A zinc finger transcription factor, ZicL, is a direct activator of Brachyury in the notochord specification of Ciona intestinalis

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Summary

In ascidian embryos, Brachyury is expressed exclusively in blastomeres of the notochord lineage and play an essential role in the notochord cell differentiation. The genetic cascade leading to the transcriptional activation of Brachyury in A-line notochord cells of Ciona embryos begins with maternally provided β-catenin, which is essential for endodermal cell specification. β-catenin directly activates zygotic expression of a forkhead transcription factor gene, FoxD, at the 16-cell stage, which in turn somehow activates a zinc finger transcription factor gene, ZicL, at the 32-cell stage, and then Brachyury at the 64-cell stage. One of the key questions to be answered is whether ZicL functions as a direct activator of Brachyury transcription, and this was addressed in the present study. A fusion protein was constructed in which a zinc finger domain of Ciona ZicL was connected to the C-terminus of GST. Extensive series of PCR-assisted binding site selection assays and electrophoretic mobility shift assays demonstrated that the most plausible recognition sequence of Ciona ZicL was CCGGCTGTG. We found the elements CACAGCTGG (complementary sequence: CCAGCTGTG) at −123 and CCAGCTGTG at −168 bp upstream of the putative transcription start site of Ci-Bra in a previously identified basal enhancer of this gene. In vitro binding assays indicated that the ZicL fusion protein binds to these elements efficiently. A fusion gene construct in which lacZ was fused with the upstream sequence of Ci-Bra showed the reporter gene expression exclusively in notochord cells when the construct was introduced into fertilized eggs. In contrast, fusion constructs with mutated ZicL-binding-elements failed to show the reporter expression. In addition, suppression of Ci-ZicL abolished the reporter gene expression, while ectopic and/or overexpression of Ci-ZicL resulted in ectopic reporter expression in non-notochord cells. These results provide evidence that ZicL directly activates Brachyury, leading to specification and subsequent differentiation of notochord cells.

Key words: Ciona intestinalis, Notochord, Brachyury, ZicL, Direct activator

Introduction

The formation of the ascidian notochord is an embryological process in which the molecular mechanisms operate from the beginning of fertilization until the terminal differentiation of specific structural gene function (for reviews, see Satoh, 2001; Satoh, 2003; Corbo et al., 2001). In ascidians, including Ciona intestinalis, C. savignyi and Halocynthia roretzi, the notochord comprises exactly 40 cells, and their lineage has been completely described (Conklin, 1905; Nishida, 1987). The 32 anterior notochord cells are derived from the A4.1 pair (A-line) of the bilaterally symmetrical 8-cell embryo and the eight posterior cells are derived from the B4.1 pair (B-line) (Fig. 1J). A key gene essential for the ascidian notochord formation is a T-box gene, Brachyury (Ci-Bra of C. intestinalis, Cs-Bra of C. savignyi and Hr-Bra of H. roretzi). In the A-line, the developmental fate of the A7.3 and A7.7 pairs is restricted at the 64-cell stage to the notochord, where Brachyury is expressed (Fig. 1I) (Yasuo and Satoh, 1993; Corbo et al., 1997). In the B-line, Ci-Bra is expressed in the B7.3 pair at the 64-cell stage, while Hr-Bra is expressed in the B8.6 pair at the 110-cell stage. Namely, the ascidian Brachyury is expressed exclusively in notochord cells, and the timing of the expression coincides with that of the developmental fate restriction except for Ci-Bra expression in B7.3. Ectopic expression of Hr-Bra (Yasuo and Satoh, 1998) or Ci-Bra (Takahashi et al., 1999a) changes the developmental fate of endodermal cells to notochord. Functional suppression of Ci-Bra (Satou et al., 2001a) or Hr-Bra (H. Takahashi, personal communication) results in the failure of notochord formation. Ci-Bra triggers the transcription of various downstream genes in notochord cells (Takahashi et al., 1999a; Hotta et al., 2000).

