Morpholino-based gene knockdown screen of novel genes with developmental function in *Ciona intestinalis*

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Summary

In the present study, we conducted an extensive analysis to identify novel genes with developmental function among *Ciona intestinalis* genes discovered by cDNA projects. Translation of a total of 200 genes expressed during embryogenesis was suppressed by using specific morpholino antisense oligonucleotides. Suppression of the translation of any of 40 genes (one-fifth of the genes tested) was thereby shown to cause specific embryonic defects. Most of these genes have counterpart(s) in mouse and human, suggesting that the present approach will be useful for identifying candidate genes essential for the development of vertebrates. Suppression of translation of 14 of these 40 genes resulted in the 'disorganized body plan' phenotype characterized by gross morphological abnormalities caused by early defects in embryogenesis. These genes encode zinc-finger, transmembrane or Pbx homeodomain proteins. The morphological features of larvae of this phenotypic class varied according to the gene suppressed, suggesting that a distinct developmental event such as tissue specification or cell cycle progression was affected in each type of larva. Suppression of the remaining 26 genes resulted in the 'abnormal tail' phenotype. Some of these genes encode proteins with known functional structures such as Zn-finger and HLH motifs. Twelve genes among them are especially interesting, because their suppression produced defects in the nervous system, as demonstrated by the loss of the sensory pigment cells or palps of the adhesive organ in the knockdown larvae. These results suggest that screening for developmental genes by the reverse genetic approach in *Ciona intestinalis* embryos is effective for identifying novel genes with developmental functions required for the development of chordates.

Supplemental data available online

Key words: *Ciona intestinalis*, Reverse genetics, Novel genes, Morpholino oligonucleotide, Translational suppression

Introduction

In vertebrates, complete genome sequences are now available for human (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001), mouse (Mouse Genome Sequencing Consortium, 2002) and *Fugu rubripes* (Aparicio et al., 2002). These genome sequences and extensive analyses of expressed sequence tags (ESTs), mostly derived from humans and mice, have demonstrated that there are many protein-coding genes, and biological function has been assigned to only a small proportion of the predicted genes. A considerable number of the remaining uncharacterized genes are thought to have special biological roles. One of the key goals of developmental genomics, therefore, is to determine the developmental function of such genes.

Urochordate ascidians are marine invertebrate chordates that have a common ancestor shared by the cephalochordate amphioxus and vertebrates. The organization of ascidian tadpole larvae shows basic features of the chordate body plan (Satoh, 1994; Corbo et al., 2001; Satoh, 2003). The ascidian genome has been proposed to contain a basic set of chordate-type genes corresponding to those present before the large-scale gene duplications occurred in the lineage leading to vertebrates (Holland et al., 1994), and thus ascidian genes have less functional redundancy than vertebrate genes. Recently, the draft genome sequence of the most-studied ascidian, *Ciona intestinalis*, has been reported (Dehal et al., 2002). Its 159 Mbp genome (17 times smaller than that of humans) contains 15,852 protein-coding genes, similar to the number in other invertebrates but only half that found in vertebrates. Vertebrate gene families are typically found in simplified form in the *Ciona intestinalis* genome, supporting the idea that ascidians contain the basic ancestral complement of genes involved in cell signaling and development (Dehal et al., 2002; Satou et al., 2003a; Wada et al., 2003; Yagi et al., 2003). In vertebrates, the function of a gene is often obscured by functional redundancy of the related genes. Therefore, *Ciona* embryos may provide an appropriate experimental system for exploring the functions of genes (Satoh et al., 2003).

The *Ciona intestinalis* cDNA project consortium has conducted comprehensive studies of gene expression profiles in fertilized eggs (Nishikata et al., 2001), cleavage stage embryos (Fujiwara et al., 2002), tailbud embryos (Satou et al., 2001b),

larvae (Kusakabe et al., 2002) and young adults (Ogasawara et al., 2002). A total of more than 480,000 ESTs have been sequenced, and the spatial expression profiles of 5000 randomly selected genes have been determined (Satou et al., 2002a; Satoh et al., 2003). The genes have been classified into three major classes according to the function of the proteins they encode (Lee et al., 1999). Class A includes genes associated with functions in many cell types, class B contains genes associated with cell-cell communication and class C contains genes that function as transcription regulatory proteins. Besides genes that fall into these three classes, there are many genes for which not enough information is available to determine their biological function. These genes have been categorized into two classes, class DI and class DII. Class DI includes sequences that match ESTs or reported proteins with unknown function (mostly from humans and mice), and class DII consists of genes with no significant sequence similarity to known genes. *Ciona intestinalis* cDNA analysis suggests that at least 2500 genes are classified into class DI, and that nearly one quarter of them show specific spatiotemporal expression patterns. Most DI-class genes are assigned counterparts of vertebrate genes with unknown function. Therefore, a comprehensive functional analysis of *Ciona intestinalis* DI-class genes would provide significant information relevant to vertebrate genes with unknown function. We therefore decided to systematically investigate the loss-of-function phenotypes of DI-class genes, starting with 200 genes in the present pilot screen.

In addition to the small size of their genome and the small number of genes, ascidians provide a simple experimental system for investigating the molecular mechanisms underlying cell-fate specification during chordate development (Satoh, 1994; Satoh, 2001). The *Ciona intestinalis* fertilized egg develops within 18 hours into a tadpole larva, through invariant bilateral cleavage, gastrulation, neurulation and tailbud formation. The lineages of embryonic cells are invariant among individuals and have been well documented (Conklin, 1905; Nishida, 1987). The tadpole larva consists of ~2600 cells that form distinct types of tissues and/or organs, including epidermis that covers the entire surface; a central nervous system with two sensory pigment cells; endoderm and mesenchyme in the trunk and notochord; dorsal nerve cord; ventral endodermal strand; and muscle in the tail (Fig. 1A). The *Ciona intestinalis* cDNA project has provided us with genes expressed in a tissue-specific manner for almost all types of tissue (Satou et al., 2001b; Kusakabe et al., 2002). These genes can be used as tissue-specific markers. Furthermore, over 5600 full-length sequences of cDNAs have already been determined (Satoh et al., 2003). These circumstances make *Ciona intestinalis* embryos a suitable system for a reverse genetic approach for identifying novel genes with developmental function.

For many years, mutant screens in the invertebrates *C. elegans* and *Drosophila* have provided us with functional information on many genes. However, reverse genetic approaches have become increasingly attractive, because they offer the possibility of taking interesting candidate genes from genome/EST databases and speedily identifying their roles. RNA-based interference (RNAi) is often useful for inhibiting gene function in *C. elegans* and *Drosophila* (Hannon, 2002). However, there are no reports yet showing that RNAi works well in ascidians. In ascidians, antisense phosphorothioate DNA oligonucleotides (S-DNAs) have been used to block embryonic gene function (Swalla and Jeffery, 1996; Nishida and Sawada, 2001). S-DNAs form RNA-DNA hybrids with the target mRNA and act as substrates for degradation of the mRNA by RNAse H (Cazenave et al., 1989). However, generally speaking, S-DNAs have nonspecific toxic side effects on embryos and therefore their specific effects are not always distinguishable. Recently, another strategy for inhibiting gene function has been shown to be extremely effective. Morpholino antisense oligonucleotides (hereafter referred as only 'morpholinos') have been shown to block translation by preventing ribosomes from binding to target mRNA (Summerton, 1999). Morpholinos cause less toxicity and effectively enable the study of maternal and zygotic gene functions in a range of model organisms (Heasman, 2002). This is also the case in ascidian embryos (Satou et al., 2001a; Wada and Saiga, 2002). Injection of a morpholino into eggs specifically targets a certain gene by suppressing translation and thereby gene function. In zebrafish embryos, it has been reported that the morpholino-induced phenotype is the same as that of a mutant of the targeted gene, although the penetrance of the phenotype is variable (Heasman, 2002). Morpholinos therefore offer a high and rapid throughput assay system for chordate functional genomics applications.

