Unraveling genomic regulatory networks in the simple chordate, *Ciona intestinalis*

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The draft genome of the primitive chordate, *Ciona intestinalis*, was published three years ago. Since then, significant progress has been made in utilizing *Ciona*’s genomic and morphological simplicity to better understand conserved chordate developmental processes. Extensive annotation and sequencing of staged EST libraries make the *Ciona* genome one of the best annotated among those that are publicly available. The formation of the *Ciona* tadpole depends on simple, well-defined cellular lineages, and it is possible to trace the lineages of key chordate tissues such as the notochord and neural tube to the fertilized egg. Electroporation methods permit the targeted expression of regulatory genes and signaling molecules in defined cell lineages, as well as the rapid identification of regulatory DNAs underlying cell-specific gene expression. The recent sequencing of a second *Ciona* genome (*C. savignyi*) permits the use of simple alignment algorithms for the identification of conserved noncoding sequences, including microRNA genes and enhancers. Detailed expression profiles are now available for almost every gene that encodes a regulatory protein or cell-signaling molecule. The combination of gene-expression profiles, comparative genome analysis, and gene-disruption assays should permit the determination of high-resolution genomic regulatory networks underlying the specification of basic chordate tissues such as the heart, blood, notochord, and neural tube.

[Supplemental material is available online at www.genome.org.]
Ciona's morphological simplicity can provide deep insights into genetic regulation of cellular processes (Jiang et al. 2005). Along with a detailed overview of these studies, each section will also use these studies as launching points to discuss other related work and the advantages and challenges inherent to each of these approaches.

Comprehensive studies of gene expression and function
Since the sequencing of the genome, the potential of Ciona as a model system has been greatly enhanced by a number of systematic annotation studies (Imai 2003; Satou and Satoh 2003; Yagi et al. 2004). Of particular importance was the recent publication of gene-expression patterns for nearly every identified regulatory gene and signaling factor in the C. intestinalis genome (Imai et al. 2004). This survey focused mostly on early stages from the zygote through gastrulation, but also included representative stages (neurula, early and late tailbud) from older embryos. This work supplements the sequencing of an extensive set of tissue and stage-specific EST libraries and expression surveys of 1043 transcripts from these libraries (Satou et al. 2002b) along with thorough annotation of predicted genes (Satou and Satoh 2003). Together, these data constitute one of the most comprehensive sets of expression patterns for any model organism and have enormous utility for studies in Ciona and other chordates.

An overview of this new expression data generated two important observations regarding early Ciona development (Imai et al. 2004). First, over 70% of the genes encoding transcription factors and signaling molecules are maternally expressed. The substantial presence of maternally loaded factors may underlie rapid determination of cell fates in early Ciona embryos. Second, only 65 transcription factors and 25 signaling factors are zygotically expressed by the early gastrula stage. As almost all embryonic tissues are specified by this time, it is suggested that "comprehensive transcriptional networks" can be constructed based on the interactions between these 90 genes. Based on this prediction, a quantitative real time PCR-based assay was conducted, measuring the response of these 90 genes to suppression of the characterized tissue-specification factors \(\beta\)-catenin, FoxD, and FGF9/16/20 (Imai et al. 2004).

The comprehensive expression screen and associated RT–PCR assays complement an extensive body of research focused on early specification of Ciona endomesodermal lineages. We have attempted to draw together much of this work into a summary network (Fig. 1C). On the right are the regulatory outputs that subdivide the vegetal hemisphere of the 110-cell Ciona embryo into endoderm and five distinct mesodermal lineages. Details of the genes that control the formation of these lineages and the regulatory interactions can be found in Supplement 1. The subdivision of distinct endomesodermal lineages depends on two crucial maternal factors, Macho-1 and \(\beta\)-catenin (Fig. 1C). \(\beta\)-catenin is translocated into the nuclei of vegetal blastomeres, where it plays an essential and apparently highly conserved role in endomesoderm specification (Imai et al. 2000). Macho-1 functions as the maternal determinant for the tail-muscle lineage, a role that probably evolved within the tunicates in association with the extremely rapid embryogenesis of a functional tail (Nishida and Sawada 2001; Sawada et al. 2005).