Early developmental events leading to specification of notochord cells have also been investigated. As in the case of vertebrate embryos, cell–cell interaction is involved; signals emanating from neighboring endodermal cells promote notochord specification (Nakatani and Nishida, 1994), and the cellular interaction leads to activation of Hr-Bra (Nakatani et al., 1996). The endoderm of Ciona embryos is specified by nuclear localization of maternally provided β-catenin (Imai et al., 2000), which triggers transcription of various downstream genes, including lhx3, otx, ttf1, FoxA, FoxD, Fgf9/16/20, and ZicL (Satou et al., 2001b; Imai et al., 2002a; Imai et al., 2002c; Imai et al., 2002b; Imai, 2003). When the nuclear localization of β-catenin is suppressed, presumptive endodermal cells change their fate.
into epidermis, and therefore, due to lack of endoderm, no notochord cells differentiate, and when the nuclear localization of β-catenin is ectopically promoted, most blastomeres, including presumptive notochord cells, change their fate into endoderm, resulting in a lack of notochord differentiation (Imai et al., 2000). One of the β-catenin downstream genes, Fgf9/16/20, is involved in the induction of mesenchyme by endoderm, while its role in notochord induction appears partial because Brachyury is expressed in Fgf9/16/20-suppressed embryos, although its activation is retarded (Imai et al., 2002a). In addition, a forkhead transcription factor gene, FoxD, and a zinc finger transcription factor gene, ZicL, are involved in the process of notochord specification. The expression of Cs-ZicL, a direct target of β-catenin, commences in A5.1, A5.2 and B5.1 at the 16-cell stage (Fig. 1G), and is maintained in A6.1, A6.3 and B6.1 at the 32-cell stage (Fig. 1H) but becomes undetectable by the 64-cell stage (Fig. 1I). FoxD is not always required for endoderm differentiation but is essential for notochord differentiation (Imai et al., 2002b). However, ZicL is expressed in A6.2, A6.4, B6.2 and B6.4 at the 32-cell stage (Fig. 1B, H), and A7.3, A7.4, A7.7, A7.8, B7.3, B7.4, B7.5, B7.7 and B7.8 at the 64-cell stage (Fig. 1C, I) (Imai et al., 2002c). A6.2, A6.4, B6.2 and B7.3 are presumptive notochord cells, while A7.3, A7.7 and B8.6 are primordial notochord cells. The expression of Cs-ZicL in the A-line cells is activated downstream of β-catenin/FoxD (Imai et al., 2002b). The Cs-ZicL expression is essential for the differentiation of A-line notochord cells but not of B-line notochord cells (Imai et al., 2002c).

Corbo et al. (1997) investigated the sequence upstream of Ci-Bra to show that the 434-bp upstream of the TATA box is sufficient as a minimal enhancer for the notochord-specific expression of a reporter gene (cf. Figs 3, 4). This enhancer contains recognition sequences of the Suppressor of Hairless [Su(H)], suggesting the possibility that the Notch signaling pathway plays a role in notochord differentiation (Corbo et al., 1998). Imai et al. (2002b) suggested that, in B-line notochord cells, FoxD activates the Notch signaling pathway, which is likely to eventually lead to the activation of Brachyury. Therefore, in B-line cells, β-catenin→FoxD→Notch→Brachyury is the most probable genetic cascade leading to notochord differentiation.

In the A-line, at least two molecular events remain to be explored to understand the genetic cascade for notochord specification: whether FoxD directly activates ZicL, and whether ZicL directly controls Brachyury expression. In the present study we investigated the latter process and provided evidence for the function of ZicL as a direct activator of Brachyury.

### Materials and methods

#### Ascidian eggs and embryos

*C. intestinalis* were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate.

#### Construction of recombinant plasmids for GST fusion proteins

cDNA fragments encoding the zinc finger motifs (ZF) of ZicL of both
**Expression and purification of the proteins**

GST fusion proteins as well as GST proteins were prepared using the DH5α strain of *Escherichia coli*. Proteins used for binding site selection analyses were purified from crude bacterial extracts using glutathione Sepharose 4B according to the manufacturer's instructions (Amersham Pharmacia). For gel mobility shift assays, proteins were eluted from the beads in binding buffer (25 mM HEPES (pH 7.5), 100 mM KCl, 10 mM ZnSO₄, 1 mM DTT, 0.1% NP-40, 5% glycerol) containing 10 mCi reduced glutathione. Purified proteins were analyzed by 10% SDS-PAGE.