In the present study, we conducted an extensive analysis of genes discovered in the *Ciona intestinalis* cDNA project. To identify genes with developmental function, translation of a total of 200 genes expressed in embryos was suppressed using specific morpholinos. Most of these 200 genes were classified into class DI, genes whose vertebrate counterparts have unknown function. The suppression of the translation of any of 40 of these genes (20% of the genes examined) caused specific embryonic defects. These results demonstrate that the *Ciona* embryo is a powerful tool for identifying developmental genes essential for formation of the chordate body plan. Furthermore, the present study might represent the first example of a screen using morpholinos as a reverse genetic tool to identify genes required for chordate development.

Materials and methods

Biological materials

Ciona intestinalis were cultivated at the Maizuru Fisheries Research Station of the Kyoto University, Maizuru, and the Education and Research Center of Marine Bio-Resources of Tohoku University, Miyagi, Japan. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were dechorionated by treatment with actinase E and sodium thioglycolate as described previously (Satou et al., 2001b). Dechorionated fertilized eggs were microinjected with morpholinos as described below. After microinjection, they were maintained in agar-coated dishes with Millipore-filtered seawater containing 50 mg/ml streptomycin sulfate at 18°C. They developed into tailbud embryos and tadpole larvae by about 13 hours and 18 hours of development, respectively.

Characterization of genes for the screen

For the 200 genes examined in the present study, clones of the corresponding cDNA that encoded the full length of the putative protein were isolated and sequenced as described previously (Satou et al., 2002b). In all of these cDNA clones, there are in-frame stop codon(s) upstream of the putative initiation codon, which supports the validity of the prediction of the initiation codon. Motifs in the putative proteins were searched with SMART (http://smart.embl-heidelberg.de/). Homology searches were performed against the

DDBJ nucleotide database with BlastX, and gene sequences with *P* values less than 10^{-15} were selected as homologs. Temporal expression profiles of genes were determined based on the results of the EST analyses (Satou et al., 2002a). A list of the 200 genes including gene collection IDs, clone IDs (used as the name of each gene here), Accession Numbers of cDNA sequences and nucleotide sequences of the corresponding morpholino are provided in Table S1 at http://dev.biologists.org/supplemental. EST information and the cDNA sequences of the 200 genes are available in our web site (http://ghost.zool.kyoto-u.ac.jp/indexr1.html).

Design of morpholinos and microinjection

Morpholinos were made to order (Gene Tools, LLC). For most of the 200 genes, a complementary morpholino was designed to target a sequence containing the putative initiation codon. For the other genes, a morpholino was designed to target a sequence of the 5′ untranslated region (Table S1 at http://dev.biologists.org/supplemental). Microinjection was carried out as described previously (Satou et al., 2001a). The amount of morpholino injected was 10 fmoles, an amount chosen based on the results of previous reports (Satou et al., 2001a) and our preliminary experiments. Ten fmoles of morpholinos dissolved in distilled water were injected into fertilized eggs and the effects on morphology were determined using stereomicroscopy about 18 hours later at the tadpole larva stage. In cases of the use of a second form of citb018a19 morpholino, 30 fmoles were injected into fertilized eggs. As controls, 10 or 30 fmoles of a morpholino against *lacZ* were injected, but these had no effect on embryogenesis, provided excess amounts were not injected.

Two researchers simultaneously, but independently, injected morpholinos into 25 or more fertilized eggs. When they obtained the same result, it was scored. When the results of the two researchers were inconsistent, a third researcher performed another injection to reach a final conclusion.

Rescue experiments with synthetic mRNA

For rescue experiments, the protein-coding region of *Ci-Bra*, cieg003h01 or citb018a19 that lacked a target sequence for morpholino was amplified by PCR and cloned into pBluescriptRN3 vector. Capped mRNA was synthesized in vitro using a Megascript T3 kit (Ambion) together with the cap analog 7mGpppG. Because the synthesized mRNA lacks the target sequence for the corresponding morpholino, translation from it is not inhibited by the morpholino. The fertilized eggs were injected with 5 pg (*Ci-Bra*) or 25 pg (cieg003h01 and citb018a19) of the synthesized mRNA together with 10 fmoles of the corresponding morpholino, allowed to develop up to the tailbud embryo stage, and examined for marker gene expression or morphology.

Whole-mount in situ hybridization and histochemistry

Whole-mount in situ hybridization was carried out as described previously (Satou et al., 2001b). The probes used were *Ci-Epi1* for epidermal cell differentiation (Chiba et al., 1998), *Ci-ETR* for nervous system development (Satou et al., 2001b), *Ci-talin* for notochord differentiation (Satou et al., 2001b; Sasakura et al., 2003) and ciad005j06 for mesenchyme cell differentiation (Satou et al., 2001b). Probes for *Ci-arr* (Nakagawa et al., 2002), *Ci-opsin1* (Kusakabe et al., 2001), *Ci-opsin3* (Nakashima et al., 2003) and *Ci-Gαi1a* (Yoshida et al., 2002) were also used for experiments with the citb018a19 gene.

Histochemistry for endoderm alkaline phosphatase (ALP) and muscle acetylcholine esterase (AchE) was performed as described (Imai et al., 2000).

Results

Application of morpholinos in a screen to identify genes required for ascidian embryogenesis

In the present study, the function of 200 *Ciona intestinalis* genes was examined using morpholinos. The genes were chosen from a collection identified through the *Ciona intestinalis* cDNA project (Satou et al., 2002b). Of them, 144 genes were selected because they belong to class DI (see Table S1 at http://dev.biologists.org/supplemental). The rest of the genes were selected because they exhibit interesting features in structure and/or expression pattern. Although the 200 genes encode a wide spectrum of proteins with various structures, about one-third of them consist of genes for Zn-finger proteins, which is concurrent with the fact that a number of this type of proteins have been shown to play important roles in development (see Table S1 at http://dev.biologists.org/supplemental). Most of the 200 genes are both maternally and zygotically transcribed, as shown by EST evidence (http://ghost.zool.kyoto-u.ac.jp/indexr1.html).

Translational suppression of each gene was achieved by microinjection of a corresponding morpholino into fertilized eggs, and the resultant embryos were examined for any defect in larval morphology to identify genes required for normal development. When morpholino against *lacZ* mRNA was injected as a negative control, all of the injected embryos developed normally (data not shown). However, injection of morpholino complementary to *Ci-Bra*, an essential regulator of notochord development in *Ciona* embryos (Corbo et al., 1997; Takahashi et al., 1999), at the same dose led to a shortened tail phenotype in almost all injected embryos (Fig. 1B). Expression of *Ci-talin*, a notochord marker (Fig. 1C) was lost in the morpholino-injected embryos (Fig. 1D). These findings are consistent with the proposed role of *Ci-Bra* in notochord formation. The specificity of the action of *Ci-Bra* morpholino was assessed by a rescue experiment with synthetic *Ci-Bra* mRNA. Co-injection of *Ci-Bra* morpholino and *Ci-Bra* mRNA abrogated the loss of *Ci-talin* expression caused by injection of *Ci-Bra* morpholino (Fig. 1E).

