

Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks

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Summary

Achieving a real understanding of animal development obviously requires a comprehensive rather than partial identification of the genes working in each developmental process. Recent decoding of genome sequences will enable us to perform such studies. An ascidian, *Ciona intestinalis*, one of the animals whose genome has been sequenced, is a chordate sharing a basic body plan with vertebrates, although its genome contains less paralogs than are usually seen in vertebrates. In the present study, we discuss the genomewide approach to networks of developmental genes in *Ciona* embryos. We focus on transcription factor genes and some major groups of signal transduction genes. These genes are comprehensively listed and examined with regard to their embryonic expression by in situ hybridization (<http://ghost.zool.kyoto-u.ac.jp/tfst.html>). The results revealed that 74% of the transcription factor genes are expressed maternally and that 56% of the genes are zygotically expressed during embryogenesis. Of these,

34% of the transcription factor genes are expressed both maternally and zygotically. The number of zygotically expressed transcription factor genes increases gradually during embryogenesis. As an example, and taking advantage of this comprehensive description of gene expression profiles, we identified transcription factor genes and signal transduction genes that are expressed at the early gastrula stage and that work downstream of β -catenin, *FoxD* and/or *Fgf9/16/20*. Because these three genes are essential for ascidian endomesoderm specification, transcription factor genes and signal transduction genes involved in each of the downstream processes can be deduced comprehensively using the present approach.

Supplemental data available online

Key words: Ascidian, *Ciona intestinalis*, Transcription factors, Signal transduction molecules, Expression profiles

Introduction

Ascidians belong to the subphylum Urochordata, which is one of the three chordate groups. Their fertilized eggs develop into tadpole-type larvae that consist of about 2600 cells that form several distinct types of tissue (reviewed by Satoh, 1994; Satoh et al., 2003). The tail of the larva contains the notochord flanked dorsally by the nerve cord, ventrally by the endodermal strand and bilaterally by three rows of muscle cells. The trunk contains a dorsal central nervous system (CNS) with two sensory organs (otolith and ocellus), endoderm, mesenchyme, trunk lateral cells (TLCs) and trunk ventral cells (TVCs). The entire surface of the larva is covered by an epidermis. This configuration of the ascidian tadpole is thought to represent one of the most simplified and primitive chordate body plans (reviewed by Satoh and Jeffery, 1995; Di Gregorio and Levine, 1998; Satou and Satoh, 1999; Corbo et al., 2001; Satoh, 2003).

Reflecting the simplicity of the larval body plan, ascidian embryogenesis is comparatively simple. In the egg, maternal determinants for muscle, endoderm and epidermis are stored, and these tissues differentiate autonomously. However, inductive signals are required for differentiation of the

notochord, mesenchyme and nervous system (Nishida, 2002). The molecular nature of inductive signals has been revealed using embryos of *Halocynthia roretzi*, *Ciona intestinalis* and *Ciona savignyi*. Although most of the developmental system is thought to be common between *Halocynthia* and *Ciona*, recent studies have also shown several differences between them. In fact, they are evolutionarily distant, because *Halocynthia* is an Enterogona ascidian and *Ciona* is a Pleurogona ascidian – these two being major orders of ascidians. Therefore, we describe here mostly the *Ciona* system in order to avoid any possible confusion.

In ascidian embryos, the developmental fate of each blastomere is restricted to one tissue at or before the 110-cell stage (Fig. 1). In accordance with this early cell fate restriction, key genes that are essential and sufficient for differentiation of each tissue are thought to be expressed in each lineage until this stage. In fact, recent studies have identified such genes for larval endomesodermal tissues: *Lhx3* for endoderm (Satou et al., 2001a), *Twist-like-1* for mesenchyme, including TLCs (Imai et al., 2003), *Brachyury* for notochord (Yasuo and Satoh, 1993; Yasuo and Satoh, 1998; Corbo et al., 1997a), and *Mesp*

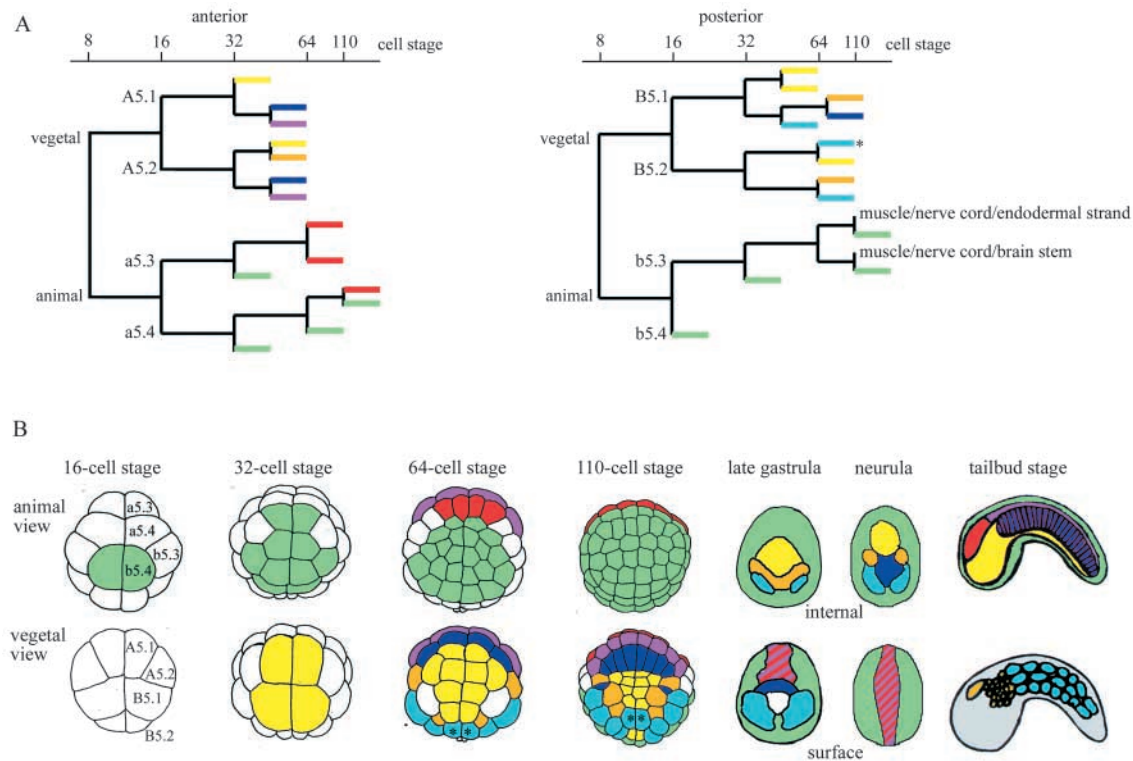


Fig. 1. Early restriction of developmental fates in the ascidian embryo. (A) Cell lineage of blastomeres during early embryogenesis. Because the embryo is bilaterally symmetrical, only the left half of the embryo is shown. Thick and colored lines indicate blastomeres whose developmental fate becomes restricted to one tissue. This is based mainly on data obtained in *Halocynthia* by Nishida (Nishida, 1987). (B) Schematic representations of the ascidian embryo. Blastomeres whose developmental fate is restricted to one tissue are in color: yellow, endoderm; orange, mesenchyme; light blue, muscle; dark blue, notochord; green, epidermis; pink, nerve cord; red, nervous system. The blastomeres marked with asterisks give rise to muscle and trunk ventral cells.

for TVCs (Satou et al., 2004). Muscle cells are autonomously differentiated by maternally supplied *macho-1* encoding a Zic-like transcription factor in *Halocynthia* (Nishida and Sawada, 2001), and its *Ciona* homolog is also important for the differentiation of muscle cells, although an additional mechanism will also be required in *Ciona* embryos (Satou et al., 2002a; Imai et al., 2002a). All the differentiation processes in each lineage are accomplished through expression of these key genes. Therefore, the investigation of how these genes are expressed in each lineage will elucidate specification mechanisms of the ascidian endomesodermal tissues, and identification of genes downstream of the key genes will lead to understanding of the differentiation process of each tissue.