**Binding site selection assay and mutational analyses**

DNA sequences optimal for binding with GST/ZicL(ZF) fusion protein were determined by a PCR-assisted binding site selection assay basically as described by Pollock and Treisman (1990). Initially, random sequence libraries were generated by a primer extension reaction with 61-mers (5'-GGCGCCTCTAAGACTGATTGAGTG(G)16 CGATACCGCACCTGGAGG-3') and 70-mers (5'-GGGCCGCTCTAGCTGTTCG(N)26 CGATACCGCACCTGGAGG-3') as template and R1 primer (5'-CCCTCGAGGCTCACGGATATG-3') in a Klenow reaction mixture. Twenty-five picomoles of double-stranded DNA fragments from the above random sequence libraries were mixed with about 8 ng of the GST fusion protein or GST protein bound on Glutathione Sepharose 4B beads (Amersham Pharmacia) in 100 μl of binding buffer consisting of 25 mM HEPES (pH 7.5), 100 mM KCl, 10 mM ZnSO₄, 1 mM DTT, 0.1% NP-40, 5% glycerol and 50 μg/ml poly[dI-dC]. After incubation on ice for 30 minutes, the beads were washed in a washing buffer (25 mM HEPES (pH 7.5), 100 mM KCl, 10 mM ZnSO₄, 1 mM DTT, 0.1% NP-40, 5% glycerol) and treated with proteinase K, and the DNA fragments were purified. A fraction of the resultant DNA fragments was amplified by PCR with primers (F1: 5'-GGCGCCTCTAAGACTGATTGAGTG-3' or F2: 5'-GGGCCGCTCTAAGACTGATTGAGTG-3') and R1 (5'-CCCTCGAGGCTCACGGATATG-3'). The PCR products were resolved by 10% polyacrylamide gel electrophoresis, and the bands corresponding to 61- or 70-bp DNA fragments were excised from the gel and eluted into TE buffer. Purified DNA fragments were subjected to the next round of selection. Ten rounds of selection were performed, with PCR amplification after each round. After the 8th and 10th cycles, the PCR products were subcloned into pGEM-T vector and analyzed.

**EMSA**

Electrophoretic mobility shift assays (EMSA) were carried out under the following conditions. Each reaction contained ~50 μM α-[32P]-labeled substrate DNA fragment and 200 ng of purified GST fusion protein or GST protein in 20 μl of binding mixture, consisting of 25 mM HEPES (pH 7.5), 100 mM KCl, 10 mM ZnSO₄, 1 mM DTT, 0.1% NP-40, 5% glycerol, and 50 μg/ml poly[dI-dC]. DNA fragments were prepared by annealing complementary oligonucleotides, which are shown in Fig. 2C and Fig. 3B, for example, ZicL-b: 5'-ACTAGTTGATCCGCGGACGGAGTGG-3' or 5'-CCCGACAGCCGGGATCATCT-3'. The resultant double-stranded DNA fragments were labeled with [α-32P]dCTP by Klenow fill-in reaction and purified with a Microspin S-200 column (Amersham Pharmacia). The binding mixtures were incubated at room temperature for 30 minutes, and subjected to electrophoresis on an 8% native polyacrylamide gel. The products were visualized by autoradiography of the dried gel. For competition reactions, unlabeled DNA fragments identical to ZicL-b (X100 molar excess) were pre-incubated with the protein for 10 minutes prior to the addition of labeled probe. In the zinc removal experiment, 25 mM EDTA was added to the binding mixture.

**Mutagenized Ci-Bra/lacZ constructs**

Mutagenized forms of the *Ci-Bra/lacZ* fusion genes were prepared by site-directed mutagenesis from constructs containing the 'full-length' (3.5 kb) or 'minimal' (483 bp) enhancer of *Ci-Bra* (Corbo et al., 1997) used as templates with Quik Change Site-Directed Mutagenesis Kit (Stratagene). The following mutagenic oligonucleotides were used to delete the ZicL-b1 and ZicL-b2 site, respectively. Essentially in each case, a putative ZicL-binding site was replaced by a sequence that reduced the in vitro binding affinity (corresponding to lower case letters). μZicL-b1: 5'-GAGCAACCCCTCAGAGCTGCTGCGCCAG-3' and μZicL-b2: 5'-CCAACAGGTGTCGACGAGTTGGCTC-3' and μZicL-b2: 5'-CCAACAGGTGTCGACGAGTTGGCTC-3'.

**Electroporation and microinjection**

Different fusion gene constructs were simultaneously electroporated into fertilized eggs as described (Corbo et al., 1997). Each electroporation used eggs from several different batches, and each construct was examined by two or more electrophorations. For microinjection, these constructs were used after linearization with HindIII. The 25-mer morpholino antisense oligonucleotide for *Ci-ZicL*, 5'-GATACATTACGACATTAC-3', was ordered (GeneTools, LLC). In vitro synthesized capped mRNA of *Ci-ZicL* was prepared from cDNA cloned into pBluescript RS3 vector using Megascript T3 kit (Ambion). Microinjection was performed as described previously (Imai et al., 2000; Imai et al., 2002c). Solutions of about 0.5 mM fusion genes, 0.5 mM morpholino and 1.2 μM synthesized mRNA were used for microinjection, and each injection contained 30 pl of solution.