A gene was considered to be positive when half or more of the specimens within the group of injected embryos exhibited developmental deficiency. Out of the 200 genes examined, 40 genes were judged to be positive by this criterion (Table 1; information on the 40 positive genes is summarized in Table 2). The remaining 160 genes were judged to be tentatively negative and excluded from the subsequent analysis. It has been reported that, in zebrafish embryos, the penetrance of the effects of morpholinos is variable and much lower than 100% in many cases (Heasman, 2002). Similarly, in our experimental system, the efficiency of morpholinos judged to be positive was variable depending on the morpholino. We estimated that the efficiency (the rate of embryos showing a developmental defect in the injected embryos) was in the range 60-100%, depending on the morpholino, although the reason for this variation is unknown. In Table 2, morpholinos with efficiency of 80% or higher were considered to have strong penetrance, while those with lower efficiency were considered to have weak penetrance.

Classification of phenotypes induced by morpholino-mediated translational inhibition

Phenotypes obtained in the present screen were classified into two major classes, the ‘disorganized body plan’ class and the ‘abnormal tail’ class (Table 1). The first major phenotypic class, the ‘disorganized body plan’ class, was characterized by gross morphological defects. Of 40 genes with a morpholino-

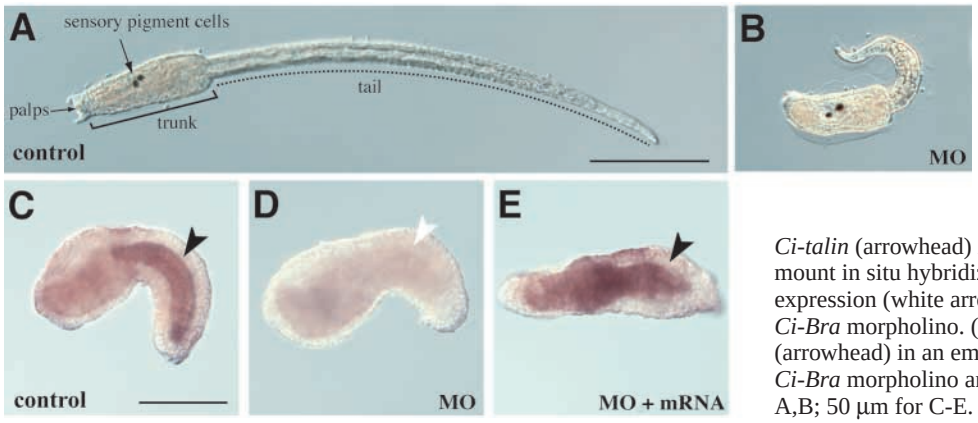

Fig. 1. Control experiments. (A) Morphology of an uninjected control larva. Trunk and tail are indicated by a bracket and a broken line, respectively. (B) Morphology of a larva developed from an egg injected with *Ci-Bra* morpholino. (C) Expression of notochord marker *Ci-talin* (arrowhead) in a tailbud embryo visualized by whole-mount in situ hybridization. (D) The absence of *Ci-talin* expression (white arrowhead) in a tailbud embryo injected with *Ci-Bra* morpholino. (E) Recovery of *Ci-talin* expression (arrowhead) in an embryo at the tailbud stage co-injected with *Ci-Bra* morpholino and its mRNA. Scale bars: 100 μm for A,B; 50 μm for C-E.

induced phenotype, 14 genes belonged to this phenotypic class (Tables 1 and 2; Fig. 2). Neither gastrulation nor neurulation occurred normally during the development of these embryos. In severe cases, even the cleavage pattern became atypical. The morphological features of the larvae of the 'disorganized body plan' phenotypic class was varied depending on the morpholino (Fig. 2).

The second major phenotypic 'abnormal tail' class was characterized by a tail with abnormal morphology. Of the 40 genes for which we detected a morpholino-induced phenotype, 26 genes gave rise to this phenotypic class (Tables 1 and 2; Fig. 3). Embryos showing a phenotype of this class were further subdivided into two groups according to the length of their tail. Embryos of the 'short tail' phenotypic group had a tail whose length was less than half that of the normal tail. Of the 26 genes in the 'abnormal tail' class, eight had this phenotype. For example, embryos injected with *Ci-Bra* morpholino (Fig. 1B) were grouped into this phenotypic class. Embryos with a longer tail were categorized into the 'bent tail' phenotypic group because of their kinked tail (e.g. Fig. 3A). Eighteen genes were associated with this phenotype.

Some embryos showing the 'abnormal tail' phenotype also exhibited two prominent phenotypes related to the formation of the nervous system. One was the 'loss of the sensory pigment cells' phenotype (e.g. Fig. 3C), which was displayed by three genes associated with the 'short tail' phenotype and six genes with the 'bent tail' phenotype. The other was the 'loss of the palps' phenotype (e.g. Fig. 3D), which was displayed by two genes that were associated with the 'bent tail' phenotype and one gene that also gave the 'loss of the sensory pigment cells' and 'short tail' phenotypes. The palps are an adhesive organ located at the anterior tip of the larva, an organ containing peripheral sensory neurons essential for signal transmission for metamorphosis.

Genes associated with the 'disorganized body plan' phenotypic class

To provide an overview of the outcome of the present screen and to illustrate the morphological deficiencies of embryos, several interesting examples are described in the following two sections. Fourteen genes were associated with the 'disorganized body plan' phenotypic class (Table 1). They consisted of 12 DI-class genes, including eight genes for Zn-finger proteins, one DII-class gene and one C-class gene that is a *Ciona intestinalis* homologue of *Pbx* (Table 2). Several intriguing examples in this phenotypic class are described.

cieg015c02 encodes a protein that contains C2H2 type Zn-finger motifs and shows sequence similarity to the human hypothetical protein XP 030938 (Table 2). ESTs for this gene were detected in fertilized eggs and cleaving embryos. Eggs injected with its morpholino developed into rough-surfaced aggregates of cells (Fig. 2A). They were not enclosed by a smooth-surfaced epidermis layer. The cells that comprised them were loosely connected, so that the aggregates were very fragile. In spite of such extreme abnormality, the expression of markers for some endodermal and mesodermal tissues was detected in them, suggesting that they did not simply expire. Rather, it seems likely that a mechanism involved in cell adhesion or epidermis formation is affected in the cieg015c02 knockdown embryos.

cieg040a13 encodes a protein that has a Zpr1 motif (Galcheva-Gargova et al., 1998) and is similar to the human Zn-finger protein 259 (Table 2). ESTs for this gene were scored in fertilized eggs, cleaving embryos and young adults. Eggs injected with morpholino complementary to this gene developed into deformed embryos (Fig. 2B). The size of the cells constituting these embryos was significantly larger than that of any cells comprising normal embryos, suggesting that the process of cell division was affected in these embryos.

Table 1. The overall outcome of the present screen

		Number of genes associated with embryonic defects		
		Phenotypic class†		
Class*	Number of genes examined	Disorganized body plan	Abnormal tail	Total
A	16	0	2	2
B	10	0	2	2
C	3	1	1	2
DI	144	12	15	27
DII	27	1	6	7
Total	200	14	26	40 (20%)

*Class of genes was determined according to Lee et al. (Lee et al., 1999). Class A, genes associated with functions in many cell types; Class B, genes associated with cell-cell communication; Class C, genes for transcription regulatory proteins; Class DI, sequences that match ESTs or reported proteins with unknown function; Class DII, sequences with no significant sequence similarity to known genes.

†Characteristics of each phenotypic class were described in the text.