Several studies addressing these issues have been reported. Maternally supplied β -catenin is important for development of vegetal blastomeres, especially for endoderm (Imai et al., 2000). Most of the genes expressed in the endodermal lineage are under the control of β -catenin, and the expression of *Lhx3*, which is one of the β -catenin downstream genes, triggers the differentiation of endoderm (Satou et al., 2001a). *FoxD* is also expressed in cells with the endodermal fate at the 16-cell and 32-cell stages under the direct control of β -catenin (Imai et al., 2002b), and *FoxD* then somehow promotes the expression of *ZicL*, another Zic-like transcription factor, in the A-line notochordal lineage (Imai et al., 2002a). *ZicL* binds to the *Brachyury* promoter and activates its expression (Yagi et al., 2004). The expression of *Brachyury* is also controlled

negatively by snail (Fujiwara et al., 1998) and positively by suppressor of hairless (Corbo et al., 1998). The expression of *Fgf9/16/20*, which represents an ancestral form of vertebrate *Fgf9*, *Fgf16* and *Fgf20*, is also regulated by β -catenin and the resultant FGF protein is required for the proper expression of *Brachyury* (Imai et al., 2002c). This FGF also works as a natural inducer for mesenchyme (Imai et al., 2002c). In response to this FGF, *Twist-like-1* is activated in the mesenchymal lineage, which triggers the differentiation of mesenchyme (Imai et al., 2003). It has recently been demonstrated that this FGF also induces neural tissues (Bertrand et al., 2003). In the TVCs, *Mesp* is expressed at the 110-cell stage under the control of both β -catenin and *macho-1* (Satou et al., 2004).

Downstream genes of several of these key genes have been identified. For example, more than 20 genes downstream of *Brachyury* have been reported (Takahashi et al., 1999; Hotta et al., 2000). Among these, genes required for the function of notochord itself [such as *Ci-trop*, which encodes tropomyosin-like protein (Di Gregorio and Levine, 1999), and *Ci- β 4Gal-T*, which encodes β 4-gal-transferase] are included. Genes that may be involved in morphogenesis of the tail, such as *Ci-pk1*, which encodes a homolog of *Drosophila* prickle, were also identified. Therefore, to understand the molecular mechanism of the ascidian development, it is important to analyze the downstream and upstream genes regulated by these key genes.

Although analyses of individual genes have clarified the

gene network behind the endomesoderm differentiation of the ascidian embryo, analyses focusing on individual genes are insufficient for elucidating the entire mechanism, because such analyses cannot cover the entire gene repertoire. To overcome this problem, genome-scale analyses are required. In this respect, ascidians also provide an ideal experimental system, because the draft genome sequence of *Ciona intestinalis* reveals that its genome contains the basic ancestral complement of genes involved in cell signaling and development (Dehal et al., 2002). The ascidian developmental genes are therefore less redundant compared with those of vertebrates, in which paralogous genes are frequently present, rendering analyses of gene function difficult.

Towards the comprehensive analysis of *Ciona* genes, a microarray covering almost the entire gene repertoire in the *Ciona* genome has been published (Azumi et al., 2003). Quantitative data can be successfully obtained by analyses using microarrays. However, for studying the development, it is also important to obtain qualitative data – without basic information about where individual genes are expressed, quantitative data cannot be effectively evaluated. Based on this line of research, we have already described the expression profiles of ~1000 genes in five developmental stages of fertilized eggs (Nishikata et al., 2001), cleaving embryos (Fujiwara et al., 2002), tailbud embryos (Satou et al., 2001b), larvae (Kusakabe et al., 2002) and young adults (Ogasawara et al., 2002).

The importance of transcriptional regulation of genes in animal development is obvious (e.g. Davidson et al., 2002; Levine and Tjian, 2003). In general, transcription factors can trans-activate other transcription factor genes and sometimes themselves, making a network of transcription factor genes. These transcription factor networks are interlinked with one another and regulated by signaling molecules, such as Wnt, TGF β and FGF. These networks make a complex body from a single cell of the fertilized egg and have been partially delineated in ascidian embryos, as described above. To expand this type of analysis to a genomewide scale and to understand ascidian development completely, the reconstruction of gene networks consisting of transcription factors and signaling genes is a good starting point. Therefore, we address the expression profiles of all transcription factor genes and major signaling genes. The *Ciona* system is ideal for this type of analysis, because the genome contains a limited number of transcription factor genes and signaling genes compared with vertebrate genomes. Another advantage is that the expression pattern of each gene can be described at the single-cell level at and before the 110-cell stage. To date, expression patterns of several transcription factor genes and signaling genes have been described, including *Brachyury* [*Ci-Bra* (Corbo et al., 1997a)], *FoxA-a* [*Ci-fkh* (Corbo et al., 1997b)], *Otx* (Hudson and Lemaire, 2001; Satou et al., 2001a), *Gsx* (Hudson and Lemaire, 2001), *MyoD* [*Ci-MDF* (Meedel et al., 1997)], *snail* (Corbo et al., 1997b; Fujiwara et al., 1998), *TTF1* (Ristoratore et al., 1999; Satou et al., 2001a), *Dll-A*, *Dll-B* and *Dll-C* (Caracciolo et al., 2000), *Lhx3* (Satou et al., 2001a), *ZicL* (Imai et al., 2002a), *FoxD* (Imai et al., 2002b), *macho-1* (Satou et al., 2002a), *engrailed* (Jiang and Smith, 2002; Imai et al., 2002d), *Fgf* genes (Imai et al., 2002c; Imai et al., 2002d), *lefty/antivin* (Imai, 2003), and *hedgehog1* and *hedgehog2* (Takatori et al., 2002). However, these

represent only a small part of all the transcription factor and major signaling genes.

Once the complete gene expression profiles of such genes are described, a variety of genome-scale analyses can be more easily performed, because the number of genes that should be examined becomes limited. In addition, because the function of individual genes can be predicted from their expression profiles, the description of the gene expression profiles also promotes such analyses.

No systematic description of the transcription factor gene expression profiles during embryogenesis has been reported yet in any animal to our knowledge. Therefore, the following description and discussion will also address three fundamental questions. (1) How many and what kind of transcription factor genes are encoded in the *Ciona* genome? (2) How many such genes are maternally or zygotically expressed? (3) Are there any specific tendencies or differences in the expression patterns between transcription factor families?