To date, the majority of research on these early networks has involved loss-of-function studies to identify potential targets. There are relatively few studies in which direct interactions between regulatory genes and their targets have been verified by the manipulation of specific binding sites within enhancers (Fig. 1C, shown by dark lines). Characterization of cell-specific enhancers for the core patterning genes, ZicL, FoxD, FGF9/16/20, Tbx6, Twist-like1, Mesp, MyoD, and NoTric, would provide the basis for establishing causal interconnections among these genes. A recent study on the Ciona Otx enhancer demonstrates the potential of such an approach (Bertrand et al. 2003).

Regulatory interactions underlying Ciona neural specification
Despite the simplicity of the Ciona larval central nervous system (~330 cells), the overall organization is comparable to the vertebrate CNS (Fig. 2B) (Meinertzhagen et al. 2004). Many aspects of early neural specification and later neuronal differentiation appear to rely on conserved chordate gene networks that have been expanded during the evolution of more complex vertebrate neural structures (Meinertzhagen et al. 2004). Thus, dissection of

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**Figure 1.** Network map for specification of endomesodermal lineages. (A) Diagram of the vegetal hemisphere of a Ciona gastrula. Each cell is color coded according to its lineage as indicated by the color labels in the center. (B) Confocal image of an early tailbud embryo stained with phalloidin. Embryonic tissues have been artificially colored in accordance with the color scheme in A to display the fate of each of the lineages. Domains of some tissues are approximate and may not be wholly accurate. (C) Summary of data on interactions between genes involved in specification of endomesodermal lineages. Boxes for each lineage are colored according to the scheme in A and B. Bold lines indicate that this interaction has been verified through the manipulation of binding sites within an enhancer. Asterisks indicate evidence for appropriate binding sites in the noncoding DNA upstream of the target genes. Dotted lines indicate tangential interaction indicated by embryological manipulations. The notochord determinant Brachyury encodes a T-box homeodomain protein. The genes required for specifying different mesodermal lineages (NoTric [previously known as Handle], Twist-like1, Mesp, MyoD) all encode bHLH transcriptional factors. Further details regarding endomesodermal differentiation can be found in Supplement 1.
gene networks in *Ciona* neurogenesis will provide crucial insights into conserved aspects of these networks in vertebrates. This potential is exemplified by a recent study on the regulation of the early neural specification gene *Otx* (Bertrand et al. 2003), which encodes a homeodomain protein expressed in both *Ciona* and vertebrate anterior neural structures.

The methodology of this study represents an optimal use of *Ciona*’s potential to dissect *cis*-regulation of gene networks. A 3.5-kb enhancer was identified for the *Ci-Otx* gene, and subsequent manipulations characterized an ~120-bp activation element (a-element). This analysis exploited two key features of the *Ciona* system as follows: (1) the *Ciona* genome is very compact, with regulatory regions often located immediately upstream of the genes they control; (2) in *Ciona*, regulatory regions can be rapidly assessed and dissected in great detail by high-throughput electroporation of reporter constructs into fertilized eggs (Corbo et al. 1997; Harafuji et al. 2002; see Fig. 3A,B). The authors utilized the 120-bp *Otx* regulatory element to identify the endogenous activator as *Ci-FGF9/16*20. Based on previous work, it was clear that *Otx* induction relied on MEK signaling (Hudson and Lemaire 2001; Hudson et al. 2003). However, identification of the endogenous factor relied on two further advantages of *Ciona*. (1) The limited number of paralogs in the *Ciona* genome reduces the number of candidate factors (six *Ciona* FGFs vs. 22 vertebrate FGFs). (2) The low cell number of early *Ciona* embryos permits stringent evaluation of candidate factors through in situ hybridization. (The subsequent publication of the comprehensive gene expression database discussed above greatly magnifies this advantage, see Fig. 4).

