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Temporal expression patterns of 39 *Brachyury*-downstream genes associated with notochord formation in the *Ciona intestinalis* embryo

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Expression of the *Brachyury* (*Ci-Bra*) gene of the ascidian *Ciona intestinalis* is initiated at the 64-cell stage. Gene expression is restricted to notochord precursor cells, and *Ci-Bra* plays a key role in notochord differentiation. In a previous study, nearly 50 cDNA clones for potential *Ci-Bra*-downstream genes that are expressed in notochord cells were isolated. The present determination, by whole-mount *in situ* hybridization, of the temporal expression patterns of 19 notochord-specific and 20 notochord-predominant genes demonstrated that the timings of initiation of the expression of various genes was not identical. The expression of several genes was initiated as early as the gastrula stage. However, the expression of most of the notochord-specific genes commenced at the neural plate stage. Partial nucleotide sequence data of these clones suggest that genes expressed earlier encode potential transcriptional factors and/or nuclear proteins, while those expressed later encode proteins implicated in cell adhesion, signal transduction, regulation of the cytoskeleton, and components of the extracellular matrix. These gene activities may be associated with changes in cell shape and adhesion during the intercalation and extension of the notochord cells.

Key words: ascidian, *Brachyury,* downstream genes, notochord, temporal expression patterns.

Introduction

Brachyury encodes a sequence-specific activator that contains a T-box DNA-binding domain (reviewed by Herrmann & Kispert 1994; Smith 1997; Papaioannou & Silver 1998). The gene was originally cloned from mice, taking advantage of a *Brachyury* mutation (Herrmann *et al.* 1990). In vertebrates, *Brachyury* is initially expressed in the presumptive mesoderm of gastrulae, and during later stages the expression is gradually restricted to the developing notochord and tail-bud (Wilkinson *et al.* 1990; Smith *et al.* 1991; Schulte-Merker *et al.* 1992; Kispert *et al.* 1995). *Brachyury* expression is critical for notochord differentiation in all vertebrates that have been studied, including mice (Rashbass *et al.* 1991), frogs (Conlon *et al.* 1996) and zebrafish (Schulte-Merker *et al.* 1994).

Ascidians (urochordates) constitute one of the three chordate groups. Because fertilized ascidian eggs develop rather quickly into tadpole-type larvae with several distinct types of tissues and organs, and because the lineage of embryonic cells is well documented, the ascidian embryo provides a good experimental system to explore the expression and function of developmental genes (reviewed by Satoh 1994, 1999). In addition, the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan (reviewed by Satoh 1995; Satoh & Jeffery 1995; Di Gregorio & Levine 1998). It contains a notochord composed of just 40 cells, of which the lineage has been completely described (Conklin 1905; Nishida 1987). Previous studies in two divergent ascidians revealed that ascidian *Brachyury* genes, *As-T* of *Halocynthia roretzi* (Yasuo & Satoh 1993, 1994) and *Ci-Bra* of *Ciona intestinalis* (Corbo *et al.* 1997), are expressed exclusively in the notochord precursor cells and that the timing of gene expression coincides with clonal restriction of the notochord lineage at the 64-cell stage (cf, Fig. 1A). In *H. roretzi*, notochord formation is induced at the 32-cell stage by signals emanating from the adjacent endoderm cells as well as neighboring notochord cells (Nakatani & Nishida 1994). Overexpression of *As-T* via its messenger ribonucleic acid (mRNA)