Table 2. Genes which showed embryonic defects when their activity was suppressed with a specific morpholino oligonucleotide

Gene collection ID*	Clone ID*	Class†	Motif‡	Homologous gene‡	Distribution of homologous genes§						Number of ESTs¶						Phenotype**			Penetrance††	Figure
					Hs	Mm	Dm	Ce	Sc	At	EG	CL	GN	TB	LV	AD	Tail	Neural	Disorganized		
CLSTR00905r1	cilv008a08	C	HOX	Pre-B-cell leukemia transcription factor 3 (*Homo sapiens*)	Hs	Mm	Dm	Ce	–	–	6	8	5	9	7	0	–	–	D	Low	Fig. 2E
CLSTR00624r1	citb002m02	DI	FBOX, WD40	KIAA0696 protein (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	At	3	5	2	4	5	0	–	–	D	High	Fig. 2F
CLSTR01520r1	cieg009f01	DI	RRM	KIAA1579 protein (*Homo sapiens*)	Hs	Mm	–	–	–	–	24	6	3	3	0	1	–	–	D	High	Fig. 2G
CLSTR01949r1	cicl024n06	DI	ZNF_U1	cDNA FLJ31121 protein (*Homo sapiens*)	Hs	Mm	Dm	Ce	–	At	11	4	1	2	0	0	–	–	D	Low	Fig. 2H
CLSTR02215r1	cicl004d05	DI	ZNF_C2H2	Hypothetical protein DKFZp572C163.1 (*Homo sapiens*)	Hs	Mm	–	Ce	–	–	0	2	2	2	11	1	–	–	D	Low	Fig. 2I
CLSTR02310r1	citb011j18	DI	ZNF_C2H2	Hypothetical protein FLJ10891 (*Homo sapiens*)	Hs	Mm	–	–	–	–	1	0	0	1	1	0	–	–	D	High	Fig. 2J
CLSTR03177r1	cieg003h01	DI	(None)	Hypothetical protein FLJ12377 (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	–	2	3	0	0	1	0	–	–	D	High	Fig. 2D
CLSTR05630r1	cieg008h18	DI	(None)	Hypothetical protein FLJ20303 (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	At	8	3	5	4	0	1	–	–	D	High	Fig. 2L
CLSTR07724r1	cieg052o01	DI	ZNF_C2H2	DNA-binding protein CPBP (*Homo sapiens*)	Hs	Mm	Dm	Ce	–	–	3	1	0	1	6	0	–	–	D	High	Fig. 2M
CLSTR09851r1	cieg015c02	DI	ZNF_C2H2	Hypothetical protein XP_030938 (*Homo sapiens*)	Hs	Mm	Dm	Ce	–	–	2	1	0	0	0	0	–	–	D	High	Fig. 2A
CLSTR09872r1	cieg015i17	DI	ZNF_C2H2	Hypothetical protein FLJ14345 (*Homo sapiens*)	Hs	Mm	–	–	–	–	1	0	0	1	0	0	–	–	D	Low	Fig. 2N
CLSTR10813r1	cicl045c22	DI	ZNF_C2H2	Hypothetical protein XP_041139 (*Homo sapiens*)	Hs	Mm	Dm	–	–	–	5	1	3	6	2	1	–	–	D	High	Fig. 2C
CLSTR11183r1	cieg040a13	DI	Zpr1	Zinc finger protein 259 (*Homo sapiens*)	Hs	Mm	Dm	Ce	(Sp)	At	1	1	0	0	0	2	–	–	D	High	Fig. 2B
CLSTR04831r1	cilv015k10	DII	(None)	No significant similarity	–	–	–	–	–	–	0	0	0	0	13	0	–	–	D	Low	Fig. 2K
CLSTR00341r1	citb035h24	DI	EFf	Calumenin (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	At	10	10	9	10	5	4	Bent	–	–	High	
CLSTR00506r1	cilv040l06	DI	TSP1	T21B6.3 protein (*Caenorhabditis elegans*)	–	–	–	Ce	–	–	1	1	0	18	17	0	Bent	–	–	High	
CLSTR06766r1	cieg029k06	DI	(None)	Hypothetical protein DKFZp761A078 (*Homo sapiens*)	Hs	Mm	Dm	–	–	–	3	2	2	1	1	0	Bent	–	–	High	
CLSTR07788r1	cieg031m04	DI	zf-DHHC	Hypothetical protein XP_029461 (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	At	3	4	1	0	1	2	Bent	–	–	High	
CLSTR09939r1	cieg017e06	DI	PKD, EGF	KIAA0319 gene product (*Homo sapiens*)	Hs	Mm	–	–	–	–	1	0	0	0	0	0	Bent	–	–	Low	
CLSTR02157r1	cilv019e03	DII	(None)	No significant similarity	–	–	–	–	–	–	0	1	0	1	3	1	Bent	–	–	Low	
CLSTR03452r1	cicl054n02	DII	TSC22	No significant similarity	–	–	–	–	–	–	9	3	3	3	3	2	Bent	–	–	Low	
CLSTR03964r1	cilv009f05	DII	(None)	No significant similarity	–	–	–	–	–	–	0	2	1	1	7	12	Bent	–	–	High	
CLSTR04736r1	cicl053g02	A	aminotran_3	GABA aminotransferase (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	At	0	3	1	0	5	1	Bent	–	–	Low	
CLSTR03300r1	cicl015f04	B	S_TKc, S_TK_X	cAMP-dependent kinase subunit (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	At	9	7	4	3	14	0	Bent	–	–	High	
CLSTR03158r1	cieg003e24	DI	FCH, SH3	KIAA0769 gene product (*Homo sapiens*)	Hs	Mm	Dm	–	Sc	–	5	8	6	2	1	2	Short	–	–	High	
CLSTR06851r1	cieg047f01	DI	ZNF_C2H2	Hypothetical protein XP_039908 (*Homo sapiens*)	Hs	Mm	Dm	–	–	–	2	0	0	0	0	1	Short	–	–	Low	
CLSTR06386r1	cieg017l16	DII	HLH	No significant similarity	–	–	–	–	–	–	4	0	1	6	2	3	Short	–	–	High	Fig. 3F
CLSTR00676r1	cilv018l17	B	RGS	Regulator of G protein signaling 20 (*Homo sapiens*)	Hs	Mm	Dm	Ce	–	–	0	0	0	7	3	1	Short	–	–	High	
CLSTR00353r1	citb018a09	DI	RING, WWE	Hypothetical protein DKFZp434O1427 (*Homo sapiens*)	Hs	Mm	Dm	Ce	(Sp)	–	9	13	6	9	1	0	Bent	SPC	–	High	
CLSTR00405r1	cieg015b23	DI	ZNF_C2H2	KIAA1629 protein (*Homo sapiens*)	Hs	Mm	–	–	–	–	2	1	1	1	1	0	Bent	SPC	–	High	Fig. 3B
CLSTR01478r1	cieg040j10	DI	(None)	KIAA0253 gene product (*Homo sapiens*)	Hs	Mm	–	–	–	–	4	0	4	1	0	0	Bent	SPC	–	Low	
CLSTR10499r1	cieg022g05	DI	SANT	SNAP190 (*Homo sapiens*)	Hs	Mm	–	Ce	–	At	5	1	2	3	0	0	Bent	SPC	–	Low	Fig. 3A
CLSTR07753r1	ciad046i23	DII	ZP	No significant similarity	–	–	–	–	–	–	0	0	0	0	3	1	Bent	SPC	–	High	
CLSTR01032r1	citb006g18	A	RPE65	β-Carotene 15,15′- dioxygenase (*Homo sapiens*)	Hs	Mm	Dm	Ce	–	At	2	3	7	20	10	0	Bent	SPC	–	High	
CLSTR13121r1	cicl057g13	DI	TPR	Hypothetical protein FLJ12890 (*Homo sapiens*)	Hs	Mm	Dm	–	–	At	2	2	11	0	0	0	Short	SPC	–	Low	Fig. 3C
CLSTR11771r1	cieg055k01	DII	(None)	No significant similarity	–	–	–	–	–	–	3	3	9	1	0	0	Short	SPC	–	High	
CLSTR03695r1	cicl002e04	C	ZNF_C2H2	Zic3 (*Homo sapiens*)	Hs	Mm	Dm	Ce	–	–	0	12	10	1	0	0	Short	SPC	–	High	Fig. 3E
CLSTR00161r1	cilv006d23	DI	BTB	Hypothetical protein MGC2628 (*Homo sapiens*)	Hs	Mm	Dm	–	–	–	12	16	8	17	11	7	Bent	PA	–	High	Fig. 3D
CLSTR13021r1	cieg058i05	DI	Spc97_Spc98	KIAA1669 gene product (*Homo sapiens*)	Hs	Mm	–	–	–	–	1	0	0	2	0	0	Bent	PA	–	High	
CLSTR11884r1	cieg049n18	DI	ZNF_C2H2, RING	Hypothetical protein XP_034481 (*Homo sapiens*)	Hs	Mm	Dm	Ce	Sc	At	9	0	0	0	1	0	Short	SPC & PA	–	High	

