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[REVIEW]

Decoding *cis*-Regulatory Systems in Ascidians

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ABSTRACT—Ascidians, or sea squirts, are lower chordates, and share basic gene repertoires and many characteristics, both developmental and physiological, with vertebrates. Therefore, decoding *cis*-regulatory systems in ascidians will contribute toward elucidating the genetic regulatory systems underlying the developmental and physiological processes of vertebrates. *cis*-Regulatory DNAs can also be used for tissue-specific genetic manipulation, a powerful tool for studying ascidian development and physiology. Because the ascidian genome is compact compared with vertebrate genomes, both intergenic regions and introns are relatively small in ascidians. Short upstream intergenic regions contain a complete set of *cis*-regulatory elements for spatially regulated expression of a majority of ascidian genes. These features of the ascidian genome are a great advantage in identifying *cis*-regulatory sequences and in analyzing their functions. Function of *cis*-regulatory DNAs has been analyzed for a number of tissue-specific and developmentally regulated genes of ascidians by introducing promoter-reporter fusion constructs into ascidian embryos. The availability of the whole genome sequences of the two *Ciona* species, *Ciona intestinalis* and *Ciona savignyi*, facilitates comparative genomics approaches to identify *cis*-regulatory DNAs. Recent studies demonstrate that computational methods can help identify *cis*-regulatory elements in the ascidian genome. This review presents a comprehensive list of ascidian genes whose *cis*-regulatory regions have been subjected to functional analysis, and highlights the recent advances in bioinformatics and comparative genomics approaches to *cis*-regulatory systems in ascidians.

Key words: bioinformatics, enhancer, promoter, transgenic embryos, transcriptional regulation

INTRODUCTION

cis-Regulatory DNA controls gene expression and contains binding sites for transcription factors (Davidson, 2001; Howard and Davidson, 2004). A large fraction of metazoan genomes are thought to encode *cis*-regulatory DNAs (Duret and Bucher, 1997; Hardison, 2000; Onyango *et al.*, 2000; Harafuji *et al.*, 2002; Cameron *et al.*, 2004). Deciphering *cis*-regulatory DNAs is important because they encode where, when, and how much each protein will be produced. Unlike protein-coding DNA sequences, however, the prediction of *cis*-regulatory elements solely by computational methods has been difficult, especially in higher eukaryotes, because the transcription factor binding sites are generally short, show degenerate sequences, and are often widely dispersed in large intergenic and intronic DNAs. In addition, characterization of the *cis*-regulatory regions of even a sin-

gle gene usually requires labor-intensive experiments. For these reasons, only a limited number of *cis*-regulatory DNAs have been elucidated in metazoans.

Recent advances in molecular developmental biology and genomics of the urochordate ascidian offer a unique opportunity to investigate *cis*-regulatory DNAs in a chordate genome. An ascidian fertilized egg develops within a day into a tadpole-like larva consisting of ≈2600 cells (Satoh and Jeffery, 1995; Satoh, 2003). The ascidian larva shares a basic body plan with vertebrates (Satoh and Jeffery, 1995; Corbo *et al.*, 2001; Satoh, 2001, 2003; Satoh *et al.*, 2003). The draft genome of the ascidian *Ciona intestinalis* was determined; its 153~159 Mbp genome is estimated to contain 15,852 protein coding genes; on average, there is one gene every 7.5 kb of euchromatic DNA (Dehal *et al.*, 2002). Because of a well-established cell lineage (Conklin, 1905; Nishida, 1987) and transparency, spatial gene expression patterns can be visualized in detail during ascidian development; extensive information is available on the expression profiles of thousands of genes in *Halocynthia roretzi*

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(Kawashima *et al.*, 2000, 2002; Makabe *et al.*, 2001) and *C. intestinalis* (Satou *et al.*, 2001a, 2002; Nishikata *et al.*, 2001; Kusakabe *et al.*, 2002; Fujiwara *et al.*, 2002; Ogasawara *et al.*, 2002). Transient transgenesis can be used for efficient expression of exogenous genes in ascidian embryos and larvae (Fig. 1) (Hikosaka *et al.*, 1992; Corbo *et al.*, 1997a; Zeller, 2004). Simple electroporation methods permit the simultaneous transformation of hundreds of synchronously developing *C. intestinalis* embryos (Corbo *et al.*, 1997a). Relatively short 5' flanking sequences are generally sufficient to recapitulate the endogenous gene expression patterns of most tissue-specific genes in ascidians. The availability of the whole genome sequences of two *Ciona* species, *C. intestinalis* and *C. savignyi*, allows the application of comparative genomics approaches to identify *cis*-regulatory DNAs (Satoh *et al.*, 2003; Johnson *et al.*, 2004).

In addition to its value in understanding gene regulation, *cis*-regulatory DNA provides a powerful tool for tissue-specific genetic manipulation in the study of ascidian development and physiology. For example, *cis*-regulatory DNAs have been used to express wild-type or mutant forms of proteins that regulate developmental or physiological processes (Corbo *et al.*, 1998; Fujiwara *et al.*, 1998; Ono *et al.*, 1999; Takahashi *et al.*, 1999a; Imai *et al.*, 2000; Satoh *et al.*, 2000; Mitani *et al.*, 2001; Okagaki *et al.*, 2001; Di Gregorio *et al.*, 2002; Keys *et al.*, 2002; Kawai *et al.*, 2005). *cis*-Regulatory DNAs were also used to demonstrate *trans*-splicing in ascidian embryos (Vandenberghe *et al.*, 2001). Neurons and neural circuits were visualized in living embryos by expressing fluorescent proteins under the control of neuron-specific promoters (Fig. 1D) (Okada *et al.*, 2001, 2002; Yoshida *et al.*, 2004).

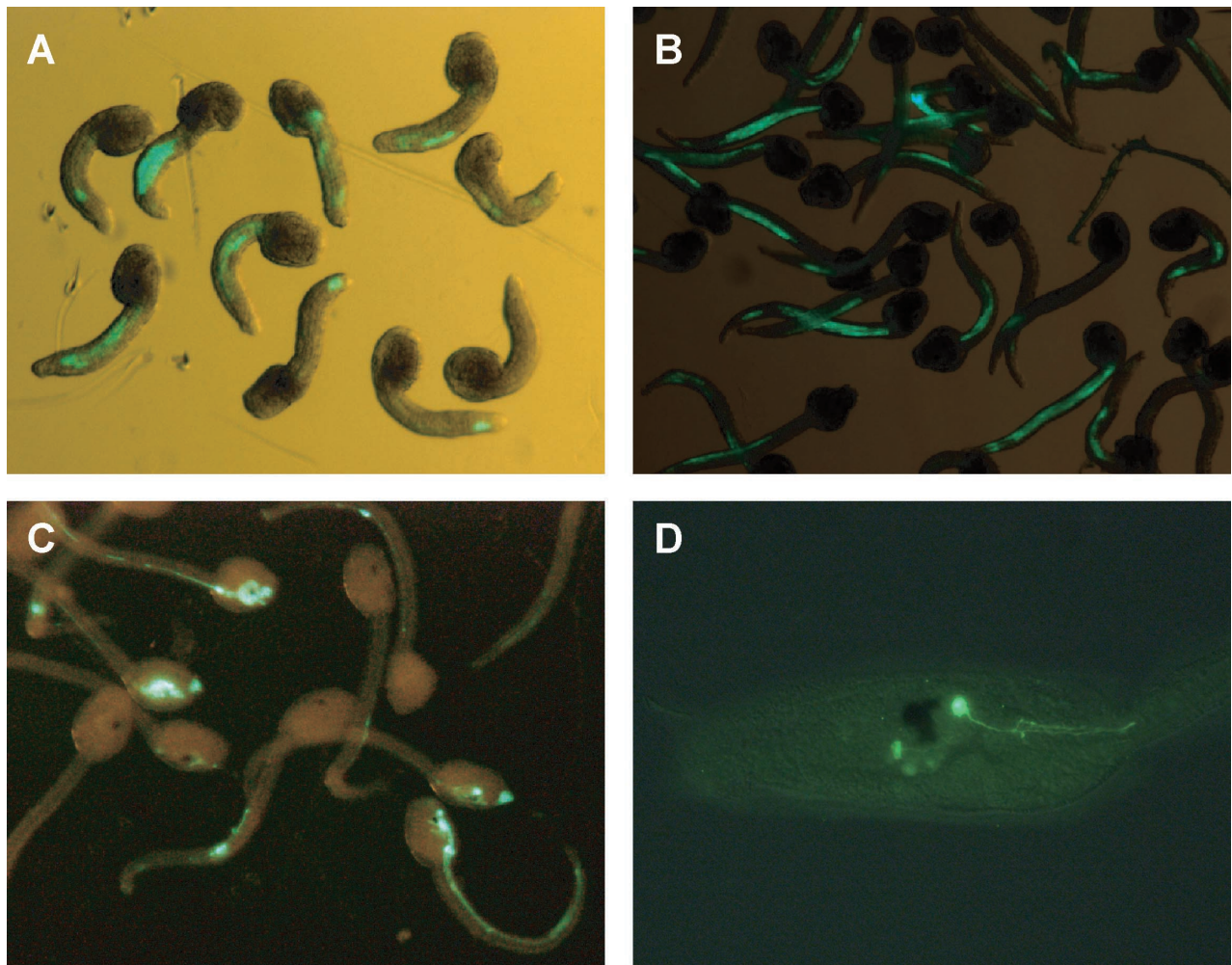


Fig. 1. Expression of *GFP* driven by various tissue-specific promoters in *C. intestinalis* embryos and larvae. Promoter-*GFP* fusion constructs were introduced into fertilized eggs by electroporation. *GFP* fluorescence was observed specifically in muscle cells of mid tailbud embryos (**A**) and tadpole larvae (**B**) developed from eggs electroporated with promoter-*GFP* fusion construct of a myosin essential light chain gene (Kusakabe *et al.*, 2004). (**C**) Nervous system-specific expression of *GFP* under the control of the promoter of a β -tubulin gene (Kusakabe *et al.*, 2004). (**D**) Visualization of neuron morphology using a neuron-type specific promoter (Yoshida *et al.*, 2004). Cell bodies and axons of GABAergic neurons were visualized by *GFP* fluorescence in a larva developed from an egg electroporated with a promoter-*GFP* fusion construct of the vesicular GABA transporter gene.

