Development 126, 5599-5609 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV7743

Regulation of *Ci-tropomyosin-like*, a Brachyury target gene in the ascidian, *Ciona intestinalis*

Anna Di Gregorio* and Michael Levine

Department of Molecular and Cell Biology, Division of Genetics and Development, 401 Barker Hall, University of California, Berkeley, CA 94720, USA

*Author for correspondence (e-mail: annadg@uclink4.berkeley.edu)

Accepted 23 September; published on WWW 24 November 1999

SUMMARY

Brachyury is a sequence-specific transcriptional activator that is essential for notochord differentiation in a variety of chordates. In vertebrates, *Brachyury* is expressed throughout the presumptive mesoderm, but becomes restricted to the notochord at later stages of development. In ascidians, such as *Ciona intestinalis*, *Brachyury* is expressed exclusively in the notochord and does not exhibit an early pan-mesodermal pattern. Subtractive hybridization screens were recently used to identify potential *Ciona* Brachyury (Ci-Bra) target genes (Takahashi, H., Hotta, K., Erives, A., Di Gregorio, A., Zeller, R. W., Levine, M. and Satoh, N. (1999). *Genes Dev*. 13, 1519-1523). Of the genes that were identified in this screen, one corresponds to a new member of the tropomyosin superfamily, *Ciona tropomyosin* (*Ci-trop*). Here we show that *Ci-trop* is specifically expressed in the developing notochord beginning at gastrulation, and expression persists in the notochord during tailbud and tadpole stages. A 3 kb region of the *Ci-trop* 5′-flanking sequence was characterized via electroporation of *lacZ* fusion genes into fertilized *Ciona* eggs. A minimal, 114 bp enhancer was identified that is sufficient to direct the expression of a heterologous promoter in the notochord. DNA binding assays indicate that this enhancer contains two sets of low-affinity Brachyury half-sites, which are bound in vitro by a GST/Ci-Bra fusion protein. Deletion of the distal sites inactivates the notochord-specific staining pattern mediated by an otherwise normal *Ci-trop/lacZ* transgene. These results suggest that *Ci-trop* is a direct target gene of Ci-Bra and that *Brachyury* plays an immediate role in the cellular morphogenesis of the notochord.

Abbreviations: bp, base pair(s); kb, kilobase(s), or 1000 base pairs; 5′-UTR, 5′ untranslated sequence; CNS, central nervous system; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; GST, glutathione S-transferase; GFP, green fluorescent protein.

Key words: *Ciona intestinalis*, Ascidian, Notochord, *Brachyury*, Tropomyosin.

INTRODUCTION

Ascidians provide an excellent opportunity for studying notochord differentiation due to detailed cell lineage information and the ease of introducing transgenic DNAs into developing embryos via simple electroporation procedures (reviewed by Di Gregorio and Levine, 1998). The ascidian notochord arises from two lineages: blastomeres of the A4.1 pair give rise to 32 of the 40 definitive notochord cells (primary lineage) and the B4.1 pair gives rise to the remaining 8 notochord cells (secondary lineage). The A-lineage precursors are clonally restricted to form notochord at the 32- to 64-cell stage, while the B-lineage precursors become fate restricted at the 64- to 110-cell stage (Satoh, 1994, and references therein). At the onset of gastrulation, there is a total of 10 notochord precursor cells (8 from the A-lineage and 2 from the B-lineage). These divide once during gastrulation and again during neurulation to establish the definitive number of notochord cells.

At the completion of neurulation the presumptive notochord is arranged in two rows of 20 cells each. During tailbud stages, these rows converge and intercalate to form a single column of notochord cells. Initially, the cells are columnar in shape (Fig. 1A), but during subsequent stages of development they undergo shape changes and acquire an extended morphology (Fig. 1B). This transformation in cell shape is responsible for elongating the tail along the anteroposterior axis.

Our long-term goal is to determine the molecular events that mediate these complex morphogenetic changes during notochord differentiation. The starting point for this effort is the Brachyury (or T) transcription factor, which has been implicated in notochord differentiation in a variety of vertebrates (e.g., Schulte-Merker et al., 1994; Kispert et al., 1995; Stott et al., 1993; Cunliffe and Smith, 1994). In frogs, *eFGF* and the *Bix* homeobox gene have been recently identified as target genes of the *Xenopus* Brachyury (Xbra) protein in the early mesoderm (Casey et al., 1998; Tada et al., 1998), but no target genes have been identified that are specifically expressed

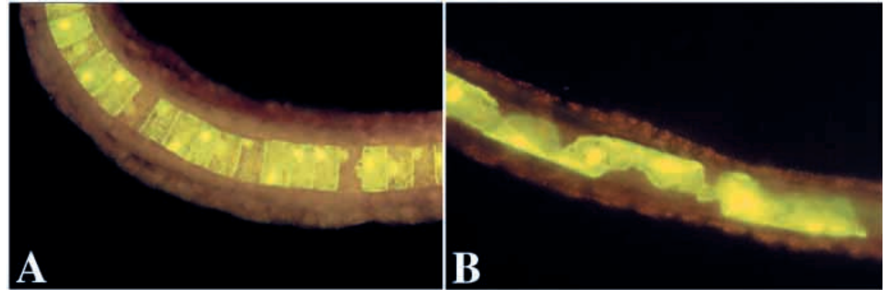

Fig. 1. Changes in cell shape during notochord differentiation. (A) The notochord cells of a mid-tailbud stage *Ciona* embryo (~12 hours after fertilization). This embryo was electroporated at the 1-cell stage with a *Ci-Bra/GFP* fusion gene containing 3.5 kb of the *Ci-Bra* 5′-flanking region. The cells are tightly linked to each other and have a columnar shape. (B) Same as A except the embryo is older (after hatching, ~18 hours after fertilization). The cells have undergone a shape change, causing nearly a two-fold elongation of the tail as compared with the younger embryo in A. In both panels, there is mosaic incorporation of the transgene and the nuclei stain stronger than the cytoplasm due to a cryptic nuclear localization signal in the GFP coding sequence.