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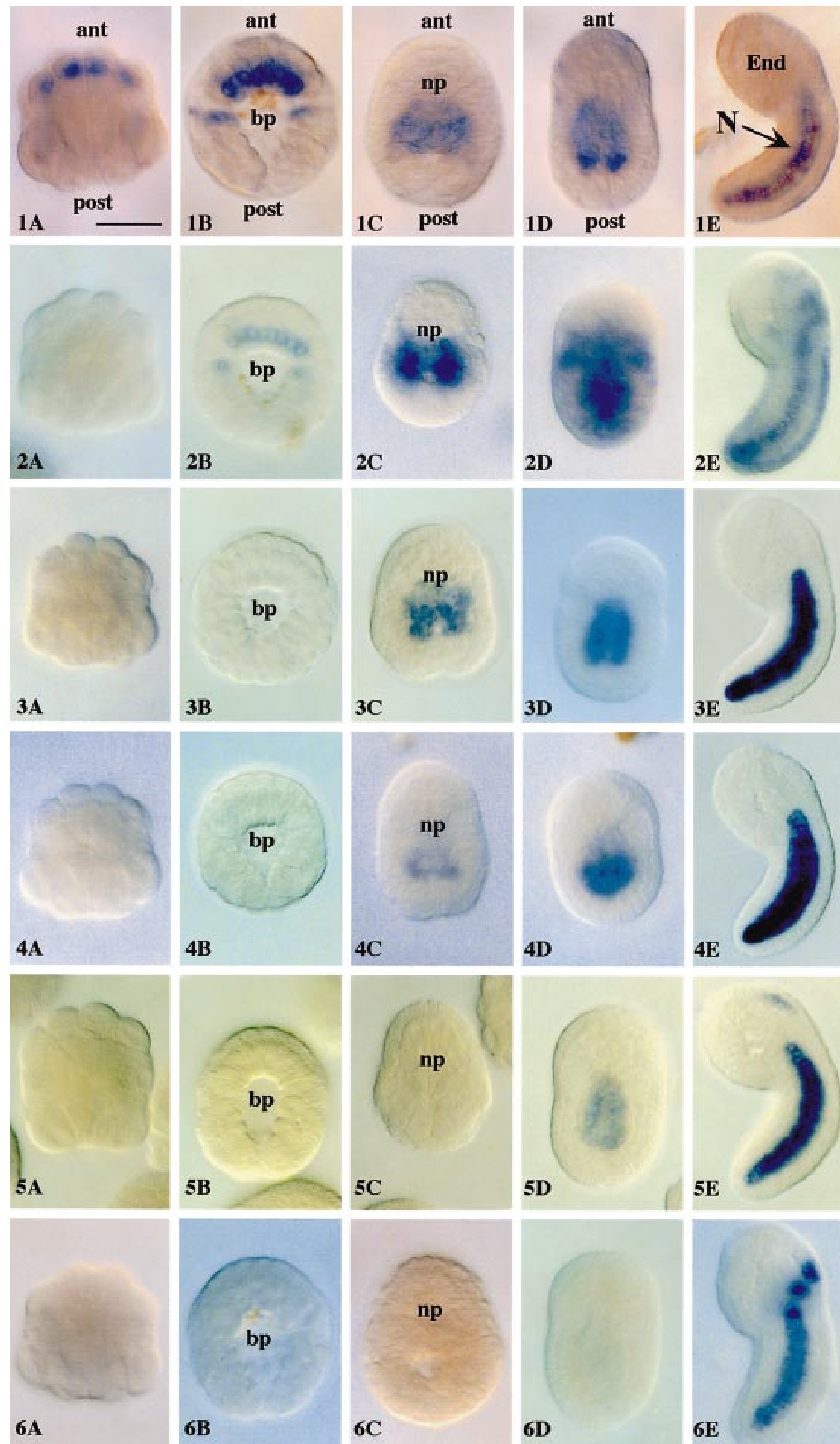
injection results in notochord formation without a requirement for the inductive event at the 32-cell stage (Yasuo & Satoh 1998). In addition, misexpression of both *As-T* (Yasuo & Satoh 1998) and *Ci-Bra* (Takahashi *et al.* 1999) causes transformation of endodermal and neuronal lineages into notochord cells. These studies demonstrate that the ascidian *Brachyury* gene is critical in determination of the notochord.



Figs 1–6. Temporal expression patterns of (1) *Ci-Bra* and (2–6) five *Ci-Bra*-downstream notochord-related genes during embryogenesis of *Ciona intestinalis*, as revealed by whole-mount *in situ* hybridization with a digoxigenin-labeled antisense probe. Genes encoded by cDNA clones: (2) 403e, (3) 406g, (4) 210d, (5) 309h, and (6) 309g. Embryos at (A) 64-cell stage, vegetal pole view; (B) gastrula stage, vegetal pole view; (C) neural plate stage, dorsal (vegetal) view; (D) neurula stage, dorsal view; and (E) tail-bud stage, side view. ant, anterior; post, posterior side of the embryo. bp, blastopore; End, endoderm; N, notochord; np, neural plate. Bar, 50 μm 1(A); applicable for all panels.