Table 1. Ascidian genes whose *cis*-regulatory regions were experimentally analyzed

Gene	Gene products	Species	Tissue or cell type	References
<i>HrMA1a</i>	actin	<i>H. roretzi</i>	larval muscle	Kusakabe <i>et al.</i> , 1995; Satoh <i>et al.</i> , 1996
<i>HrMA1b</i>	actin	<i>H. roretzi</i>	larval muscle	Kusakabe <i>et al.</i> , 1995; Satoh <i>et al.</i> , 1996
<i>HrMA2</i>	actin	<i>H. roretzi</i>	larval muscle	Kusakabe <i>et al.</i> , 1995
<i>HrMA4a</i>	actin	<i>H. roretzi</i>	larval muscle	Hikosaka <i>et al.</i> , 1992, 1993, 1994; Kusakabe <i>et al.</i> , 1995; Satou and Satoh, 1996
<i>HrMA4b</i>	actin	<i>H. roretzi</i>	larval muscle	Kusakabe <i>et al.</i> , 1995
<i>HrMA5</i>	actin	<i>H. roretzi</i>	larval muscle	Kusakabe <i>et al.</i> , 1995
<i>HrMA6</i>	actin	<i>H. roretzi</i>	larval muscle	Kusakabe <i>et al.</i> , 1995
<i>HrMHC1</i>	myosin heavy chain	<i>H. roretzi</i>	larval muscle	Araki and Satoh, 1996
<i>HrEpiB</i>	UDP glucose-4-epimerase	<i>H. roretzi</i>	epidermis	Ueki and Satoh, 1995
<i>HrEpiC1</i>	unknown; trefoil domain	<i>H. roretzi</i>	epidermis	Ishida and Satoh, 1999
<i>HrEpiD</i>	SEC61	<i>H. roretzi</i>	epidermis	Ueki and Satoh, 1995
<i>As-T</i>	Brachyury	<i>H. roretzi</i>	notochord	Takahashi <i>et al.</i> , 1999b
<i>As-T2</i>	Tbx6	<i>H. roretzi</i>	larval muscle	Mitani <i>et al.</i> , 2001
<i>HrTyr</i>	tyrosinase	<i>H. roretzi</i>	pigment cells	Toyoda <i>et al.</i> , 2000
<i>HrTyrp (HrTRP)</i>	tyrosinase-related protein	<i>H. roretzi</i>	pigment cells	Wada <i>et al.</i> , 2002; Toyoda <i>et al.</i> , 2004
<i>syf</i>	synaptotagmin	<i>H. roretzi</i>	neurons, epidermis	Katsuyama <i>et al.</i> , 2002
<i>Hroth</i>	Otx	<i>H. roretzi</i>	anterior nervous system, endoderm	Oda-Ishii and Saiga, 2003
<i>HrGan</i>	G protein α subunit	<i>H. roretzi</i>	larval muscle, mesenchyme, trunk lateral cells (TLC)	Iwasa <i>et al.</i> , 2003
<i>MocuMA1</i>	actin	<i>M. oculata</i>	larval muscle	Kusakabe <i>et al.</i> , 1996
<i>MoccMA1a</i>	actin (pseudogene)	<i>M. occulta</i>	larval muscle	Kusakabe <i>et al.</i> , 1996
<i>MoccMA1b</i>	actin (pseudogene)	<i>M. occulta</i>	larval muscle	Kusakabe <i>et al.</i> , 1996
<i>Ci-Bra</i>	Brachyury	<i>C. intestinalis</i>	notochord	Corbo <i>et al.</i> , 1997a, 1998; Fujiwara <i>et al.</i> , 1998; Takahashi <i>et al.</i> , 1999b; Yagi <i>et al.</i> , 2004b
<i>Ci-Sna</i>	Snail	<i>C. intestinalis</i>	larval muscle, mesenchyme, central nervous system (CNS)	Corbo <i>et al.</i> , 1997b; Fujiwara <i>et al.</i> , 1998; Erives <i>et al.</i> , 1998; Erives and Levine, 2000; Boffelli <i>et al.</i> , 2004
<i>Ci-fkh (Ci-FoxA-a)</i>	FoxA (HNF-3 β , Forkhead)	<i>C. intestinalis</i>	notochord, endoderm, endodermal strand, nerve cord (floor plate)	Erives <i>et al.</i> , 1998; Di Gregorio and Levine, 1999; Di Gregorio <i>et al.</i> , 2001; Boffelli <i>et al.</i> , 2004
<i>Ci-trop</i>	tropomyosin-like	<i>C. intestinalis</i>	notochord	Di Gregorio and Levine, 1999
<i>CiHox3</i>	Hox3	<i>C. intestinalis</i>	visceral ganglion	Locascio <i>et al.</i> , 1999
<i>Cititf1</i>	NK-2 class	<i>C. intestinalis</i>	endoderm	Ristoratore <i>et al.</i> , 1999; Fanelli <i>et al.</i> , 2003
<i>Ci-Tnl</i>	troponin I	<i>C. intestinalis</i>	larval muscle, heart [trunk ventral cells (TVC)]	Vandenberghe <i>et al.</i> , 2001; Cleto <i>et al.</i> , 2003; Davidson and Levine, 2003; Johnson <i>et al.</i> , 2004
<i>Ci-Dll-A</i>	Distalless	<i>C. intestinalis</i>	anterior nervous system	Harafuji <i>et al.</i> , 2002
<i>29h10</i>	unknown	<i>C. intestinalis</i>	endoderm, TVC	Davidson and Levine, 2003
<i>Ci-Hndx (Ci-NoTrlc)</i>	Hand-like	<i>C. intestinalis</i>	TVC, TLC, endoderm	Davidson and Levine, 2003
<i>Ci-NPP</i>	phosphodiesterase	<i>C. intestinalis</i>	endoderm, TVC	Davidson and Levine, 2003
<i>Ci-TnT</i>	troponin T	<i>C. intestinalis</i>	larval muscle, TVC, brain vesicle	Davidson and Levine, 2003
<i>Ci-TPO</i>	thyroid peroxidase	<i>C. intestinalis</i>	endostyle (juvenile)	Sasakura <i>et al.</i> , 2003
<i>Ci-otx</i>	Otx	<i>C. intestinalis</i>	anterior nervous system, endoderm	Bertrand <i>et al.</i> , 2003
<i>Ci-msxb</i>	Msx	<i>C. intestinalis</i>	mesenchyme, muscle, endodermal strand, pigment cells, CNS, pharynx (stomodaeum), neural gland	Russo <i>et al.</i> , 2004
<i>Ci-arr</i>	arrestin	<i>C. intestinalis</i>	ocellus photoreceptor cells	Yoshida <i>et al.</i> , 2004
<i>Ci-Gai1</i>	G protein α subunit	<i>C. intestinalis</i>	neurons in CNS, papilla	Yoshida <i>et al.</i> , 2004
<i>Ci-vAChTP</i>	vesicular acetylcholine transporter	<i>C. intestinalis</i>	cholinergic neurons	Yoshida <i>et al.</i> , 2004
<i>Ci-vGAT</i>	vesicular GABA transporter	<i>C. intestinalis</i>	GABAergic neurons	Yoshida <i>et al.</i> , 2004
<i>Ci-Musashi</i>	Musashi	<i>C. intestinalis</i>	ventral endostyle, peripharyngeal band, oral siphon, pharyngeal gill	Awazu <i>et al.</i> , 2004
<i>Ci-MLC2</i>	myosin essential light chain	<i>C. intestinalis</i>	larval muscle	Kusakabe <i>et al.</i> , 2004
<i>Ci-MRLC2</i>	myosin regulatory light chain	<i>C. intestinalis</i>	larval muscle	Kusakabe <i>et al.</i> , 2004
<i>Ci-tubulin-β2</i>	β -tubulin	<i>C. intestinalis</i>	neurons	Kusakabe <i>et al.</i> , 2004
<i>Ci-IkB</i>	IkB	<i>C. intestinalis</i>	maternal, notochord	Kawai <i>et al.</i> , 2005
<i>Ci-pitx</i>	Pitx	<i>C. intestinalis</i>	anterior neural boundary/stomodaeum, epidermis, ocellus photoreceptors, left visceral ganglion, left endoderm	Christiaen <i>et al.</i> , 2005
<i>Cs-Noto9</i>	ribosome binding protein 1	<i>C. savignyi</i>	notochord	Johnson <i>et al.</i> , 2004
<i>Cs-Syt</i>	synaptotagmin	<i>C. savignyi</i>	brain, nerve cord, epidermis	Johnson <i>et al.</i> , 2004
<i>Cs-Tnl</i>	troponin I	<i>C. savignyi</i>	larval muscle	Johnson <i>et al.</i> , 2004
<i>Cs-Tub</i>	α -tubulin	<i>C. savignyi</i>	brain, nerve cord, epidermis	Johnson <i>et al.</i> , 2004

In spite of their simple genomes, ascidians share basic gene repertoires and many characteristics, both developmental and physiological, with vertebrates (Dehal *et al.*, 2002; Satoh, 2003; Satoh *et al.*, 2003; Meinertzhagen *et al.*, 2004; Campbell *et al.*, 2004). Therefore, decoding *cis*-regulatory systems in ascidians will contribute toward elucidating the genetic regulatory systems underlying the developmental and physiological processes of vertebrates. As of December 2004, functional analyses of *cis*-regulatory regions have been published for as many as 50 genes in ascidians (Table 1). This review presents a comprehensive list of ascidian genes whose *cis*-regulatory regions have been subjected to functional analysis, and highlights the recent advances in bioinformatics and comparative genomics approaches to *cis*-regulatory systems in ascidians.

EXOGENOUS DNA TRANSFER INTO ASCIDIANS

Foreign gene transfer into ascidian embryos was first reported in 1992 (Hikosaka *et al.*, 1992). Hikosaka and his colleagues microinjected a plasmid DNA containing the bacterial β -galactosidase gene (*lacZ*) connected with the chicken β -actin promoter and the Rous sarcoma virus enhancer into fertilized eggs of *C. savignyi*. The microinjection was performed into eggs with an intact chorion (vitelline coat). The *lacZ* expression was observed in various cell-types of tailbud embryos and larvae, irrespective of injection of linear or circular form of the plasmid. The same paper also reported muscle-specific reporter expression by the promoter of a muscle actin gene (*HrMA4a*) of *H. roretzi* (Kusakabe *et al.*, 1992). In this experiment, the bacterial gene encoding chloramphenicol acetyltransferase (*CAT*) was used as the reporter and the promoter-reporter fusion plasmid was microinjected into *H. roretzi* fertilized eggs without chorions. In subsequent studies, microinjection into *H. roretzi* eggs has been usually performed through the chemically softened chorion (Hikosaka *et al.*, 1994; Kusakabe *et al.*, 1995).

