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Analyzing gene regulation in ascidian embryos: new tools for new perspectives

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Abstract Ascidians are marine protochordates at the evolutionary boundary between invertebrates and vertebrates. Ascidian larvae provide a simple system for unraveling gene regulation networks underlying the formation of the basic chordate body plan. After being used for over a century as a model for embryological studies, ascidians have become, in the past decade, an increasingly popular organism for studying gene regulation. Part of the renewed appeal of this system is the use of electroporation to introduce transgenic DNAs into developing embryos. This method is considerably more efficient than conventional microinjection assays and permits the simultaneous transformation of hundreds of embryos. Electroporation has allowed the identification and characterization of *cis*-regulatory DNAs that mediate gene expression in a variety of tissues, including the notochord, tail muscles, CNS, and endoderm. Electroporation has also provided a simple method for misexpressing patterning genes and producing dominant mutant phenotypes. Recent studies have used electroporation to create "knock-out" phenotypes by overexpressing dominant negative forms of particular proteins. Here we review the past and present uses of electroporation in ascidian development, and speculate on potential future uses.

Key words ascidian · body plan · transgenesis · electroporation · gene regulation

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Introduction

Ascidians or tunicates, commonly known as sea squirts, are marine invertebrates that belong to the most primitive branch of the chordate phylum, the Urochordates. The swimming larva (~1 mm long) is comparable to the amphibian tadpole; the tail contains an axial notochord flanked by muscles, a dorsal neural tube, and a ventral endodermal strand. Even though the ascidian tadpole represents the most elementary chordate body plan, it appears to contain rudiments of most vertebrate tissues. For example, the larva of *Ciona intestinalis*, the most cosmopolitan ascidian species, contains approximately 2,600 cells, organized into six different tissues: 40 notochord cells, 36 muscle cells, ~800 epidermal cells, ~500 endodermal cells, and ~900 mesenchyme cells, which are the precursors of most of the adult tissues (Satoh, 1994). The central nervous system (CNS) is composed of only ~330 cells, including just 86 neurons. About 60% of the cells in the CNS comprise the sensory vesicle, located in the dorsal region of the larval trunk (Meinertzhagen and Okamura, 2001; Okada et al., 2001).

Ascidians develop quickly, and a swimming tadpole hatches within 24 h after fertilization. The transcription of zygotic genes begins at the 16-cell stage, about 4 h after fertilization. The clonal restriction of larval tissues, such as the tail muscles and notochord, occurs shortly thereafter, between the 32-cell and 64-cell stage (Satoh, 1994). Gastrulation begins at the 110-cell stage, and neurulation rapidly follows after a few more cell divisions. These processes are driven by the same type of cell shape changes and movements (e.g. convergence and extension) as those seen in vertebrates (Satoh, 1978).

The morphological parallels between ascidian larvae and vertebrate tadpoles were first noted by Kowalevsky in 1866. We now know that the ascidian tadpole employs the same mechanisms of tissue specification as those used by vertebrates, which are composed of millions of

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cells. For example, the same evolutionarily conserved gene, *Brachyury*, has been shown to play a central role in notochord development in both ascidian and vertebrate embryos (Herrmann et al., 1990; Yasuo and Satoh, 1993; Takahashi et al., 1999). Similarly, the anterior-posterior and dorsal-ventral patterning of the neural tube are controlled by related regulatory genes in ascidians and vertebrates (Satou and Satoh, 1999 and references therein). All these similarities indicate that, in spite of their morphological simplicity, the ascidian tissues are specified by the same basic gene networks that drive vertebrate embryogenesis.

Ascidians as a model system for genomics and proteomics

Ascidians also present an opportunity to assess gene function without the genetic redundancy seen in many vertebrates (e.g. *Xenopus*; Thiebaud and Fischberg, 1977). Ascidians possess one of the smallest and most compact genomes among all chordates. For instance, the pufferfish, *Fugu*, contains the smallest known vertebrate genome. It is composed of ~350 Mb and contains ~30,000 genes (Brenner et al., in preparation). The genome of what is considered to be the most primitive living ascidian, *Ciona intestinalis*, is about half the size and complexity of *Fugu* (~160 Mb and ~15,000 genes; Simmen et al., 1998; Rokhsar et al., in preparation). This is quite similar to the *Drosophila* genome (~130 Mb and ~14,000 genes; Adams et al., 2000; Rubin et al., 2000).