**Whole-mount in-situ hybridization and histochemical detection of β-galactosidase activity**

Expression of transgenes was visualized by whole-mount in-situ hybridization (Satou et al., 2001a) or histochemical detection of β-galactosidase activity (Corbo et al., 1997). For histochemical detection, embryos were fixed for 15 minutes in 0.5 M NaCl, 0.1 M MOPS (pH 7.5) containing 4% paraformaldehyde, rinsed in PBT and incubated in PBT containing 1 mM MgCl₂, 3 mM K₃[Fe(CN)₆], 250 μM X-Gal. Whole-mount in-situ hybridization and histochemical detection of β-galactosidase activity

**Results**

**Characterization of *Ci-ZicL* cDNA**

cDNAs for *ZicL* were originally characterized for *C. savignyi* (Zs-ZicL) (Imai et al., 2002c). Because the present study was carried out mainly using *C. intestinalis*, we first characterized cDNA clones for the *C. intestinalis* ortholog, *Ci-ZicL*. *Ci-ZicL* cDNA clones were identified by searching a database of EST projects of *C. intestinalis* (http://ghost.zool.kyoto-u.ac.jp/index1.html) with the *Cs-ZicL* cDNA sequence. One of the clones, cicl002e04, encoding a full-length protein, was used in the following experiments. This *Ci-ZicL* cDNA consisted of 1539 nucleotides (DDBJ/EMBL/GenBank accession no. AK113645), encoding a polypeptide of 388 amino acids with five zinc finger (ZF) motifs. Alignment of the amino acid residues of the ZF motifs (Fig. 1A) indicated that *Ci-ZicL* and *Cs-ZicL* share 93% amino acid identity in this region. As shown in Fig. 1B-D, the spatiotemporal expression pattern of *Ci-ZicL* was identical to that of *Cs-ZicL*. We also
confirmed that the function of Ci-ZicL in notochord formation is identical to that of Cs-ZicL. In agreement with the requirement for Cs-ZicL in notochord formation (Imai et al., 2002c), suppression of Ci-ZicL function with specific morpholino abolished endogenous Ci-Bra activation (Fig. 1E,F) following the loss of notochord differentiation. The expression pattern of Ci-FoxD, which regulates the transcription of Ci-ZicL, was also identical to that of Cs-FoxD (data not shown).

Characterization of binding sequences of Ci-ZicL

Fusion proteins of GST/Ci-ZicL(ZF) and GST/Cs-ZicL(ZF) were obtained using pGEX-4T-1, and purified (see Materials and methods). SDS-PAGE (Fig. 2A) confirmed the purification of the protein with bands of the expected mobility. Recognition sequences or binding elements of Ci-ZicL(ZF) and Cs-ZicL(ZF) were identified by PCR-assisted binding site selection assays and EMSAs. Identical results were obtained for both proteins, and therefore we describe here only that for Ci-ZicL(ZF). dsDNAs consisting of the selected 16-bp or 26-bp sequences were examined after five, eight, or 10 rounds of selection. The results for 16-bp and 26-bp dsDNAs appeared similar, although the consensus among the 26-bp dsDNAs was weaker than that among the 16-bp dsDNA (data not shown). Fig. 2B shows the raw numbers of nucleotides obtained after the selection with 16-bp random dsDNAs. Eight and 10 rounds of selection yielded a similar consensus sequence, and this was the case for both GST/Ci-ZicL(ZF) and GST/Cs-ZicL(ZF), which were aligned. The bottom sequence in each table represents the compiled most favored sequence at each nucleotide position. The underlined ggatc is identical to the 3' end of primer F1. (C,D) A mutation analysis of the binding sequence of GST/Ci-ZicL(ZF). Eleven types of oligonucleotides (C) were examined by a gel-shift assay (D). The boxed uppercase letters of the ZicL-b oligonucleotides indicate the consensus ZicL-binding sequence. The shaded lowercase letters in the µA-µJ oligonucleotides indicate mutated nucleotides in each mutant oligonucleotide. The binding seemed specific because it was not detected under conditions of zinc-removed incubation (lane 1), pre-incubation with x100 molar excess of unlabeled competitor DNA or replacement with GST protein (data not shown).
ZicL is a Brachyury activator to T) in the core region of the recognition sequence resulted in loss of the binding. Altogether, these findings indicated that the most probable binding sequence of GST/ZicL(ZF) is 5'-CCCGCTGTG-3', and its core, GCTG, is critical for the binding.