Microinjection into eggs with intact chorions has been used for transgenesis of two widely used *Ciona* species, *C. intestinalis* (Kusakabe *et al.*, 1996) and *C. savignyi* (Hikosaka *et al.*, 1992, 1993; Deschet *et al.*, 2003), and two molgulid ascidians, *Molgula oculata* and *Molgula occulta* (Kusakabe *et al.*, 1996). However, microinjection into intact *C. intestinalis* eggs has been generally regarded as difficult (Zeller, 2004), and microinjection is usually performed into dechorionated eggs in this species (e.g. Sasakura *et al.*, 2003a). Microinjection either before or after insemination can produce transgene expression in *Ciona* embryos. Microinjection of DNA constructs into a particular blastomere has been achieved with dechorionated embryos of *C. savignyi* (Hikosaka *et al.*, 1993) and with *H. roretzi* embryos with chemically softened chorions (Okada *et al.*, 2002; Kat-suyama *et al.*, 2002).

Either circular or linearized forms of plasmid DNA constructs can be injected into ascidian eggs to obtain exoge-

nous gene expression. In *C. savignyi*, Hikosaka *et al.* (1992) reported that both the efficiency and spatial patterns of *lacZ* expression were comparable between larvae developed from eggs injected with circular and linearized DNAs. In experiments using the *Halocynthia* tyrosinase gene promoter, however, when circular plasmids were injected, it was necessary to increase the quantity of injected DNA from a concentration of 5.0 ng/ μ l, which was sufficient for linearized plasmids, to 50 ng/ μ l to obtain reporter *lacZ* expression (Toyoda *et al.*, 2004). Furthermore, a *lacZ*-fusion construct whose expression was not detected when it had been injected in a linearized form was capable of driving gene expression when it had been injected in a circular form (Toyoda *et al.*, 2000).

Electroporation has become the most commonly used method to introduce exogenous DNAs into *Ciona* embryos (Corbo *et al.*, 2001; Zeller, 2004). In 1997, Corbo and his colleagues first reported the electroporation of exogenous DNA into *C. intestinalis* embryos (Corbo *et al.*, 1997a). The same method has also been applied to transgenesis of *C. savignyi* embryos (Nakatani *et al.*, 1999). Electroporation is performed with dechorionated fertilized eggs. This method is faster, simpler, and more efficient than microinjection and permits the simultaneous transformation of hundreds of synchronously developing embryos. Like microinjection, electroporation has been used both to analyze *cis*-regulatory DNAs and to assess gene function (Corbo *et al.*, 2001; Di Gregorio and Levine, 2002; Zeller, 2004). However, there are some limitations on application of electroporation: first, introduction of DNA into particular blastomeres is difficult; second, because dechorionated embryos do not develop normal tunics and become sticky, larvae that develop from electroporated eggs do not swim well. This problem can be a disadvantage for studies of neural function and swimming behavior.

Most transgenic studies in ascidians conducted so far are "transient" transgenesis. This is mainly due to difficulties in long term culturing and also due to the low frequency of germline transmission of transgene constructs. However, some laboratories have overcome these difficulties, and stable transgenic lines of ascidians have been established (Deschet *et al.*, 2003; Sasakura *et al.*, 2003a). Deschet and her colleagues generated stable transgenic lines of *C. savignyi* using a technique in which the endonuclease I-SceI was coinjected into fertilized eggs with a transgene construct (Deschet *et al.*, 2003). Sasakura and his colleagues used a *Tc1/mariner* superfamily transposon, *Minos*, to achieve germ-line transgenesis of both *C. intestinalis* (Sasakura *et al.*, 2003a) and *C. savignyi* (Matsuoka *et al.*, 2004). *Minos* has high activity in *Ciona* (Sasakura *et al.*, 2003b); it was efficiently integrated into the genome of germ cells and stably transmitted to subsequent generations (Sasakura *et al.*, 2003a). Enhancer trapping in *Ciona* by *Minos* was demonstrated and led to the identification of *Musashi* orthologue enhancers (Awazu *et al.*, 2004).

CELL/TISSUE-SPECIFIC *CIS*-REGULATORY REGIONS

Larval Muscle

Ascidian larvae develop unicellular, striated muscle cells on each side of the tail. The number of muscle cells differs among species: 36 in *C. intestinalis* and 42 in *H. roretzi* (Nishida, 1987). Twenty-eight of the 42 muscle cells of the *H. roretzi* tadpole are derived from the B4.1 blastomeres (B-line), four from the A4.1 blastomeres (A-line), and 10 from the b4.2 blastomeres (b-line) of the bilaterally symmetrical 8-cell embryo (Nishida, 1987). The B-line muscle cells differentiate autonomously, whereas the A- and b-line muscle cells are specified conditionally (Nishida, 1992, 2002). Developmental mechanisms of the larval muscle have been a central subject in embryology of ascidians since E. G. Conklin proposed a maternal cytoplasmic determinant for muscle differentiation (Conklin, 1905; Nishida, 1992, 2002; Nishida and Sawada, 2001).

Muscle actin gene promoters are the first ascidian *cis*-regulatory DNAs whose function was analyzed (Hikosaka *et al.*, 1992, 1993, 1994; Kusakabe *et al.*, 1995; Satou and Satoh, 1996; Kusakabe, 1997). In *H. roretzi*, at least seven muscle actin genes are expressed in larval muscle (Kusakabe *et al.*, 1995). Five of them (*HrMA2*, *HrMA4a*, *HrMA4b*, *HrMA5*, and *HrMA6*) form a tandem cluster in the genome (Kusakabe *et al.*, 1992). The other two genes (*HrMA1a* and *HrMA1b*) form another cluster in which they are closely linked in a head to head arrangement and share a 340-bp bi-directional promoter (Kusakabe *et al.*, 1995). Microinjection of *lacZ* fusion constructs of each of the seven *HrMA* genes demonstrated that short upstream regions (<200 bp) are sufficient for the muscle-specific expression of each gene (Hikosaka *et al.*, 1994; Kusakabe *et al.*, 1995; Satou and Satoh, 1996; Satoh *et al.*, 1996). Another muscle-specific promoter identified and analyzed in *Halocynthia* is that of a myosin heavy chain gene, *HrMHC1* (Araki and Satoh, 1996). The 242-bp upstream region (from -132 to +110) of *HrMHC1* is sufficient to drive muscle-specific reporter gene expression. In *C. intestinalis*, promoter function has been analyzed for a number of muscle-specific genes, including genes encoding troponin I (*Ci-TnI*), troponin T (*Ci-TnT*), calcium-ATPase (*Ci-SercaA*), myosin essential light chain (*Ci-MLC2*), and myosin regulatory light chain (*Ci-MRLC2*) (Vandenbergh *et al.*, 2001; Cleto *et al.*, 2003; Davidson and Levine, 2003; Kusakabe *et al.*, 2004). A muscle-specific enhancer was identified and characterized in the upstream region of the *snail* gene of *C. intestinalis* (Erives *et al.*, 1998; Erives and Levine, 2000). Muscle-specific promoters were also identified in *Molgula* (Kusakabe *et al.*, 1996).

E-box (CANNTG) and CArG box (CC(A/T)₆GG) sequences are conserved in the *HrMA* promoters (Kusakabe *et al.*, 1992; Kusakabe *et al.*, 1995). An E-box is also present in the minimal promoter of *HrMHC1* (Araki and Satoh, 1996). Deletion or mutation of these motifs, however, did not severely impair muscle-specific activity of the promoters (Hikosaka *et al.*, 1994; Satou and Satoh, 1996; Satoh *et al.*,

1996; Araki and Satoh, 1996). Instead, two *cis*-regulatory elements, B- and M-regions, were shown to be necessary and sufficient for muscle-specific transcription from the *HrMA4a* promoter (Satou and Satoh, 1996). The sequences of the B- and M-regions are 5'-TCGCACTTC-3' and 5'-GTGATAACAACCTG-3', respectively. Both motifs were present in the promoter regions of three *H. roretzi* troponin I genes (Yuasa *et al.*, 2002). A 12-bp sequence (5'-TTTT-TTCTTTCA-3'), apparently distinct from the B- and M-regions of *HrMA4a*, is critical for the promoter activity of *HrMHC1* (Araki and Satoh, 1996).

Analysis of a *C. intestinalis* *snail* (*Ci-Sna*) gene enhancer revealed that AC-core E-box sequences (CAA-CTG) and motifs recognized by T-box transcription factors (T-binding motif; GTGNNA) are important for gene expression in muscle lineage cells (Erives *et al.*, 1998; Erives and Levine, 2000). Interestingly, the M-region of *HrMA4a* consists of an AC-core E-box and a T-binding motif, and the B-region also contains a T-binding motif (Erives and Levine, 2000). T-binding motifs are also found in the promoters of *HrMA1a*, *HrMA1b*, *HrMHC1*, and *As-T2*, the *Halocynthia* *Tbx6* gene (Erives and Levine, 2000; Mitani *et al.*, 2001). Mutation or deletion of T-binding motifs reduced activity of these promoters. Candidate transcription factors that recognize T-box motifs in these muscle-specific regulatory regions are CiVegTR and Tbx6 (As-T2) (Mitani *et al.*, 1999; Erives and Levine, 2000; Mitani *et al.*, 2001). Both the *Halocynthia* and *Ciona* *Tbx6* genes are expressed in differentiating muscle cells (Yasuo *et al.*, 1996; Mitani *et al.*, 1999; Takatori *et al.*, 2004). Ectopic and/or over expression of *Halocynthia* *Tbx6* promoted the ectopic expression of *HrMA4* and *HrMHC1* in epidermal cells, and its inhibition of the *Tbx6* function resulted in specific downregulation of *HrMA4* and *HrMHC1* transcription (Mitani *et al.*, 1999). Thus, *Tbx6* seems to be a major transcriptional activator in embryonic muscle cells.

The myogenic bHLH transcription factors, such as MyoD, bind to E-box sequences and are essential for the expression of many muscle-specific genes in vertebrates (Olson, 1990; Weintraub *et al.*, 1991). The ascidian genome contains only one myogenic bHLH family gene: *AMD1* in *Halocynthia* (Araki *et al.*, 1994) and *CiMDF* in *Ciona* (Meedel *et al.*, 1997). The myogenic bHLH gene is expressed in developing muscle cells of ascidian embryos. Sequence comparisons between *C. savignyi* and *C. intestinalis* depicted highly conserved noncoding sequences in the 5' flanking region of the troponin I (*TnI*) gene (Johnson *et al.*, 2004). Functional analyses of the *C. savignyi* *TnI* enhancer demonstrated that the conserved sequences contain *cis*-regulatory elements important for muscle-specific transcription (Johnson *et al.*, 2004). One such conserved region contains a potential binding site for a myogenic bHLH factor; this sequence (5'-TGCAGCTG-3') perfectly matches a muscle-specific motif, M9, found by a computational analysis of muscle-specific promoters of *C. intestinalis* (Fig. 2) (Kusakabe *et al.*, 2004). Therefore, CiMDF may directly activate

transcription of *Tnl*. A number of promoters that lack E-boxes, however, can drive transcription in muscle cells of ascidian embryos (Hikosaka *et al.*, 1994; Satou and Satoh, 1996; Satoh *et al.*, 1996; Araki and Satoh, 1996; Kusakabe *et al.*, 2004). Hence, the role of the myogenic bHLH factor in ascidian embryonic muscle cells remains ambiguous.

