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 CCCGCTGTG. 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

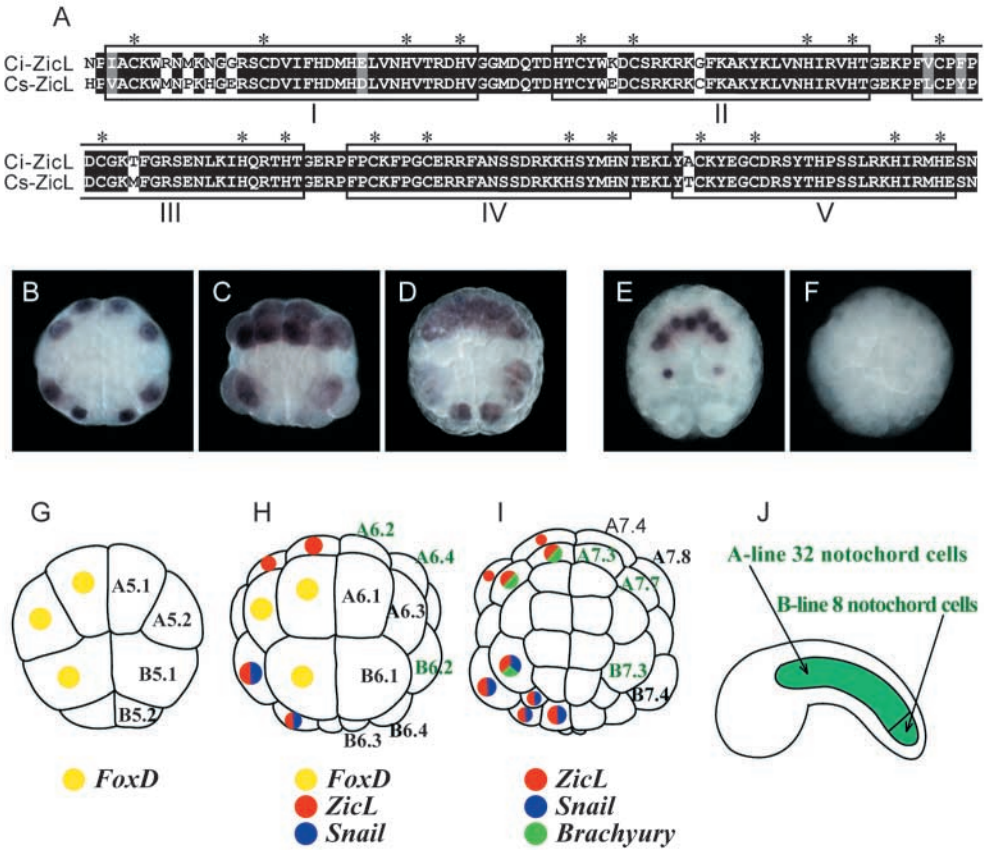

Fig. 1. (A) Alignment of the amino acid residues of the central region of Ci-ZicL with those of Cs-ZicL. The five zinc finger motifs are boxed, and conserved CCHH are indicated by asterisks. This region was used to produce a GST/ZicL(ZF) fusion protein. (B-D) Zygotic expression of *Ci-ZicL*, as revealed by whole-mount in-situ hybridization. (B) A 32-cell, (C) a 64-cell and (D) a 110-cell stage embryo, vegetal view. (E,F) The effect of suppression of *Ci-ZicL* function on expression of *Ci-Bra*, in (E) a control and (F) a *Ci-ZicL*-suppressed embryo at the 110-cell stage. (G-J) Schematic representations of the expression of *FoxD*, *ZicL*, *Snail* and *Brachyury* genes. Embryos at (G) the 16-cell, (H) 32-cell, (I) 64-cell and (J) tailbud stage, viewed from (G-I) the vegetal pole and (J) the lateral side. Blastomeres are named according to the nomenclature of Conklin (1905). Blastomeres whose names are colored green in (H) and (I) are those that give rise to notochord cells.