In a previous study, we attempted the isolation of potential *Ci-Bra* target genes (Takahashi *et al.* 1999). A 2.6 kb genomic DNA fragment from the 5′-flanking region of the *forkhead/HNF-3β* gene of *C. intestinalis* (*Ci-fkh*) is sufficient to direct the expression of a *lacZ* reporter gene in these tissues after electroporation into 1-cell embryos (Corbo *et al.* 1997). Attaching the *Ci-Bra* coding sequence to the *Ci-fkh* promoter region resulted in misexpression of the *Ci-Bra* gene in ectopic tissues. The resulting fusion gene causes extensive transformation of the endoderm into notochord, whereby mutant tail-bud embryos contain a large mass of notochord tissue in midtail regions. The efficiency of the electroporation method allowed us to obtain large quantities of mutant embryos, thereby facilitating subtractive hybridization reactions using mRNA extracted from wild-type and mutant embryos at the neurula and early tail-bud stages. A subtractive cDNA library was prepared that contained mRNA that are overexpressed in the mutant embryos relative to the wild-type controls. The library contained 923 cDNA clones, 501 of which were shown to be independent. Of the 501 independent cDNA clones that were surveyed, nearly 50 genes were specifically or predominantly expressed in the notochord cells. The potential *Ci-Bra* target genes encode components of the extracellular matrix, as well as proteins implicated in cell adhesion, signal transduction, and regulation of the cytoskeleton. Therefore, we propose that those genes define one or more signaling pathways, which control changes in cell shape and adhesion during the intercalation and extension of the notochord (cf, Miyamoto & Crowther 1985). Even though the expression of all of these genes is induced by *Ci-Bra* misexpression, it remains to be determined whether these genes are direct or indirect targets of *Ci-Bra*. As a first step in studies to understand the *Ci-Bra*-downstream genetic cascade, the present study determined the temporal expression patterns of 19 notochord-specific and 20 notochord-predominant genes that are induced by *Ci-Bra* misexpression.

Materials and Methods

Ascidian eggs and embryos

Eggs and sperm of *C. intestinalis* were obtained surgically from the gonoduct. After insemination, eggs were dechorionated by immersing them in seawater that contained 1% sodium thioglycolate (Wako Pure Chemical Industries Ltd, Osaka, Japan) and 0.05% actinase E (Kaken Pharmaceutical Co. Ltd, Tokyo, Japan). Naked eggs and embryos were then reared at about 18°C in agar-coated dishes with Millipore-filtered seawater (MFSW) containing 50 μg/mL streptomycin sulfate. Under our culture conditions, the first cleavage occurred about 1 h after insemination. Embryos developed to the 64-cell, gastrula, neural plate, neurula, and early tail-bud stages about 4, 5.5, 7, 8, and 9 h after fertilization, respectively. Tadpole larvae began to swim at about 18 h of development. Eggs and embryos at appropriate stages were fixed for *in situ* hybridization.

cDNA probes for Ciona *notochord-related genes*

Details of isolation and characterization of cDNA clones for *Ci-Bra*-downstream genes that are expressed in notochord cells of *C. intestinalis* tail-bud embryos were reported in Takahashi *et al.* (1999). In the present study, we examined the temporal expression patterns of 39 notochord-related genes (cf, Table 2). Nineteen genes represented by cDNA clones 103f, 107g, 110g, 210d, 212g, 309h, 402c, 403e, 406g, 407e, 408h, 409d, 411d, 502a, 505a, 507d, 507h, 801h, and 807d are expressed specifically in notochord cells of the early tail-bud embryo. The other 20 genes, represented by 002d, 003b, 003d, 005d, 006c, 007d, 010b, 111a, 204d, 208e, 209c, 211g, 308d, 309g, 504g, 603d, 608g, 706e, 708g, and 903g, are predominantly expressed in notochord cells of the early tail-bud embryo (hereafter in this report we designate the clone number as the gene name). Determination of the sequences of about 500 nucleotides of both the 5′ and 3′ regions suggested that 21 of the 39 genes show sequence similarity to reported proteins, but the other 18 have no sequence similarity to known proteins (Takahashi *et al.* 1999; cf, Table 2).