Materials and methods

Ascidian eggs and embryos

Ciona intestinalis adults were obtained from the Maizuru Fisheries Research Station of Kyoto University. They were maintained in aquaria in our laboratory at Kyoto University under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from gonoducts. After insemination, eggs were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 μ g/ml streptomycin sulfate.

Gene lists

The list for transcription factor genes was obtained by a combination of three methods. First, genes for basic helix-loop-helix (bHLH), homeodomain (HD), Fox, ETS, bZIP, nuclear receptor (NR), HMG, T-box transcription factors or specific categories of zinc-finger transcription factors encoded in the *Ciona* genome were previously annotated comprehensively (Satou et al., 2003a; Wada et al., 2003; Yagi et al., 2003; Yamada et al., 2003). Because nucleotide sequences of several genes that were recently duplicated are almost identical, we treated these gene groups as single genes in this study (*Twist-like1a/Twist-like1b*, *Tbx6b/Tbx6c/Tbx6d*, *FoxD-a/FoxD-b* and *FoxN1/4-a/FoxN1/4-b*). Second, results of Interpro searches of the *Ciona* proteome were also used. *Ciona* proteins with an Interpro domain categorized into transcription factors by gene ontology terms (GO:3700 or its children) were added to the list. Finally, we performed homology searches of the *Ciona* proteins against the human proteome (IPI, 2003.8.6 version). When the best-hit human protein of each *Ciona* protein was annotated as a transcription factor, the corresponding gene was added to the list. General transcription factors such as TFIIA were excluded manually from the list. As shown in Table 1 and Table S1 (at <http://dev.biologists.org/supplemental>), the list includes 394 transcription factor genes. Known transcription factor genes that are not included in this list include some zinc-finger genes and genes that do not have any recognizable motifs or homologs identified as transcription factors in other animals. As for zinc-finger genes, genes for nuclear receptors, Zic-related genes and genes encoding homologs that are identified as transcription factors in humans are all included. Zinc-finger motifs themselves do not always indicate that a gene encodes a transcription factor, because even proteins with a C2H2 motif, which is one of the best known zinc-finger motifs, are not necessarily transcription factors. Therefore, of 600 zinc-finger genes in the *Ciona intestinalis* genome (L. Yamada and N. Satoh, unpublished), only 86 genes that certainly encode a transcription factor are included in the current list.

The list of signaling genes consists of genes for receptor tyrosine

Table 1. Transcription factor genes in *Ciona intestinalis*

Gene family	Total number of genes	Number of genes found in the gene collection	Number of genes whose cDNA was amplified by PCR	Number of genes not analyzed in the present study
bHLH	44	32	7	5
bZIP	26	24	0	2
Ets	15	13	2	0
Fox	27	21	3	3
HMG	21	19	1	1
Homeobox	92	62	12	18
Nuclear receptor	18	16	1	1
T-box	8	6	1	1
Others	138	131	1	6
Totals	389	324	28	37

kinase pathways including ligands such as Fgfs and intracellular signaling molecules such as MAPK, Notch, Wnt, TGFβ, Hedgehog and JAK/STAT pathways that were annotated previously (Table 2, Table S2 at <http://dev.biologists.org/supplemental>) (Satou et al., 2002c; Satou et al., 2003b; Hino et al., 2003).

cDNA clones and whole-mount in situ hybridization

Most of the cDNA clones were obtained from our EST collection (Satou et al., 2002b). IDs for the cDNA clones used are shown in Tables S1 and S2 (<http://dev.biologists.org/supplemental>). We attempted to amplify cDNAs for genes not found in the EST collection by PCR with perfect-match primers, which were designed based on the genome sequence. The templates for the PCRs were obtained from cDNA libraries constructed in UniZAP phagemid (Stratagene) derived from eggs, cleaving embryos, gastrulae, tailbud embryos, larvae and juveniles.

All the DIG-RNA probes were synthesized by in vitro transcription with T7 RNA polymerase. The detailed procedure has been described previously (Satou and Satoh, 1997).

Relative quantification of mRNA by RT-PCR using real-time PCR method

In the present study, we used 25-mer morpholino oligos (referred to as ‘morpholinos’; Gene Tools, LLC) for Ci-β-catenin (5’-CTGTT-CATCATCATTTCAGCCATGC-3’), *Ci-Fgf9/16/20* (5’-CATAGAC-ATTTTCAGTATGGAAGGC-3’) and *Ci-FoxD-a/b* (5’-GCACAC-AACACTGCACTGTCATCAT-3’). After insemination, fertilized eggs were dechorionated and microinjected with 15 pmole of morpholinos and/or synthetic capped mRNAs in 30 pl of solution using a micromanipulator (Narishige Science Instrument Laboratory, Tokyo) as described (Imai et al., 2000). Injected eggs were reared at about 18°C in MFSW containing 50 mg/l streptomycin sulfate. Embryos injected with these morpholinos exhibited a same phenotype as *Ciona savignyi* embryos in which these gene functions were inhibited with specific morpholinos (Imai et al., 2000; Imai et al., 2002b; Imai et al., 2002c).

Relative quantifications of mRNA were performed by the real-

time PCR method with SYBR-green chemistry using an ABI prism 7000 (Applied Biosystems). The amplification of a specific product in each reaction was confirmed by determining a dissociation curve. When the dissociation curve indicated multiple PCR products, the primer pairs were redesigned until a specific amplification could be achieved. Primers used in this study are shown in Table S3 (<http://dev.biologists.org/supplemental>) and the detailed procedure has been described previously (Imai, 2003). Only genes exhibiting more than threefold difference in the amount of mRNAs by comparison between morpholino-injected embryos and control embryos were counted as positive ones. The results for the positive genes were further confirmed by another independent experiment.

Results and discussion

Transcription factor genes in the *Ciona intestinalis* genome

First, we listed the transcription factor genes encoded in the *Ciona intestinalis* genome. As shown in Tables 1 and S1 (<http://dev.biologists.org/supplemental>), this list contains all transcription factors with basic helix-loop-helix (bHLH), homeodomain (HD), Fox, ETS, bZIP, HMG, nuclear receptor (NR), T-box and specific categories of zinc fingers. In previous studies, the genes encoding factors with these specific motifs were completely surveyed and molecular phylogenetically analyzed (Satou et al., 2003a; Wada et al., 2003; Yagi et al., 2003; Yamada et al., 2003). In addition, we also used an Interpro search and a Blastp search against the current human proteome (IPI, 2003.8.6 version), which found transcription factors without any of the motifs described above and co-factors of transcription factors such as groucho. However, general transcription factor genes such as *TFIIA* and *TFIID* were excluded from the list, because these genes are not the focus of the present study. As a result, 389 transcription factor genes were listed.