in the vertebrate notochord. *Brachyury* homologs have been isolated and characterized in two distantly related ascidians, *Halocynthia roretzi* and *Ciona intestinalis* (Yasuo and Satoh, 1994; Corbo et al., 1997a). Both genes are expressed exclusively in the developing notochord, beginning with clonal restriction at the 32- to 64-cell stage. Several lines of evidence suggest that the ascidian *Brachyury* genes are essential for notochord formation. Microinjection of *As-T* (*Halocynthia Brachyury*) mRNA into neuroectodermal and endodermal blastomeres results in the formation of ectopic notochord cells in *Halocynthia* (Yasuo and Satoh, 1998). Moreover, misexpression of the *Ciona Brachyury* (*Ci-Bra*) coding region under the control of the heterologous *forkhead* (*Ci-fkh*) promoter region results in the formation of mutant tadpoles that contain a mass of ectopic notochord-like cells, due to the transformation of endoderm into notochord (Takahashi et al., 1999). The mutant tadpoles were used for subtractive hybridization screens to identify notochord-specific genes that are upregulated in response to the overexpression of *Ci-Bra*.

One of the genes isolated in this screen is a member of the tropomyosin superfamily, *Ci-trop*. This gene was selected for detailed analysis since it is specifically expressed in the developing notochord beginning at gastrulation, shortly after the first appearance of Ci-Bra gene products. A 3 kb region of the *Ci-trop* 5′-flanking sequence directs the expression of a *lacZ* reporter gene in the developing notochord of electroporated *Ciona* embryos. This *Ci-trop*/*lacZ* fusion gene is upregulated in electroporated embryos, which also express transgenes that cause misexpression of Ci-Bra and an expansion of the notochord. *In vitro* binding assays identified two sets of Ci-Bra half-sites within a critical 114 bp region of the *Ci-trop* 5′-flanking sequence. This 114 bp sequence is sufficient to direct the expression of a heterologous promoter in the developing notochord. Deletion of the distal sites disrupts the notochord-specific expression of an otherwise normal *Ci-trop/lacZ* fusion gene. These results provide both *cis* and *trans* evidence that Ci-Bra directly regulates the *Ci-trop* target gene and thereby influences notochord differentiation.

MATERIALS AND METHODS

Ascidians

Adult *Ciona intestinalis* were collected from marinas in Half Moon Bay and Bodega Bay in Northern California, or purchased from either Marinus Inc., Long Beach (CA) or the Marine Biological Laboratory in Woods Hole (MA). Details on the rearing and handling of embryos, dechorionation and *lacZ* stainings are described by Corbo et al. (1997a).

Isolation of cDNA and genomic clones for *Ci-trop*

A subtractive cDNA library was prepared from mRNAs that are overexpressed in *Ciona* embryos after electroporation with a *Ci-fkh/Ci-Bra* fusion gene. Details are described in Takahashi et al. (1999). One of the cDNA clones was found to encode a member of the tropomyosin superfamily (see Fig. 2). We therefore called this gene *Ciona tropomyosin-like* (*Ci-trop*). The original *Ci-trop* cDNA clone is 1055 bp long, and a RACE assay (Corbo et al., 1997a) was used to identify the 5′ end. This involved the use of the following primer: (5′-3′)-AACCGCTCAATATCGGTGTCGTTAGTGG, yielding a 280 bp product. DNA sequence analysis (Sambrook et al., 1989) identified just 16 bp of 5′ sequence beyond the extent of the cDNA clone. It therefore appears that the *Ci-trop* cDNA contains the entire protein coding region and a short 5′-UTR sequence. Two genomic DNA clones were isolated by screening a *Ciona* genomic library with a 300 bp *Pst*I-*Bsp*EI ^{32}P-labeled fragment containing the 5′ region of the cDNA clone. Southern blot and DNA sequence analysis indicate that these clones contain 5′-flanking sequences that reside upstream of the *Ci-trop* transcription unit.

Whole-mount in situ hybridizations

In situ hybridizations were carried out on whole-mount, staged *Ciona* embryos as described by Corbo et al. (1997a), using digoxigenin-labeled RNA probes synthesized from the cDNA clone, a 1055 bp *Eco*RI-*Pst*I fragment cloned in pBlueScript SK+. The RNA antisense probe was prepared by transcribing the *Not*I linearized template with T7 RNA polymerase (Biolabs); the sense probe (control) was prepared by transcribing an *Xho*I-linearized DNA template with T3 RNA polymerase (Biolabs).

Transgenes and electroporations

All of the *lacZ* transgenes that were prepared from the *Ci-trop* 5′-flanking sequence are shown in Table 1. The first transgene was prepared by filling the ends of a *Pst*I-*Bsp*EI genomic DNA fragment and then inserting this into a blunted *Bam*HI site within the polylinker of the pSP1.72-27 vector (see Corbo et al., 1997a). This fragment contains 456 bp upstream of the start of the *Ci-trop* cDNA sequence and 110 codons of the *Ci-trop* coding region fused in-frame with the *lacZ* reporter gene (indicated as –0.45 kb in Table 1). Subsequently, a 2.2 kb *Bam*HI-*Sac*I fragment was fused upstream of the previous construct, thereby creating the −3 kb *Ci-trop*/*lacZ* fusion. This fusion gene directs robust *lacZ* expression in the notochord of electroporated embryos. All the other constructs shown in Table 1 were prepared with the indicated restriction enzymes using the −3 kb *Ci-trop*/*lacZ* fusion gene.