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-FoxD*, 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

C. intestinalis and *C. savignyi* were prepared by PCR using the following primers and cDNAs as templates: Ci-ZicL(ZF), 5′-CGCGGATCCACCAATCCGATTGCTTGC-3′ and 5′-CCGCTCGAGGGGAACTGTTGTCGATTACG-3′, cicl002e04; Cs-ZicL(ZF), 5′-CGCGGATCCTCGCATCCAGTCGCTTG-3′ and 5′-CCGCTCGAGTTGTGACGAATTGATGACG-3′, BAB68356.

The resultant cDNA fragments were digested with *Bam*HI and *Xho*I, and inserted into pGEX-4T-1 expression vector (Amersham Pharmacia).

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 μM $ZnSO_4$, 1 mM DTT, 0.1% NP-40, 5% glycerol) containing 10 mM 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′-GGCCGCTCTAGAACTAGTGGATC-$(N)_{16}$CGATACCGTCGACCTCGAGGG-3′) and 70-mers (5′-GGCCGCTCTAGACTGCTGTTCG$(N)_{26}$CGATACCGTCGACCTCGAGGG-3′) as template and R1 primer (5′-CCCTCGAGGTCGACGGTATCG-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 μM $ZnSO_4$, 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 μM $ZnSO_4$, 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′-GGCCGCTCTAGAACTAGTGGATC-3′ or F2: 5′-GGCCGCTCTAGACTGCTGTTCG-3′ and R1: 5′-CCCTCGAGGTCGACGGTATCG-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 (EMSAs) were carried out under the following conditions. Each reaction contained ~50 mM α-^{32}P-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 μM $ZnSO_4$, 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′-ACTAGTGGATCCCGCTGTG-3′ + 5′-CCGCACAGCGGGATCCACT-3′. The resultant double-stranded DNA fragments were labeled with [α-^{32}P]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 kbp) 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′-GAGCAACCCTCAgAcgTcGATGCCACCACCTACGGC-3′ + 5′-GCCGTAGGTGGTGGCATCgAcgTcTGAGGGTTGCTC-3′ and μZicL-b2: 5′-CACAAGGTGTTCGATCgAcgTcTGAAAGTAAACATAGAGC-3′ + 5′-GCTCTATGTTTACTTTCAgAcgTcGATCGAACACCTTGTG-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 electroporations. For microinjection, these constructs were used after linearization with *Hin*dIII. The 25-mer morpholino antisense oligonucleotide for *Ci-ZicL*, 5′-GATCAACCATTACATTAGAATACAT-3′, was order-made (GeneTools, LLC). In vitro synthesized capped mRNA of *Ci-ZicL* was prepared from cDNA cloned into pBluescript RN3 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 nM 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 $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$ and 250 μM X-gal.

Results

Characterization of *Ci-ZicL* cDNA

cDNAs for *ZicL* were originally characterized for *C. savignyi* (*Cs-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/indexr1.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