So far, representative members have been isolated for several evolutionarily conserved families of DNA-binding proteins, such as T-box (e.g. Yasuo and Satoh, 1993; Corbo et al., 1997a; Takada et al., 1998; Erives and Levine, 2000), homeobox (e.g. Di Gregorio et al., 1995; Wada et al., 1998; Ristoratore et al., 1999; Caracciolo et al., 2000), helix-winged-helix (e.g. Corbo et al., 1997b; Olsen and Jeffery, 1997; Shimauchi et al., 1997), zinc-finger (e.g. Swalla et al., 1993; Swalla and Jeffery, 1996; Corbo et al., 1997b; Wada and Saiga, 1999; Nishida and Sawada, 2001), and basic helix-loop-helix (e.g. Meedel et al., 1997). It is likely that the regulatory networks defined by these genes will help identify and decipher the more complex and often redundant genetic circuits found in vertebrates. In fact, there is emerging evidence that the evolution of new or more complex body structures depends on the functional recruitment of preexisting genes into new pathways and the creation of *ex novo* interactions among preexisting gene products, rather than an increase in gene number (Carroll et al., 2001).

Three different ascidians are currently being subjected to genome-wide analyses (for details, see Satoh, 2001): *Ciona intestinalis* (Joint Genome Institute, CA, USA; National Institute of Genetics, Japan) *Halocynthia roretzi* (National Institute of Genetics, Japan) and *Ciona savignyi* (Whitehead Institute, Massachusetts Institute of Technology, USA). Within the next year, complete assemblies should be available for all three genomes. The basic "core proteome" of the fruit fly, that is, the number of distinct protein families, is 8065 (Rubin et al., 2000). Considering that the *Ciona* and the *Drosophila* genomes are very similar in size and complexity, we anticipate that the ascidian core proteome will be similar in size to that of *Drosophila*.

Another important advantage of the compact *Ciona* genome is that key *cis*-regulatory DNAs tend to map near the core promoter (e.g. Hikosaka et al., 1994; Corbo et al., 1997a; Erives et al., 1998; Di Gregorio and Levine, 1999). This has facilitated the isolation and characterization of several enhancers that mediate tissue-specific and spatially-restricted patterns of gene expression during embryogenesis. In the best studied cases, enhancers map within the first few kilobases of the 5′-flanking region, whereas comparable enhancers are located more than 10 kb 5′ or 3′ of the corresponding genes in vertebrates (e.g. notochord and CNS enhancers in the *HNF-3β* gene; Sasaki and Hogan, 1996; Di Gregorio et al., 2001). Transient expression assays provide an effective tool for analyzing enhancers since transgenic DNA does not have to persist in developing embryos for extended periods of time: in fact, definitive tadpole tissues are formed after just 10 cleavages and 12 h of development (Satoh, 1994).

All your eggs in one cuvette ...

Ascidian eggs are usually transparent, although in some species a regionalized pigmentation is observed, as in the famous case of the "yellow crescent", which is associated with the muscle determinant first identified by Conklin (1905) and recently characterized by Nishida and Sawada (2001). The molecular mechanisms controlling the development of solitary ascidians are currently being studied in four different genera: *Halocynthia*, *Ciona*, *Phallusia* and *Molgula*. *Halocynthia* possesses large and easily injectable eggs (~300 microns in diameter), while *Ciona* has smaller eggs (~170 microns in diameter). This is one of the reasons that prompted the development of electroporation as a faster and easier method to achieve transformation (Corbo et al., 1997a). The technique does not seem to work well for *Halocynthia* eggs but has been successfully used in *Boltenia villosa* (Dr. Billie J. Swalla, personal communication) and on *Phallusia* sp. (Dr. Sacha Glardon, personal communication).