The 5' minimal enhancer of Ci-Bra contains two Ci-ZicL-binding elements

Corbo et al., (1997) showed that the 434 bp upstream of the TATA box (483 bp upstream of the putative transcription start site) of Ci-Bra is sufficient as a minimal enhancer for the notochord-specific expression of reporter genes (GFP and lacZ) (Fig. 3A). The minimal Ci-Bra enhancer consists of three regions. The most proximal region (∼188) includes three E-boxes and is involved in ectopic reporter expression in mesenchyme and muscle cells. This region is also required for expression in notochord cells. The slightly more distal region between −299 and −188 contains two Su(H) binding motifs and is essential for activation of Ci-Bra in notochord cells (see also Corbo et al., 1998). The distal region between −434 and −299 includes a Snail binding motif and is associated with repression of the ectopic expression in mesenchyme and muscle cells (see also Fujiwara et al., 1998; Erives et al., 1998). In a report by Corbo et al. (1997), the TA TA box (shown by a dotted line in Fig. 3A) was regarded as +1. In the present study, the 5'-end residue of the Ci-Bra cDNA sequence that is registered in the database related to the genome sequence was regarded as +1 of the transcription start site. The TA TA box is therefore located at −44 to −49. In the enhancer there are four putative Snail binding sites (sna1 to sna4), two putative Su(H) binding sites [Su(H)1 and Su(H)2] and three E-box sequences (E1 to E3).
described above. These possible ZicL-binding sequences were located at −123, −168, −731, −1053, −1279 and −1996, and also at −241, which was around the Su(H)2 site, with six out of nine nucleotide identity (Fig. 3B). The −123 sequence, CACAGCTGG (complementary sequence: CCAGCTGTC), and the −168 sequence, CCAGCTGTC, were identical and shared eight out of nine nucleotide identity with the determined binding sequence (underlined); notably, six successive nucleotides, including the core sequence GCGT, were identical. The −123 and −168 elements overlap with E3 and E2, respectively.

We examined whether these sequences were recognized by GST/Ci-ZicL(ZF). As shown in Fig. 3C, the −123 (lane 3) and −168 (lane 4) sequences bound to the fusion protein with a similar affinity as ZicL-b (lane 2), while −731 (lane 6) bound less tightly. Therefore, it is highly likely that Ci-ZicL recognizes the −123 and −168 upstream sequences of Ci-Bra (hereafter designated ZicL-b1 and ZicL-b2, respectively), suggesting the possibility that Ci-ZicL acts as a direct activator of Ci-Bra.

Furthermore, sequences resembling the Ci-ZicL binding element were also present in the upstream region of the presumptive C. savignyi Brachyury gene (Fig. 3D). The upstream sequence of Cs-Bra has been characterized (Y.S. and K. S. Imai, unpublished). Possible ZicL-binding sites were found at −764 and −792 upstream of Cs-Bra. The more proximal one (corresponding to ZicL-b1 of C. intestinalis) had nine out of nine base identity with the binding consensus sequence, while the other had six out of nine base identity. Therefore, ZicL-binding sites, at least ZicL-b1, are likely to function for Cs-Bra expression, too.

**The ZicL-binding elements are required for the Ci-Bra enhancer activity**

To determine the significance of ZicL-binding sites in the Ci-Bra enhancer in vivo, a series of Ci-Bra/lacZ fusion genes in which the Ci-Bra upstream sequence was fused in frame with lacZ with or without mutations in the potential ZicL-binding sites were introduced into fertilized eggs. The 3.5-kb genomic DNA fragment upstream from the Ci-Bra transcription start site contains the cis-regulatory information required for authentic Ci-Bra expression, since it mediates a precise, notochord-specific expression pattern of reporter genes in transgenic embryos (Corbo et al., 1997), while the 483-bp region appeared to possess ‘minimal’ enhancer activity.

p(−483)Ci-Bra/lacZ, possessing the 483-bp ‘minimal’ or p(−3.5k)Ci-Bra/lacZ, possessing the 3.5-kbp ‘full-length’ Ci-Bra enhancer, were mutated at either one or both of the potential ZicL-binding sites; the ZicL-b1 site, CACAGCTGG (CCAGCTGTC) to CAGAgCGTC, and the ZicL-b2 site, CCAGCTGTC to CGAAGgCTG. Using these mutated constructs, we examined the effects of the mutations in the ZicL-binding sites on the expression of the reporter gene.