Computational analyses of 5' flanking regions of 22 muscle-specific genes of *C. intestinalis* identified several DNA motifs associated with muscle-specific gene expression (Fig. 2) (Kusakabe *et al.*, 2004). One of these motifs, M2, was distributed preferentially in regions from –200 to –100 bp relative to the translational start sites (Fig. 2B). Mutations of M2 sites in the *Ci-MLC2* and *Ci-MRLC2* promoters greatly reduced gene expression in muscle cells (Fig. 2C). When M2 sites were located upstream of a basal promoter, the reporter *GFP* was specifically expressed in muscle cells (Fig. 2C). Motif M2 contains a consensus binding sequence for a CREB transcription factor. CREB and ATF proteins together constitute a subfamily of the basic leucine zipper (bZip) protein family. A recent study demonstrated that mammalian CREB binds directly to the retinoblastoma protein gene (*RB*) promoter and activates *RB* transcription in differentiating myoblasts by recruiting MyoD and the p300 coactivator (Magenta *et al.*, 2003). This MyoD-mediated transactivation is independent of the E-boxes and of the direct binding of MyoD to the promoter sequences. Interestingly, there are no E-box sequences in the *Ci-MLC2* and *Ci-MRLC2* promoters. In the human *RB* promoter, the CREB binding site is located at positions from –192 to –187 relative to the transcription start site (Magenta *et al.*, 2003). This location is similar to regions where M2 sites are frequently found in the *Ciona* muscle-specific promoters. These observations suggest that a *Ciona* CREB or CREB-related protein regulates muscle-specific transcription by a mechanism similar to that found in *RB* activation in mammalian skeletal muscle (Kusakabe *et al.*, 2004).

Differentiation of primary muscle cells in ascidian embryos is triggered by maternally provided mRNA encoding a Zic-like zinc-finger protein, macho-1 (Nishida and Sawada, 2001). *Ciona* macho-1 recognizes a nucleotide sequence, 5'-GCCCCCGCTG-3', that resembles the mammalian Zic binding sites (Yagi *et al.*, 2004a). Potential Zic binding sequences were found in 5' flanking regions of transcription factor genes, *Tbx6b* and *snail*, both of which are specifically expressed in muscle lineage cells. So far, however, macho-1-binding sequences have not been reported in *cis*-regulatory regions of muscle-specific differentiation genes.

Notochord

The ascidian larva has a notochord comprising 40 cells, whose lineage has been described completely (Conklin, 1905; Nishida, 1987). The 32 anterior notochord cells are derived from the A4.1 blastomeres (A-line) of the bilaterally symmetrical 8-cell embryo and the eight posterior cells are derived from the B4.1 blastomeres (B-line). As the notochord is a key feature of chordates, the elucidation of the

genetic circuitry underlying its formation in ascidian embryos is central to understanding molecular developmental mechanisms underlying chordate evolution (Satoh and Jeffery, 1995; Di Gregorio and Levine, 1998; Satou and Satoh, 1999).

Notochord lineage-specific promoters and enhancers have been identified and characterized for both developmental regulatory genes (Corbo *et al.*, 1997a; Takahashi *et al.*, 1999b; Di Gregorio *et al.*, 2001) and a downstream differentiation gene (Di Gregorio and Levine, 1999). The first notochord-specific *cis*-regulatory DNA identified in chordates is an enhancer of the *C. intestinalis* *Brachyury* gene (*Ci-Bra*) (Corbo *et al.*, 1997a). *Brachyury* encodes a sequence-specific activator that contains a T-box DNA binding domain and is critical for notochord differentiation in vertebrate embryos (Hermann *et al.*, 1990; Smith, 1999). *Brachyury* is expressed exclusively in the *H. roretzi* (Yasuo and Satoh, 1993) and *C. intestinalis* (Corbo *et al.*, 1997a) notochord precursor cells. The spatial and temporal patterns of *Brachyury* expression coincide with the developmental fate restriction of the notochord lineages. Regulatory mechanisms of notochord-specific activation of *Ci-Bra* have been extensively studied (Corbo *et al.*, 1997a; Fujiwara *et al.*, 1998; Corbo *et al.*, 1998; Takahashi *et al.*, 1999b; Yagi *et al.*, 2004b). The minimal *Ci-Bra* enhancer for notochord specific-expression is 434 bp in length (Corbo *et al.*, 1997a). The *Ci-Bra* enhancer contains recognition sequences of Suppressor of Hairless [Su(H)] (Corbo *et al.*, 1998) and Snail (*Ci-Sna*) (Fujiwara *et al.*, 1998). Su(H) activates the *Ci-Bra* enhancer in all the mesodermal lineages, including the notochord, muscle, and trunk mesenchyme (Corbo *et al.*, 1998). The *Ci-Sna* gene is expressed in the tail muscle cells and trunk mesenchyme, and *Ci-Sna* directly represses *Ci-Bra* expression (Corbo *et al.*, 1997b; Fujiwara *et al.*, 1998). A forkhead transcription factor gene, *FoxD*, and a zinc finger transcription factor gene, *ZicL*, are also involved in the process of notochord specification in *Ciona* (Imai *et al.*, 2002a, 2002b; Yagi *et al.*, 2004b). *FoxD* activates the expression of *ZicL* in the A-line notochord cells (Imai *et al.*, 2002a). *ZicL* expression is essential for the differentiation of A-line notochord cells but not of B-line notochord cells (Imai *et al.*, 2002b). *ZicL* directly binds to the proximal upstream region of *Ci-Bra* that is required for the basal activation of the gene (Yagi *et al.*, 2004b). Several experiments consistently suggested that *ZicL* is a direct activator of *Ci-Bra* (Yagi *et al.*, 2004b). In B-line notochord cells, the Notch signaling pathway, which is downstream of *FoxD*, is likely to be involved in the activation of *Brachyury* (Imai *et al.*, 2002a).

The function of *cis*-regulatory regions of the *Brachyury* gene has also been analyzed in *H. roretzi* (Takahashi *et al.*, 1999b). Deletion analysis of the 5' flanking region of the *H. roretzi* *Brachyury* gene (*As-T*) demonstrated that a module between –289 and –250 bp is responsible for notochord-specific expression of a *lacZ* reporter. The 5' flanking region of *As-T* contains a potential T-binding motif (ACCTAGGT) around –160 bp. Takahashi *et al.* (1999b) suggest that the

T-binding motif is responsible for autoactivation of the gene.

Brachyury is a key regulator of notochord formation in ascidian embryos (Yasuo and Satoh, 1998; Takahashi *et al.*, 1999a; Satou *et al.*, 2001b). *Ci-Bra* triggers the transcription of various downstream genes in notochord cells (Takahashi *et al.*, 1999a; Hotta *et al.*, 2000). The upstream regulatory region of a *Ci-Bra* target gene, *Ci-tropomyosin-like (Ci-trop)*, has been characterized (Di Gregorio and Levine, 1999). A minimal, 114 bp enhancer is sufficient to direct the expression of the heterologous promoter in the notochord. This enhancer contains *Ci-Bra* binding sites and deletion of the sites inactivates notochord-specific activity of a *Ci-trop/lacZ* transgene. Thus, Brachyury seems to directly activate notochord-specific downstream genes in ascidian embryos.

Another *cis*-regulatory DNA that might be involved in notochord development is an element in the upstream region of *Ci-lkB*, a gene encoding $\text{l}\kappa\text{B}$ homologue of *C. intestinalis* (Kawai *et al.*, 2005). When the GFP fusion protein of *Ci-rel1*, a NF- κB /Rel homologue of *C. intestinalis*, was overexpressed in the notochord, the expression of *Ci-lkB* was remarkably enhanced in notochord cells at the beginning of the tailbud stage. The 1.0-kb upstream region specifically drives *lacZ* reporter expression in the notochord, while mutation or deletion of a κB consensus sequence in this upstream region results in decreased *lacZ* expression in the notochord and ectopic *lacZ* expression in mesenchyme and epidermis. *Ci-rel1* can directly bind to this κB consensus sequence *in vitro*. Thus, transcriptional regulation mediated by the NF- κB /Rel signaling pathway may be involved in notochord formation in ascidian embryos.

Heart

Trunk ventral cells (TVCs) of the ascidian larva are precursors for the adult heart and derived from the B7.5 blastomere pairs of the 110-cell stage embryo (Hirano and Nishida, 1997; Satou *et al.*, 2004). The ascidian heart first appears after metamorphosis as a tube with a single layered myoepithelium that is continuous to a single layered pericardial wall (Ichikawa and Hoshino, 1967; Davidson and Levine, 2003). Conserved migration patterns and expression of *Nkx* indicate that early heart specification is conserved among chordates (Davidson and Levin, 2003). Several genes are expressed in both the larval muscle and TVCs (Satou *et al.*, 2001a; Kusakabe *et al.*, 2002; Davidson and Levine, 2003). The ascidian *Mesp* gene is specifically and transiently expressed in B7.5 cells and is essential for the specification of heart precursor cells, which express *Nkx*, *HAND*, and *HAND-like (NoTrlc)* genes, suggesting that a mechanism for heart specification beginning with *Mesp* through *Nkx* and *HAND* is conserved among chordates (Satou *et al.*, 2004).

In *Halocynthia*, the promoter of muscle actin genes can drive *lacZ* expression in the TVCs (Kusakabe *et al.*, 1995). Davidson and Levine (2003) identified tissue-specific enhancers for five genes [*Ci-TnI*, *Ci-TnT*, *Ci-NPP*, *29h10*, and *Ci-Hndx (Ci-NoTrlc)*] expressed in the *C. intestinalis*

TVCs. Bioinformatics analysis identified shared sequence motifs within the 5' flanking regions of *Ci-NPP* and *Ci-Hndx*, which show overlapping expression in the TVCs and endoderm (Davidson and Levine, 2003). The shared motifs include putative binding sites for *Nkx*, *Tinman*, and *GATA* factors. A genomic DNA fragment containing the putative *Ci-Hndx* enhancer predicted by the shared motif distribution directed an authentic pattern of *lacZ* expression in the TVCs, trunk lateral cells, which are mesenchymal cells derived from A7.6 blastomeres, and endoderm.