Ascidian gametes can be easily obtained from naturally spawned adults or by surgical extraction (Fig. 1A). All solitary ascidians are hermaphrodites, and both types of gametes are released at the same time; however, most species are completely or partially self-sterile (Rosati and De Santis, 1978; Satoh, 1994 and references

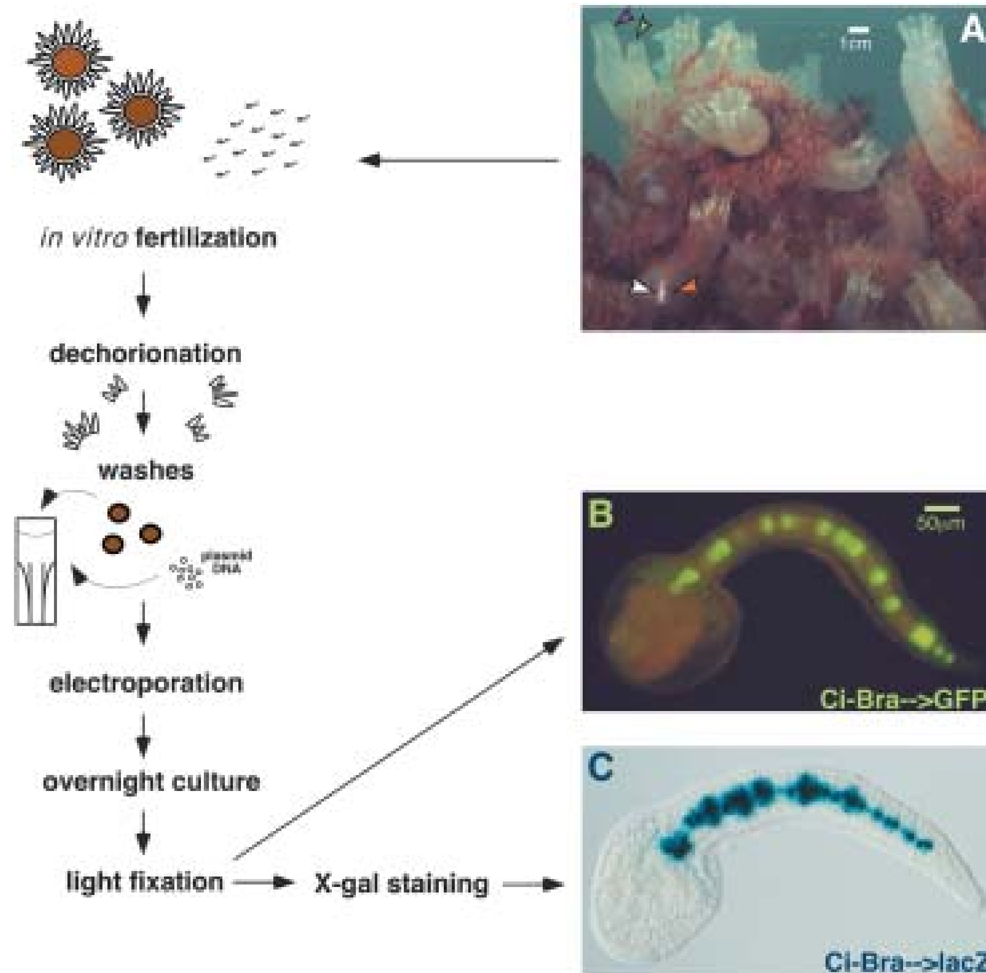

Fig. 1 The "nitty-gritty" of an electroporation experiment. (**A**). A group of adult *Ciona intestinalis*, the solitary ascidian species with a world-wide distribution. Different individuals are usually found in shallow sea water, alone or, more frequently, in groups of up to hundreds of animals. The adult body consists of two siphons, a wider oral siphon, which inhales the sea water (indicated by the *purple arrowhead*) and a smaller, atrial siphon (indicated by the *green arrowhead*) through which gametes and waste materials are expelled. Every individual produces both female and male gametes; the spermiduct is indicated by the *white arrowhead* and the oviduct by the *orange arrowhead*. Surgically removed gametes can be used for *in vitro* fertilization (see *schematic on the left*): the procedure requires only a couple of minutes, and is followed by a few quick rinses. The zygotes are then dechorionated (see text), washed thoroughly, quickly mixed with the circular plasmid DNA (25–200 µg), diluted in ~0.5 ml of 0.77 mol/l mannitol, and transferred to pulser cuvettes. With a pulse of 50 Volts (1 milliFarad), a time constant ranging between 15 and 21 milliseconds usually leaves most of the zygotes intact and ensures a good survival rate. Electroporated zygotes are quickly transferred to agarose-coated Petri dishes containing filtered sea water, which can be supplemented with antibiotics to prevent contamination. The embryos are cultured overnight at temperatures ranging from 15 °C to 18 °C. Within this range, the tailbud stages are reached in 15 or 13 h, respectively (Whittaker, 1977). After the desired stage is reached, a light fixation is carried out at room temperature, using either formaldehyde in sea water (Corbo et al., 1997a) or paraformaldehyde in PBS (Okada et al., 2001). Depending on the reporter gene used, the embryos can be either mounted and observed immediately, as in the case of the Green Fluorescent Protein (GFP) reporter (panel **B**), or subjected to staining procedures from a few hours to a few days (panel **C**), or even fixed for standard *in situ* hybridization procedures. Larvae shown in panels **B** and **C** have been electroporated with the *Ciona intestinalis Brachyury* (*Ci-Bra*) 434-bp notochord enhancer, driving *GFP* and *LacZ*, respectively. In both cases, not all of the 40 notochord cells express the reporter gene, due to a mosaic segregation of the transgene. Gametes, plasmids, and cuvettes are not drawn to scale.