The cDNA were inserted into the *Eco*RI/*Xho*I site of the plasmid vector pBluescript II SK(+) (Stratagene, La Jolla, CA, USA). Digoxigenin (DIG)-labeled sense and antisense probes were synthesized following the instructions of the suppliers of the kit (DIG RNA Labelling Kit; Boehringer Mannheim, Heidelberg, Germany). Their final sizes were reduced to approximately 150 nucleotides by alkaline hydrolysis.

In situ *hybridization*

Whole-mount specimens were hybridized *in situ* using DIG-labeled antisense and sense RNA probes essentially according to the method described by Corbo *et al.* (1997). Briefly, embryos were fixed in 4% paraformaldehyde in 0.1 M 3-(N-Morpholino) propanesulfonic acid (MOPS) buffer (pH 7.5), 0.5 M NaCl. After being thoroughly washed with phosphate-buffered saline containing 0.1% Tween 80 (PBT), the embryos were treated with 2 μg/mL proteinase K (Sigma Chemical Co., St Louis, MO, USA) in PBT for 30 min at 37°C, then postfixed with 2% paraformaldehyde in PBT for 30 min at room temperature. After 1.5 h of

prehybridization at 55°C, the embryos were allowed to hybridize with the DIG-labeled probes at a concentration of 1 μg/mL for at least 18 h at 55°C. After hybridization, the embryos were washed 10 times for 15 min each with the hybridization solution at 60°C. Thereafter, the samples were incubated for 2 h with 500 μL anti-DIG-alkaline phosphate conjugate, and color was developed according to the protocol of Boehringer Mannheim. After dehydration, some of the specimens were made transparent by placing them in 100% xylene. The samples were mounted on glass slides with entellan neu (Merck, Darmstadt, Germany) and photographed.

Results

Temporal expression patterns of 19 notochord-specific genes

With the aid of *in situ* hybridization of whole-mount specimens, we first examined the appearance and distribution of transcripts of each of the 19 potential *Ci-Bra* downstream genes that are expressed specifically in notochord cells of *C. intestinalis* tail-bud embryos. As summarized in Table 1, the timings for the initiation of gene expression were not identical; two genes initiated their expression as early as the mid-gastrula stage, while the expression of 12 genes began at the neural plate stage and that of five genes at the neurula stage. No genes were expressed before the initiation of gastrulation.

The 403e gene, for example, began to be expressed at the mid-gastrula stage (Fig. 2B). The timing of initiation of the 403e gene expression was about 1.5 h behind that of *Ci-Bra* expression at the 64-cell stage (Fig. 1A). At this stage, the 403e transcript was evident only in 10 notochord precursor cells (Fig. 2B). Although in later stages this gene seemed to be expressed in several neuronal cells as well as epidermal cells (Fig. 2C–E), we categorized it as notochord specific, because its initial expression was restricted to the notochord precursor cells. Partial nucleotide sequence data suggest that the 403e gene encodes a nuclear protein with a LIM domain (Table 2). The transcript of another gene (507h) also became detectable in the notochord precursor cells at the gastrula stage. The 507h gene encodes a protein with no sequence similarity to any known protein.

The present analysis demonstrated that the expression of 12 of the 19 *Ci-Bra*-downstream notochord-specific genes (63%) is initiated at the neural plate stage (Table 1; Figs 3,4); genes 107g, 110g, 406g, 408h, 502a, 807d, 103f, 407e, 210d, 212 g, 801h and 505a were categorized into this group. For example, 406g may encode a nuclear protein associated with cell division control (Table 2). The transcript of this gene became detectable in almost all of the primordial notochord cells at the neural plate stage (Fig. 3C), and later was restricted to notochord cells in the tail-bud embryo (Fig. 3E). On the other hand, 210d encoded a protein with no sequence similarity to any known protein. The 210d transcript was first evident in a fraction of the primordial notochord cells that were located in the posterior region of the notochord cell field (Fig. 4C,D), and later the transcript was found in all of the notochord cells (Fig. 4E). Partial nucleotide sequence data of the notochord-specific genes that initiate their expression at the neural plate stage suggested that these genes encode proteins that act as cell surface receptors, or play roles in signal transduction or regulation of the cytoskeleton.

