNAs from, respectively, gonads and larvae. A collaborative EST project between the University of California at Santa Barbara (W.C.S.'s laboratory) and Stanford University (Arend Sidow's laboratory) is sequencing cDNAs from embryonic stages of *C. savignyi*. At the time of writing, approximately 2500 tailbud-stage *C. savignyi* cDNAs have been sequenced at the termini. Future efforts with the *C. savignyi* EST project will focus on additional embryonic stages, including gastrula and neurula.

(b) *Polymorphic strains and meiotic maps*

Mutational screens so far have been performed on wild-caught ascidians. The most pressing need currently is for laboratory strains of ascidians that have desirable features (e.g. free from obvious recessive mutations, rapid growth in culture, high frequency of self-fertilization), as well as developing two or more strains that are highly polymorphic. The success of mapping efforts will improve with greater degrees of polymorphism between the lines, although the rate of meiotic recombination in *Ciona* is an important variable for which there is currently no information.

For the same purpose, projects aimed at developing polymorphic inbred lines of *C. intestinalis* and *C. savignyi* are currently in progress in Santa Barbara, Naples and Kyoto. The ability of *Ciona* spp. to self-fertilize could help reduce the number of generations required to make the lines isogenic. However, experience in all three projects has revealed reduced viability upon inbreeding. Indeed, self-fertilized lines have a very high mortality rate as juveniles, and those individuals that are viable typically grow much more slowly than outbred (wild) animals.

Initial lines proved to be unacceptably slow growing after three generations of self-fertilization.

In the case of *C. savignyi*, a new inbreeding project is underway using additional founder animals (more than 60) from both southern California and Puget Sound (Washington State). Random amplified polymorphic DNA (RAPD) analysis is used both to assess the success of inbreeding and to examine the degree of polymorphism among wild *C. savignyi* populations. The results on polymorphism with a limited set of oligonucleotide primers have been encouraging, showing extensive polymorphism between animals collected at various locations in southern California and even greater polymorphism between Japanese and Californian *C. savignyi*. Once polymorphic strains are established, the goal is to construct a meiotic map of the *C. savignyi* genome with 300–500 single-strand conformation polymorphism markers (Orita *et al.* 1989) generated from 3′ untranslated regions of ESTs. We reason that mapped ESTs will be much more useful than mapped anonymous markers, such as amplified fragment length polymorphisms (AFLPs), RAPDs or microsatellites, for mapping mutations. The mapped ESTs may also provide insight into the evolution of chordate genomes by pointing to regions of synteny between the invertebrate and vertebrate subphyla.

Aimed at establishing less polymorphic, genotyped, clonal strains of *C. intestinalis*, two projects are in progress in Naples and Kyoto. Five polymorphic microsatellite sequences have been isolated in the genome of *C. intestinalis* from three populations in the Gulf of Naples. These microsatellites were able to detect high levels of genetic polymorphism, revealing 28 alleles in 60 individuals. Microsatellite and RAPD markers are now tested for their use in the establishment of clonal strains (Procaccini *et al.* 2000). At the University of Kyoto, characterization of genetic polymorphism in seven natural populations in Japan has represented the foundation for the establishment of an inbred strain, whose heterozygosity is surveyed by RAPD markers (Kano *et al.* 2000).

(c) *Insertional mutagenesis and transgenic procedures*

Our ability to generate and propagate mutant lines in ascidians opens up new opportunities to study the embryology and physiology of these primitive chordates. However, unless methods can be developed that allow for cloning of mutated genes, the mutant lines are of limited value. Recent experiences in zebrafish genetics provide a model for how to proceed with this challenging problem, and clearly show that no single approach is appropriate for all situations. Perhaps the most direct way of linking phenotype to genotype is via insertional mutagenesis, which allows for the direct cloning of flanking genomic DNA, and the (usually) straightforward identification of the disrupted gene. In insertional mutagenesis, the mutagen is exogenous DNA. Although inserted DNA is a less efficient mutagen than ENU, it serves as a molecular tag to aid in the isolation of the mutated gene by facilitating the sequencing of the mutagenized gene. For this purpose, insertional mutagenesis tests in Nori Satoh's laboratory at Kyoto University are surveying rates of integration and transmission of transposon-based transgenic vectors (S. Chiba and N. Satoh, personal communication).

Insertional mutagenesis strategies in vertebrates have suffered from low mutagenesis rates (Amsterdam *et al.* 1999), requiring that many animals be raised and screened for each mutant recovered. Thus, chemical mutagenesis remains the strategy of choice when seeking to recover mutants at a high frequency, or when hoping to approach saturation. In zebrafish, a number of chemically mutated loci have been identified by examining likely candidate genes (e.g. Kishimoto *et al.* 1997; Schulte-Merker *et al.* 1997), although a growing list of mutant loci has been identified by mapping linked markers such as RAPDs and AFLPs followed by chromosome walking (e.g. Donovan *et al.* 2000; Zhang *et al.* 1998). It is this later step, chromosome walking, that is particularly labour-intensive and time-consuming. The development of zebrafish genomic tools such as meiotic maps of cloned genes, ESTs and anonymous markers (e.g. RAPDs and AFLPs), large insert genomic libraries, and radiation hybrid panels are valuable tools for gene mapping (Talbot & Hopkins 2000). A fully sequenced genome will of course be the greatest asset in mapping mutations. Two *C. intestinalis* genome projects have recently been announced in Japan and the United States (N. Satoh and M. Levine, personal communications).

Conventional transgenic procedures have had a tremendous impact upon our comprehension of the regulation of gene expression. Although transient expression of electroporated transgenes is routinely and efficiently performed in ascidians, no stable transgenic lines, such as ones with fluorescent markers or inducible vectors, have been reported yet. To this aim, we have embarked on tests to explore the possibility to integrate microinjected or electroporated transgenes in *C. intestinalis* and *C. savignyi* genomes. In this context, promoters that drive expression in germ cells, such as *vasa* (Fujimura & Takamura 2000) could enhance transgene transmission through the germ line.

5. CONCLUDING REMARKS

The next few years will be crucial in developing the full genetic potential of ascidians for studies of vertebrate origins and chordate developmental genetics. The embryological and genetic simplicity of ascidians may be key for understanding complex vertebrate features and for obtaining insight into the evolution of the chordates. One of the key limitations of the use of ascidians genetically is the requirement that laboratories be in close proximity to the ocean to have access to running seawater. Although there is no fundamental reason why *Ciona* could not be raised in closed aquaria, such a system has yet to be developed and it would probably be costly and labour intensive. Nevertheless, the wide distribution of *Ciona* may favour the establishment of integrated networks among laboratories exploiting the system with modern tools, thus making it a competitive model. The time seems to be ripe to move ahead in ascidian developmental genetics.

We thank P. Cirino, S. Chiba, S. Kano, A. Toscano, P. Lemaire, M. Levine and N. Satoh for kindly sharing unpublished information. P.S. received support from the European Commission, the European Molecular Biology Organization, The Royal Society and the Company of Biologists. Work on *C. savignyi* is supported by grants from the US National Institutes of Health and the National Science Foundation to W.C.S.

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