Table 2. *Ciona* signaling genes analyzed in the present study

Pathway	Total number of genes	Number of genes found in the gene collection	Number of genes whose cDNA was amplified by PCR	Number of genes not analyzed in the present study
Hedgehog	6	6	0	0
JAK/STAT	5	5	0	0
NFκB	3	3	0	0
Notch	18	14	3	1
RTK, MAPK	42	35	2	5
TGFβ	19	19	0	0
Wnt	25	20	2	3
Totals	118	102	7	9

Table 3. Terms used for description of in situ hybridization results

Developmental stage	Terms
Fertilized eggs	<i>pem</i> -like localized signal was detected. Unlocalized signal was detected. No signal was detected
Late gastrulae and neurulae	Epidermis, nervous system (including nerve cord), muscle, notochord, mesenchyme, endoderm, unidentifiable
Tailbud embryos	Epidermis, nervous system, nerve cord, muscle, notochord, mesenchyme, endoderm, unidentifiable

In the 389 transcription factor genes, only zinc-finger genes that are readily annotated as a transcription factor gene are included. The *Ciona* genome contains 514 additional zinc-finger genes (L. Yamada and N.S., unpublished). Because the zinc-finger motif itself cannot be used as evidence that a gene encodes a transcription factor, we cannot know exactly how many transcription factors are included among the 514 zinc-finger proteins. Therefore, the number of transcription factor genes encoded in the *Ciona* genome would range from 389 to 903 (389+514). The nematode genome contains about 500 transcription factor genes, about one-third of which encode a zinc-finger protein (Ruvkun and Hobert, 1998). The fly genome contains about 700 transcription factor genes, about half of which encode a zinc-finger protein (Adams et al., 2000). However, the human genome contains about 3000 transcription factor genes (Lander et al., 2001). Therefore, the *Ciona* genome appears to contain a comparable number of transcription factor genes to genomes of other invertebrates. This is consistent with our previous comprehensive annotations of specific families of transcription factor genes in the *Ciona intestinalis* genome (Satou and Satoh, 2003). For example, 46 bHLH genes are encoded in the *Ciona* genome, 38 in the nematode genome and 58 in the fly genome, but about 130 in the human genome (Satou et al., 2003a). Ninety-two HD transcription factor genes are encoded in the *Ciona* genome, 101 in the nematode genome and 113 in the fly genome, but about 270 in the human genome (Wada et al., 2003). Therefore, our list appears to cover almost all transcription factor genes in the *Ciona intestinalis* genome.

As shown in the previous studies (Satou et al., 2003a; Wada et al., 2003; Yagi et al., 2003; Yamada et al., 2003), the *Ciona* genome does not have any orthologs of several transcription factor genes found in the vertebrate genome (Table S4). For example, among bHLH genes, no clear orthologs for *NeuroD*, *Beta3*, *Oligo*, *SCL*, *NSCL*, *SRC*, *Bmal* and *Clock* were found in the *Ciona* genome. Some of these genes were lost in the *Ciona* genome and the others were probably acquired in the course of vertebrate evolution after the divergence of ascidians and vertebrates. Therefore, these missing orthologs were not examined in this study.

The expression profiles of the transcription factor genes

Of 389 genes, cDNA clones for 324 genes were found in our EST collection (Table 1) (Satou et al., 2002b; Satoh et al., 2003). We tried to amplify cDNA fragments for the remaining 65 genes from cDNA libraries derived from eggs, cleaving embryos, gastrulae, tailbud embryos, larvae and juveniles to obtain cDNA fragments of 28 genes (Table 1). In other words, we could not amplify cDNAs for 37 genes in spite of using perfect-match primer pairs. Our EST collection contains 480,000 ESTs, and 225,238 of these entries were obtained from cDNA libraries from eggs to tailbud embryos (Satou et al., 2002b). Because such extensive EST analyses and PCR

amplifications of cDNAs failed to show evidence of the gene expression, most of these genes are unlikely to be expressed during embryogenesis. It is also possible that some of these genes are pseudogenes or ones mispredicted by gene-prediction programs. The following analysis was therefore performed for 352 (324+28) transcription factor genes, and their embryonic expression profiles were examined by whole-mount in situ hybridization.

In situ hybridization was carried out with fertilized eggs, 16-cell embryos, 32-cell embryos, 64-cell embryos, early gastrulae (110~180 cell embryos), late gastrulae, neurulae, early tailbud embryos and mid-late tailbud embryos. The presence or absence of maternal transcripts was classified into three categories as shown in Table 3. Zygotic expression up to the 64-cell stage was described at the single-cell level according to Conklin's nomenclature (Conklin, 1905). Zygotic expression at the early gastrula was also described similarly, but names of blastomeres at the 110-cell stage were used even when the expression after the 110-cell stage was described. This is because cell divisions after the 110-cell stage are rapid and non-synchronous, which makes a large scale precise sampling of 110-cell embryos difficult. Therefore, for example, when the present assay says that a given gene is expressed in B7.1 at the early gastrula stage, this gene is expressed in B7.1 and/or its descendants in the embryo between the 110-cell stage and the 180-cell stage. Zygotic expression after the early gastrula stage was described at the tissue level (Table 3). Trunk ventral cells (TVCs) and trunk lateral cells (TLCs) have been traditionally regarded as a part of mesenchyme and it is often difficult to distinguish these cells from B7.7- and B8.5-derived mesenchyme at the tailbud stage, especially when all of these lines of the mesenchyme were stained. Therefore, we described TVCs and TLCs as mesenchyme, although we also describe which mesenchyme expresses a particular gene as long as we can distinguish between the mesenchymes (this can be seen at <http://ghost.zool.kyoto-u.ac.jp/tfst.html>). Zygotic transcripts in ascidian embryos can be easily discriminated from maternal transcripts, because the first zygotic expression signals are observed in the nucleus.

All the results are shown in Tables S5-S13. Figure 2A-D shows four examples of in situ hybridization of transcription factor genes. *Lhx3* is not expressed maternally or at the 16-cell stage, but is expressed in A6.1, A6.3 and B6.1 at the 32-cell stage, in A7.1, A7.2, A7.3, A7.5, A7.7, B7.1, B7.2 and B7.3 at the 64-cell stage, and in A7.1, A7.2, A7.5, B7.1, B7.2 and B7.5 in the early gastrula. *Lhx3* is not expressed in the late gastrula, but is then expressed in cells of the nervous system in neurula and tailbud embryos (Fig. 2A). This expression pattern is very similar to that of the *Lhx3* gene in a closely related species, *Ciona savignyi* (Satou et al., 2001a). *neurogenin* is also not expressed maternally or at the 16-cell stage, but is transiently expressed in A6.1, A6.3 and B6.1 at the 32-cell stage. The expression is diminished transiently at the 64-cell stage but the gene is expressed in cells of the

nervous system at the late-gastrula stage and thereafter (Fig. 2B). *Jun* is not expressed maternally or in early embryos until the 64-cell stage, and is expressed in B7.7 at the early gastrula stage and in its descendants in late gastrula. The expression is diminished at the neurula stage, and is again detected in part of the mesenchyme in the tailbud embryo (Fig. 2C). *C/EBPβ/δ/ε* is ubiquitously expressed in eggs and no zygotic signal was detected during embryogenesis (Fig. 2D). All of the photographs are available on our website (<http://ghost.zool.kyoto-u.ac.jp/tfst.html>).