Endoderm

As in the case of vertebrate embryos, the endoderm of ascidian embryos is specified autonomously, and specified endoderm then induces notochord specification (Nishida, 1993, 2002; Satoh, 2001). The autonomy is dependent on maternal determinants that are prelocalized in the cytoplasm of eggs and early embryos (Nishida, 1993). In *Ciona* embryos, the nuclear accumulation of β -catenin is most likely the first step of endoderm specification (Imai *et al.*, 2000). β -catenin is a downstream component of the Wnt signaling pathway and, to exert its function, enters the nucleus and activates downstream genes through TCF/LEF1, which is a HMG box transcription factor (Willert and Nusse, 1998; Sharpe *et al.*, 2001). The nuclear accumulation of β -catenin in *Ciona* presumptive endodermal cells regulates many downstream genes (Imai *et al.*, 2000; Imai, 2003).

Citiff1 is a potential β -catenin target and an early and specific marker of endoderm development in *C. intestinalis* (Ristoratore *et al.*, 1999; Satou *et al.*, 2001c). *cis*-Regulatory regions responsible for the endoderm specific expression of *Citiff1* have been isolated and characterized (Ristoratore *et al.*, 1999; Fanelli *et al.*, 2003). The 315-bp genomic fragment spanning from -355 to -41 relative to the transcription start site is sufficient to activate a heterologous promoter in a pattern essentially identical to that of the endogenous *Citiff1* gene (Fanelli *et al.*, 2003). This enhancer contains at least three distinct regulatory regions; two of which are responsible for activation of transcription in the endoderm and in the mesenchyme, respectively, while the third is a negative control region that represses transcription in mesenchyme.

The *Ciona forkhead/HNF-3 β* gene [*Ci-fkh*; also referred to as *Ci-FoxA-a* (Yagi *et al.*, 2003)] is expressed in the notochord, endoderm, and rudimentary floor plate of the CNS during embryonic development (Corbo *et al.*, 1997b; Di Gregorio *et al.*, 2001). A regulatory sequence (AS) located ~1.7 kb upstream of the transcribed region is shown to be essential for expression in these tissues (Di Gregorio *et al.*, 2001). The analysis of various truncated and deleted *Ci-fkh* promoter sequences showed that transcriptional repression is an essential component of *Ci-fkh* regulation. Removal of repressor sites from the AS resulted in the persistence of the early *Ci-fkh* expression pattern in the lateral ependymal cells of the spinal cord. A series of internal deletions allowed the uncoupling of a region responsible for notochord/endo-

derm expression from the *cis*-regulatory elements controlling expression in the CNS. Partial uncoupling of the notochord and endoderm expression patterns was obtained by mutating a T-box recognition sequence in the distal enhancer. This mutation specifically disrupted expression in the endoderm, whereas staining in the notochord was essentially unaffected. DNA binding assays showed that a GST fusion protein containing the T-domain of the *Ciona* Brachyury protein (Ci-Bra) binds this T-box element, although a different member of the T-box family is probably responsible for regulating *Ci-fkh* expression in the endoderm. DNA binding assays revealed a number of high affinity Ci-Fkh recognition sequences in the 5' regulatory region. Mutations in one of these sites, adjacent to the AS, diminish the expression mediated by an otherwise normal *Ci-fkh/lacZ* transgene, thereby providing evidence for autoregulation (Di Gregorio *et al.*, 2001).

Epidermis

Restriction to epidermal cell fate occurs as early as at the 16-cell stage in ascidian embryogenesis (Conklin, 1905; Nishida, 1987). Differentiation of epidermis is autonomous and controlled by maternal determinants (Nishida, 1994, 2002). *HrEpiA*, *HrEpiB*, *HrEpiC*, and *HrEpiD* are epidermis-specific genes of the *H. roretzi* embryo, which show different temporal expression patterns during embryogenesis. Putative *cis*-regulatory elements shared by *HrEpiB* and *HrEpiD* were investigated by microinjecting *lacZ*-fusion constructs into *H. roretzi* fertilized eggs (Ueki and Satoh, 1995). The 5' upstream regions from -345 to +200 of *HrEpiB* and -166 to +108 of *HrEpiD* are sufficient for epidermis-specific expression of the reporter gene. The 5' flanking regions of *HrEpiB* and *HrEpiD* share several sequence motifs, which might be responsible for regulation of gene expression during epidermal cell differentiation. The 5' upstream sequences of *HrEpiC* that are associated with specific expression patterns of this gene were also analyzed (Ishida and Satoh, 1999). Restriction site mapping and sequencing of genomic clones showed that the *H. roretzi* genome contains two copies of *HrEpiC* genes, *HrEpiC1* and *HrEpiC2*, aligned tandemly in about 8 kb of the genome. A 103-bp 5' flanking region was sufficient for the minimal epidermis-specific expression of *HrEpiC1*. The region between -281 bp and -198 bp of the 5' flanking region was associated with the amplification of the minimal expression of the reporter gene in the epidermis and also with activating *HrEpiC1* on schedule at the 64-cell stage.

Nervous system

The ascidian larva has a central nervous system (CNS) derived from the dorsal neural tube and consisting of about 330 cells, of which about 100 cells are neurons (Meinertzhagen and Okamura, 2001). The expression patterns of developmental regulatory genes along the anteroposterior axis are conserved in the developing CNS between ascidians and vertebrates, suggesting that developmental mech-

anisms of the CNS are conserved among chordates (Wada *et al.*, 1998; Hudson and Lemaire, 2001; Wada and Satoh, 2001; Imai *et al.*, 2002c). The anterior brain vesicle (sensory vesicle) of the CNS contains two sensory organs, an eyespot (ocellus) and a gravity sense organ (otolith), which are responsible for the swimming behavior of the larva (Tsuda *et al.*, 2003). The ascidian larva also has a peripheral nervous system consisting of the adhesive organ and epidermal neurons in the trunk and tail (Takamura, 1998).

A promoter of a synaptotagmin gene (*syt*) was identified and characterized in *H. roretzi* (Katsuyama *et al.*, 2002), and it has been used to label motor neurons and to express wild-type and mutant forms of neuronal proteins (Ono *et al.*, 1999; Okada *et al.*, 2001, 2002). The 3.4-kb upstream region of the *H. roretzi syt* gene can drive reporter gene expression in neuronal and epidermal cells, but not elsewhere. Deletion analysis of the *syt* promoter suggested that *syt* expression in neurons and in the embryonic epidermis depends on distinct *cis*-regulatory regions. In *C. intestinalis*, *cis*-regulatory regions of four genes, *Ci-Gai1*, *Ci-arr*, *Ci-vAChTP*, and *Ci-vGAT*, each of which is expressed in distinct sets of neurons in the central nervous system, were isolated and characterized (Yoshida *et al.*, 2004). *Ci-Gai1* is widely expressed in various types of neurons (Yoshida *et al.*, 2002), and *Ci-arr*, *Ci-vAChTP*, and *Ci-vGAT* are expressed in ocellus photoreceptor cells, cholinergic neurons, and GABAergic neurons, respectively (Nakagawa *et al.*, 2002; Takamura *et al.*, 2002; Yoshida *et al.*, 2004). The function of an upstream regulatory region of a pan-neuronal gene, *Ci-tubulin-β2*, was also examined in *C. intestinalis* (Kusakabe *et al.*, 2004). The reporter gene driven by the 5' flanking region of *Ci-tubulin-β2*, *Ci-Gai1*, *Ci-arr*, and *Ci-vAChTP* recapitulated the endogenous gene expression patterns, while the *Ci-vGAT* promoter can drive *GFP* expression in particular subsets of neurons expressing the endogenous gene. Deletion analysis revealed that the *Ci-Gai1* promoter consists of multiple regulatory modules controlling expression in different types of cells.

The ocellus and otolith in the brain vesicle each contain a single pigment cell, which is important for sensory functions (Sakurai *et al.*, 2004). The promoters of two pigment-cell specific genes, *HrTyr* (tyrosinase) and *HrTyrp* (tyrosinase-related protein), have been identified and characterized in *H. roretzi* (Toyoda *et al.*, 2000, 2004; Wada *et al.*, 2002). A 1.8-kb 5' flanking region of *HrTyr* is sufficient for specific reporter gene expression in pigment cell precursors (Toyoda *et al.*, 2000). Deletion analyses of the 5' region revealed that the upstream region from -152 to the translation start site can drive gene expression in the pigment cells. An essential positive *cis*-regulatory element lies close to position -152, positive elements are also located in the region between -1.8 and -1.69 kb, and an additional regulatory sequence, which may act to restrict expression to the pigment cell lineage, is present between positions -250 and -170 (Toyoda *et al.*, 2000). The 73-bp 5' flanking region from -73 to -1 of *HrTyrp* is sufficient for gene expression in

the pigment cells, although regions more upstream seem to be required for stronger expression (Toyoda *et al.*, 2004). The *H. roretzi* *Otx* gene *Hroth* activates transcription of *HrTyrr* by binding either or both of sequences at –113/–108 and –90/–73 (Wada *et al.*, 2002). The human *Tyrr2* gene also has an *Otx* binding site and is activated by OTX2 (Takeda *et al.*, 2003). Therefore, *Otx* may regulate expression of *Tyrr* genes in both vertebrates and ascidians. Because putative binding sites for Pax-3 are scattered in the upstream regions of *HrTyrr* and *HrTyr* (Toyoda *et al.*, 2000, 2004) and overexpression of *HrPax3/7* induces ectopic tyrosinase enzyme activity (Wada *et al.*, 1997), *HrPax3/7* might activate transcription of *HrTyrr* and *HrTyr*. In vertebrates, Pax-3 may affect expression of tyrosinase family gene via the microphthalmia protein (Mitf), a basic helix-loop-helix protein transcription factor that is essential for melanocyte development (Goding, 2000). Interestingly, however, the minimal promoters of *HrTyr* and *HrTyrr* do not contain an M-box sequence, which is a potential binding site for Mitf and is conserved in the vertebrate tyrosinase family genes.