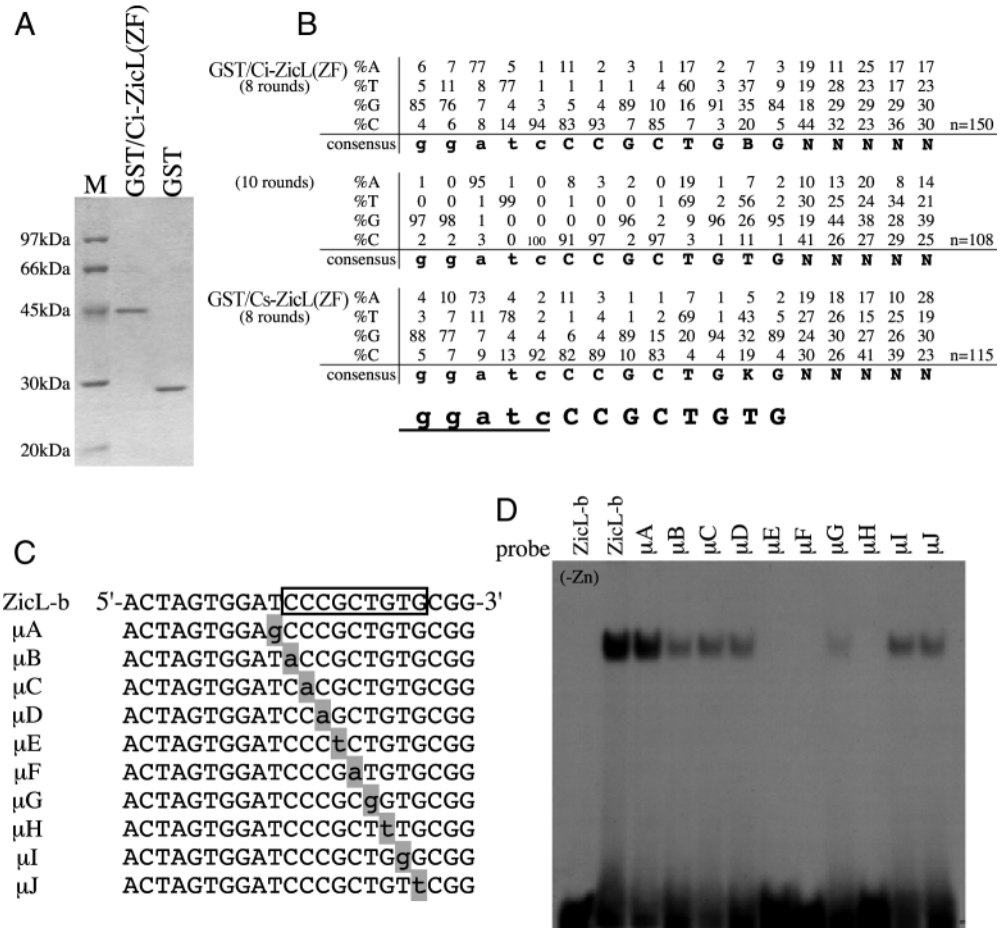

Fig. 2. Characterization of nucleotide sequences recognized by GST/ZicL(ZF) fusion protein. (A) SDS-PAGE of purified GST/Ci-ZicL(ZF) fusion protein (lane 2) and GST protein (lane 3). The molecular mass relative to the Marker (lane 1) shows the successful production of the fusion protein. (B) Compilation of the GST/Ci-ZicL(ZF) and GST/Cs-ZicL(ZF) binding sequences. DNA sequences that bound to the fusion proteins were selected and determined as described in Materials and methods. Cloned random sequences, after eight or 10 rounds of selection for GST/Ci-ZicL(ZF) and eight rounds for GST/Cs-ZicL(ZF), 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).

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 endogeneous *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). This analysis demonstrated that most of the sequences selected had CCGCTGTG at the 3′ terminus of the primer F1 (ggatc).

A double-stranded oligonucleotide, ‘ZicL-b’ (Fig. 2C, upper: 5′-ACTAGTGGATCCCGCTGTGCGG-3′) was synthesized. The specific binding of the fusion protein to the suggested sequence was confirmed by EMSA with ZicL-b (Fig. 2D, lane 2). We performed a series of mutation analyses to determine whether a part of the primer F1 sequence was included in the binding sequence, and confirmed that CCCGCTGTG was required for effective binding (data not shown). In addition, to examine the significance of each of the nucleotides for binding, we further performed EMSAs with various oligonucleotides that had a nucleotide substitution (A↔C or T↔G transversion) in these sequences. The results are shown in Fig. 2C,D. The T to G mutation (Fig. 2C, μA) in the region neighboring the above-mentioned recognition sequence did not significantly alter the binding affinity to GST/Ci-ZicL(ZF) (Fig. 2D). Mutations μB, μC, μD, μI and μJ, in which a nucleotide near one of the ends of the recognition sequence is substituted, reduced the binding affinity. Moreover, mutations μE (G to T), μF (C to A), μG (T to G) and μH (G