The heterologous fusion genes shown in Table 2 were prepared with an *Xho*I-*Mun*I genomic DNA fragment from the *Ciona forkhead* (*Ci-fkh*) promoter region, which includes the first 0.45 kb from the start

of the cDNA sequence. The −0.45 kb *Ci-fkh/lacZ* fusion gene directs only sporadic staining in trunk mesenchyme (A. D. G., unpublished data). With the exception of the first two constructs, all the fusion genes shown in Table 2 were prepared by PCR amplification using *Pfu* (Stratagene) or *Taq* (Perkin-Elmer) DNA polymerases to isolate the indicated *Ci-trop* 5′ genomic DNA fragments, as well as the fusion genes shown in Fig. 5E,F. All the sequences have been checked using the chain-termination method (Sambrook et al., 1989). The −2.6 kb *Ci-fkh/Ci-Bra* fusion gene used in Fig. 4B is described in Takahashi et al. (1999). Aliquots containing 100 μg of a given *Ci-Bra/lacZ* fusion gene were electroporated into fertilized, dechorionated *Ciona* eggs, as described by Corbo et al. (1997a). All the fusion genes were tested in parallel on several different batches of embryos and the results that are presented were observed in at least three independent experiments.

Gel-shift assays

The following double-stranded 25-mer oligonucleotides were used for binding assays (only the 5′-3′ strand is shown):

-CTAGTCACACCTAGGTGTGAAATTC- (Mouse Bra)
-CTAGACATGACTGAAGTTGAAATTC- (Mouse MUT)
-CGTTTTATCACTATCCTGTGAAGTA- (Ci-Bra Proximal)
-GTCTCTCGCACCCGGCACCTCTTTC- (Ci-Bra Distal)
-GTCGCACGACTCCGGACCGGCTTTC- (DistMUT)
-CTGCCCTGTTTGTTTTAGTTACGA- (HNF3W; 24-mer, modified from Overdier et al., 1994).

Oligonucleotides were annealed, radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and purified by chromatography using Biogel P-10 columns. Protein-DNA complexes were formed at room temperature for 15 minutes as described by Corbo et al. (1998), using 3×10^5 cts/minute of ^{32}P-labeled oligonucleotides per reaction and ~20 ng of a GST/Ci-Bra fusion protein. This fusion protein contains amino acid residues 1 to 248 from Ci-Bra, which includes the entire T-domain that mediates DNA binding. The pGEX-Ci-Bra plasmid was kindly provided by Albert Erives. The GST/Ci-Bra fusion protein was prepared according to Guan and Dixon (1991).

RESULTS

A recent subtractive hybridization screen led to the identification of 38 different cDNAs that are specifically expressed in the notochord of developing *Ciona* embryos and represent putative *Ci-Bra* target genes (Takahashi et al., 1999). One of these cDNAs is expressed in notochord precursor cells just after the onset of gastrulation. The early expression of this

A

```
Ciona        .........MENIKMKIAKLKAESDEKENQICDLSDQLKKVTEERKQFEEVNRSLSNKIS   51
Homo         M...AGLNSLEAVKRKIQALQQQADEAEDRAQGLQRELDGERERREKAEGDVAALNRRIQ   57
Rattus       M...AGSSSLEAVRRKIRSLQEQADAAEERAGSLQRELDQERKLRETAEADVASLNRRIQ   57
Ciona CTm1   .........LQGLQKRLKATEDELDTSQEKLRTAIENLENAEKKAADAEQEVASLNRRIT   93
Hydra        MVVETGVNKLVRLQGKIEGINSKIDEADLRRANAKSSIVEASSRLEKAEGEVASFQRRIR   60

Ciona        TNDTDIERL.ELQNEELKRKSENAERELDEVQRELKKLQSEHTASAERCDDLTEELKLRK  110
Homo         LVEEELDRAQERLATALQ.KLEEAEKAADESERGMKVIENRAMKDEEKMEIQEMQLKEAK  116
Rattus       LVEEELDRAQERLATALQ.KLEEAEKAADESERGMKVIESRAQKDEEKMEIQEIQLKEAK  116
Ciona CTm1   LVEEELDRAQERLTISLS.KLEEAEKAADESERGRKVIENRSLKDEERLEVQEIQLTEAK  151
Hydra        LVQQNLNDVTER.AQMLQSKVDNLEDVSESVKQARNQYEEEEAESDEKIQNLEEEVKVKK  119

Ciona        MDLDEVTTNYDDAIARIKVLDGDNCRLDDKVQALEDEAKQLRESGQDMDGILKALEAKET  170
Homo         HIAEEADRKYEEVARKLVILEGELERAEERAEVSELKCGDLEEELKNVTNNLKSLEAASE  176
Rattus       HIAEDADRKYEEVARKLVIIESDLERAEERAELSEGQVRQLEEQLRIMDQTLKALMAAED  176
Ciona CTm1   NIAEDADRKYVEVARKLVMVEAELERGEERAELAESKAIELEEELKIVANNLKSLEASAE  211
Hydra        RELEENEIKLREKERRNVVVHRDIEAATVKADAIEKRIEILENTIKNGLESIKDLEEREG  179

Ciona        TYGNNEIQAEDQIRSLKMALEESECRREALENEAKKYQADIEKLELDLDKEMAEKEEAKA  229
Homo         KYSEKEDKYEEEIKLLSDKLKEAETRAEFAERTVAKLEKTIDDLE...EKLAQAKEENVG  233
Rattus       KYSQKEDKYEEEIKVLSDKLKEAETRAEFAERSVTKLEKSIDDLE...EKVAHAKEENLS  233
Ciona CTm1   KYAAKEGIFEEEIKTLSDKLKDSETRAEFAEKSVVKLEKNIDELE...DSLYAEKCKIKA  268
Hydra        RTNEKENNIAEQISFLDNKFKEVEIRIEAAERNCNVLEHNIKETS...EEIKTWKHNTHA  236

Ciona        ......ELDRVLSELGEM....                                       241
Homo         LHQ...TLDQTLNELNCI....                                       248
Rattus       MHQ...MLDQTLLELNNM....                                       248
Ciona CTm1   ISE...DMDVTLQGIGDL....                                       283
Hydra        IEEEIAAMDDVGDDDPVILFYL                                       258
```