The function of *cis*-regulatory regions has also been analyzed for several transcription factor genes that are expressed in the central nervous system, including ascidian homologs of *snail* (Corbo *et al.*, 1997b; Erives *et al.*, 1998; Boffelli *et al.*, 2004), *forkhead/HNF-3 β* (Di Gregorio *et al.*, 2001; Boffelli *et al.*, 2004), *Msx* (Russo *et al.*, 2004), *Otx* (Oda-Ishii and Saiga, 2003; Bertrand *et al.*, 2003), *Hox3* (Locascio *et al.*, 1999), *Pitx* (Christiaen *et al.*, 2005), and *Distalless* (Harafuji *et al.*, 2002). Most of these transcription factor genes show complex expression patterns in multiple germ layers.

The *Ciona snail* gene *Ci-Sna* is expressed in the tail muscle, trunk mesenchyme, and CNS, including the brain vesicle and lateral endodermal cells of the nerve cord (Corbo *et al.*, 1997b). A 3.0-kb 5' flanking region of *Ci-Sna* is sufficient to direct the expression of a heterologous promoter in the CNS, muscle, and mesenchyme (Erives *et al.*, 1998). Expression in the CNS persisted when the B4.1 enhancer, which is necessary for expression in the primary muscle and mesenchyme, had been removed from the 3.0-kb 5' flanking sequence. Similarly, mutations of AC-core E-boxes in the B4.1 enhancer resulted in the loss of reporter expression in the primary muscle cells while still activating the gene expression in the brain vesicle and nerve cord (Erives *et al.*, 1998). Recently, a CNS-specific enhancer of *Ci-Sna* was identified by an intraspecies sequence comparison and succeeding experimental analysis (Boffelli *et al.*, 2004). Thus, different *cis*-regulatory elements control the *Ci-Sna* expression in the CNS and mesoderm.

The *Ciona forkhead/HNF-3 β* gene *Ci-fkh* (*Ci-FoxA-a*) is expressed in the posteriormost region of the brain vesicle and in the ventral endodermal cells of the spinal cord (Di Gregorio *et al.*, 2001). The ventral endodermal cells have been proposed to represent a rudimentary floor plate (Corbo *et al.*, 1997b; Olsen and Jeffery, 1997; Shimauchi *et al.*, 1997). Expression of the *Ci-fkh/lacZ* fusion construct con-

taining 3 kb of the 5' flanking region in the nerve cord is restricted to the ventral endodermal cells, while truncated constructs containing less than 1.7 kb of the 5' flanking region exhibit expression in the lateral endodermal cells as well as loss of expression in the ventral cells (Di Gregorio *et al.*, 2001). These results suggest that distal regions of the *Ci-fkh* promoter contain one or more repressor elements responsible for restricting expression to the floor plate. Removal of these elements causes ectopic expression in the two lateral rows of endodermal cells. The same region also contains sequences that are important for the activation of *Ci-fkh*. The sequence interval between –1.77 and –1.65 kb contains a *cis*-regulatory sequence responsible for the normal expression in the ventral endodermal cells. Deletion of a 20-bp upstream sequence from –1.65 to –1.63 kb causes the expanded *lacZ* expression in the lateral endodermal cells. This 20-bp region contains divergent binding sites for Ci-Sna, which is transiently expressed in the lateral endodermal cells (Corbo *et al.*, 1997b). The Ci-Sna repressor binds to this region, excludes expression from the lateral endodermal cells, and restricts *Ci-fkh* expression to the ventral endodermal cells (Di Gregorio *et al.*, 2001). Deletion analyses of the *Ci-Sna* promoter also showed that the 5' flanking regions from –0.96 to –0.9 kb and from –0.61 to –0.55 kb are important for expression in the CNS.

The *Ciona msx* gene *Ci-msxb* has a complex expression pattern during embryogenesis (Aniello *et al.*, 1999). At the beginning of gastrulation, the transcript appears in the precursors of mesenchyme cells, muscle, nerve cord, endodermal strand, CNS, and primordial pharynx (stomodaeum). In the larva, *Ci-msxb* is expressed in the primordial pharynx and in the neck, connecting the brain vesicle and the visceral ganglion. In the juvenile, the expression is restricted to the pharynx and neural gland. A 3.8-kb upstream region of *Ci-msxb* contains regulatory elements that can recapitulate the endogenous gene expression pattern (Russo *et al.*, 2004). The region extending from position –239 to –97 of the *Ci-msxb* promoter contains *cis*-regulatory elements responsible for restricted expression of the *lacZ* reporter in the nervous system. A 30-bp sequence, N3, in this region shows specific binding of nuclear proteins and can direct transcription from basal promoters in the nervous system.

In ascidians, *otx* is the earliest gene known to be expressed in the prospective neural tissue. It plays an important role in early neural development (Wada *et al.*, 1996, 2002, 2004; Wada and Saiga, 1999; Hudson and Lemaire, 2001; Satou *et al.*, 2001c). Bertrand *et al.* (2003) identified a *cis*-regulatory element necessary for activation of *Ciona otx* in the precursor cells of the anterior neural plate (a6.5 blastomeres). This element (a-element) is located between –1541 and –1418. When the a-element was placed upstream of the basal promoter of *Ci-Bra*, *Ci-Gsx*, or *HrMA4a*, it directed transcription in neural precursors at the 110-cell stage. In the absence of a basal promoter, the a-element had no activity, showing that it acts as an enhancer rather than as a promoter. Extensive analyses of function of

the a-element established that neural fate is induced by FGF9/16/20, acting via a combination of maternal GATA (GATAa) and Ets (Ets1/2) transcription factors, which synergistically bind and activate the a-element (Bertrand *et al.*, 2003). The function of *cis*-regulatory regions has been also examined in the *H. roretzi otx* gene, *Hroth* (Oda-Ishii and Saiga, 2003). Three upstream sequences of 10, 16, and 70 bp, respectively, are shown to be responsible for *Hroth* expression in the brain vesicle.

Expression of the paralogous group 3 *Hox* gene (*CiHox3*) of *C. intestinalis* is confined to the anteriormost region of the visceral ganglion of the larval CNS (Locascio *et al.*, 1999). *In vivo* analysis of *CiHox3* promoter function revealed that a *cis*-regulatory element(s) necessary for expression in the CNS is present in the 80 bp of the 5' flanking region from -1943 to -1864 of *CiHox3*. This enhancer activates transcription not only in the visceral ganglion, but also in the brain vesicle. Furthermore, deletion of upstream regions of *CiHox3* resulted in both the loss of CNS expression and the gain of ectopic expression in mesenchyme. Therefore, there seem to be both positive and negative regulatory elements in the *CiHox3* upstream region.

Pituitary homeobox (*pitx*) genes are critical molecular determinants of various processes of craniofacial development, including pituitary organogenesis, in vertebrates. The *C. intestinalis pitx* gene *Ci-pitx* produces two distinct mRNA variants, *Ci-pitxa/b* and *Ci-pitxc*, which are expressed in mutually exclusive embryonic domains (Christiaen *et al.*, 2005). The *Ci-pitxa/b* isoform is expressed in the anterior neural boundary (ANB) at the tailbud stage and in the tail muscle and the stomodæum (oral siphon rudiment/primordial pharynx). On the other hand, *Ci-pitxc* is expressed asymmetrically in the epidermis, left visceral ganglion, and left posterior trunk endoderm as well as in ocellus photoreceptor cells. Separate promoters and regulatory elements regulate the transcription of these two variants. Interspecific comparison of *pitx* locus between *C. intestinalis* and *C. savignyi* identified 10 conserved noncoding regions (Christiaen *et al.*, 2005). Among these regions, two regions, D1 and P2, were shown to contain *cis*-regulatory modules, each of which drives gene expression in a distinct subset of cells in the ABN/stomodæal expression domain.

HETEROLOGOUS BASAL PROMOTERS

Activity of *cis*-regulatory elements has been demonstrated by connecting them with heterologous basal promoters derived from various ascidian genes. The most widely used basal promoter is the *Ci-fkh* basal promoter (Erives *et al.*, 1998; Di Gregorio *et al.*, 2001). The *Ci-fkh* basal promoter is a TATA-less promoter that can be activated by different promoters in various tissues (Erives *et al.*, 1998; Di Gregorio and Levine, 1999; Harafuji *et al.*, 2002; Davidson and Levine, 2003; Boffelli *et al.*, 2004; Johnson *et al.*, 2004; Christiaen *et al.*, 2005). This basal promoter was used for a genome wide-enhancer screen (Harafuji *et al.*, 2002) and for

functional evaluation of conserved noncoding genomic sequences (Boffelli *et al.*, 2004).

The B4.1 enhancer of *Ci-Sna* activates transcription from the basal promoter of *Ci-Bra* only when fused in the *syn* orientation (Erives *et al.*, 1998). In contrast, the same enhancer activates transcription in both orientations when it is placed upstream of the *Ci-fkh* basal promoter. Erives *et al.* (1998) pointed out that the *Ci-Bra* promoter contains a perfect TATAAA sequence while the *Ci-fkh* promoter appears to be TATA-less. The *Ci-Bra* basal promoter is also activated by enhancers of *Ci-fkh* (Di Gregorio *et al.*, 2001) and *Ci-otx* (Bertrand *et al.*, 2003).

Other basal promoters that have been used for functional assays of heterologous *cis*-regulatory sequences include those of *Ci-Sna* (Di Gregorio *et al.*, 2001), *CiHox3* (Fanelli *et al.*, 2003; Russo *et al.*, 2004), *HrMA4a* (Bertrand *et al.*, 2003), and *Ci-Gsx* (Bertrand *et al.*, 2003). Another example of promoter activation by heterologous *cis*-regulatory sequences is enhancer trapping in the *Ciona* genomes using the *Minos* transposon. In germline transgenesis using a *Minos* vector containing an upstream region of *CiTPO*, which is an endostyle-specific gene encoding thyroid peroxidase, Sasakura and his colleagues obtained enhancer trap lines of *C. intestinalis* (Sasakura *et al.*, 2003; Awazu *et al.*, 2004) and *C. savignyi* (Matsuoka *et al.*, 2004).

INTERSPECIFIC ACTIVITY OF *CIS*-REGULATORY DNA

Studies on the activity of *cis*-regulatory DNAs in different species, both closely- and distantly-related, can provide clues to understand the diversity and evolution of organisms. Interchangeability of *cis*-regulatory regions also has a practical application because it allows us to use a regulatory DNA from one species as a molecular tool to express genes and proteins in another species. It has been shown that *cis*-regulatory regions are usually interchangeable between *C. intestinalis* and *C. savignyi* (Nakatani *et al.*, 1999; Deschet *et al.*, 2003; Matsuoka *et al.*, 2004; Johnson *et al.*, 2004).