*Gene collection ID and clone ID of genes follow those in '*Ciona intestinalis* cDNA collection release 1' (Satou et al., 2002b).

†Class of genes was determined according to Lee et al. (Lee et al., 1999).

‡Motifs and homologous genes were determined as described in Materials and methods.

§Distribution of homologous genes in other model organisms. Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; At, *Arabidopsis thaliana*. Dashes indicate the absence of a homologous gene.

¶Number of ESTs at each developmental stage was determined in the EST project (Satou et al., 2002a). EG, fertilized egg stage; CL, cleavage stage; GN, gastrula and neurula stages; TB, tailbud embryo stage; LV, larva stage; AD, young adult stage.

**Tail indicates 'abnormal tail' phenotypic class, which is subdivided into 'bent tail' (bent) or 'short tail' (short) phenotypic subclasses. Neural indicates additional defects found in the nervous system of embryos with 'abnormal tail' phenotype. SPC, 'loss of sensory pigment cells phenotype'; PA, 'loss of the palps phenotype'. Disorganized or D indicates 'disorganized body plan' phenotypic class.

††Penetrance of morpholino-induced abnormality was determined as described in the text.

Two distinct genes, cicl045c22 and cieg003h01, gave similar, remarkable phenotypes. cicl045c22 encodes a protein that contains C2H2 type Zn-finger motifs and is similar to the human hypothetical protein XP_041139 (Table 2). ESTs for this gene were detected at all the developmental stages examined. Eggs injected with cicl045c22 morpholino or cieg003h01 morpholino developed into flattened larvae with similar appearance (Fig. 2C,D). Here, the results of suppression of cieg003h01 function are described in detail.

cieg003h01 encodes a protein that has multiple putative transmembrane motifs and is similar to the human hypothetical protein FLJ12377 (Table 2) and its homologs are present in the genomes of mice, flies, worms, plants and yeasts (Table 2). ESTs for this gene were detected at the stages of fertilized egg, cleaving embryo and larva, and its maternal message is distributed evenly in the egg cytoplasm (data not shown). Eggs injected with cieg003h01 morpholino developed into flattened larvae with trunk-like and tail-like regions (Fig. 2D, Fig. 4D), although the cleavage pattern became atypical as early as the eight-cell stage. The trunk-like region appeared to comprise a layer of epidermal cells with no visible development of the endoderm and nervous system (Fig. 2D). Whole-mount in situ hybridization revealed the expression of *Ci-Epi1*, an epidermal cell differentiation marker (Fig. 4A,B), and histochemistry demonstrated the production of muscle AchE (Fig. 4C,D). In ascidian embryos, the differentiation of epidermis and muscle takes place autonomously dependent on maternally provided egg cytoplasmic information (e.g. Nishida, 2002). This suggests that the genetic cascades leading to the differentiation of epidermis and muscle are independent of cieg003h01. However, in the trunk-like region of experimental embryos, neither the endoderm assessed by histochemical detection of ALP (Fig. 4K,L) nor the nervous system assessed by *Ci-ETR* expression (Fig. 4G,H) differentiated. In the tail-like region, the differentiation of notochord was inhibited (Fig. 4I,J) and that of mesenchyme was partially suppressed (Fig. 4E,F). The differentiation of notochord and mesenchyme is dependent on the endoderm in *Ciona* embryos (Imai et al., 2000; Imai et al., 2002a). The specificity of the action of cieg003h01 morpholino was assessed by a rescue experiment with synthetic cieg003h01 mRNA. Co-injection of the morpholino and the mRNA abrogated the loss of ALP expression caused by the injection of cieg003h01 morpholino (Fig. 4N). In some cases, the co-injection resulted in the formation of larvae with a deformed but distinct tail region (Fig. 4M).

Interestingly, the morphology of larvae injected with cieg003h01 morpholino or cicl045c22 morpholino was similar to that of *Ciona* larvae injected with morpholino that targets β-catenin, an essential regulator of the animal-vegetal specification of the ascidian embryo (Imai et al., 2000).

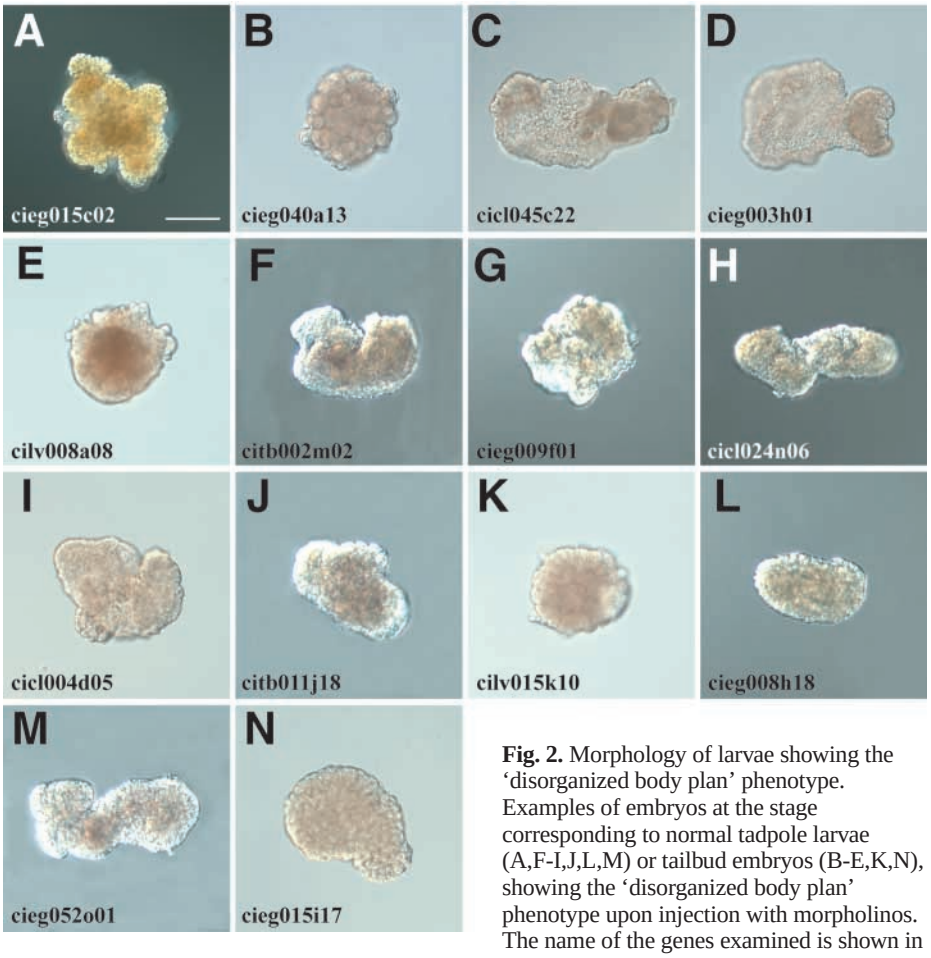

Fig. 2. Morphology of larvae showing the 'disorganized body plan' phenotype. Examples of embryos at the stage corresponding to normal tadpole larvae (A,F-I,J,L,M) or tailbud embryos (B-E,K,N), showing the 'disorganized body plan' phenotype upon injection with morpholinos. The name of the genes examined is shown in the bottom left-hand corner of each panel. See text for details. Scale bar: 50 μm.