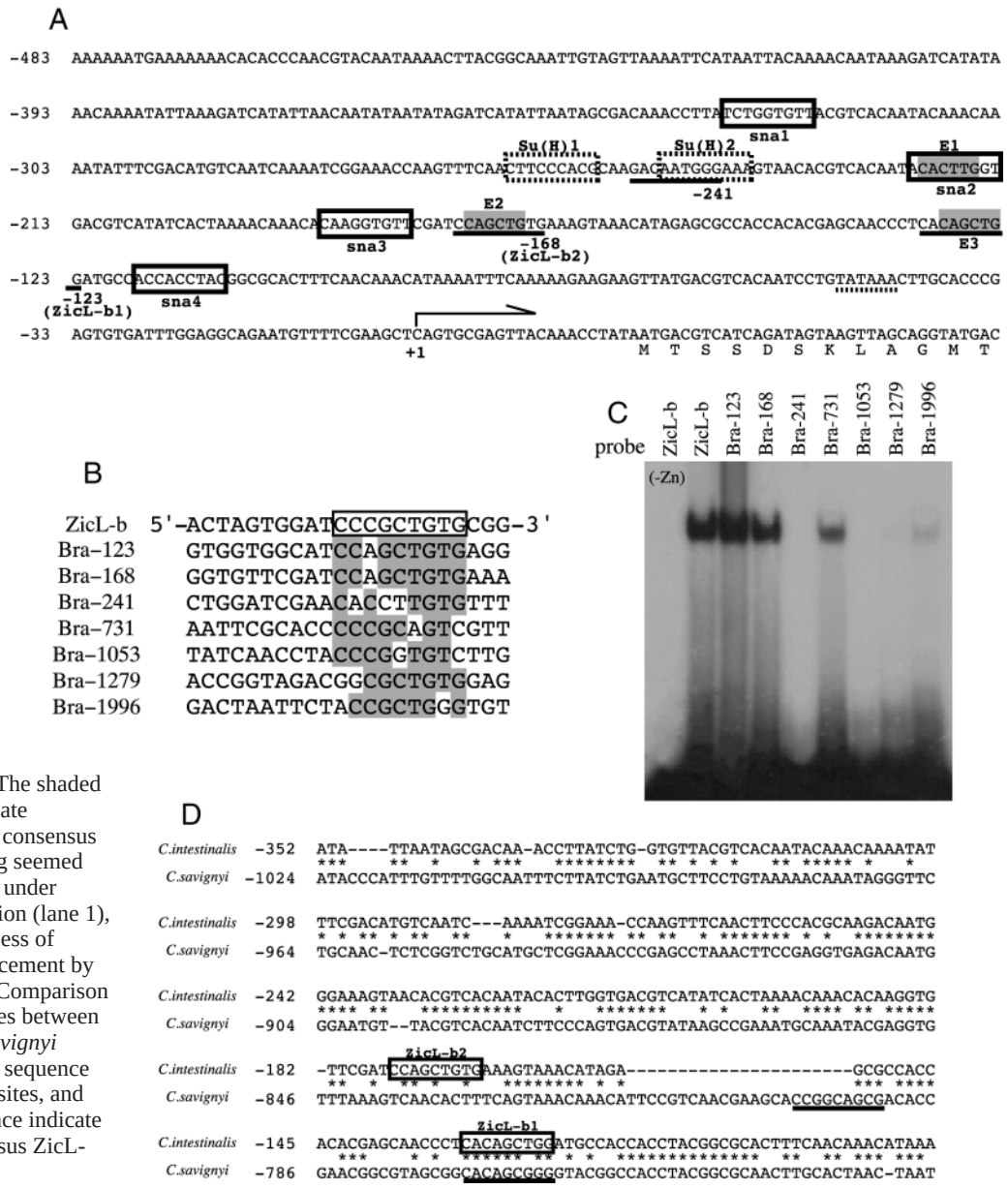

Fig. 3. ZicL-binding sequences in the *cis*-regulatory region of the *Ci-Bra* gene. (A) DNA sequence of the minimal 483-bp *Ci-Bra* enhancer (see Corbo et al., 1997; Fujiwara et al., 1998). Solid unfilled boxes, dotted unfilled boxes, filled boxes and the dotted line indicate Ci-Snail-binding sites, Su(H)-binding sites, E box sequences and a TATA element, respectively. The arrow represents the presumptive transcription start site. Solid lines represent elements with sequence similarity to the consensus ZicL-binding sequence. (B,C) A gel shift assay of the binding of GST/Ci-ZicL(ZF) to oligonucleotides corresponding to various locations in the *Ci-Bra* transcription regulatory region. Oligonucleotide sequences shown in (B) were examined in a gel-shift assay (C). The boxed letters of ZicL-b indicate the consensus ZicL-binding sequence. The shaded letters in Bra-123 to Bra-1996 indicate nucleotides identical to those in the consensus ZicL-binding sequence. 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 by GST protein (data not shown). (D) Comparison of the *Brachyury* upstream sequences between *Ciona intestinalis* (upper) and *C. savignyi* (lower). Boxes in the *C. intestinalis* sequence indicate presumptive ZicL-binding sites, and solid lines in the *C. savignyi* sequence indicate sequences that resemble the consensus ZicL-binding sequence.

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 TATA 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 TATA 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).

We searched the Ci-ZicL-binding element in the 2-kbp region upstream of *Ci-Bra* and found six elements where more than seven out of the nine nucleotides were identical to the GST/Ci-ZicL(ZF) binding consensus sequence determined as

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: CCAGCTGTG), and the –168 sequence, CCAGCTGTG, were identical and shared eight out of nine nucleotide identity with the determined binding sequence (underlined); notably, six successive nucleotides, including the core sequence GCTG, 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 [CCAGCTGTG] to CAgAcgTcG, and the ZicL-b2 site, CCAGCTGTG to CgAcgTcTG. 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; Fujiwara 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,J, 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-b1 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).

These results were further confirmed by examination at the tailbud stage (Fig. 5D-G; corresponding to Fig. 4H-O), with the same reason described above. In the present experiments,