We compared the results with those of genes analyzed in the previous studies, including *Brachyury* (*Ci-Bra*) (Corbo et al., 1997a), *FoxA-a* (*Ci-fkh*) (Corbo et al., 1997b), *Otx* (Hudson and Lemaire, 2001; Satou et al., 2001a), *Gsx* (Hudson and Lemaire, 2001), *MyoD* (*Ci-MDF*) (Meedel et al., 1997), *snail* (Corbo et al., 1997b; Fujiwara et al., 1998), *TTF1* (Ristoratore et al., 1999; Satou et al., 2001a), *Dll-A*, *Dll-B*, *Dll-C* (Caracciolo et al., 2000), *Lhx3* (Satou et al., 2001a), *ZicL* (Imai et al., 2002a), *FoxD* (Imai et al., 2002b), *macho-1* (Satou et al., 2002a), *engrailed* (Jiang and Smith, 2002; Imai et al., 2002d). Our descriptions are basically same as the previous ones, indicating that the present assay is reliable. However, slight discrepancies were found in two genes. *TTF1* was found to be expressed in the brain and endoderm at the mid-late tailbud stage in the present study, but the expression in the brain was not found in the previous study (Ristoratore et al., 1999), although the expression in the earlier stages is same. *Gsx* is transiently expressed in the brain at the early tailbud stage, and the expression was not observed at the neurula stage and at the

mid-late tailbud stage in the present study, while the previous study found the gene to be expressed in the brain from the neurula stage to the mid-late tailbud stage (Hudson and Lemaire, 2001). We performed in situ hybridization for these genes twice; both independent experiments reproduced the same results. This may be due to the difference of the population, Japanese animals and European animals, or developmental stages of the embryo used in the present study may be slightly different from those in the previous studies.

Maternally expressed transcription factor genes

How many transcription factor genes are maternally expressed in ascidian eggs? A result of our analysis is shown in Table S5. Because detection of maternal messages by in situ hybridization is possibly difficult, especially when the amount of the message is around the level of the limit of detection (e.g. Fig. 2D), we evaluated our results with EST information derived from the egg cDNA library. For this analysis, only 324 genes that have at least one EST in our whole EST collection derived from 12 different libraries (Satou et al., 2002b) were used. As shown in Table S14 (at <http://dev.biologists.org/supplemental>), of the 10 genes with mRNA localized in the posterior region of the egg [such as *posterior end mark* (*pem*) (Yoshida et al., 1996)], seven genes have one or more corresponding ESTs derived from the egg cDNA library and the remaining three genes do not have any corresponding ESTs. The maternal expression of these 10 genes is most reliable, because of their localized signal. This suggests that nearly 30% of maternal genes are likely to represent genes with

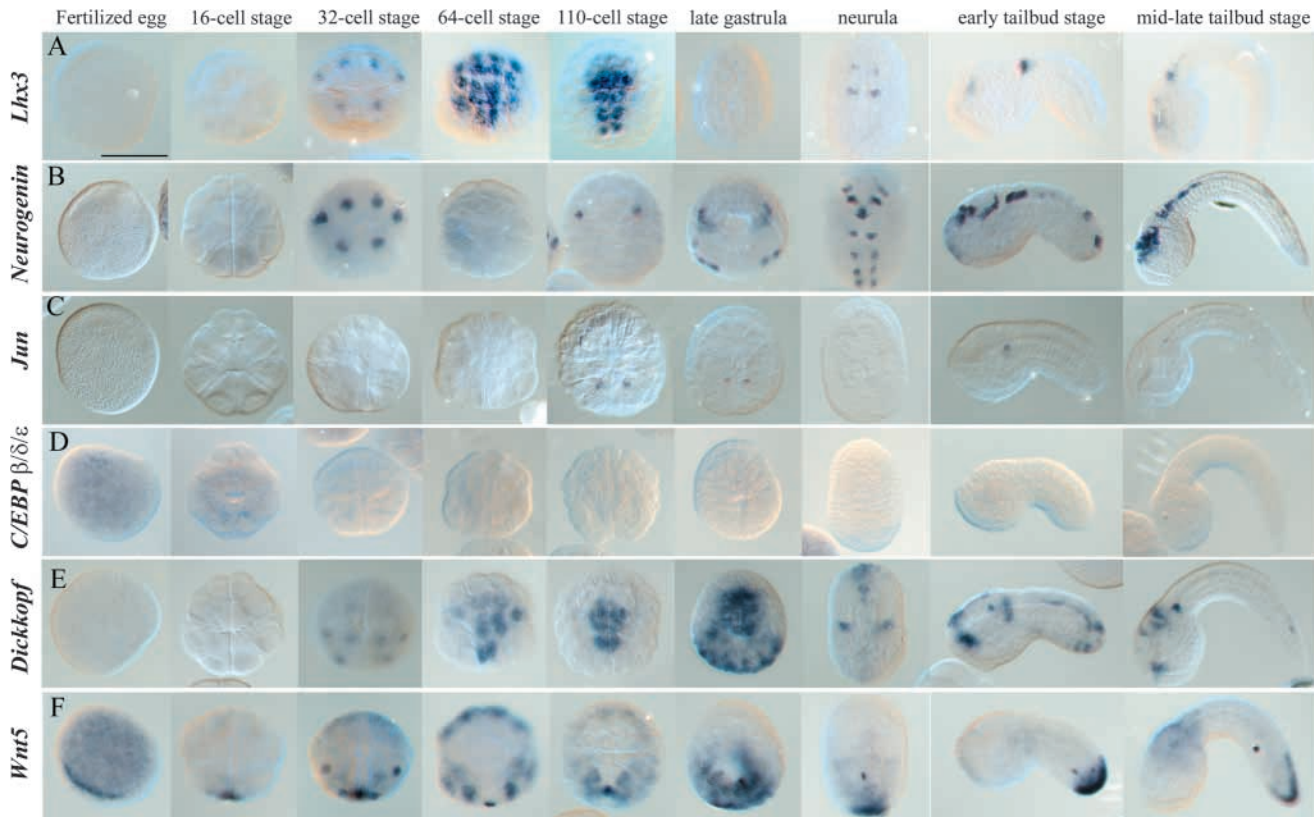


Fig. 2. Examples of expression profiles of transcription factor genes (A, *Lhx3*; B, *neurogenin*; C, *Jun*; D, *C/EBPβ/δ/ε*) and signaling genes (E, *Dickkopf*; F, *Wnt5*). Scale bar: 100 μm.

rare maternal message and are not included in the EST data. Therefore, if our in situ data are correct, a similar percentage of the EST coverage is expected for genes with unlocalized mRNA. In fact, of 224 transcription factor genes with unlocalized maternal mRNA, 163 genes (73%) have one or more ESTs and the remaining 61 genes (27%) do not. A similar percentage of the presence of the corresponding ESTs indicates accuracy of the result. Of 90 genes in the 'genes not maternally expressed' class, 84 genes (93%) do not have any corresponding ESTs and the remaining six genes (7%) have one or two corresponding ESTs. The number of the corresponding ESTs for each of these six genes was two at maximum and 1.3 on average, while the number of the ESTs for each gene with maternal expression is 30 at maximum and 3.2 on average, suggesting a possibility that these six genes are maternally expressed at low level below the limit of detection by in situ hybridization. The evaluation with the EST data overall supports the reliability of the in situ result.