Expression of the remaining five notochord-specific genes was initiated at the neurula stage (Table 1). For example, 309h, of which the transcript became detectable at the neurula stage (Fig. 5D), may encode an integral membrane protein associated with a sulfate transporter protein (Table 2). The expression of four other genes, 402c, 409d, 507d and 411d, also began at this stage. In the present analysis, none of the potential *Ci-Bra*-downstream, notochord-specific genes started to be expressed at the early tail-bud or later stages.

Temporal expression patterns of 20 notochord-predominant genes

Twenty potential *Ci-Bra*-downstream genes were expressed predominantly in notochord cells of the tail-bud embryos. In addition, transcripts were detectable in nerve cord cells, neuronal cells, or muscle cells, depending on the gene. Thus, these genes were expressed in the notochord and its neighboring cells.

Table 1. Timing of initiation of expression of *Ci-Bra*-downstream notochord-related genes during embryogenesis of the ascidian *Ciona intestinalis*

Genes	No. genes	Stage of initiation of the gene expression				
		64-cell	Gastrula	Neural plate	Neurula	Tail-bud
Notochord-specific genes	19	0	2	12	5	0
Notochord-predominant genes	20	0	6	3	7	4
Total	39	0	8	15	12	4

Table 2. Timing of initiation of *Ci-Bra*-downstream notochord-related gene expression in *Ciona* embryos

Developmental stage	Clone	Sequence similarity	Possible features	Figure
Gastrula	**403e**	LIM-only protein	LIM domain	2
	507h			
	006c		Serpin proteins, transcriptional regulatory	
	308d			
	504g	Myosin heavy chain	Structure	
	706e			
	211g		Inhibin β B chain signature, C1q domain. speract receptor repeat	
	903g			
Neural plate	**107g**			
	110g	ATP-citrate (pro-S-)-lyase	C2HC-type zinc-finger signature, laminin-type EGF-like (LE) domain	
	406g	Cell division control protein 45	Nuclear protein integral membrane signature	3
	408h	Prothrombinase precursor (fibrinogen-like protein)	Fibrinogen β and γ chains C-terminal domain	
	502a	Cellular tumor antigen P53	EF-hand calcium-binding domain, recoverin family signature	
	807d			
	103f	HSPG	LDL-receptor class A (LDLRA) domain	
	407e	LIM-only protein	LIM domain	
	210d			4
	212g			
	801h	Extensin precursor (proline-rich glycoprotein)		
	505a	Sulfate adenylyltransferase (SAT)	Pheromone A receptor signature, tyrosine-specific protein phosphatases proteins	
	003b	Agrin	Cell adhesion, calcium-binding EGF-like domain pattern proteins	
	010b	Collagen α1 (XI) chain		
	603d	Max interacting protein 1 (MXI1 protein)	myc-type, 'helix-loop-helix' dimerization domain proteins	
Neurula	**402c**			
	409d			
	507d			
	309h	Sulfate transporter	Membrane, sulfate transporter proteins, integral membrane proteins	5
	411d			
	002d	Ezrin/radixin/moesin	Cell adhesion, membrane	
	608g	Protein-tyrosine phosphatase MEG1	Structure, extracellular	
	209c			
	007d			
	111a	Reticulocalbin 1	EF-hand calcium-binding	
	204d	P-selectin	Cell adhesion, selectin superfamily complement-binding repeat, sushi domain	
	708g			
Initial tail-bud	003d			
	005d	Tensin	Cell adhesion	
	208e			
	309g	*N*-acetyl-lactosamine synthase		6

Bold type, notochord-specific genes; roman type, notochord-predominant genes.
ATP, adenosine triphosphate; HSPG, heparan sulfate proteoglycan; LDL, low density lipoprotein; MEG1, megakaryoblastic cell line cDNA1.