therein). The *in vitro* fertilization procedure is extremely simple and takes place in just a few minutes. Fertilized eggs are washed thoroughly, in order to remove sperm and debris. Ascidian eggs are enveloped by two different types of accessory cells, test cells located near the egg and outer follicle cells. Follicle cells are removed by the procedure known as dechorionation; this treatment is necessary to facilitate the uptake of transgenic DNAs. The follicle cells can be removed either manually, with the aid of sharp metal needles, or, more simply, chemically, using either the purified hatching enzyme or more widely available proteinases (Satoh, 1994). Chemical dechorionation of a large number of zygotes requires ~5–10 minutes of proteinase treatment. The procedure

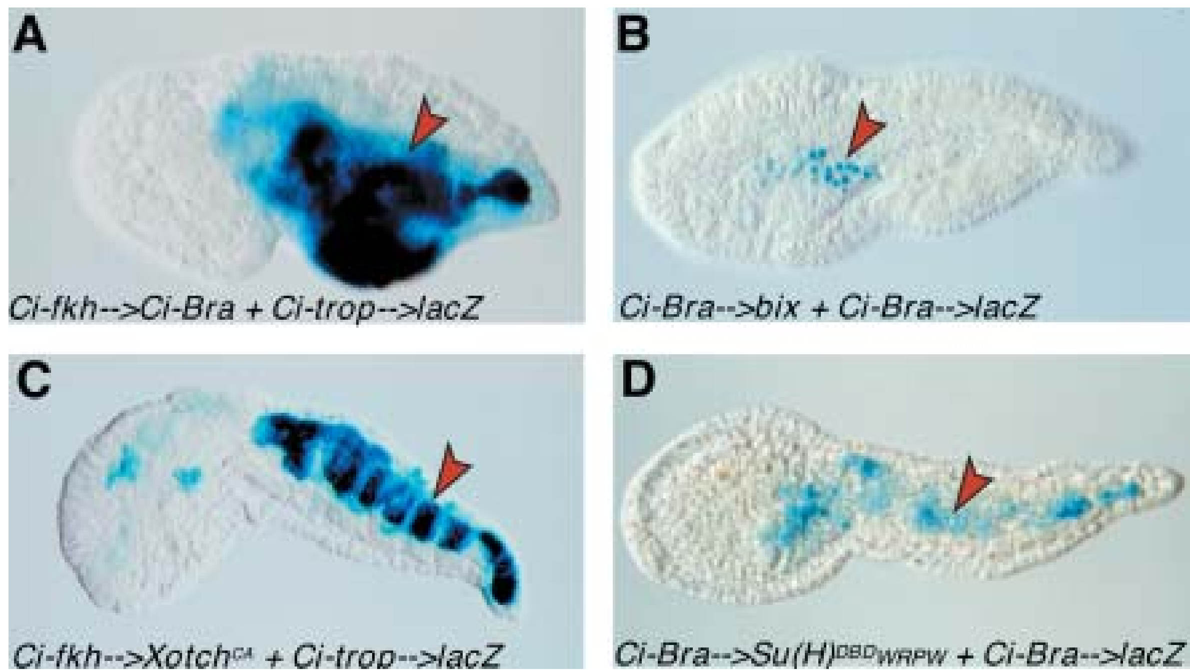

Fig. 2 Co-electroporation and misexpression experiments. Mid-tailbud larvae co-electroporated at the 1-cell stage with 100 μg of two different transgenes. After electroporation, embryos were incubated at 15 °C for 15.5 h, fixed, and stained with the X-gal substrate to detect β-galactosidase activity. Electroporation and staining conditions are the same in all the panels. The *red arrowhead* indicates notochord and notochord-like cells. (**A**) Larva expressing the *Ci-fkh→Ci-Bra* misexpression plasmid and the *Ci-fkh→lacZ* reporter plasmid. Note that the larval body plan is dramatically altered by the misexpression of *Ci-Bra*. In the tail region there is an ectopic mass of notochord-like cells that express the *lacZ* reporter gene (compare with the control larva in Fig.1C). Reproduced from Di Gregorio and Levine (1999). (**B**) Larva at the same stage as **A**, co-electroporated with the misexpression plasmid −3.5 kb *Ci-Bra→bix* and the reporter plasmid −3.5 kb *Ci-Bra→lacZ*. The *Ci-Bra→bix* transgene targets the *Xenopus* gene *bix* to the notochord precursors, disrupting notochord formation and, as a consequence, preventing tail elongation (Di Gregorio et al., 2002). (**C**) Larva co-electroporated with the *Ci-fkh→Xotch*^{CA} misexpression plasmid and the *Ci-trop→lacZ* reporter plasmid. This mutant phenotype is weaker, but similar to that shown in (**A**). See text for details, and Corbo et al., (1998). (**D**) Larva co-electroporated with the *Ci-Bra-→Su(H)*$_{DBD}$*WRPW* misexpression plasmid and the *Ci-Bra→lacZ* reporter plasmid. This mutant phenotype is milder but comparable to that shown in (**B**). See text for details, and Corbo et al. (1998).