These two methods used in the present study have merits and demerits, respectively. The EST analysis could miss rarely expressed genes if the number of the ESTs is not large enough, while the EST analysis does not have a limit of detection if the number of the ESTs is large enough. The above comparison between results of the in situ hybridization and the EST analysis indicates our EST collection still misses some rarely expressed genes, although *C. intestinalis* is ranked sixth in the number of ESTs among all animals (NCBI EST database, May 7, 2004). However, in situ hybridization has a limit of detection and the result has to be evaluated visually, which means it is less objective than the EST analysis. However, in situ hybridization has the advantage of being able to test any genes accurately, even if the genes are rarely expressed. Considering the advantages and disadvantages of both methods, the inconsistency (3+61+6=70 genes; 22%) of both results probably corresponds to the rarely maternally expressed genes and this inconsistency may never be reconciled even in future studies. Nevertheless, the overall consistency (78%) supports the overall reliability of the in situ result. Accordingly, 52% of transcription factor genes (163+7=170 genes) are maternally expressed, 20% (3+61=64 genes) are maternally expressed at a low level (detectable by in situ hybridization), 2% (6 genes) are maternally expressed at a very low level (undetectable by in situ hybridization) and 26% (84 genes) are not maternally expressed.

Empirically, expression below the limit of detection by in situ hybridization is not so important for ascidian embryogenesis. Therefore, we discuss the maternal expression based on the results of the in situ hybridization of the 352 transcription factor genes, including 28 genes whose cDNA was isolated by PCR (these were not included in the comparison with the EST data). As summarized in Fig. 3A, 74% (259 genes) of transcription factor genes are expressed maternally, of which 3% (10 genes) are localized in the posterior region of the egg, and the remaining 26% (93 genes) of transcription factor genes were not detected maternally. In other words, a significant percentage of the *Ciona* transcription factor genes (74%) are expressed maternally above the limit of detection by in situ hybridization. It is thought that the dependency of development on maternally supplied information is higher in the ascidian than in other animals, because the ascidian egg shows a 'mosaic' mode of early

embryogenesis. Therefore, this high percentage of maternally expressed transcription factor genes may be specific for ascidian development, but this notion should be tested by comparing results from other model animals in future. Conversely, if the unveiled maternal determinants are a transcription factor like the *macho-1* gene in *Halocynthia*, candidates are only 259 genes described here.

Figure 3B shows the number and the ratio of maternally expressed genes for each transcription factor family consisting of at least 15 member genes. The ratio of maternally expressed genes among HMG (16 genes, 80%) and NR genes (14 genes, 82%) are markedly higher than the average (74%) and that of HD genes (44 genes, 59%) is markedly lower than the average. Although the number of genes in each gene family is not large enough to obtain statistically meaningful conclusions, maternal usage of transcription factor genes tends to be different for each transcription factor subfamily. Because the number of transcription factor genes in one animal is limited, this should be further examined in other animals in future.

Zygotically expressed transcription factor genes

Of 352 transcription factor genes examined, 199 (56%) genes are expressed zygotically during *Ciona* embryogenesis (Fig. 3C; Tables S6-S13). Of these 199 genes, 120 genes (34%) are also expressed maternally and the remaining 79 genes (22%) are expressed only zygotically. Of genes that are not expressed zygotically, 14 genes (4%) are not expressed maternally. Therefore, only 14 genes are not used during embryogenesis. Fig. 3D shows the number of zygotically expressed genes at each developmental stage. At the 16-cell, 32-cell, 64-cell, early gastrula, late-gastrula, neurula, early tailbud and mid-late tailbud stage, 13, 23, 47, 58, 90, 129, 175 and 180 genes are zygotically expressed, respectively. The number of genes expressed gradually increases as development proceeds. This may be simply explained by a hypothesis that the construction of a body with more complex elements requires more transcription factor genes.

Figure 3E,F shows the number and the ratio of the expressed gene number of each of seven major transcription factor families. Each transcription factor family may have a tendency in its zygotic expression according to the developmental stage. Fox transcription factor genes show a tendency for the genes to begin to be expressed at earlier stages, while the bHLH, HD and Ets families show a tendency for initiation of their expression to occur at later stages. A lower proportion of bZip, HMG and NR family genes are zygotically expressed during *Ciona* embryogenesis. Therefore it is possible that some transcription factor family may be largely assigned specific roles in development, although the number of genes in each gene family is not large enough to obtain statistically meaningful conclusions. Because the number of transcription factor genes in one animal is limited, this should be further examined in other animals in future.

Genes for signaling molecules

We previously identified most of the major signaling molecule genes in the *Ciona intestinalis* genome, including all ligands and major components of the Wnt, TGF β , Fgf, Notch, Hedgehog and JAK/STAT pathways [Tables 2, S2 (<http://dev.biologists.org/supplemental>)] (Satou et al., 2002c; Satou et al., 2003b; Hino et al., 2003). Embryonic expression