When p(−483)Ci-Bra/lacZ or p(−3.5k)Ci-Bra/lacZ was introduced via microinjection and the lacZ expression was assessed by in-situ hybridization, the reporter reproduced the spatial expression pattern of endogenous Ci-Bra at the 64-cell stage (Fig. 4A,D) and at the 110-cell stage (Fig. 4B,E). The reporter expression directed by the 483-bp upstream sequence was weaker. However, when both ZicL-binding sites were mutated, reporter expression was completely abolished (Fig. 4C,F). Introduction of the fusion genes by electroporation gave identical results. Each of these examinations was confirmed with 50 or more embryos. These results indicated that the potential ZicL-binding sites in the Ci-Bra enhancer are essential for the initiation of Ci-Bra transcription.

We also examined the requirement for these ZicL-binding sites at the tailbud stage, because the Ci-Bra enhancer has been assayed at this stage (Corbo et al., 1997; Corbo et al., 1998; Fujiiwara et al., 1998) and the expression of the reporter gene after the prolonged embryogenesis is detected with better sensitivity even by β-galactosidase staining. Of 528 embryos developed from eggs electroporated with p(−483)Ci-Bra/lacZ, 43% showed strong reporter expression in almost all notochord cells and 26% in a part of the notochord cells (Fig. 4G,H). In contrast, as shown in Fig. 4G,I, the expression level of p(−483)Ci-Bra/lacZ was markedly reduced when either ZicL-b1 (123-bp upstream of the transcription start site) or ZicL-b2 (168-bp upstream) was mutated. In particular, mutation of ZicL-b2 suppressed the expression of the reporter gene, and only 4% of the manipulated embryos showed partial expression of lacZ (Fig. 4G,I). In addition, the ZicL-b1/ZicL-b2 double mutation abolished the reporter expression completely (Fig. 4G,K).

When p(−3.5k)Ci-Bra/lacZ was electroporated into fertilized eggs, more than 80% of the resultant embryos exhibited strong lacZ expression in almost all notochord cells at the tailbud stage, as described previously (Fig. 4G,L) (Corbo et al., 1997). Mutation in ZicL-b2 of p(−3.5k)Ci-Bra/lacZ did not cause a significant decrease of the reporter expression (Fig. 4G,N). However, mutation in ZicL-b1 affected the expression level; about 53% of embryos still exhibited the reporter expression in most notochord cells (Fig. 4G), but the intensity of the expression was clearly weaker than that in controls (compare Fig. 4M with Fig. 4L). The proportion of embryos with weak and partial reporter expression or without expression increased. Moreover, when both ZicL-b1 and ZicL-b2 were mutated in p(−3.5k)Ci-Bra/lacZ, the reporter expression was reduced further (Fig. 4G,O).

**Overexpression of Ci-ZicL promotes ectopic expression of Ci-Bra/lacZ reporter**

We lastly examined whether or not Ci-ZicL is responsible for Ci-Bra expression in a manner dependent on these elements in vivo. If Ci-ZicL binds to these elements to activate the transcription of Ci-Bra, overexpression of Ci-ZicL by microinjection of its synthetic mRNA into fertilized eggs should direct ectopic activation of the reporter gene driven by the Ci-Bra enhancer in blastomeres of non-notochord lineages, and conversely, suppression of Ci-ZicL should inactivate the reporter expression.

As shown in Fig. 5A, injection of Ci-ZicL mRNA caused ectopic transactivation of the co-injected reporter gene p(−3.5k)Ci-Bra/lacZ at the 110-cell stage (compared with Fig. 4E). In contrast, p(−3.5k)Ci-Bra/lacZ with double mutation of ZicL-b1 and ZicL-b2 was not activated by co-injection of Ci-ZicL mRNA (Fig. 5B). Moreover, the reporter expression was lost by suppression of endogeneous Ci-ZicL with specific morpholino (Fig. 4C).
ZicL is a Brachyury activator