Ascidians are divided into two major orders, Enterogona and Pleurogona. *Ciona* belongs to the former order, which has a single gonad, and *Halocynthia* to the latter, which has a pair of gonads. These two orders diverged early during the evolutionary history of ascidians (Wada *et al.*, 1992; Wada, 1998; Swalla *et al.*, 2000). Nonetheless, *Halocynthia* *cis*-regulatory regions tested so far often show authentic expression patterns in *Ciona*, and *vice versa*. For example, a *Halocynthia* muscle actin promoter is specifically activated in tail muscle cells of *C. savignyi* (Hikosaka *et al.*, 1993) and *C. intestinalis* (Corbo *et al.*, 1997a). The -3.5 kb upstream region of *Ci-Bra* drives a *lacZ* reporter in notochord cells of *H. roretzi* (Takahashi *et al.*, 1999b). When the *Ci-Bra* promoter was deleted down to -250 bp, however, the reporter gene was not expressed in the notochord of *H. roretzi*, but it was expressed in the notochord of *C. intestinalis*. Thus some alterations seem to have occurred in organization of promoters during evolution of *Ciona* and *Halocynthia*.

cynthia (Takahashi *et al.*, 1999b). Promoters of muscle actin genes from *M. oculata* and *M. occulta*, which are diverged members of the order Pleurogona (Hadfield *et al.*, 1995; Swalla *et al.*, 2000), can also drive reporter gene expression in muscle cells of *C. intestinalis* (Kusakabe *et al.*, 1996).

Evolutionary changes of promoter activity have been examined between two closely-related species, *M. oculata* and *M. occulta*, with different modes of development (Kusakabe *et al.*, 1996). *Molgula oculata* shows indirect development with a tadpole larva (urodele development), while *M. occulta* has lost the tailed larva, a mode known as anural development. The anural larva of *M. occulta* lacks a neural sensory organ and a tail with a differentiated notochord and muscle cells. *Molgula occulta* produces notochord and muscle precursor cells during embryonic development but they remain undifferentiated in the posterior region of the larva (Swalla and Jeffery, 1990). The urodele species *M. oculata* has a larval muscle actin gene *MocuMA1*, which is single-copy and intronless (Kusakabe *et al.*, 1996). The anural species *M. occulta* has two paralogous muscle actin genes, *MoccMA1a* and *MoccMA1b*, which are also intronless and likely to be orthologous to *MocuMA1*. The coding regions of *MoccMA1a* and *MoccMA1b* genes contain critical deletions and/or insertions that would make their translated proteins nonfunctional actins, suggesting they are pseudogenes. A fusion gene construct of the 702-bp upstream region of *MocuMA1* fused with *lacZ* is expressed in the tail muscle cells of urodele embryos. Interestingly, this construct is also expressed in vestigial muscle cells of the *M. occulta* anural larva, suggesting that transcription factors responsible for muscle-specific expression of muscle actin genes have been retained in *M. occulta* embryos. The function of muscle actin pseudogene promoters in the anural species *M. occulta* also has been investigated by microinjecting promoter-*lacZ* fusion constructs into *C. intestinalis* eggs. The results indicate that both *MoccMA1a* and *MoccMA1b* promoters retain muscle-specific activity although it is reduced in *MoccMA1b*. These analyses on muscle actin genes and their promoters suggest that the regression of muscle cell differentiation in the anural embryo of *M. occulta* is mediated by loss-of-function mutations of muscle actin genes rather than by changes in muscle-specific transcription factors.

The activity of ascidian *cis*-regulatory DNAs has been examined in vertebrates. Locascio *et al.* (1999) generated transgenic mouse embryos bearing a *lacZ* reporter connected with *CiHox3* regulatory regions. When a 2.3-kb genomic fragment including the region involved in neural-specific *CiHox3* expression in *Ciona* embryos was used, the transgenic embryos consistently gave reporter expression in a pattern reminiscent of mouse *Hox* regulatory elements. However, when they tested a smaller promoter fragment that was responsible for the specific reporter gene expression in the *Ciona* nervous system, the *lacZ* construct did not generate any specific pattern of expression in transgenic mouse embryos. Conversely, when *lacZ* constructs of the mouse *kreisler*-dependent enhancer regions, involved in the

expression of *Hoxa3* and *Hoxb3* in the hindbrain, were electroporated into *Ciona* embryos, no nervous system-specific expression was observed (Locascio *et al.*, 1999). These results suggest that the regulation of *Hox3* gene expression in the mouse and ascidian CNS does not appear to be mediated by the same highly conserved elements. Mice and ascidians might use different regulatory elements and components for *Hox3* expression, and/or similar components might be involved but they have diverged and are unable to function across a large evolutionary distance (Locascio *et al.*, 1999).

BIOINFORMATICS AND COMPARATIVE GENOMICS

Given the increasing number of sequenced metazoan genomes, the prediction of *cis*-regulatory elements by computational methods has become an attractive approach to identify *cis*-regulatory DNAs on a genomic scale (Ohler and Niemann, 2001; Markstein and Levine, 2002; Halfon and Michelson, 2002). In addition to its phylogenetic proximity to vertebrates, the compactness of the genome and the manipulability of gene expression and function make ascidians attractive model organisms to study *cis*-regulatory sequences by computational approaches. Furthermore, the availability of the whole-genome sequences from two *Ciona* species and intraspecies polymorphisms allows comparative genomics approaches to identify *cis*-regulatory DNAs. Recently published studies using different approaches demonstrate that bioinformatics and comparative genomics can help identify *cis*-regulatory DNAs in the ascidian genome (Boffelli *et al.*, 2004; Christiaen *et al.*, 2005; Johnson *et al.*, 2004; Kusakabe *et al.*, 2004).

Genes regulated by the same transcription factors are expected to share *cis*-regulatory elements in their flanking and/or intronic noncoding regions. DNA motifs over-represented in 5' flanking regions of potentially co-regulated genes were identified by computational analyses of 5' flanking regions of 50 tissue-specific genes from genome databases of *C. intestinalis* and *C. savignyi* (Kusakabe *et al.*, 2004) (Fig. 2). Three groups of potentially co-regulated genes (photoreceptor, pan-neuronal, or muscle-specific gene groups) were selected according to their spatial expression patterns, which were determined by whole-mount *in situ* hybridization. Several DNA motifs were distributed predominantly in upstream regions of the co-regulated gene groups (Fig. 2A). Some of the motifs show substantial similarities to binding sites for known transcription factors, while others show no distinct similarities to known transcription factor binding sites. Furthermore, some of the muscle-specific motifs are enriched in regions that are located specific distances from the translational start site (Fig. 2B). For example, one motif, called M2, is preferentially distributed to regions from -200 to -100 bp relative to the translational start site. Another motif, M1, is more frequently found in the distal half than in the proximal half of the 1000-bp upstream regions. An M9 site, which contains a GC-core E-box, is

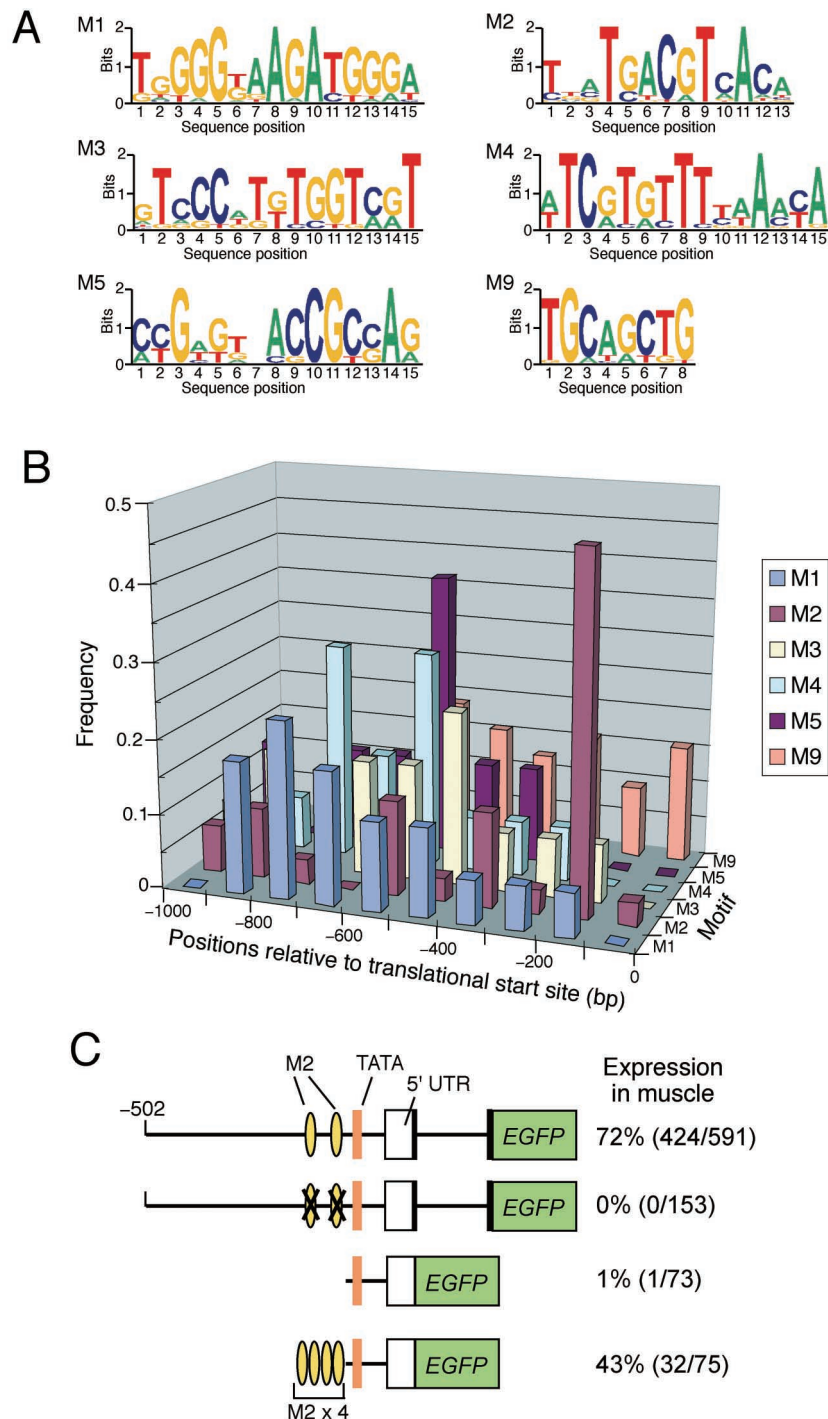


Fig. 2. Computational discovery of DNA motifs associated with muscle-specific gene expression in *C. intestinalis* larvae (Kusakabe *et al.*, 2004). **(A)** Motif logos of the six over-represented motifs (M1, M2, M3, M4, M5, and M9). The height of the stack of letters is proportional to the information content, and the relative frequency of each base is given by its relative height. A perfect match would have a score of two bits. **(B)** Positional distributions of the occurrence of six over-represented motifs found in upstream regions of the muscle-specific gene set. The positions of base 1 of the motifs were binned in 100-bp intervals. 'Frequency' represents the proportion of the number of motifs found in a 100-bp interval to the total number of motifs found in the 1000-bp upstream region. **(C)** Function of M2 sites in muscle-specific expression of a myosin essential light chain gene (*Ci-MLC2*). Schematic diagram showing the structure of wild type and mutant *GFP* constructs. A 643-bp genomic DNA, containing a 5' flanking sequence, the first exon, the first intron, and a 5' portion of the second exon of *Ci-MLC2*, is connected with an enhanced GFP coding sequence (*EGFP*). The exons are indicated by an open box (noncoding region) and solid boxes (coding region). Two M2 sites and the putative TATA-box are indicated by yellow ellipses and an orange box, respectively. Numbers at the 5' end of the wild-type construct indicate nucleotide positions relative to the translational start site (+1). The percentages of positive embryos were indicated at the right side of the drawing representing each construct. Numbers in the parentheses indicate the number of positive and total embryos scored for each constructs.