B

Percent Identity:

	Ciona	*Homo*	*Rattus*	*Ciona* CTm1	*Hydra*
Ciona	----	28.2	29.0	24.0	20.6
Homo	28.2	----	78.2	58.0	30.8
Rattus	29.0	78.2	----	55.8	27.5
Ciona CTm1	24.0	58.0	55.8	----	27.4
Hydra	20.6	30.8	27.5	27.4	----

Fig. 2. Ci-trop sequence. (A) The top line shows the amino acid sequence of the Ci-trop protein (‘*Ciona*’), based on the nucleotide sequence of the cDNA (GenBank accession number: AF155191). It is compared with the fibroblast tropomyosin TM30p1 from humans (‘*Homo*’ MacLeod et al., 1987); the rat fibroblast tropomyosin 5B (‘*Rattus*’ Goodwin et al., 1991); CTm1, a muscle tropomyosin from *Ciona intestinalis* (‘*Ciona*’, Meedel and Hastings, 1993) and a cnidarian tropomyosin sequence (‘*Hydra*’, accession number S32041). Identity in three or more positions is indicated in black, similar residues are shaded in gray. Positions where amino acid residues are identical or similar in all 5 proteins are indicated by asterisks. (B) Conservation of tropomyosin sequences. The Ci-trop protein shares nearly 30% amino acid identity with the fibroblast tropomyosins of rats and humans. It shares just 24% identity with the *Ciona* muscle tropomyosin and 21% identity with the tropomyosin of *Hydra*.

gene suggested that it might be directly regulated by Ci-Bra. Here we present a detailed analysis of this gene, focusing on its expression pattern throughout embryogenesis and on the structure of its 5′-regulatory region.

A new member of the tropomyosin superfamily

The cDNA clone isolated in the subtractive screen contains a 1055 bp insert. This sequence includes a single extended ORF of 242 codons. The sequence of the putative protein is presented in Fig. 2A. It is related to a non-muscle tropomyosin (Pittenger et al., 1994). The newly identified gene, hereafter called *Ci-trop*, shares 23 invariant and several conservative amino acid residues with previously identified non-muscle tropomyosins and with the muscle-type tropomyosin isolated from the *Ciona* body-wall muscle (Meedel and Hastings, 1993). It shares nearly 30% overall identity with fibroblast tropomyosins identified in rats and humans (Fig. 2B). Ci-trop contains a number of structural motifs that are common to other tropomyosins, including a predicted coiled-coil secondary structure and several leucine-zipper motifs which are likely to be responsible for dimerization (e.g. Bikle et al., 1993).

Expression of *Ci-trop* during *Ciona* embryogenesis

Ci-trop expression was monitored by in situ hybridization using a full-length antisense RNA probe and whole-mount preparations of staged *Ciona* embryos. *Ci-trop* expression is first detected during gastrulation (Fig. 3A). Staining is detected in all three tiers of invaginating notochord precursor cells. Neurulation commences ~1.5 hours later, and at this time *Ci-trop* expression is detected in all of the invaginated notochord cells (Fig. 3B). This staining pattern is resolved into two rows of notochord precursor cells at later stages of neurulation (Fig. 3C). During tailbud stages, these two rows converge and intercalate to form a single column of cells (Fig. 3D). *Ci-trop* staining persists after the completion of the tailbud stages and can be detected in tadpoles (Fig. 3E).

Previous studies have shown that electroporation of a *Ci-fkh/Ci-Bra* fusion gene in *Ciona* embryos results in the ectopic expression of *Ci-Bra* in the endoderm and neural tube. These tissues are at least partially transformed into notochord tissue (Fig. 3F). Hybridization of the mutant tadpoles with the *Ci-trop* probe reveals a substantial expansion of the *Ci-trop* expression pattern (Fig. 3F; compare with E). This expanded staining pattern is consistent with the possibility that *Ci-trop* is an early target gene of Ci-Bra (see below).

Characterization of the *Ci-trop* 5′-flanking region

Genomic DNA clones containing *Ci-trop* 5′-flanking sequences were isolated and a region spanning ~3 kb upstream of the cDNA sequence was chosen for analysis.

Table 1.

Construct	NOTOCHORD	EPIDERMIS	MESENCHYME
*Bam*HI -3 kb — *lacZ*	+++	+	-
*Ppu*MI -2.7 kb — *lacZ*	+++	+	-
*Pst*I -1.8 kb — *lacZ*	+++	++	-
*Apo*I -1.5 kb — *lacZ*	+++	++	-
*Bst*EII -1.4 kb — *lacZ*	+++	++	+/-
*Eco*47III -1.3 kb — *lacZ*	-	++	+
*Psh*AI -1.1 kb — *lacZ*	-	++	+/-
*Sna*BI -1 kb — *lacZ*	-	++	++
*Sac*I -0.8 kb — *lacZ*	-	+	+
*Pst*I -0.45 kb — *lacZ*	-	-	+/-
*Bam*HI -3 kb, Δ *Sgr*AI (-2.2 kb) – *Psh*AI (-1.1 kb) — *lacZ*	-	++	++
*Ppu*MI -2.7 kb, Δ *Bst*EII (-1.4 kb) – *Psh*AI (-1.1 kb) — *lacZ*	-	+++	-
*Pst*I -1.8 kb, Δ *Bst*EII (-1.4 kb) – *Psh*AI (-1.1 kb) — *lacZ*	-	+++	++

Table 2.