we took advantage of cleavage-arrested embryos to minimize other influences of Ci-ZicL overexpression, because Ciona ZicL plays crucial roles in the differentiation of not only notochord but also muscle and nerve cord, and overexpression of Ci-ZicL causes severe embryological defects, which were already manifested by production of ball-shaped embryos at the 110-cell stage (cf. Fig. 5A,B) (Imai et al., 2002c). We injected p(−3.5k)Ci-Bra/lacZ with or without Ci-ZicL mRNA into fertilized eggs, and arrested cleavage at the 110-cell stage with cytochalasin B (2 μg/ml), and then performed histochemical detection of lacZ expression at the time when control embryos reached the early tailbud stage. A 110-cell stage embryo contains 10 notochord precursor cells, which divide twice to form 40 notochord cells in the tailbud embryo. Embryos injected with p(−3.5k)Ci-Bra/lacZ alone showed the reporter expression, in some cases in cells corresponding to notochord lineage (Fig. 5D) and in some other cases in notochord, muscle and mesenchyme cells, where Ci-ZicL is expressed, as noted above (Fig. 5E). However, co-injection of Ci-ZicL mRNA caused ectopic transactivation of the reporter gene in most blastomeres, including the notochord lineage (Fig. 5F). In contrast, double mutation of ZicL-b1 and ZicL-b2 resulted in loss of lacZ expression despite Ci-ZicL overexpression (Fig. 5G).
The question we explored in the present study is whether Ci-ZicL is a primary enhancer of Ci-Bra. The present study substantiates the findings of the study of Corbo et al. (Corbo et al., 1997) reported that the most proximal region (~188) of the minimal Ci-Bra enhancer is mainly involved in ectopic expression of a reporter gene in notochord lineage cells. Overexpression of the p(–188)Ci-Bra/lacZ construct in notochord cells [fig. 5A of Corbo et al. (Corbo et al., 1997)]. The present study substantiates the findings of the study of Corbo et al. (Corbo et al., 1997) by pointing out the significance of the proximal-most region of Ci-Bra is associated with enhancement of the gene expression in notochord cells, although their data demonstrated the reporter expression of the p(–188)Ci-Bra/lacZ construct in notochord cells [fig. 5A of Corbo et al. (Corbo et al., 1997)]. The present study substantiates the findings of the study of Corbo et al. (Corbo et al., 1997) by pointing out the significance of the proximal-most region of Ci-Bra is associated with enhancement of the gene expression in notochord cells, although their data demonstrated the reporter expression of the p(–188)Ci-Bra/lacZ construct in notochord cells [fig. 5A of Corbo et al. (Corbo et al., 1997)].

Corbo et al. (Corbo et al., 1997) reported that the most proximal region (~188) of the minimal Ci-Bra enhancer is mainly involved in ectopic expression of a reporter gene in notochord lineage cells. Overexpression of the p(–188)Ci-Bra/lacZ construct in notochord cells [fig. 5A of Corbo et al. (Corbo et al., 1997)].

Discussion

Ci-ZicL is a primary enhancer of Ci-Bra

The question we explored in the present study is whether Ci-ZicL is involved in the direct activation of Ci-Bra transcription in C. intestinalis embryos. The PCR-assisted binding site selection assays and EMSAs indicated that CCCGCTGTG is the most probable binding sequence of Ci-ZicL. Two CCAGCTGTG elements were found in the proximal region of the upstream sequence of Ci-Bra (at -123 and at -168), a region shown in a previous study to be required for the basal activation of the gene (Corbo et al., 1997; Corbo et al., 1998; Fujiwara et al., 1998). The Ci-ZicL zinc finger domain bound to these elements in vitro. Mutations in these elements of Ci-ZicL enhancer/lacZ resulted in loss of the reporter gene expression. Overexpression of Ci-ZicL, promoted ectopic reporter gene expression of p(–3.5k)Ci-Bra/lacZ, and suppression of Ci-ZicL did not direct the reporter expression. In addition to blastomeres of notochord lineage, the expression was weakly detected in blastomeres giving rise to muscle and mesenchyme (Fig. 4A,B,D,E), where ZicL is expressed. This also applies to some cleavage-arrested tailbud stage embryos (Fig. 5E). However, this expression completely disappeared when ZicL-binding sites were mutated (Fig. 4C,F, and Fig. 5G), as in the case of notochord lineage blastomeres. All of these results support the notion that Ci-ZicL is a direct activator of Ci-Bra.