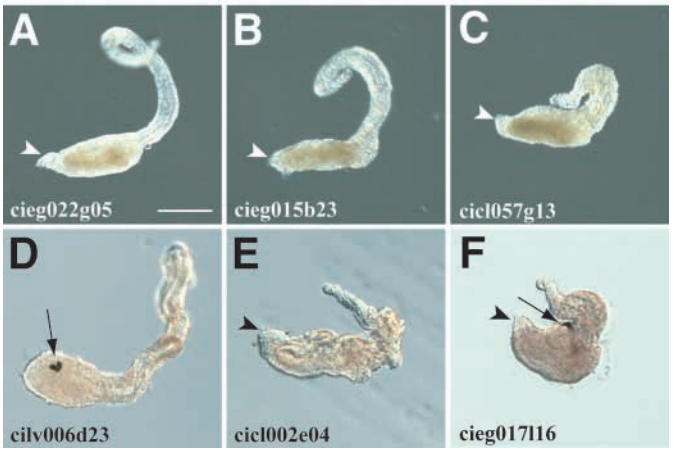

Fig. 3. Morphology of larvae showing the 'abnormal tail' phenotype. The arrowhead in A-C,E,F shows palps, and the arrow in D,F shows the sensory pigment cells. The name of the gene examined is shown in the bottom left-hand corner of each panel. See text for details. Scale bar: 50 μm.

Furthermore, the profile of differentiation of the six tissues we examined was identical in cieg003h01 morpholino-injected embryos and β-catenin morpholino-injected embryos. These findings raise the interesting possibility of a relationship between cieg003h01, cicl045c22 and β-catenin.

cilv008a08 is the single *Ciona intestinalis* counterpart of vertebrate Pbx genes (Wada et al., 2003) (Table 2). ESTs for this gene were detected in all the developmental stages examined (Table 2). Eggs injected with cilv008a08 morpholino developed into ball-shaped larvae (Fig. 2E). Pbx proteins are TALE class homeodomain transcription factors that act as co-factors for Hox class homeodomain transcription factors and play multiple roles during development according to their partner proteins (Mann and Chan, 1996). The severe defects observed in the cilv008a08 morpholino-injected embryos suggest that cilv008a08 may play a similar crucial role.

In addition to the genes described above, nine genes gave the 'disorganized body plan' phenotype. Although they are not described in detail here, embryos injected with morpholino against each of them are shown in Fig. 2F-N.

Genes associated with the 'abnormal tail' phenotypic class

Of the 40 positive genes, 26 genes belonged to the 'abnormal tail' phenotypic class. They consisted of 15 DI-class genes, six DII-class genes, two A-class genes, two B-class genes and one C-class gene, and included six genes encoding Zn-finger proteins (Table 2).

cieg022g05 encodes a protein that has four SANT motifs (Aasland et al., 1996) and shows sequence similarity to the human SNAP190 protein, the function of which is unknown (Table 2). ESTs for this gene were detected in all the developmental stages examined. Eggs injected with cieg022g05 morpholino developed into larvae that had bent tails and lacked the sensory pigment cells (Fig. 3A). Remarkably similar phenotypes were obtained when eggs were injected with morpholino that targets cieg015b23, which encodes a protein similar to the human KIAA1629 protein (Fig. 3B), or cicl057g13, which encodes a protein similar to the human hypothetical protein FLJ12890 (Fig. 3C), although the average length of the tail varied depending on the morpholino. On the other hand, injection of a morpholino designed against cilv006d23, which encodes a protein with a BTB motif (Zollman et al., 1994) that is similar to the human hypothetical protein MGC2628 (Table 2), also led to larvae with a bent tail; the larvae developed the sensory pigment cells but lacked the palps (Fig. 3D).

cicl002e04 encodes a protein that belongs to the Zic family of C2H2-type zinc-finger proteins (Table 2). ESTs for this gene were detected in embryos at the cleavage, gastrula/neurula and tailbud stages. Zic family genes play crucial roles in development in a wide range of animals (Nagai et al., 1997). Eggs injected with cicl002e04 morpholino developed into larvae that had short tails and had palps but no sensory pigment cells (Fig. 3E). Recently, Zic family genes were isolated from *Ciona savignyi* (*Cs-ZicL*) (Imai et al., 2002b) and *Halocynthia roretzi* (*HrzicN*) (Wada and Saiga, 2002), and translational suppression