Construct	NOTOCHORD	EPIDERMIS	MESENCHYME
-1.8 kb *Pst*I — *Bst*EII -1.4 kb (deleted) — -0.45 kb *Ci-fkh* — *lacZ*	-	-	+/-
-1.8 kb *Pst*I — *Eco*47III -1.3 kb — -0.45 kb *Ci-fkh* — *lacZ*	+++	-	+/-
-1.76 kb — *Eco*47III — -0.45 kb *Ci-fkh* — *lacZ*	+++	-	+/-
-1.73 kb — *Eco*47III — -0.45 kb *Ci-fkh* — *lacZ*	+++	-	+/-
-1.47 kb — *Eco*47III — -0.45 kb *Ci-fkh* — *lacZ*	++	-	+/-
-1.4 kb *Bst*EII — *Eco*47III — -0.45 kb *Ci-fkh* — *lacZ*	+	-	+/-
-1.34 kb — *Eco*47III — -0.45 kb *Ci-fkh* — *lacZ*	-	-	+/-
*Bst*EII — -0.45 kb *Ci-fkh* — *lacZ*	-	-	+/-
-0.45 kb *Ci-fkh* — *lacZ*	-	-	+/-

This 'full-length' *Ci-trop* genomic DNA fragment directs the expression of a *lacZ* reporter gene in the notochord of electroporated embryos (Fig. 4A; red arrowhead). This transgene also exhibits weak, variable expression in the epidermis (Fig. 4A; green arrowhead), which is not a normal site of *Ci-trop* expression based on in situ hybridization assays (Fig. 3). It is conceivable that the *Ci-trop* regulatory region contains one or more repressor elements that normally exclude expression in epidermal cells; these elements may not be present in the first 3 kb of the 5′-flanking region (see Discussion).

The preceding in situ hybridization assays showed that *Ci-trop* expression is upregulated in mutant tadpoles that contain the *Ci-fkh/Ci-Bra* fusion gene (Fig. 3F). To determine whether the −3 kb *Ci-trop/lacZ* reporter gene contains putative Ci-Bra response elements, it was co-electroporated into *Ciona* embryos along with the *Ci-fkh/Ci-Bra* fusion gene (Fig. 4B). The *lacZ* reporter gene exhibits an expanded pattern of staining, and includes the transformed endodermal and neuroectodermal tissues (yellow arrowhead, Fig. 4B).

These results indicate that the 3 kb *Ci-trop* promoter region contains elements that directly or indirectly respond to the Ci-Bra activator. Subsequent experiments were conducted to determine whether *Ci-trop* is directly regulated by Ci-Bra. As a first step towards identifying the minimal *Ci-trop* promoter region, different DNA fragments from the 5′-flanking sequence were attached to the *lacZ* reporter and electroporated in *Ciona* embryos. The 1.4 kb 5′-sequence is sufficient to direct *lacZ* staining in the notochord (Fig. 5B). This staining pattern is similar to that observed with the full-length, 3 kb *Ci-trop* promoter region (Figs 5A, 4A; Table 1). The removal of 114 bp, between −1.4 and −1.3 kb, causes a virtual loss of the notochord staining pattern (Fig. 5C). Variable staining is detected in the tail epidermis and mesenchyme. Further evidence that the region between −1.4 and −1.3 kb is important for *Ci-trop* expression was obtained by deleting sequences between −1.4 kb and −1.1 kb within the full-length 3 kb reporter gene (Fig. 5D). This internal deletion causes loss of notochord staining (compare with Fig. 5A).

Identification of minimal sequences necessary for notochord expression

The preceding analyses indicate that the region between −1.4 kb and −1.3 kb is crucial for notochord expression. Additional experiments were done to determine whether this interval is sufficient for such expression. Different DNA fragments from the *Ci-trop* 5′-regulatory region were attached to the heterologous *Ci-fkh* promoter (summarized in Table 2). This promoter sequence includes the first 450 bp of the *Ci-fkh* 5′-flanking region. Previous studies have shown that it directs weak and sporadic expression of a *lacZ* reporter gene in the trunk mesenchyme (A. D. G. et al., unpublished data; see Table 2). A 530 bp DNA fragment located between −1.8 and −1.3 kb upstream of the *Ci-trop* transcription unit directs robust expression of the *Ci-fkh/lacZ* fusion gene in the notochord (Fig. 6A). Removal of 114 bp from the 3′ end of the fragment

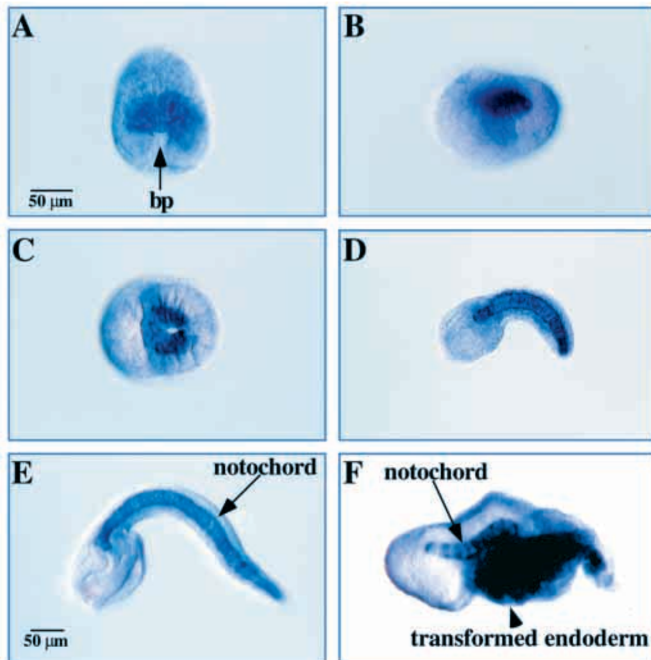

Fig. 3. Expression pattern of *Ci-trop* during embryogenesis. The *Ci-trop* cDNA was used to prepare a digoxigenin-labeled antisense RNA probe. The probe was hybridized to whole-mount preparations of staged *Ciona* embryos. Embryos were histochemically stained with anti-digoxigenin antibodies. (A) Gastrulating embryo. Staining is detected in all the notochord precursor cells. The blastopore (bp) is indicated by an arrow. (B) Neurulating embryo, side view; anterior is to the left, dorsal is up. *Ci-trop* transcripts are detected in each of the invaginating notochord precursor cells. (C) Dorsal view of a late neurula-stage embryo, with anterior to the left. Staining is detected in the two rows of notochord precursor cells. (D) Lateral view of an early tailbud, ~11 hours after fertilization. The two rows of notochord cells merged to form the notochord rudiment. (E) Lateral view of a tadpole, ~14 hours post-fertilization. (F) Same as E except that the embryo was electroporated at the 1-cell stage with a *Ci-fkh*/*Ci-Bra* fusion gene. This causes a transformation of endoderm into notochord. *Ci-trop* transcripts are detected in both the notochord (arrow) and transformed endoderm (arrowhead).