At this point, the authors successfully addressed a pivotal obstacle in unraveling signaling networks, i.e., how cells of various lineages differentially interpret a broad signal. In *Ciona*, *FGF9/16/20* is known to mediate induction of the three following lineages: notochord, mesenchyme, and neural cells (Imai et al. 2002; Hudson et al. 2003; see Figs. 1,2). To reveal the mechanistic basis for a neural-specific response to this signal, the authors used phylogenetic footprinting. The availability of both *C. intestinalis* and *C. savignyi* genome assemblies provides an opportunity to identify potential regulatory DNAs by simply aligning orthologous sequences from the two species (Johnson et al. 2004). Alternatively, the high level of polymorphism between individuals from different populations of *C. intestinalis* can also be used to identify regulatory sequences (Boffelli et al. 2004). The authors used the cross-species comparison to reveal a concentration of potential transcriptional factor binding motifs for Ets and Gata factors. They were then able to rapidly confirm the importance of these sites through mutational analysis and by creating versions of the a-element containing only Ets or Gata sites. This analysis showed that the FGF signal causes general activation of *Otx*, while the requirement for Gata coactivation limits this response to the emerging neural lineage. Once again, the ability to perform such in-depth analysis relied on the ability to test reporter constructs by high-throughput electroporation (Fig. 3C). We wish to emphasize that reporter gene activity is monitored in the “zero” generation, just hours after electroporation of 1-cell embryos as compared with the lengthy process of creating germ-line transformation in vertebrate systems. Mosaic expression is not a problem, due to the efficient incorporation of the fusion genes and the small number of cell divisions leading to the formation of the tadpole. Additionally, it is now possible to establish stable transgenic lines through injection or electroporation of recombinant genes flanked by the Minos transposon (Sasakura et al. 2003; Matsuoka et al. 2005). This technique has been used for the identification of regulatory DNAs via enhancer trapping (Awazu et al. 2004).

The results discussed above provide a definitive regulatory network for activation of *Otx* in the anterior neural lineage (the a-lineage). The posterior CNS in *Ciona* is derived from two distinct lineages, the A-line and b-line (Fig. 2A,B). The regulatory networks specifying these two neural lineages are less well understood and may involve cell-autonomous mechanisms and
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ever, such networks are only the first step in understanding deciphering the basic gene networks in the discussed previously, these enhancer studies will set the stage for comprehensive gene-expression studies and functional assays and stomodaeum (Christiaen et al. 2005). In combination with Imai 2003; Oda-Ishii et al. 2005), tail muscles (Yagi et al. 2005), Levine, in prep.), endoderm (Satou et al. 2001; Fanelli et al. 2003; Imai 2003; Oda-Ishii et al. 2005), tail muscles (Yagi et al. 2005), and stomodaemum (Christiaen et al. 2005). In combination with the comprehensive gene-expression studies and functional assays discussed previously, these enhancer studies will set the stage for deciphering the basic gene networks in the Ciona embryo. However, such networks are only the first step in understanding Ciona development. The next step is to investigate the roles of downstream genes in influencing the cellular components underlying tissue morphogenesis. In other words, how do interconnected networks of regulatory factors and cell-signaling molecules control the detailed behavior of individual cells? As discussed in the next section, Ciona is also an excellent model system for exploring these downstream cellular processes.

Notochord morphogenesis

During Ciona notochord development, many of the genes downstream of Brachyury appear to control cellular processes such as adhesion, intercalation, and cell shape (Hotta et al. 2000). Studies on notochord differentiation and morphogenesis highlight the broad range of tools that are available for the dissection of complex morphogenetic processes (Di Gregorio et al. 2002; Keys et al. 2002). A recent study used forward genetics (Fig. 5) combined with detailed analysis of mutant cell behavior to characterize the role of Prickle, a component of the planar polarity pathway, in the morphogenesis of the notochord (Jiang et al. 2005).

The authors began with a screen for spontaneous mutations, focusing on defects in notochord formation. Because the larval body is largely disposable, Ciona embryos with severe defects in larval structures (including the notochord and dorsal nerve cord) can still metamorphose into reproductive juveniles. The authors isolated the aimless (aim) mutant based on severe defects in larval tail formation. A variety of genomic tools identified aim as a mutation in the conserved cell polarity gene, Prickle. This gene was previously identified in a comprehensive subtractive hybridization screen for notochord-specific genes downstream of Brachyury (Takahashi et al. 1999).