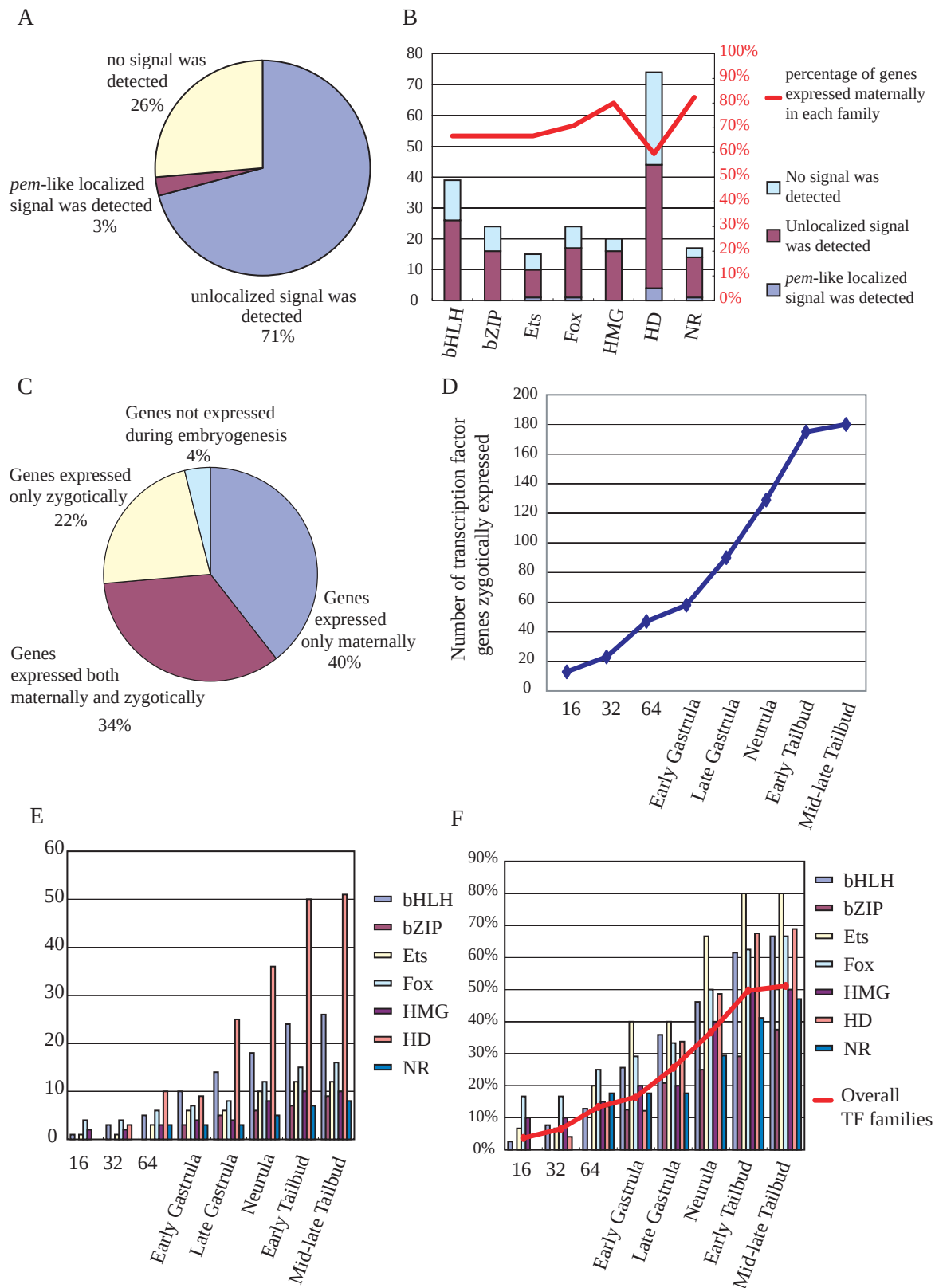


Fig. 3. Maternal and zygotic expression of *Ciona* transcription factor genes. (A) Maternal expression of transcription factor genes. (B) The numbers (bars and the left scale) and the ratios (red line and the right scale) of maternal expression of each transcription factor family. (C) Maternal and zygotic expression of transcription factor genes. (D) Genes zygotically expressed at each developmental stage. (E) The numbers of zygotic expression of each transcription factor family. (F) The ratios of zygotic expression of each transcription factor family. The line indicates the total proportion of genes in seven transcription factor families expressed zygotically at each developmental stage.

profiles of these genes are also described here. The initial list includes 118 genes, of which cDNAs for 109 genes were obtained for analysis similar to that performed for transcription factor genes (Table 2). The analysis revealed that 83 signaling genes are expressed maternally and six genes of them are localized in the posterior end of the embryo (Table S5 at <http://dev.biologists.org/supplemental>). Sixty-eight genes are zygotically regulated at the transcription level (Tables S6–S13 at <http://dev.biologists.org/supplemental>). For example, *Dickkopf* is not expressed maternally or at the 16-cell stage, but is expressed in A6.1, A6.3 and B6.1 at the 32-cell stage, in A7.1, A7.2, A7.5, B7.1 and B7.2 at the 64-cell and early gastrula stages, and in endodermal cells and cells of the nervous system at the late gastrula stage and thereafter (Fig. 2E). *Wnt5* is maternally expressed and localized to the posterior pole of the embryo, as is *pem* (Fig. 2F). The zygotic expression of *Wnt5* begins in B6.1 and B6.2 at the 32-cell stage, and is seen subsequently in A7.4, A7.8, B7.3, B7.4, B7.7 and B7.8 at the 64-cell stage, B7.7, B8.5, B8.7, B8.8, B8.15 and B8.16 at the early gastrula stage, muscle and posterior epidermis at the late gastrula and neurula stages, and parts of epidermis at the tailbud stage (Fig. 2F).

We compared these descriptions with those of genes analyzed in the previous studies, including *Fgfs* (Imai et al., 2002c; Imai et al., 2002d), *lefty/antivin* (Imai, 2003), *hedgehog1* and *hedgehog2* (Takatori et al., 2002). Our descriptions are basically same as the previous ones, indicating that the present assay is reliable. However, an apparent discrepancy in one gene was found. *hedgehog1* was not found to be zygotically expressed in the previous study, but in the present study the gene was found to be zygotically expressed in the 32-cell stage embryo and in embryos from the early gastrula stage to the mid-late tailbud stage, which was confirmed by two independent experiments. But the expression was very weak, and therefore we speculate that recent advances in the in situ hybridization techniques and methodology have made this expression detectable, although the precise reason is unknown.

Possible transcription factor networks

In ascidian development, the developmental fate of almost all blastomeres is restricted to one tissue at or before the 110-cell stage, as described above. The present comprehensive description demonstrated that only 65 transcription factor genes and 25 signaling genes are zygotically expressed until the early gastrula stage. Namely, the *Ciona* embryo requires only 65 zygotically expressed transcription factor genes and 25 signaling genes for the embryonic tissue specification. Therefore, we may be able to reconstruct comprehensive transcriptional networks by analyzing this limited number of genes in future.

Figure S1 shows transcription factor genes zygotically expressed during early embryogenesis in individual tissue lineages at each developmental stage. For example, the ascidian endoderm is determined by *Lhx3*, which is activated directly or indirectly by maternal β -catenin. *Lhx3* is first expressed in cells with endodermal fate at the 32-cell stage (Table S7; Fig. S1). The present analysis showed that only 12 transcription factor genes are zygotically expressed in this lineage of cells at the 16-cell stage and only 15 genes, except *Lhx3* itself, are expressed at the 32-cell stage (see Fig. S1 at <http://dev.biologists.org/supplemental>). Therefore, analyzing

genetic relationships among only 18 genes (=12+15–9; nine genes are expressed both at the 16 and 32-cell stage) clarifies a zygotic gene network beginning with maternal β -catenin and leading to activation *Lhx3* in the endodermal lineage for its specification.

Table S15 (see <http://dev.biologists.org/supplemental>) shows transcription factor genes whose expression overlaps that of individual transcription factor genes and signaling genes zygotically expressed in early embryogenesis at each developmental stage (i.e. possible direct transcriptional regulators of each of transcription factor genes and signaling genes are shown, on the assumption that each transcription factor protein has the same life span as its mRNA). For example, Table S15 (<http://dev.biologists.org/supplemental>) shows that 13 transcription factor genes are expressed in the blastomeres where *Brachyury* is first expressed at the 64-cell stage. In fact, one of these 13 genes, *ZicL*, was recently shown to be a direct activator of *Brachyury* (Yagi et al., 2004), suggesting that this type of comprehensive description will become an important milestone for future studies of the ascidian development.

Genes downstream of β -catenin, *FoxD* and *Fgf9/16/20*

As a first step to reconstruct the transcriptional gene network involved in making the ascidian endomesoderm specification, we chose three important genes; β -catenin, *FoxD* and *Fgf9/16/20*. As described above, β -catenin is essential for endoderm differentiation, and therefore required for forming tissues such as notochord and mesenchyme, which are induced by endodermal cells (Imai et al., 2000). *FoxD* is expressed under the control of β -catenin and essential for specification of notochord and TLCs (Imai et al., 2002b; Imai et al., 2003). *Fgf9/16/20* is also expressed under the control of β -catenin and induces mesenchyme and is partially responsible for inducing notochord (Imai et al., 2002c). Therefore, genes under the control of these three genes should be involved in notochord specification. Genes under the control of β -catenin and *FoxD* but not *Fgf9/16/20* should be involved in specification of notochord or TLCs. Genes under the control of β -catenin and *Fgf9/16/20*, but not *FoxD* should be involved in specification of mesenchyme or cells of the nervous system. Genes under the control of β -catenin but not *FoxD* and *Fgf9/16/20* should be involved in specification of endoderm.