eliminates this expression (Fig. 6B; Table 2). The analysis of progressively smaller DNA fragments identified this 114 bp region as the minimal notochord-specific enhancer (Table 2). When attached to the *Ci-fkh/lacZ* fusion gene it is sufficient to direct expression in the notochord (Fig. 6C). It is possible that there are additional notochord-specific regulatory sequences in the Ci-trop promoter region since sequences between −1.73 kb and −1.4 kb are important for optimal expression (see Table 2).

DNA binding assays

Previous studies have identified the sequence AGGTGTGA as a high-affinity half-site for the mouse Brachyury protein (Kispert and Herrmann, 1993). PCR selection assays suggest that optimal recognition sequences contain two copies of this half-site arranged as inverted repeats (summarized in Fig. 7B; see Kispert and Herrmann, 1993; Kispert et al., 1995).

The nucleotide sequence of the minimal, 114 bp notochord enhancer includes five putative Brachyury half-sites; four of these are indicated by red horizontal arrows in Fig. 7A. Two of these half-sites are arranged as inverted repeats in a proximal portion of the enhancer (‘Ci-Bra Prox’; red arrows in Fig. 7A,B). It shares 10/20 matches with the optimal mouse palindromic recognition sequence (‘Mouse Bra’, Fig. 7B). Two additional Brachyury half-sites are arranged in tandem in a distal region of the 114 bp enhancer (‘Ci-Bra Distal’; red arrows in Fig. 7A,B). The first half-site shares a 7/10 match with the consensus sequence, and the second shares a 5/10 match (circled in Fig. 7B). Moreover, a search of the first 1.4 kb of the *Ci-trop* 5′ flanking sequence identified another potential Brachyury half-site ~1.1 kb upstream of the transcribed region. This latter site, ‘Ci-Bra #3’ (Fig. 7B), shares an 8/10 match with the mouse consensus half-site.

Gel-shift assays were done to determine whether a GST/Ci-Bra fusion protein binds to the putative Brachyury recognition sequences identified in the *Ci-trop* promoter region. The GST fusion protein contains the entire Ci-Bra DNA-binding domain (see Methods). The mouse palindromic recognition sequence was used as a control in these experiments (‘Mouse Bra’, Fig. 7B). It was labeled with ^{32}P, mixed with the GST/Ci-Bra fusion protein and fractionated on a polyacrylamide gel (Fig. 7C). The

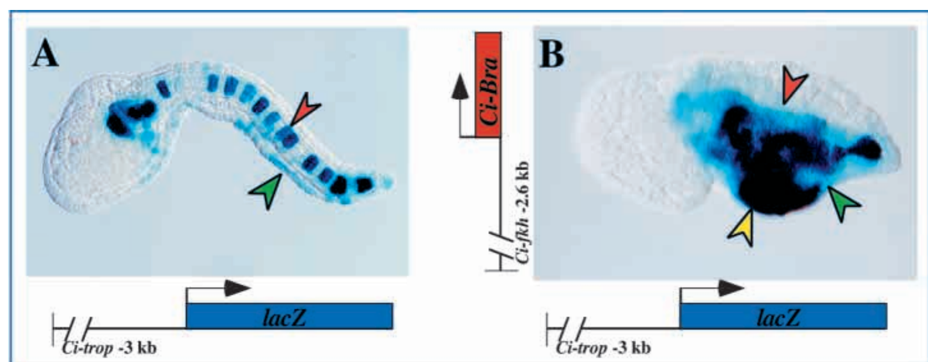

Fig. 4. Expression of the 3 kb *Ci-trop*/*lacZ* fusion gene in mutant embryos. Embryos were electroporated at the 1-cell stage with a *lacZ* fusion gene containing ~3 kb of 5′-flanking sequence from the *Ci-trop* gene. Electroporations were done at the 1-cell stage and embryos were allowed to develop to late tailbud stages, ~14 hours after fertilization. The embryos were histochemically stained with X-gal. They are oriented with anterior to the left and dorsal up. (A) The −3 kb *Ci-trop*/*lacZ* fusion gene directs strong notochord staining (red arrowhead). Staining is also detected in the tail epidermis (green arrowhead) of ~50% of the electroporated embryos. (B) Same as A except that the embryo was co-electroporated with a *Ci-Bra* fusion gene containing 2.6 kb of 5′-flanking sequence from the *Ci-forkhead* (*Ci-fkh*) gene (the diagram at left summarizes the transgene). The transgene results in the misexpression of *Ci-Bra* in endodermal and neuronal tissues. There is at least a partial transformation of endoderm into notochord (Takahashi et al., 1999; see Fig. 3F). The *Ci-trop*/*lacZ* fusion gene is expressed in the notochord (red arrowhead), transformed endoderm (yellow arrowhead), and regions of the tail epidermis (green arrowhead).

fusion protein results in the appearance of two shifted complexes (Fig. 7C, lane 3). Neither complex is observed in the absence of protein (lane 1), nor when a GST nonfusion protein is added to the labeled DNA fragment (lane 2). It is conceivable that the two complexes observed in lane 3 (Fig. 7C) represent monomeric (lower band) and dimeric (upper band) forms of the GST/Ci-Bra fusion protein. Two distinct, but smaller complexes are observed when thrombin is added to the reaction mix. Thrombin cleaves the GST moiety, suggesting that both shifted complexes contain the Ci-Bra peptide. These complexes are lost upon addition of a 100-fold molar excess of the unlabeled mouse sequence (data not shown; see below).