To explore the precise role of Prickle in notochord forma-
tion, the authors compared the behavior of isolated notochord precursor cells from mutant and wild-type embryos. Early lineage determination in Ciona embryos permits the isolation of individual blastomeres from defined lineages. Confocal analysis led to the observation that mutant cells exhibit loss of localized bipolar protrusions. The transparency and low cell number of Ciona embryos make it feasible to observe cell behavior in vivo. The authors exploited high-throughput Ciona transgenesis to target tagged transcripts of Prickle and the conserved cell polarity gene Dishevelled to the developing notochord. Careful observation of wild-type and mutant transgenic embryos provided evidence that Prickle mediates the localization of Dishevelled along the medio-lateral axis of intercalating notochord cells.

The power of Ciona for determining the genetic basis of cellular processes is only now being exploited. There is great potential in applying similar techniques to other aspects of Ciona development, including directed cell migration of heart and blood cells, cell movements underlying gastrulation, and morphogenesis of the neural tube.

Emerging resources and techniques

There are a number of significant resources and techniques that will promote further research. Some of these tools are cur-
1. DNA oligo microarrays covering the whole transcriptome are under development to replace the existing cDNA microarray, which has only about 85% coverage (Azumi et al. 2003). Although the gene expression survey discussed above (Imai et al. 2004) represents a powerful resource, it suffers from inherent problems associated with in situ hybridization, namely, that the quality of probes vary, leading to background noise that may obscure the true expression pattern. For example, in comparing the gene expression survey (Imai et al. 2004) with the study on Macho-1 targets (Yagi et al. 2004), it becomes clear that a large number of transcription/signaling factors expressed by the 32-cell stage (and subsequently down-regulated by Macho-1 suppression) were not detected by in situ hybridization until after the 110-cell stage. Extensive microarray screens using staged embryos and isolated blastomeres will provide a more complete assessment of the gene networks underlying embryogenesis.

2. Although the Forkhead promoter has been used to visualize the activities of a variety of tissue-specific enhancers, it is far from ideal, since it sometimes causes spurious expression patterns (Di Gregorio et al. 2001). The Brachyury promoter may be better suited for such enhancer analysis (Bertrand et al. 2003), but there is still a great need for additional core promoters.

3. Recently completed EST libraries of early juvenile stages should help launch studies into the differentiation of critical post-larval structures such as the endostyle, branchial gill slits, and heart. Many of the putative regulatory genes that are not expressed in the embryo might be essential for post-metamorphic events.

4. Establishment of ascidian stock centers will overcome the main obstacle to a robust Ciona research community, which is year-round access to gravid adults. Most research takes place in “land-locked” laboratories, and it is not always easy to find animals (particularly in winter months) that provide embryos for electroporation assays. To overcome this, centers where wild-type and stable transgenic lines are developed and maintained are under development in both Japan (Shimoda) and...
the USA (UC Santa Barbara). Such centers will be critical for providing gravid animals year round and for distribution of stable transgenic lines. Particularly useful lines might include Gal4 UAS transgenes for expression of constructs in particular lineages and lines with GFP-tagged lineages for easy assessment of mutant phenotypes.

Conclusions

The Ciona system possesses virtually every modern analytical tool for the comprehensive determination of the genomic regulatory networks underlying development. We anticipate that some of the current deficiencies, such as access to gravid animals and the availability of inexpensive microarrays, will be remedied in the near future. It is easy to envision complete networks governing the specification, differentiation, and morphogenesis of some of the current deficiencies, such as access to gravid animals and the availability of inexpensive microarrays, will be remedied in the near future. It is easy to envision complete networks for unraveling the more elaborate genetic interactions used for the construction of homologous structures in vertebrates. These networks should provide the foundation for unraveling the more elaborate genetic interactions used for the construction of homologous structures in vertebrates. In principle, it should be possible to integrate gene expression profiles, interlocking networks of regulatory genes and cell-signaling pathways to identify the on/off state of every gene in the Ciona genome in every blastomere at each stage in embryogenesis.

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