often found downstream of the most proximal M2 site. As already mentioned in a previous section of this article, *in vivo* functional analysis of M2 sites in muscle-specific promoters demonstrated that M2 sites are critical for the muscle-specific expression of some genes (Fig. 2C), suggesting the validity of computational prediction of *cis*-regulatory elements.

Sequence comparisons between the genomes of organisms separated by varying degrees of evolutionary distance currently serve as an essential means to identify genes as well as *cis*-regulatory elements (Ansari-Lari *et al.*, 1998; Nobrega *et al.*, 2003; Thomas *et al.*, 2003; Boffelli *et al.*, 2003, 2004). Both intraspecific and interspecific comparisons have been used to identify noncoding regulatory elements in *Ciona*. The genome of *C. intestinalis* shows a high rate of allelic polymorphism, with an average 1.2% of the nucleotides differing between chromosome pairs of a single individual (Dehal *et al.*, 2002). This high degree of allelic variation, more than 15-fold that noted in humans, is probably a consequence of the large effective population size of *C. intestinalis*. Boffelli *et al.* (2004) exploited sequence variation within *C. intestinalis* to computationally identify regions subjected to fast and slow rates of evolution. They determined the extent of sequence polymorphism in several *C. intestinalis* subpopulations collected at multiple locations worldwide. Regions with low mutation rates efficiently demarcated functionally constrained sequences: these include a set of noncoding *cis*-regulatory elements as well as the location of coding sequences (Boffelli *et al.*, 2004). By *in vivo* reporter assays using *C. intestinalis* embryos, slow-

evolving regions in the 5' flanking regions of *Ci-fkh* and *Ci-Sna* were shown to contain tissue-specific *cis*-regulatory elements, while fast-evolving regions failed to drive gene expression.

The genetic distance between the two *Ciona* species, *C. intestinalis* and *C. savignyi*, is so large that unconstrained sequences do not display more similarity than expected by chance (Johnson *et al.*, 2004) (Fig. 3). Nevertheless, the two species are morphologically quite similar to each other, and they are virtually identical in embryogenesis. In fact, interspecific hybrids can be obtained, and are easily reared to the larval stage (Byrd and Lambert, 2000). This suggests that the essential mechanisms of early development are conserved between the two species. Johnson *et al.* (2004) compared nucleotide sequences of eight *C. intestinalis* and *C. savignyi* loci, including *troponin I*, *synaptotagmin*, α -*tubulin*, *Noto9*, *forkhead (FoxA-a)*, *snail*, *tropomyosin-like*, and *Brachyury*. Coding exons showed a high degree of sequence similarity, whereas untranslated regions, introns, and intergenic regions rarely contained conservation beyond what was expected by chance (Fig. 3). Against this background of extremely low noncoding sequence similarity, the intergenic regions of these loci contain short significantly conserved sequences located 5' to the predicted start of transcription. *LacZ* reporter constructs that contained these conserved noncoding sequences recapitulated endogenous expression patterns of the genes in both species. As a case study for the sequence-guided dissection of gene regulation and expression, Johnson *et al.* (2004) further conducted a detailed functional and computational analysis for the tropo-

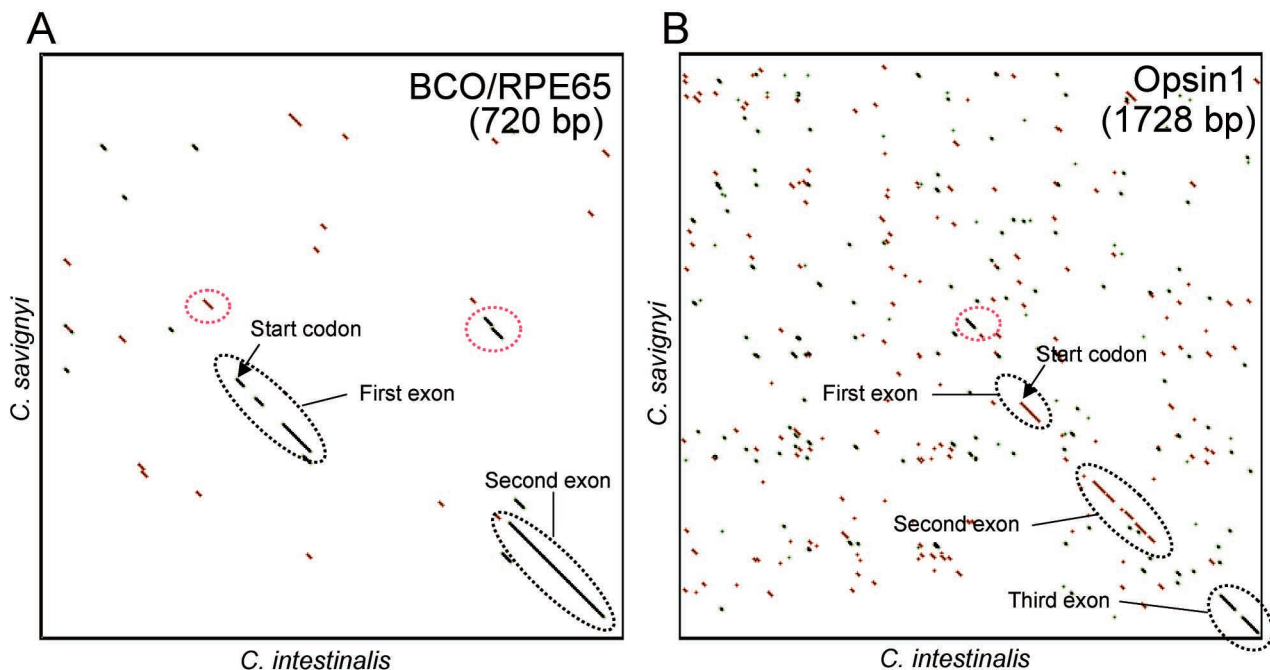


Fig. 3. Comparison of 5' flanking regions of two brain-specific genes, *BCO/RPE65* (Nakashima *et al.*, 2003) (A) and *opsin1* (Kusakabe *et al.*, 2001) (B), between *C. intestinalis* and *C. savignyi* by dot-matrix plots. In contrast to the well-conserved protein-coding exons (black dotted circles), introns, untranslated regions, and intergenic regions show almost no similarity. Conserved sequences found in these noncoding regions (red dotted circles) are good candidates for *cis*-regulatory elements.

nin I gene (*Tnl*). They identified a 363-bp minimally sufficient regulatory region (MSRR) of *Tnl*, which contains four highly conserved sites and is sufficient for strong muscle-specific expression in embryos. Deletion and mutation analyses of *Tnl* MSRR demonstrated that the four short conserved regions contain *cis*-regulatory elements important for the muscle-specific gene expression. One of the highly conserved regions contains putative binding sites for myogenic bHLH transcription factors, and mutations of these sites greatly diminish muscle-specific activity. Interestingly, as described in a previous section, this conserved region also contains a sequence that perfectly matches the muscle-specific motif M9 identified by an independent computational study of *Ciona* muscle-specific promoters (Kusakabe *et al.*, 2004). Sequence comparisons between orthologous loci of *C. intestinalis* and *C. savignyi* have also been used to identify *cis*-regulatory regions of *Otx* (Bertrand *et al.*, 2003), *Brachyury* (Yagi *et al.*, 2004a), *Musashi* (Awazu *et al.*, 2004), and *Pitx* (Christiaen *et al.*, 2005). These studies consistently illustrate the value of the interspecific comparison of the *Ciona* genomes for analysis of genetic regulatory systems.

FUTURE PERSPECTIVES: GENOME-WIDE APPROACHES TO GENETIC REGULATORY NETWORKS

As discussed above, *in silico* approaches have become of practical use for the analysis of *cis*-regulatory DNAs in ascidians. It has been possible to perform genome-wide searches for tissue-specific enhancers in *Ciona* by simply attaching random genomic DNA fragments to a basal promoter and then electroporating these into developing embryos (Harafuji *et al.*, 2002). Genome-wide gene expression profiles can be obtained from high-throughput *in situ* hybridization (Makabe *et al.*, 2001; Satou *et al.*, 2001a, 2002; Nishikata *et al.*, 2001; Kusakabe *et al.*, 2002; Fujiwara *et al.*, 2002; Ogasawara *et al.*, 2002) and DNA microarray (Ishibashi *et al.*, 2003; Azumi *et al.*, 2003, 2004) analyses. The sequences and distribution patterns of DNA motifs shared by a group of co-expressed genes can be incorporated into a model promoter structure, and the model can then be used for genome-wide prediction of co-regulated promoters (Berman *et al.*, 2002; Markstein *et al.*, 2002; Halfon *et al.*, 2002; Bulyk *et al.*, 2004; Wenick and Hobert, 2004). This strategy could lead to identification of the co-regulated gene batteries, which could reveal the molecular bases of cell and tissue identity. A genome-wide analysis identified 389 transcription factor genes in the *C. intestinalis* genome (Imai *et al.*, 2004). Among these genes, cDNA clones are available for 352 genes, and their expression profiles have been analyzed. Use of these transcription factor resources should facilitate identification of transcription factors that recognize *cis*-regulatory elements. The genome-wide application of these resources and technologies will break new ground in the field of gene network studies in chordates.

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