is followed by serial washes in filtered sea water in order to remove the debris of follicle cells and excess enzyme. Subsequently, the fertilized eggs are mixed with transgenic DNA, transferred to pulser cuvettes, and subjected to an electric pulse (see legend to Fig. 1 for details). After electroporation, the embryos are allowed to develop at 15 – 18 °C until the desired stage, fixed, and then observed with a fluorescence microscope (GFP reporter; Fig. 1B) or stained to detect beta-galactosidase activity (*lacZ* reporter; Fig. 1C). Electroporation is performed immediately after fertilization in order to insure uniform incorporation of transgenic DNA by both blastomeres arising from the first cleavage.

Co-electroporation and misexpression experiments

Over the past decade, transgenesis has been successfully used in ascidians to characterize *cis*-regulatory DNAs that mediate tissue-specific patterns of gene expression in larval tissues such as muscle, notochord, endoderm, epidermis, and CNS (e.g. Hikosaka et al., 1994; Corbo at al., 1997a; Erives et al., 1998; Ristoratore et al., 1999; Di Gregorio et al., 2001). It is possible to transform individual embryos with two different transgenes. This "co-electroporation" strategy is very useful for labeling specific tissues with a reporter gene while simultaneously assessing the consequences of altering the expression or function of a desired patterning gene. The availability of tissue-specific enhancers and full-length cDNAs opens the possibility to produce mutant phenotypes *via* ectopic expression of regulatory genes. For example, electroporation has been exploited to assess the role of the *Ciona Brachyury* (*Ci-Bra*) gene in notochord development and differentiation (Takahashi et al., 1999). The *Ci-Bra* gene encodes an evolutionarily conserved, sequence-specific transcription factor that is normally expressed in a notochord-specific fashion (Yasuo and Satoh, 1993; Corbo et al., 1997a). To test its role in larval development, the *Ci-Bra* gene was placed under the control of the 5′-flanking region of another gene, the *Ciona forkhead/HNF-3β* homolog, *Ci-fkh*. *HNF-3β* is expressed in the notochord, floor plate, and endodermal derivatives of mouse em-