In situ hybridization of whole-mount specimens demonstrated that these 20 potential *Ci-Bra*-downstream genes were expressed within a relatively broad time window, in contrast to the general pattern, in which notochord-specific downstream genes are mainly expressed at the neural plate stage, as mentioned above (Table 1). Namely, the expression of six genes was initiated at the mid-gastrula stage, while the expression of three, seven and four genes commenced at the neural plate, neurula, and tail-bud stages, respectively.

Genes 006c, 211g, 308d, 504g, 706e and 903g began to be expressed at the mid-gastrula stage (Tables 1,2). Partial nucleotide sequence data suggest that the 006c gene may encode a protein associated with transcriptional regulation, while the 504g gene may encode a structural protein with sequence similarity to the myosin heavy chain.

Transcripts of genes 003b, 010b and 603d became detectable in the primordial notochord cells at the neural plate stage. The 003b gene may encode a protein associated with cell adhesion, and 010b may encode the collagen α1 chain (Table 2).

The expression of seven genes, 002d, 007d, 111a, 204d, 209c, 608 g and 708 g, was initiated at the neurula stage (Tables 1,2). For example, 002d may encode an ezrin/radixin/moesin (ERM)-like protein that is involved in cell adhesion (Table 2).

The expression of four genes, 003d, 005d, 208e and 309 g, was initiated at the early tail-bud stage (Tables 1,2). For example, no transcripts of the 309g gene were detected at early developmental stages up to the neurula stage (Fig. 6A–D). The transcript was first evident in notochord cells as well as a few neuronal cells in the early tail-bud embryo (Fig. 6E). The 309g gene may encode N-acetyl-lactosamine synthase (Table 2). The 005d gene may encode tensin, which is involved in cell adhesion (Table 2).

Discussion

Differentiation of notochord cells in the ascidian embryo has been studied intensively (reviewed by Satoh 1994, 1999). The studies include analyses of cell lineage (Conklin 1905; Nishida 1987), specification mechanisms at the cellular level (Nakatani & Nishida 1994; Nakatani *et al.* 1996) and at the molecular level (Yasuo & Satoh 1993, 1998; Corbo *et al.* 1997, 1998; Fujiwara *et al.* 1998), behavior of notochord cells in living *C. intestinalis* embryos investigated with time-lapse video photography (Miyamoto & Crowther 1985), and ultrastructural observations of changes in cell shape and notochord-sheath formation (reviewed by Cloney 1990). Altogether, these studies have revealed that the notochord of the ascidian embryo is formed as follows.

The ascidian larval notochord consists of exactly 40 cells aligned at the midline, longitudinally in single file in the tail. Thirty-two of the 40 notochord cells in the anterior and middle portion of the tail are derived from the A4.1 pair of the 8-cell embryo, and the other eight cells, in the posterior region, from the B4.1 pair. At the 64-cell stage, the A7.3 and A7.7 pairs are restricted to give rise to notochord cells, and at the 110-cell stage, A8.5, A8.6, A8.13, A8.14 and B8.6 pairs are primordial notochord cells, giving rise to 40 cells after two divisions. Therefore, every notochord cell has a history of nine divisions from the zygotic stage until the ultimate differentiation. The positions of the notochord cells derived from each precursor cell are not predetermined within the tail. A cell lineage study revealed that the presumptive notochord cells from the left and right sides of the embryo can mix randomly at the midline (Nishida 1987). However, the A-line and B-line precursor cells do not interdigitate with one another.

In *H. roretzi*, specification of notochord cells is induced at the 32-cell stage by signals emanating from the adjacent endoderm cells as well as neighboring notochord precursor cells (Nakatani & Nishida 1994). Then, at the 64-cell stage, the *Brachyury* gene (*As-T*) is expressed exclusively in the primordial notochord cells (Yasuo & Satoh 1993, 1994). *Ci-Bra* is also expressed exclusively in the notochord precursor cells in the 64-cell-stage *Ciona* embryo (Corbo *et al.* 1997). Molecules that may possibly be responsible for the ascidian *Brachyury* activation are bFGF for *As-T* (Nakatani *et al.* 1996) and Suppressor of Hairless protein for *Ci-Bra* (Corbo *et al.* 1998). During this stage, *Ciona* snail represses *Ci-Bra* expression in para-axial mesoderm cells of the mesenchyme and muscle (Fujiwara *et al.* 1998). The expression of *As-T* or *Ci-Bra* is sufficient for gene expression that is associated with the differentiation of notochord cells and the morphological formation of the notochord (Yasuo & Satoh 1998; Takahashi *et al.* 1999; G. Satoh *et al.* unpubl. data, 1999). Studies of the formation of the notochord in living *C. intestinalis* embryos with time-lapse video photography have demonstrated that cell proliferation, growth, migration, rearrangement, shape changes, and alteration of the extracellular environment occur as the notochord is transformed from a loose plate or mass of cells during neurulation and initial tail-bud formation into an elongated rod surrounded by a sheath of fibrous materials during larval formation (Miyamoto & Crowther 1985). The basal surfaces of the notochord cells rest on a basal lamina termed the ‘notochordal sheath’ that completely surrounds the notochord. The