We checked whether or not each of transcription factor and signaling genes that are expressed at the early gastrula stage is down- or upregulated by suppression of any of these three genes. For this, the real-time PCR method was adopted, because the quantity of each mRNA can be more precisely measured by this method than by microarray analysis when analyzing a limited number of genes. Only when at least two independent experiments indicated more than threefold difference in the amount of the gene transcript between control embryos and experimental embryos, the gene was counted as one that is affected by suppression of any of the three genes (Table S16; Fig. S2).

As summarized in Fig. 4, 26 transcription factor genes and 11 signaling genes were downregulated and one transcription factor gene was upregulated as a result of suppression of β -catenin. Five transcription factor genes and one signaling gene were downregulated, and two transcription factor genes and

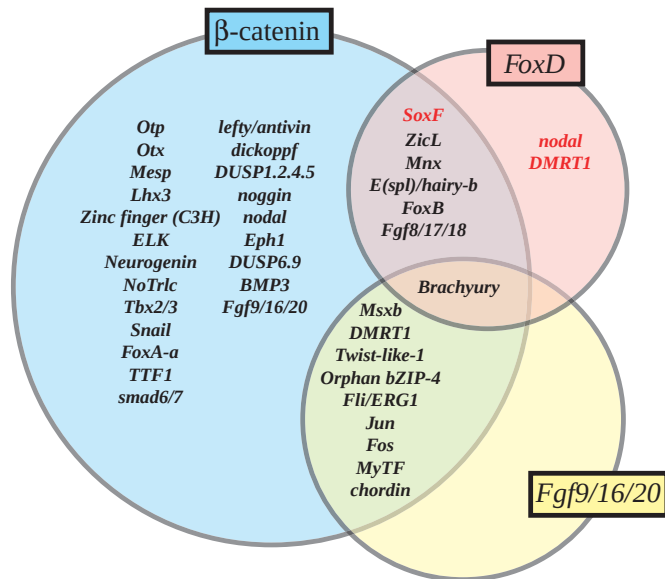


Fig. 4. Comprehensive lists of transcription factor genes and major signal transduction genes that are downstream of β -catenin, *FoxD* and *Fgf9/16/20* at the early gastrula stage. Genes indicated by black and red letters are down- and upregulated by suppression of each gene, respectively. See the text for a detailed description of the gene expression profile.

one signaling gene were upregulated by suppression of *FoxD*; and nine transcription factor genes and one signaling gene were downregulated by suppression of *Fgf9/16/20*. All genes that we previously identified as downstream genes of any of these three genes by in situ hybridization were included (see below), validating this analysis.

Only *Brachyury* is found to be affected by all three genes (Fig. 4). As described, genes under the control of these three genes should be involved in notochord specification. In fact, *Brachyury* has been proven to be an essential gene for notochord specification (Yasuo and Satoh, 1993; Corbo et al., 1997a; Yasuo and Satoh, 1998).

Among genes downstream of β -catenin and *FoxD* but not *Fgf9/16/20*, which are likely to be involved in specification of notochord and/or TLCs, *ZicL*, which is essential for notochord specification via activation of *Brachyury* transcription, was included (Imai et al., 2002a; Yagi et al., 2004). Among these genes, *Mn timer* is specifically expressed in the notochord lineage, *E(spl)/hairy-b* is expressed in the secondary-lineage (B-line) notochord cells and a part of nerve cord cells, and *FoxB* and *Fgf8/17/18* genes are expressed in cells of the TLC lineage. The expression pattern coincides well with the expected functions of these genes.

Genes downstream of β -catenin and *Fgf9/16/20* but not *FoxD* are probably involved in specification of mesenchyme, including TLCs and/or cells of the nervous system. Among these genes, *Twist-like-1*, *Orphan bZIP-4*, *Fli/ERG1*, *Jun*, *Fos* and *chordin* are expressed in mesenchymal cells. *Ciona savignyi* *Twist-like-1* has been proven to be essential for the specification of mesenchyme (Imai et al., 2003). *Myelin transcription factor (MyTF)* is expressed in cells of the TLC lineage. *Chordin* is also expressed in notochord cells and a part of muscle cells. *DMRT1* and *Msxb* genes are expressed in cells of the nervous system.

Genes downstream of β -catenin are probably involved in other vegetal cell specification, including specification of endoderm. *Lhx3*, which is one of these genes, is known to play an essential role in differentiation of endoderm (Satou et al., 2001a). The gene for TTF1, which is also known to be involved in endoderm specification, is also included in this group (Ristoratore et al., 1999). The common feature of this class of genes is that all genes except nodal are expressed in one or more vegetal cells (Tables S5-S13).

Thus, the mode of regulation summarized in Fig. 4 and the expression pattern summarized in Fig. S1 (see <http://dev.biologists.org/supplemental>) of each gene will strongly predict its function. Therefore the present data will also become another important milestone for future studies of gene function. We cannot know from this analysis whether all expression of a given gene was affected or a part of the expression was affected. For example, it was shown by in situ hybridization that the expression of *ZicL* in A-line cells is affected by β -catenin but the expression in B-line cells is not (Imai et al., 2002a). In spite of this weakness, *ZicL* was still detected to be suppressed by β -catenin morpholino in the present real-time PCR experiment, suggesting usefulness of this analysis in constructing frameworks of gene networks. In future studies, similar real-time PCR experiments using embryos at different stages should be performed. Once genes are identified as downstream of β -catenin, *FoxD* and/or *Fgf9/16/20*, the function of the genes should be determined and their epistatic relationships will be determined in a similar way. Thus, these approaches will reveal frameworks consisting of transcription factor genes and signaling genes for the complete understanding of the molecular mechanisms behind ascidian endomesoderm specification.

Conclusion

As discussed here, the expression profiles of *Ciona intestinalis* transcription factor genes and major signaling genes have been almost completely determined from the egg to the mid-late tailbud stage embryo. The patterns thus revealed answer several fundamental questions about how the transcription factor genes are used to build up the basic chordate body plan. In the ascidian embryo, almost all blastomeres are specified and determined at or before the 110-cell stage. The present analysis performed at the single-cell level has shown that 65 transcription factor genes are zygotically expressed at or before this stage. In other words, the ascidian embryo requires only 65 transcription factor genes to be zygotically expressed for specification of larval tissues. This number is smaller than expected. Thus, further analysis of this very limited number of transcription factor genes will allow elucidation of the complete transcriptional networks that are essential for the tissue specifications at the single-cell level.

Possible regulatory relationships among the transcription factor genes can be deduced from the present study, exemplified by Table S15, although we have not yet reconstructed any gene networks. These possible relationships can be actually confirmed by mid-to-high throughput analysis such as real-time PCRs adopted in the present study.

To obtain a real understanding of the molecular mechanisms behind animal development, comprehensiveness is essential. Quantitative comprehensiveness can be easily attained using modern methods such as microarrays and real-time PCR.

However, because animal development is controlled spatially and temporally, qualitative comprehensiveness is also required. To obtain these data is tedious, and therefore to start studies with a subset of genes would be a good alternative. We present here such comprehensive qualitative data about transcription factor genes and signaling genes in several major signaling pathways. These data will provide an important scaffold for achieving complete understanding of ascidian embryogenesis.

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