bryos (Sasaki and Hogan, 1996). *Ci-fkh* exhibits a similar pattern of expression: notochord, ventral ependymal cells of the nerve cord, trunk endoderm, and endodermal strand. Therefore, the *Ci-fkh* 5′-regulatory region can be used to direct ectopic expression of *Ci-Bra* in the CNS and endoderm. This results in a severe mutant phenotype, whereby endodermal and neuronal precursors are partially transformed into notochord (Takahashi et al., 1999; Fig. 2A). In this experiment, the *Ci-Bra→lacZ* reporter gene was used to monitor the formation of supernumerary notochord tissue. These results are comparable with those obtained in *Halocynthia* by Yasuo and Satoh (1998) based on the microinjection of *in vitro* synthesized *As-T* mRNA (the *Brachyury* homolog in *Halocynthia*). In *Ciona intestinalis*, a few rounds of electroporation provided a sufficient number of mutant tadpoles to perform subtractive hybridization screens. Thirty-eight genes were isolated that are directly or indirectly activated by Ci-Bra in the developing notochord (Takahashi et al., 1999; Hotta et al., 2000). More recently, the same approach has been used to study the function of *β-catenin* in *Ciona savignyi* (Imai et al., 2000).

Electroporation has been used to disrupt gene activity through the overexpression of dominant negative proteins. For example, repressor forms of the *C. intestinalis* Suppressor of Hairless [Ci-Su(H)] protein (Corbo et al., 1998) were expressed in the developing notochord using the *Ci-Bra* 5′ *cis*-regulatory region. Ci-Su(H) is a DNA-binding protein that appears to function as an activator of *Ci-Bra* (Corbo et al., 1998). Figure 2D shows a larva expressing a repressor form of Ci-Su(H), obtained by fusing the Su(H) DNA-binding domain with the minimal repression domain of the *Drosophila Hairy* gene. The fusion protein disrupts tail morphogenesis and reduces the expression of a *Ci-Bra→lacZ* reporter gene. A similar approach has been used by Mitani et al. (2001) to investigate the function of *As-T2*, a *Tbx6*-related gene in *Halocynthia roretzi* involved in muscle differentiation.

Electroporation has been used to examine cellular morphogenesis. In particular, evidence has been obtained that cell shape changes and intercalary movements in the notochord are essential for tail elongation. The homeobox repressor *Bix* (*Brachyury inducible homeobox*) was expressed in the *Ciona* notochord using the *Ci-Bra* 5′-flanking region. In *Xenopus*, overexpression of *Bix* suppresses *Xbra* expression and blocks notochord differentiation (Tada et al., 1998). In *Ciona*, Bix blocks the cellular differentiation of the notochord and causes an arrest in tail elongation (Fig. 3; Di Gregorio et al., 2002).

Electroporation assays have been used to determine whether orthologous genes isolated from other organisms can mimic the activities of ascidian genes. For example, the misexpression of *Brachyury* orthologs isolated from an hemichordate and a starfish (both nonchordate invertebrates) induces mutant phenotypes that are similar to those produced by the misexpression of *Ci-Bra* (Satoh et al., 2000). Similarly, a constitutively active form of *Xenopus Notch*, *Xotch*^{CA}, induces an expansion of the notochord when misexpressed in the endoderm, CNS, and notochord of *Ciona* embryos using *Ci-fkh cis*-regulatory DNA.

Fig. 3 Mosaic incorporation and the analysis of intermediate phenotypes. (**A**) Mid-tailbud stage larva that was co-electroporated at the 1-cell stage with the misexpression plasmid −3.5 kb *Ci-Bra→bix* and the reporter plasmid −3.5 kb *Ci-Bra→lacZ*, then stained with X-gal. The *red arrowhead* indicates the partial notochord formed by those notochord precursors which did not incorporate the transgenes (wild-type clone); the *pink arrowhead* indicates notochord precursors showing a mutant phenotype, due to the incorporation of both transgenes. The transgenes used in this experiment are the same as in Fig. 2B. (**B**) Detail of panel **A** at a higher magnification, to show the characteristic cuboidal shape of the normally developed notochord cells (*red arrowhead*), as compared to that of cells transformed by the *Ci-Bra→bix* transgene (*pink arrowhead*). About half of the 40 definitive notochord cells have incorporated the transgenes; note that not all of the cells expressing the reporter gene (*blue*) are notochord cells, since the reporter plasmid used in this experiment, −3.5 kb *Ci-Bra→lacZ*, occasionally shows ectopic expression in mesenchyme cells and in some of the muscle cells.