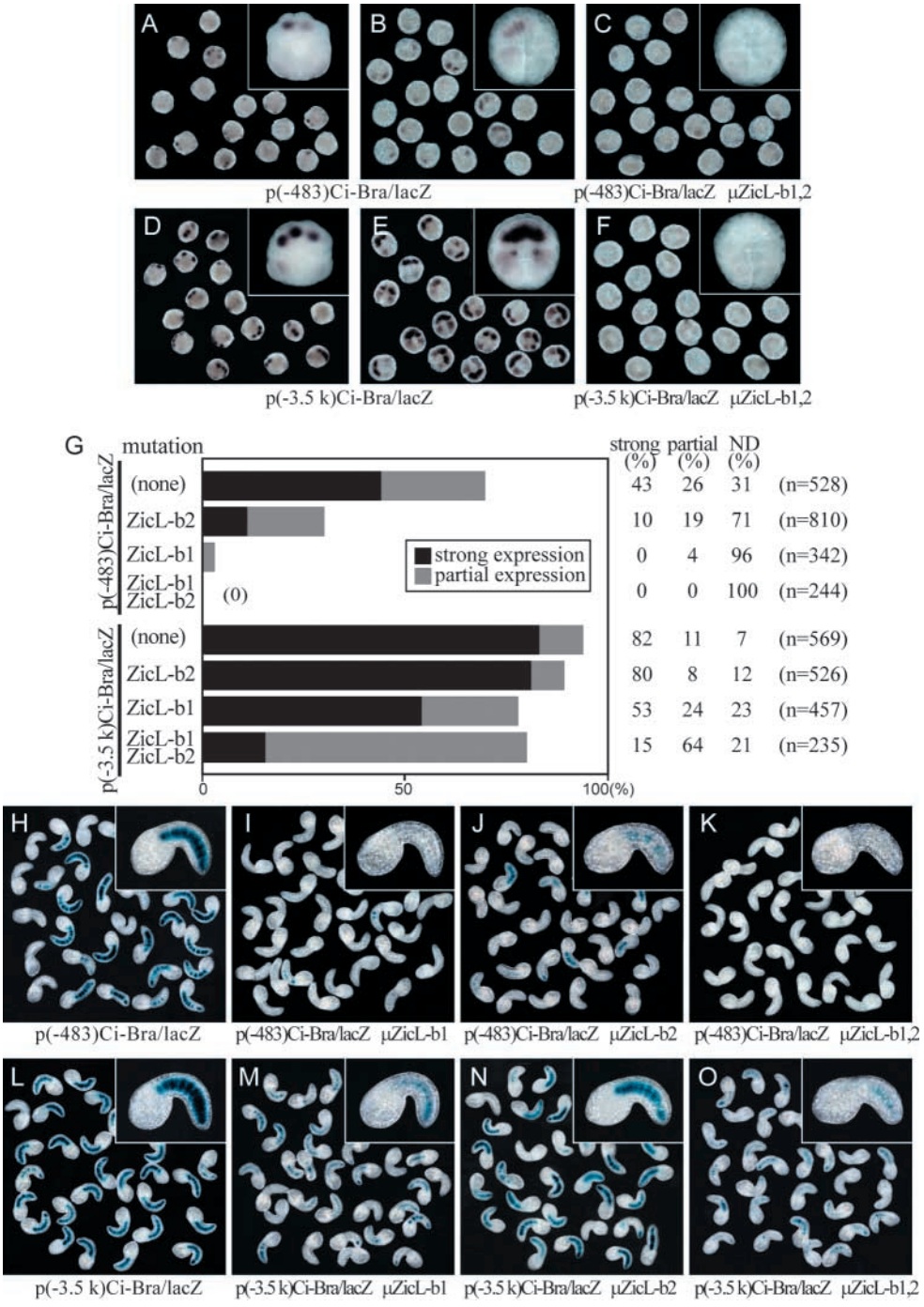

Fig. 4. Requirement of potential ZicL-binding sites for *Ci-Bra* expression in notochord cells. Reporter gene expression in embryos microinjected with (A-C) p(–483)Ci-Bra/lacZ and (D-F) p(–3.5k)Ci-Bra/lacZ, revealed by whole-mount in-situ hybridization. (A,B,D,E) Constructs without mutation or (C,F) ZicL-b1/ZicL-b2 double-mutations were introduced and examined at the (A,D) 64-cell stage or (B,C,E,F) 110-cell stage. (G) The frequency of the tailbud stage embryos with the reporter gene expression, revealed by histochemical detection of β-galactosidase activity. The electroporated *Ci-Bra/lacZ* fusion genes with the mutated ZicL-binding sites are listed on the left. Reporter gene expression in embryos electroporated with (H-K) p(–483)Ci-Bra/lacZ and (L-O) p(–3.5k)Ci-Bra/lacZ. (H,L) Constructs without mutation, (I,M) ZicL-b1 mutated, (J,N) ZicL-b2 mutated or (K,O) ZicL-b1/ZicL-b2 double-mutated. High-magnification images of the embryos are shown in insets.

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). These results were confirmed with