Additional binding assays were done with *Ci-trop* DNA sequences containing the distal and proximal elements in the 114 bp enhancer, as well as the Ci-Bra #3 sequence (see Fig. 7B). The GST/Ci-Bra fusion protein was separately mixed with each of these three sequences after labeling with ^{32}P (Fig. 7C). The distal sequence containing two putative Ci-Bra half-sites arranged in tandem forms a single shifted complex with the GST/Ci-Bra fusion protein (Fig. 7C, lane 7). This complex migrates with about the same mobility as the lower complex obtained with the mouse palindromic sequence (lane 3). A single, smaller complex is observed when thrombin is added to the reaction mix (lane 8; compare with lane 4). These results are consistent with the possibility that Ci-Bra primarily binds as a monomer to the distal sequence (see below). Additional evidence for this possibility stems from the analysis of the Ci-Bra #3 sequence (lanes 15 and 16). This sequence includes just a single putative Ci-Bra half-site, and forms a single, smaller complex with the Ci-Bra/GST fusion protein. The proximal sequence forms a single, large complex with the GST/Ci-Bra fusion protein (Fig. 7C, lane 11). This complex migrates with about the same mobility as the larger of the two complexes obtained with the mouse palindromic sequence (lane 3). Again, addition of thrombin reduces the size