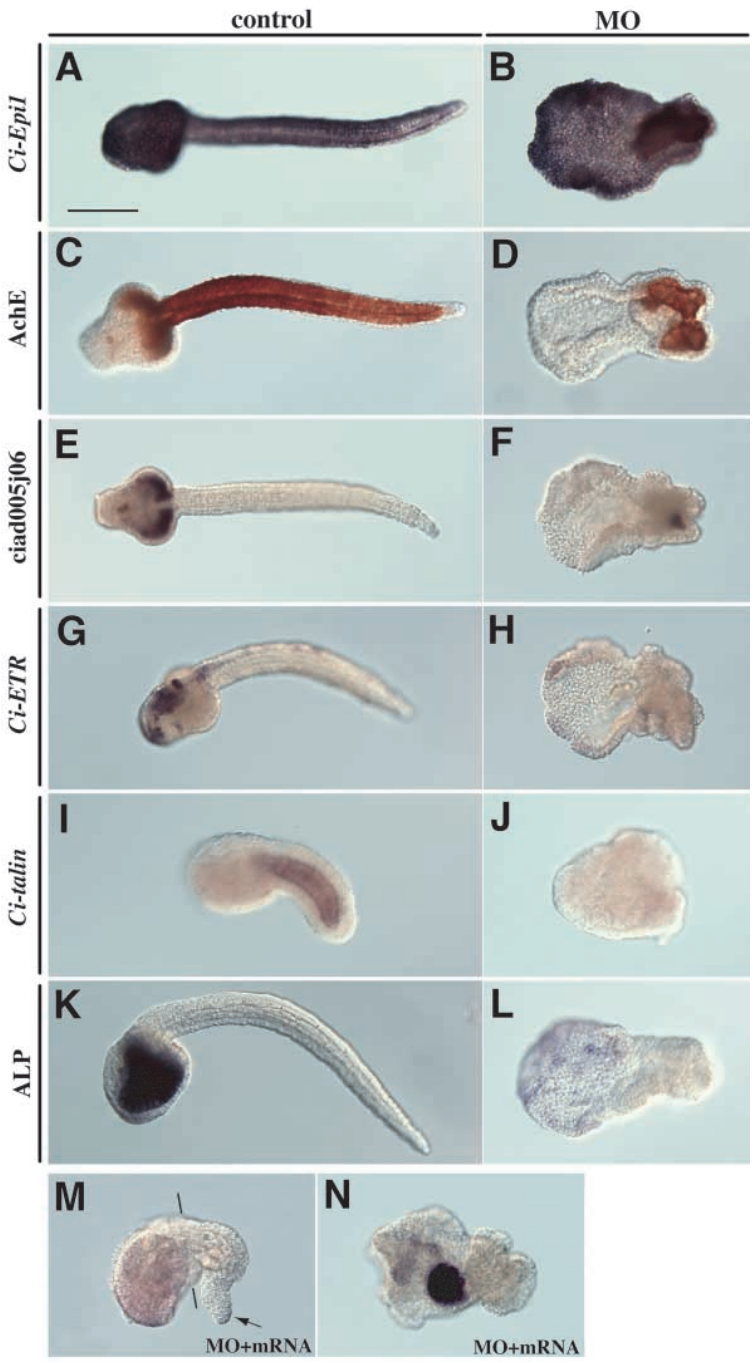

Fig. 4. Effects of suppression of cieg003h01 function with specific morpholino. (A,C,E,G,I,K) Control embryos. (B,D,F,H,J,L) Embryos injected with specific morpholino against the gene only. (M,N) Embryos co-injected with morpholino and synthetic mRNA. Expression of epidermis marker, *Ci-Epi1* (A,B); muscle marker, AchE (C,D); mesenchyme marker, ciad005j06 (E,F); nervous system marker, *Ci-ETR* (G,H); notochord marker, *Ci-talin* (I,J); and endoderm marker, ALP (K,L,N). In M, the arrow indicates the tip of the tail and bars show the border between the trunk and tail. All embryos were fixed at the stage corresponding to tailbud stage. See text for details. Scale bar: 50 μm.

experiments using a specific morpholino showed that larvae injected with *Cs-ZicL* or *HrzicN* morpholino exhibited defects in notochord and neural tube development. A molecular phylogenetic analysis of ascidian Zic family genes suggests that ciclo02e04 is one of the multiple *Ciona intestinalis* counterparts of *Cs-ZicL* and *HrzicN* (Yamada et al., 2003). Consistent with this, the knockdown phenotypes reported for *Cs-ZicL* and *HrzicN* were similar not only to each other but also to those of animals injected with ciclo02e04 morpholino.

Here, we describe in detail the results obtained with citb018a09. citb018a09 encodes a protein that has a Ring finger motif and a WWE domain, which shows sequence similarity to the human hypothetical protein DKEZp434O1427 with unknown function (Table 2). ESTs for this gene were detected in all the embryological stages but not in the adult (Table 2). Eggs injected with citb018a09 morpholino (a form that was produced against 25 nucleotides, including the first ATG codon) developed into larvae that had lacked the sensory pigment cells (Fig. 5B). Whole-mount in situ hybridization and histochemistry revealed the occurrence in the morpholino-injected embryos of differentiation markers for epidermis (*Ci-Epi1*), endoderm (ALP), muscle (AchE), notochord (*Ci-talin*) and mesenchyme (ciad005j06) (data not shown). Expression of pan-neural marker (*Ci-ETR*) indicated that citb018a09 is not required for neural tube formation (Fig. 5D,E). Because citb018a09 is likely to be involved in the formation of the sensory organs, we also examined the effects of its functional suppression on the photosensory organ. The expression of four genes involved in the function of the photosensory organ, *Ci-opsin3* (Fig. 5F,G), *Ci-arr* (Fig. 5H,I), *Ci-opsin1* (data not shown) and *Ci-Gαi1a* (data not shown) appeared in citb018a09-morpholino-injected embryos as in normal tailbud embryos. Co-injection of citb018a09 morpholino and citb018a09 mRNA failed to rescue the formation of pigment cells. However, a second form of citb018a09 morpholino which was produced against the 5′UTR also gave the same results as the first morpholino. These results suggest that this gene is not likely to be involved in the formation or function of the photosensory organ, but rather in pigment cell formation.

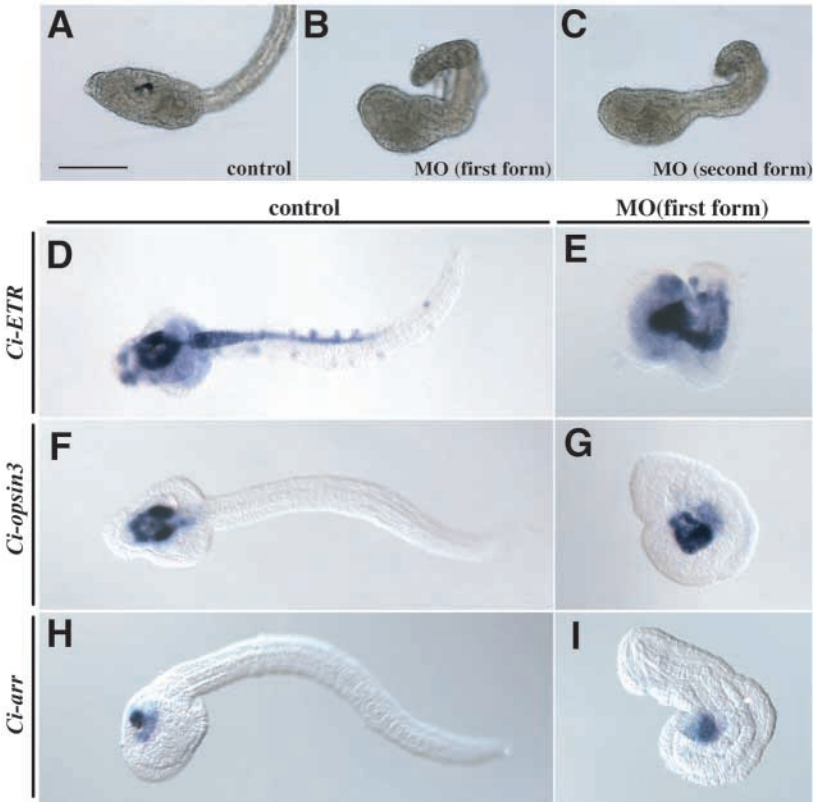

Fig. 5. Effects of suppression of citb018a09 function with specific morpholino. (A-C) Morphology of larvae injected with control *lacZ* morpholino (A), or the first (B) or second (C) forms of citb018a09-specific morpholino. (D-I) Whole-mount in situ hybridization showing the expression of a marker gene for the whole nervous system, *Ci-ETR* (D,E), and two genes involved in the function of the photosensory organ, *Ci-opsin3* (F,G) and *Ci-arr* (H,I). (D,F,H) Control embryos. (E,G,I) Embryos injected with the first form of citb018a09-specific morpholino. See text for details. Scale bar: 50 μm.

Besides the six genes described above, 20 other genes showed the ‘abnormal tail’ phenotype. Of them, 14 genes gave rise to this phenotype but not the ‘loss of the sensory pigment cells’ or ‘loss of palps’ phenotypes. For example, depletion of cieg017l16, which encodes a protein with an HLH motif, resulted in larvae that had a short tail and a bent tail, respectively, but that developed the sensory pigment cells and the palps (Fig. 3F).