notochordal sheath contains many circumferentially orientated and longitudinally orientated filaments (Cloney 1969).

Previous and present studies add further information on the process of notochord formation in the ascidian embryo. Namely, *Ci-Bra* expression in turn causes expression of a great number of genes that may be associated with formation of the notochord. As shown by the present study, the timings of initiation of expression of potential *Ci-Bra*-downstream notochord-related genes are not identical. The expression of several genes is initiated as early as the gastrula stage, about 1~1.5 h after *Ci-Bra* expression. Although the timing of expression of the *Ci-Bra* protein must be examined further, this time lag seems reasonable for inducing the activation of Ci-Bra direct target genes. However, the expression of most of the notochord-specific genes is initiated at the neural plate stage, suggesting that this is the stage critical for activation of structural genes associated with notochord formation.

Partial nucleotide sequence data of these clones as well as the present determination of timing of gene expression demonstrated that genes expressed earlier encode potential transcriptional factors and/or nuclear proteins (such as 403e). Those expressed later (such as 406g, 003b, 010b, 002d and 005d) encode proteins implicated in cell adhesion, signal transduction, and regulation of the cytoskeleton, and components of the extracellular matrix, which may be associated with changes in cell shape and adhesion during the intercalation and extension of the notochord cells. These results suggest the possibility that *Ci-Bra* may directly activate a few target genes that encode other types of transcription factors. The transcription factors then activate the notochord-specific structural genes that are expressed around the time of neurulation. Therefore, most of the structural genes that are expressed around the time of neurulation are likely indirect targets of *Ci-Bra*. Keeping in mind such a possibility, details of the *Ci-Bra*-downstream genetic cascade for notochord formation in the ascidian embryo should be studied further in the future.

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The nucleotide sequence data reported in this paper will appear in the homepage (http://hoya.zool.kyoto-u.ac.jp/introduction/dev-evo/target).

References

Cloney, R. A. 1969. Cytoplasmic filaments and morphogenesis: The role of the notochord in ascidian metamorphosis. *Z. Zellforsch.* **100**, 31–53.

Cloney, R. A. 1990. Urochordata–Ascidiacea. In *Reproductive Biology of Invertebrates* (Eds K. G. Adiyodi & R. G. Adiyodi), pp. 391–451. Oxford & IBH Publishing, New Delhi.

Conklin, E. G. 1905. The organization and cell lineage of the ascidian egg. *J. Acad. Natl Sci.* **13**, 1–119.

Conlon, F. L., Sedgwick, S. G., Weston, K. M. & Smith, J. C. 1996. Inhibition of *Xbra* transcription activation causes defects in mesodermal patterning and reveals autoregulation of *Xbra* in dorsal mesoderm. *Development* **122**, 2427–2435.

Corbo, J. C., Fujiwara, S., Levine, M. & Di Gregorio, A. 1998. Suppressor of Hairless activates *Brachyury* expression in the *Ciona* embryo. *Dev. Biol.* **203**, 358–368.

Corbo, J. C., Levine, M. & Zeller, R. W. 1997. Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis. Development* **124**, 589–602.

Di Gregorio, A. & Levine, M. 1998. Ascidian embryogenesis and the origins of the chordate body plan. *Curr. Opin. Genet. Dev.* **8**, 457–463.

Fujiwara, S., Corbo, J. C. & Levine, M. 1998. The Snail repressor establishes a muscle/notochord boundary in the *Ciona* embryo. *Development* **125**, 2511–2520.