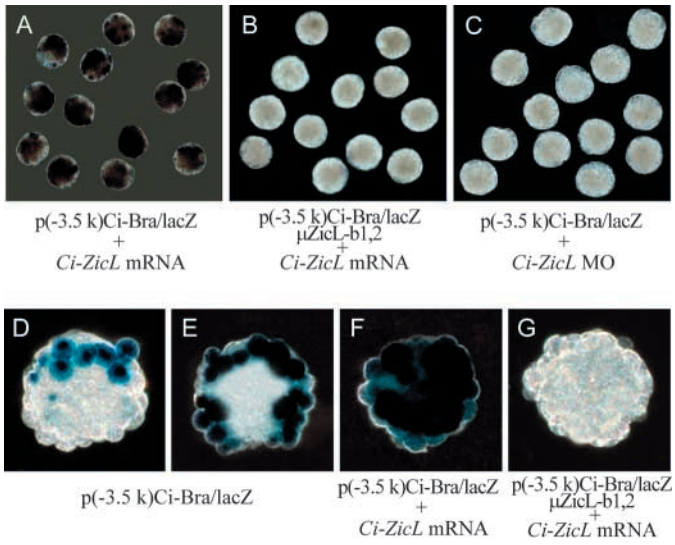

Fig. 5. Misexpression of *Ci-ZicL* promotes ectopic lacZ expression in blastomeres of non-notochord lineages, while suppression of *Ci-ZicL* does not activate lacZ expression. (A) p(–3.5k)Ci-Bra/lacZ and *Ci-ZicL* mRNA, (B) p(–3.5k)Ci-Bra/lacZ with ZicL-b1/ZicL-b2 double-mutation and *Ci-ZicL* mRNA, or (C) p(–3.5k)Ci-Bra/lacZ and *Ci-ZicL* morpholino antisense oligonucleotides were injected into fertilized eggs, and the reporter expression was examined at the 110-cell stage by whole-mount in-situ hybridization. (D,E) p(–3.5k)Ci-Bra/lacZ, (F) p(–3.5k)Ci-Bra/lacZ and *Ci-ZicL* mRNA, or (G) p(–3.5k)Ci-Bra/lacZ with ZicL-b1/ZicL-b2 double-mutation and *Ci-ZicL* mRNA were injected into fertilized eggs, embryos were allowed to develop to the 110-cell stage and cleavage was arrested for about 6 hours (equivalent to the early tailbud stage), and then the reporter gene expression was examined by histochemical detection of β-galactosidase activity.

40 or more embryos. Therefore, it is highly likely that Ci-ZicL directly activates *Ci-Bra* transcription.

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-Bra* 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*.

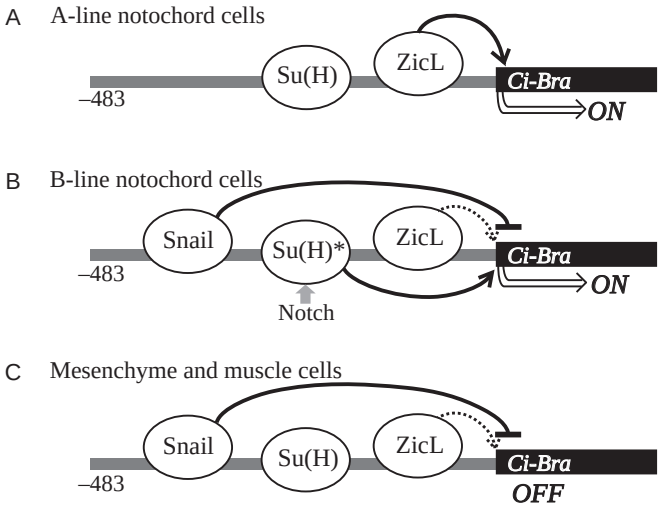

Fig. 6. Schematic representation of the transcriptional control of *Ci-Bra*. See the text for details.

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 mesenchyme and muscle 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)]. 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* for its transcription in notochord cells (Fig. 4G-K). Based on the data obtained from these two studies (Corbo et al., 1997) (this study), together with those from other studies (Corbo et al., 1998; Erives et al., 1998; Fujiwara et al., 1998; Imai et al., 2002c), we propose a basic scenario for the transcriptional control of *Ci-Bra* as follows (Figs 1, 6).

Ciona eggs are maternally provided with a large quantity of β-catenin mRNA and protein (Imai et al., 2000). Nuclear localization of β-catenin in vegetal blastomeres after fertilization triggers the genetic cascade for endodermal cell differentiation by activating *FoxA*, *otx*, *ttf1*, and other genes (Satou et al., 2001b; Imai, 2003). *FoxD* is a direct target gene of β-catenin and, as shown in Fig. 1, is expressed transiently in A5.1 and A5.2 cells at the 16-cell stage and A6.1 and A6.3 cells at the 32-cell stage (Imai et al., 2002b), although the protein distribution has not yet been determined. Overexpression of *FoxD* promotes ectopic *ZicL* expression, and suppression of *FoxD* function results in the loss of *ZicL* expression (Imai et al., 2002c). Therefore, *ZicL* is activated downstream of *FoxD*.