Corbo et al. (Corbo et al., 1997) reported that the most proximal region (~188) of the minimal Ci-Bra enhancer is mainly involved in ectopic expression of a reporter gene in notochord lineage cells. While the slightly more distal region between ~299 and ~188 (including two Su(H) binding motifs) is essential for activation of Ci-Bra in notochord cells (Corbo et al., 1998), and the distal region between ~434 and ~299 (including a Snail binding motif) is associated with repression of the ectopic expression (Fujiwara et al., 1998; Erives et al., 1998) (Fig. 3A). Their analyses, however, could not definitively determine whether the proximal-most region of Ci-Bra is associated with enhancement of the gene expression in notochord cells, although their data demonstrated the reporter expression of the p(–188)Ci-Bra/lacZ construct in notochord cells [fig. 5A of Corbo et al. (Corbo et al., 1997)].
One question here is whether FoxD directly activates the transcription of ZicL or not. One possibility is that, as is evident in Fig. 1G,H, although A6.2, A6.4 and B6.2 do not show FoxD expression, they are daughter cells of A5.1, A5.2 and B5.1 with FoxD expression. Therefore, FoxD is expressed in A5.1, A5.2 and B5.1 at the 16-cell stage, and the gene product is inherited by the A6.2, A6.4 and B6.2 daughter cells at the 32-cell stage, where it activates ZicL transcription. Another possibility is that FoxD in A6.1, A6.3 and B6.1 activates genes for inducer molecule(s) for signal transduction to promote ZicL expression in A6.2, A6.4 and B6.2 at the 32-cell stage. Once ZicL is transcribed and translated, the gene product would activate Ci-Bra via ZicL binding elements in the proximal-most region of Ci-Bra. This may be a primary genetic cascade for differentiation of A-line notochord cells (Fig. 6A).

The slightly more distal region between –299 and –188 (including two Su(H) binding motifs) and the distal region between –434 and –299 (including a Snail binding motif) are primarily associated with Ci-Bra expression in B-line notochord cells (Fig. 6B). That is, because Ci-ZicL is also expressed in B-line mesenchyme and muscle cells (B7.3, B7.4, B7.7 and B7.8), Ci-Bra could be expressed in these cells. However, the snail gene is expressed in the same cells with Ci-ZicL expression and Snail represses the Ci-Bra expression. Therefore, although the ZicL binding element shown here is not present in this region, the Ci-Bra expression domain in the primitive streak are present within 500 bp upstream of the transcription start site of this gene (Clements et al., 1996; Yamaguchi et al., 1999a). A part of the Brachyury expression domain in the primitive streak depends on Wnt3a (Yamaguchi et al., 1999b) and the downstream effectors Leftf and Tcf1 (Galceran et al., 2001). TCF-binding sites have been found in the mouse Brachyury promoter, and their mutation led to loss of Brachyury expression and Wnt responsiveness (Arnold et al., 2000; Yamaguchi et al., 1999b). However, cis-regulatory elements responsible for the notochord-specific expression of the mouse Brachyury have not been well characterized.

In Xenopus embryos, Xbra and FGF constitute an autoregulatory loop (Latinkic et al., 1997; Casey et al., 1998). The transcription of Xbra2, via the region 381 bp upstream of the transcription start site, is activated by FGF and low concentrations of activin but is suppressed by high concentrations of activin (Latinkic et al., 1997). This suppression is mediated by paired-type homeobox genes, goosecoid and Mix.1 (Latinkic et al., 1997; Latinkic and Smith, 1999). The Xbra expression also requires zygotic Wnt activity (Vonica and Gumbiner, 2002).

At present, however, no conserved trans-regulatory system has been shown to govern the Brachyury transcription in notochord cells in ascidians and vertebrates.

The complexity of ZicL function

As mentioned above, Ci-ZicL appears to function as a direct
activator of Ci-Bra. However, the expression and functions of Ciona ZicL are multiple and complex, and ZicL expression does not always lead to Brachyury activation (Imai et al., 2002c) [see also Wada and Saiga (Wada and Saiga, 2002), for multiple functions of HrzicN]. First, Cs-ZicL is expressed not only in the A-line notochord lineage but also in the A-line nerve-cord lineage and B-line muscle lineage. Although the details of ZicL function in the specification and subsequent differentiation of nerve cord cells are obscure, ZicL function in B-line muscle cells appears to be coordinated with the function of another zinc finger transcription factor gene, Ci-macho1 (Satou et al., 2002). Halocynthia macho-I appears to have the potential to promote the entire genetic cascade for muscle cell differentiation (Nishida and Sawada, 2001). On the other hand, the potential of Ci-macho1 to promote the genetic cascade for muscle cell differentiation appears partial, and requires the collaborative functioning of ZicL (Imai et al., 2002c). In future studies, it should be determined whether the ZicL binding element is present in the enhancer region of muscle-specific structural genes and serves to activate their transcription.

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References