Herrmann, B. G. & Kispert, A. 1994. The *T* genes in embryogenesis. *Trends Genet.* **10**, 280–286.

Herrmann, B. G., Labeit, S., Poustka, A., King, T. R. & Lehrach, H. 1990. Cloning of the *T* gene required in mesoderm formation in the mouse. *Nature* **343**, 617–622.

Kispert, A., Ortner, H., Cooke, J. & Herrmann, B. G. 1995. The chick *Brachyury* gene: Developmental expression pattern and response to axial induction by localized activin. *Dev. Biol.* **168**, 406–415.

Miyamoto, D. M. & Crowther, R. J. 1985. Formation of the notochord in living ascidian embryos. *J. Embryol. Exp. Morphol.* **86**, 1–17.

Nakatani, Y. & Nishida, H. 1994. Induction of notochord during ascidian embryogenesis. *Dev. Biol.* **166**, 289–299.

Nakatani, Y., Yasuo, H., Satoh, N. & Nishida, H. 1996. Basic fibroblast growth factor induces notochord formation and the expression of *As-T*, a *Brachyury* homolog, during ascidian embryogenesis. *Development* **122**, 2023–2031.

Nishida, H. 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme: III. Up to the tissue restricted stage. *Dev. Biol.* **121**, 526–541.

Papaioannou, V. E. & Silver, L. M. 1998. The T-box gene family. *Bioessays* **20**, 9–19.

Rashbass, P., Cooke, L. A., Herrmann, B. G. & Beddington, R. S. P. 1991. A cell autonomous function of *Brachyury* in *T/T* embryonic stem cell chimaeras. *Nature* **353**, 348–351.

Satoh, N. 1994. *Developmental Biology of Ascidians*. Cambridge University Press, New York.

Satoh, N. 1995. Towards a molecular understanding of developmental mechanisms underlying the origin and evolution of chordates. In *Biodiversity and Evolution* (Eds R. Arai, M. Kato & Y. Doi), pp. 267–290. National Science Museum Foundation, Tokyo.

Satoh, N. 1999. Cell fate determination in the ascidian embryo. In *Cell Lineage and Fate Determination* (Ed. S. A. Moody), pp. 59–74. Academic Press, San Diego.

Satoh, N. & Jeffery, W. R. 1995. Chasing tails in ascidians: Developmental insights into the origin and evolution of chordates. *Trends Genet.* **11**, 354–359.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. & Nüsslein-Volhard, C. 1992. The protein product of the zebrafish homologue of the mouse *T* gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116**, 1021–1032.

Schulte-Merker, S., van Eeden, F. J. M., Halpern, M. E., Kimmel, C. B. & Nüsslein-Volhard, C. 1994. *no tail* (*ntl*) is the zebrafish homologue of the mouse *T* (*Brachyury*) gene. *Development* **120**, 1009–1015.

Smith, J. 1997. *Brachyury* and the T-box genes. *Curr. Opin. Genet. Dev.* **7**, 474–480.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. & Herrmann, B. G. 1991. Expression of a *Xenopus* homolog of *Brachyury* (*T*) is an immediate-early response to mesoderm induction. *Cell* **67**, 79–87.

Takahashi, H., Hotta, K., Erives, A. *et al.* 1999. *Brachyury*-downstream notochord differentiation in the ascidian embryo. *Genes Dev.* **13**, (in press).

Wilkinson, D. G., Bhatt, S. & Herrmann, B. G. 1990. Expression pattern of the mouse *T* gene and its role in mesoderm formation. *Nature* **343**, 657–659.

Yasuo, H. & Satoh, N. 1993. Function of vertebrate *T* gene. *Nature* **364**, 582–583.

Yasuo, H. & Satoh, N. 1994. An ascidian homolog of the mouse *Brachyury* (*T*) gene is expressed exclusively in notochord cells at the fate restricted stage. *Develop. Growth Differ.* **36**, 9–18.

Yasuo, H. & Satoh, N. 1998. Conservation of the developmental role of *Brachyury* in notochord formation in a urochordate, the ascidian *Halocynthia roretzi*. *Dev. Biol.* **200**, 158–170.