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 there (Fig. 6C and Fig. 1H,I) (Fujiwara et al., 1998; Erives et al., 1998). Fujiwara et al. pointed out that binding sites sna1 and sna2, but not sna3 and sna4, are critical for suppressing the *Ci-Bra* expression (Fujiwara et al., 1998).

In B-line notochord cells of *Ciona* embryos, the Notch signaling pathway, which is downstream of *FoxD*, is likely to be involved in the activation of *Brachyury* (Imai et al., 2002b). That is, only B6.2 or B7.3 receives an inductive signal (probably Notch signaling pathway) from B6.1 or B7.1 (endodermal cell), which overcomes the Snail repression to govern *Ci-Bra* expression in B7.3 (Fig. 6B and Fig. 1I). However, because the ZicL-b1/ZicL-b2 double mutation abolishes the reporter gene expression in B-line notochord cells, ZicL may also be required for *Ci-Bra* expression in B-line notochord cells.

Other factors controlling *Ci-Bra* transcription

As shown in the present study, double mutation of both ZicL binding elements in the –483 bp minimal enhancer and –3.5 kbp full-length enhancer of *Ci-Bra* completely abolished the reporter gene expression (Fig. 4C,F,K). However, the same mutation in the 3.5-kbp enhancer region of *Ci-Bra* did not completely abolish the expression, but rather affected the level of the reporter gene expression when examined at the tailbud stage (Fig. 4G,O). This suggests that elements other than the two proximal ZicL-recognition sites are also involved in regulating the transcriptional activity of *Ci-Bra*. A possible ZicL-binding sequence found at –731, although with less intense binding affinity to the GST/Ci-ZicL(ZF) (Fig. 3B), and/or other *cis*-element(s) might be involved in regulating the *Ci-Bra* transcription, particularly in the late process.

Factors other than ZicL may also be involved in regulating the transcriptional activity of *Ci-Bra*. Previous studies have suggested that the FGF signal transduction pathway is involved in notochord specification in *Ciona* embryos (Imai et al., 2002a), where *Fgf9/16/20* is expressed in blastomeres involved in notochord differentiation, and *Brachyury* expression is partially downregulated by *Fgf9/16/20* suppression. In addition, in early embryos of *Ciona* as well as other ascidians, *FoxA* (*forkhead*; *HNF-3β*) is likely to play roles in endoderm differentiation and notochord differentiation (Olsen and Jeffery, 1997; Shimauchi et al., 1997). Factors other than these two may also be involved in the transcriptional activation of *Ciona Brachyury*. Therefore, the present results may be interpreted as indicating that ZicL is a primary and essential activator of *Ci-Bra*, leaving open the possibility that other factors also play roles in *Ci-Bra* activation.

Transcriptional control of *Brachyury* in other animal groups

Cis-regulatory elements for specific expression of *Brachyury* have been investigated in another ascidian, *H. roretzi* (Takahashi et al., 1999b). The notochord-specific expression of *Hr-Bra* depends on a module between –289 and –250 of the upstream sequence of the gene. The *Halocynthia* genome also contains two types of *Zic*-related genes, *macho-1* (Nishida and Sawada, 2001) and *zicN* (Wada and Saiga, 2002). *HrzicN* is expressed in blastomeres of muscle, notochord, anterior mesenchyme and nerve cord, and the gene seems to be responsible for the differentiation of these types of cell, and a model in which HrzicN may activate *Hr-Bra* together with FGF/BMP signaling pathway is proposed (Wada and Saiga, 2002). The results of the present study partially support that model. However, the Ci-ZicL binding element shown here is not present in this region of *Hr-Bra*. Therefore, although the *ZicL–Brachyury* network appears to function in *Halocynthia* notochord specification, details of the *Halocynthia* network remain to be elucidated.

In mouse embryos, *Brachyury* is expressed in the primitive streak (non-axial mesoderm) and in the node or notochord (axial mesoderm). The regulatory sequences required for the expression of *Brachyury* 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 *Lef1* 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 (Latinkić 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 (Latinkić et al., 1997). This suppression is mediated by paired-type homeobox genes, *goosecoid* and *Mix.1* (Latinkić et al., 1997; Latinkić 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-1* 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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