Mosaic incorporation: turning a potential problem into an advantage

After a variable number of cell divisions, a transgene might segregate into only one of the 2 daughter cells

originating from a single precursor. Depending on the number of cell divisions, a variable number of cells within a tissue will come to lack the transgene (see Fig. 3). In some organisms such a situation could be puzzling, but in ascidians the embryonic lineages are well defined, and the formation of advanced-stage embryos requires only a small number of cleavages. For example, mosaic expression is sometimes observed for the *Ci-Bra→GFP* or *Ci-Bra→lacZ* transgenes, so that only a subset of the 40 notochord cells express the reporter gene (Fig. 1C). The 40 notochord cells arise from 2 different pairs of blastomeres, A4.1 (primary lineage) and B4.1 (secondary lineage). Upon clonal restriction at the 64-cell stage there are 4 descendants of the A4.1 blastomeres. These undergo another three rounds of division prior to intercalation in neurula-stage embryos. The remaining 8 cells arise from two B4.1 descendants that are not clonally restricted until the 110-cell stage. These two cells undergo two rounds of cleavage prior to neurulation. In the example shown in Fig. 1C, the secondary notochord cells, which correspond to the 8 posterior-most cells in the notochord, do not express the reporter gene. It therefore appears that the transgene was lost from the B4.1 lineage but persisted in the A4.1 lineage.

This type of mosaic incorporation can provide insights into cell autonomy when assessing the role of a patterning gene in a complex morphological process. Fortunately, different transgenes tend to segregate into the same blastomeres when co-electroporated. Consequently, the cells that express the reporter gene are likely to also express whatever protein (wild-type or mutant) that is being studied. Figure 3 shows an example of such a situation. The larva was co-electroporated with the *Ci-Bra→bix* transgene (see above) and the *Ci-Bra→lacZ* reporter gene. During the cleavages leading to notochord formation, the transgenes have been lost from about half of the notochord precursors (the wild-type clone) and incorporated only by the cells that appear stained (the mutant clone). As a result, the wild-type notochord precursors formed a partial notochord (red arrowhead), while the mutant precursors clustered together and failed to acquire the characteristic cuboidal shape of the normal notochord cells (pink arrowhead). The analysis of these mosaic tadpoles provided evidence that intercalation is not required for the formation of a partial notochord (Di Gregorio et al., 2002). In neurulae, prospective notochord cells are arranged into two rows of 20 cells apiece. Normally, these cells converge to form a single row of cells, and then each cell elongates along the anterior-posterior axis. In this experiment, intercalation was blocked by expressing the Bix repressor in one of the two rows of notochord cells in neurulae (stained cells in Fig. 3). Nonetheless, the wild-type cells that lack Bix expression appear to undergo normal cell shape changes.

Future directions

Chemical mutagenesis screens have been successfully performed in *Ciona savignyi* (Nakatani et al., 1999) and *Ciona intestinalis* (Sordino et al., 2001). These studies have identified several interesting mutants, including some that exhibit defects in notochord differentiation. Electroporation might prove to be useful for genetic rescue experiments and "epistasis" tests. Perhaps the affected gene(s) could be identified by electroporating pools of large genomic DNA fragments. Moreover, it might be possible to achieve partial rescue of notochord mutants by forcing expression of Ci-Bra target genes identified in previous screens, such as *Ci-ERM*, *Ci-Prickle*, etc. (Hotta et al., 2000). It might also be possible to use electroporation to generate mutant phenotypes *via* RNA interference or insertional mutagenesis.

Enhancers are not the only *cis*-regulatory DNA sequences that can be analyzed using the tools currently available. Other regulatory DNAs, such as insulators and silencers, could be identified and characterized *via* electroporation. This would involve the use of transgenes containing two different core promoters linked to divergently transcribed reporter genes (Cai and Levine, 1995; 1997).

The ease and efficiency of the electroporation method permits the genome-wide search for tissue-specific enhancers in ascidians. Such studies are currently underway in *C. intestinalis*. Random genomic DNA fragments placed upstream of a minimal *Ci-fkh→lacZ* reporter gene have been systematically tested for enhancer activity in electroporated embryos (Harafuji et al., submitted). This type of approach is not feasible in systems such as *Drosophila* and mice, which require germline transformation to assess reporter gene expression. It might even be possible to use electroporation in ascidians to identify evolutionarily conserved enhancers from other chordates, such as zebrafish, frogs, and mice.

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