Discussion

Genes involved in multiple developmental events were identified in the present study

In the present screen, 200 *Ciona intestinalis* genes expressed during development were examined with respect to their possible function using specific antisense morpholinos. Forty genes were thereby shown to produce visible embryonic defects when their translation was suppressed. The phenotypes obtained in the present study were classified into two major classes, the ‘disorganized body plan’ class and ‘abnormal tail’ class. The first major class of phenotype, the ‘disorganized body plan’ class, is characterized by gross morphological defects. Because embryos with a phenotype of this class exhibited abnormality from a considerably early stage of development, genes of this class may be involved in an early step of embryogenesis or a fundamental cellular process. However, possible causes for the ‘abnormal tail’ phenotypes include failure of the differentiation and/or morphogenetic movement of tissues composing the tail, such as the notochord, muscle, dorsal nerve cord, ventral endodermal strand or posterior epidermis, although further analysis will be required to distinguish among these possibilities. Consistent with this idea, embryos injected with *Ci-Bra* morpholino also showed the ‘short tail’ subclass phenotype and expression of a notochord maker was lost in the injected embryos. Among larvae with the ‘abnormal tail’ phenotype, ‘loss of pigment cells’ phenotype and ‘loss of palp’ phenotype were detected. The sensory pigment cells are components of the photoreceptor

and gravity sensor that are formed in the sensory vesicle. In the ascidian embryo, a primary defect in neural tube formation (e.g. failure of neural tube closure) often results in winding of the tail. The embryos showing both the 'bent tail' and 'loss of the sensory pigment cells' phenotypes may represent such cases. The palps are an adhesive organ located at the anterior tip of larvae, and this organ contains peripheral sensory neurons essential for signal transmission for metamorphosis. Previous studies have shown that correct cellular inductive interactions are required for the palp formation (Lemaire et al., 2002). Such interactions may be disrupted in embryos with the 'loss of palps' phenotype. Altogether, the genes identified in the present screen will provide tools for investigating multiple and distinct steps of ascidian morphogenesis.

Evaluation of the present screen for novel developmental genes in *Ciona intestinalis* embryos

It has been shown that morpholinos often exhibit nonspecific side effects on embryogenesis in zebrafish (Heasman, 2002). One of the conceivable reasons for this is unexpected targeting of other genes by the morpholinos. However, this is not likely to have been the case in the present screen, because we confirmed that, for each morpholino that induced any phenotype studied here, there was no gene that had a complementary sequence around or upstream of the initiation codon in the *Ciona intestinalis* genome other than the original target gene (data not shown). Another possible reason for this type of side-effect of morpholinos is nonspecific effects of morpholinos or contaminants. As mentioned above, however, the alteration of the morphology induced by a given morpholino seemed to be specific and distinct from that induced in embryos injected with *Ci-Bra* morpholino. Further support for the specificity of the action of morpholinos was obtained by rescue experiments. Therefore, it is likely that the phenotypes obtained in the present study were caused by the specific actions of the morpholinos rather than nonspecific side effects of morpholinos. The successful identification here of two genes that are expected to be involved in development, a Zic family gene (cicl002e04) and a homologue of Pbx (cilv008a08), also suggest that the present screen was effective.

In the present simple screen of visible features, morpholino-injected embryos were judged to be positive or tentatively negative based on the observation of the morphology of the resultant larvae. As a result, 160 genes (out of the 200 genes examined) were judged to be tentatively negative. However, the present method may overlook some genes with developmental functions. If we examine the effects of suppressing gene activity with more sophisticated techniques and specific probes that can detect changes at the cellular and molecular level, we may be able to more efficiently identify genes with developmental functions using the present methodology.

Comparison with other genetic screens

Large-scale screens for developmental genes using a reverse genetic approach have been conducted in the nematode *C. elegans*. A systematic analysis of *C. elegans* chromosome I by RNAi resulted in the functional identification of 13.9% of the genes analyzed (Fraser et al., 2000), whereas a similar analysis of cell division-related genes on chromosome III showed that ~6% of genes analyzed were necessary for cell division (Gönczy et al., 2000). More recently, a systematic functional analysis of most of the *C. elegans* genes encoded in the genome using RNAi demonstrated that 10.3% of the examined genes showed specific phenotypes (Kamath et al., 2003). In zebrafish, it has been estimated from chemical mutagenesis screens that ~3-5% of the genes encoded in the genome can be mutated to yield some developmental defects that can be identified by a visual screen of embryos (Haffter et al., 1996). Compared with the findings of those screens, the efficiency of the present *Ciona intestinalis* morpholino-mediated experimental system, i.e. 20% of genes analyzed, seems appreciably higher. However, an important difference between the *C. elegans* studies and the present *Ciona* study should be noted. In the above-mentioned studies, a non-biased pool of genes was screened, while in the present study the genes to be tested were selected based on the features of their expression pattern and/or structure before the application of the screen. This probably accounts for the difference in the efficiency between the two screens.

In the past several years, chemical mutagenesis screening was performed in *Ciona savignyi* (Moody et al., 1999; Nakatani et al., 1999) and *Ciona intestinalis* (Sordino et al., 2000). Interestingly, the spectrum of phenotypes found in those studies seems similar to that observed in the present screen. For example, Moody et al. (Moody et al., 1999) reported that the most common phenotype they obtained was 'rounded', which seems to be classified into 'short tail' according to our categorization. The other phenotypes they described were 'short tail', 'kinked axis (similar to 'bent tail' in the present study)', 'no pigment cells' and 'early arrest (probably categorized into 'disorganized body plan')', all of which are similar to certain of the phenotypes found in the present study, although embryos with the last phenotype show diverse features depending on the morpholino, as discussed above, and thus are not necessarily functionally alike. The similarities of the phenotypes obtained by the chemical mutagenesis screen and our screen may indicate that defects in distinct developmental steps converge to produce several types of abnormal embryos with particular morphological features. Another notable, but probably rare, possibility is that homologous genes are responsible for the similar phenotypes found in the various classes of abnormal manipulated animals. In this respect, the genes identified in the present study represent candidate genes for the mutants obtained in the chemical mutagenesis. Further analysis is required to test these possibilities.

Ciona intestinalis embryos provide a powerful tool to identify novel developmental genes

Analysis of the draft genome sequence and the ESTs of *Ciona intestinalis* suggests that, among a total of 15,852 genes, ~10,000 genes are common to worms, flies and humans, 2600 genes are probably chordate-specific, and another 3400 have presently been found only in *Ciona intestinalis* (Dehal et al., 2002). As shown in Table 2, most of the DI-class genes identified in the present screen have vertebrate counterparts with unknown function. Therefore, determining the function of *Ciona intestinalis* DI-class genes may facilitate our understanding of the function of their vertebrate counterparts. There are at least 2500 DI-class genes in *Ciona intestinalis* (Satou et al., 2002a). A simple estimate from the results of the present study is that about one-fifth of DI-class genes that are

expressed during embryogenesis may be shown to have a developmental function by a morpholino-based approach. It has been shown that most of the genes homologous between ascidians and vertebrates have a conserved function during development (Corbo et al., 2001; Satoh, 2001). Therefore, developmental genes suggested to have a pivotal function based on *Ciona intestinalis* studies should be analyzed in *Xenopus* or zebrafish embryos, or should be targeted in mouse embryos.

The cDNA project has characterized transcripts for more than three-quarters of *Ciona intestinalis* genes (Satou et al., 2002b). In addition, more than 5600 full-length sequences of cDNAs have already been determined. Together with the draft genome sequence, such information should help us to design effective morpholinos. For example, in the case of a gene that gives rise to multiple transcripts by alternative splicing, it is possible for us to design morpholinos more carefully by taking this point into account. The cDNA database also provides a rough estimate of the temporal expression pattern, i.e. the count of the ESTs at each developmental stage (Satou et al., 2003b). Furthermore, the spatial expression profiles of 5000 randomly selected genes have been determined by whole-mount in situ hybridization (Satou et al., 2002a). These expression profiles may help us to interpret the phenotypes of morpholino-injected embryos. In conclusion, *Ciona intestinalis* embryos provide a powerful experimental system for identifying genes with novel developmental function required for the formation of the chordate body plan (Satoh et al., 2003).

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