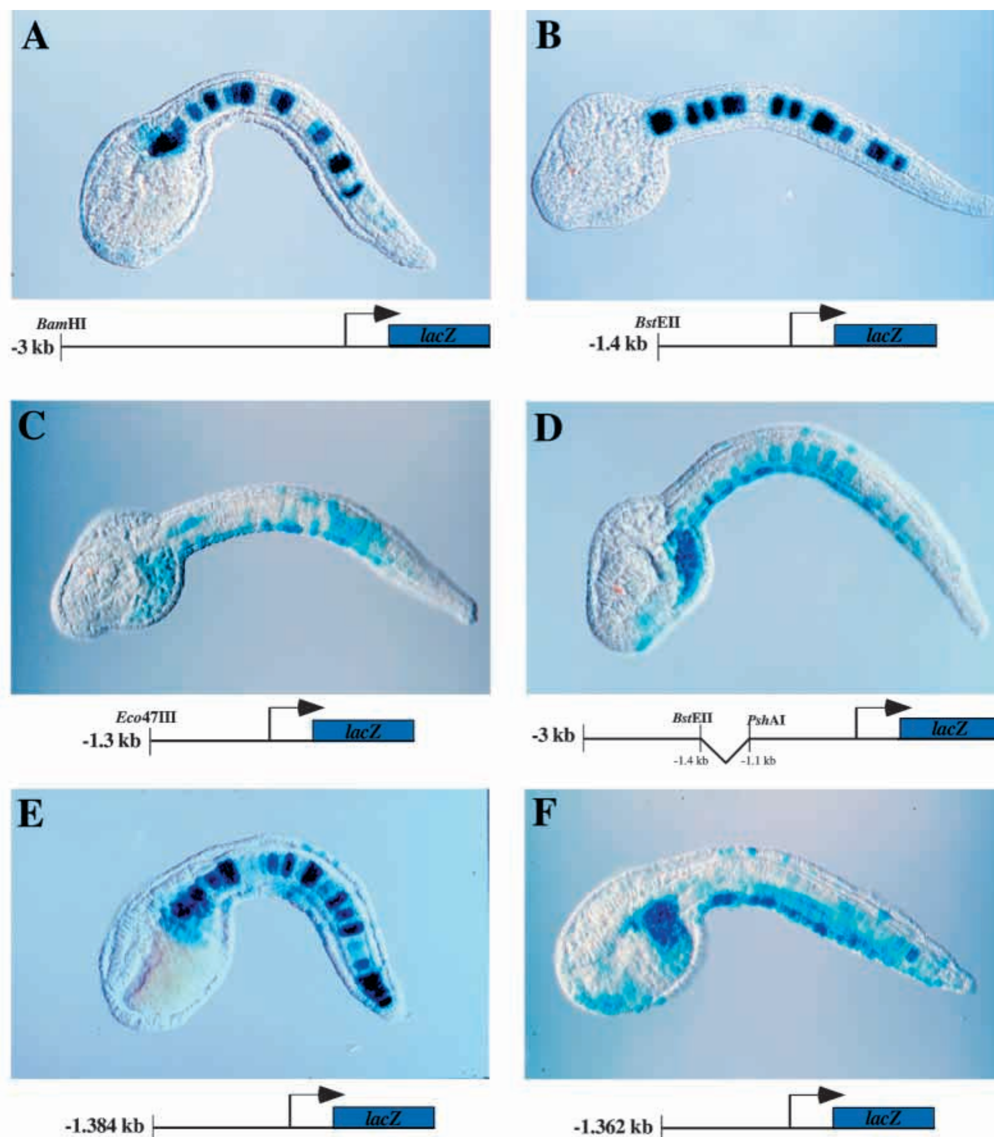

Fig. 5. Truncation analysis of the *Ci-trop* 5′-regulatory region. Different regions of the *Ci-trop* 5′-flanking sequence were attached to a *lacZ* reporter gene. The *Ci-trop/lacZ* fusion genes were electroporated into 1-cell embryos, and grown to tailbud stages prior to histochemical staining with X-gal. Embryos are oriented with anterior to the left and dorsal up. (A) Lateral view of a *Ciona* larva electroporated with the 'full-length' −3 kb *Ci-trop/lacZ* fusion gene (see diagram beneath the embryo and Fig. 4A). The fusion gene directs strong notochord staining. Note that due to mosaic incorporation of the fusion gene, not all of the 40 notochord cells are stained. (B) Staining pattern obtained with a *lacZ* fusion gene that contains 1.4 kb of 5′-flanking sequence from the *Ci-trop* gene. Strong staining is detected in the notochord, similar to that obtained with the full-length fusion gene. (C) Staining obtained with a −1.3 kb *Ci-trop/lacZ* fusion gene. The removal of just 114 bp essentially abolishes expression in the notochord (compare with B). Staining persists in the tail epidermis and trunk mesenchyme. (D) A 300 bp internal deletion was created within the full-length *Ci-trop/lacZ* fusion gene, between −1.4 kb and −1.1 kb (see diagram below). There is a loss of staining in the notochord, and persistence of expression in the epidermis and mesenchyme. The internal deletion includes the 114 bp sequence eliminated in C. (E) Staining pattern obtained with a lacZ fusion gene containing 1384 bp of 5′-flanking sequence from *Ci-trop* (see diagram below). Strong expression is detected in the notochord. (F) Truncating just 22 bp between −1384 bp and −1362 bp causes a loss of transgene expression in the notochord (compare with E; see red oval in Table 2). Staining is detected in the tail epidermis and trunk mesenchyme.

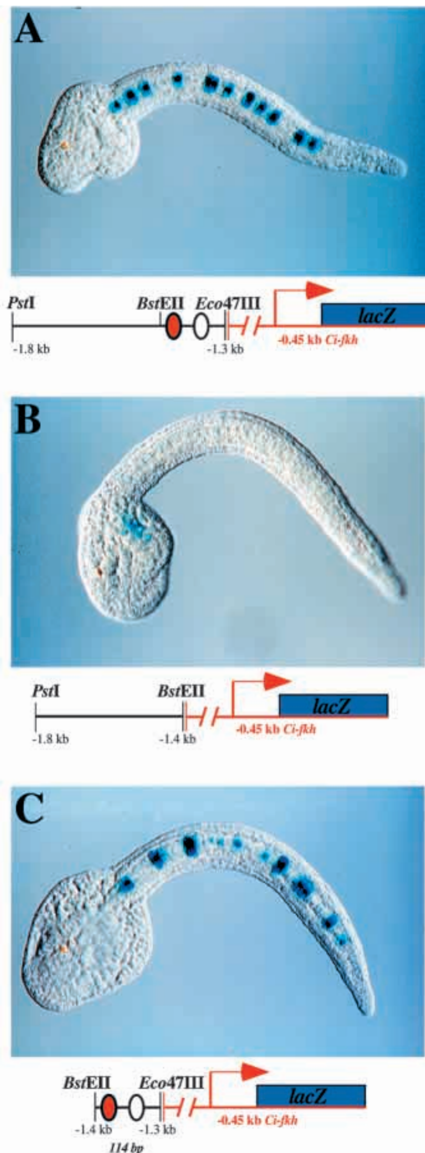

Fig. 6. Identification of the minimal notochord-specific enhancer. Embryos were electroporated at the 1-cell stage with *Ci-fkh/lacZ* fusion genes containing different pieces of *Ci-trop* 5′-flanking sequences. Larvae were histochemically stained with X-gal and are oriented with anterior to the left and dorsal up. (A) A ~500 bp region of the *Ci-trop* 5′-flanking sequence (from −1.8 kb to −1.3 kb) is able to direct notochord-specific expression when placed upstream of the heterologous *Ci-fkh* promoter (see diagram below; the red and white ovals represent Ci-Bra recognitions sequences). (B) Same as A except that 114 bp were deleted from the 3′ end of the *Ci-trop* DNA fragment (see diagram below). The resulting 400 bp sequence, from −1.8 kb to −1.4 kb, is not sufficient to direct notochord expression. (C) The 114 bp *Ci-trop* DNA fragment that was removed in B was placed upstream of the *Ci-fkh/lacZ* fusion gene (see diagram below). This sequence contains at least two Ci-Bra recognition sequences (ovals) and is sufficient to direct notochord-specific staining.

of the complex (lane 12; compare with lane 4). These results suggest that Ci-Bra binds as a dimer to the proximal element.

Additional control experiments were conducted to confirm the specificity of protein-DNA complexes (Fig. 7D). As seen previously, GST/Ci-Bra forms a predominant, small complex with the distal sequence (lane 2, Fig. 7D; see unfilled arrowhead to the left of the autoradiogram). A weaker, large complex is also observed (filled arrowhead), suggesting that a small fraction of the distal element might bind to a dimeric form of Ci-Bra. A 5-fold molar excess of either the distal or proximal competitor DNA diminishes the shifted complexes (lanes 3 and 5; compare with lane 2), while a 50-fold excess of either competitor nearly eliminates the complexes (lanes 4 and 6). Increasing amounts of control DNAs do not impede the formation of shifted complexes (Fig. 7D; lanes 7-10). Neither a 5-fold nor 50-fold excess of a mutant form of the distal sequence that lacks key residues in the Ci-Bra binding sites (see Materials and methods) diminishes the shifted complexes (lanes 7 and 8, respectively). Similarly, a 'random' DNA sequence, a putative HNF3β binding site, does not interfere with the formation of protein-DNA complexes (lanes 9 and 10). Similar results were obtained with the proximal sequence (Fig. 7D; lanes 12-20). The large protein-DNA complex is eliminated by increasing amounts of either the cold proximal competitor DNA (lanes 13 and 14) or the cold distal DNA (lanes 15 and 16). In contrast, increasing amounts of a mutant form of the mouse recognition sequence ('MouseMUT') does not intefere with the formation of the shifted complex (lanes 17 and 18). The HNF3β sequence also fails to block the shifted complex (lanes 19 and 20). These results indicate that the GST/Ci-Bra fusion protein specifically binds to the distal and proximal elements in the minimal, 114 bp notochord enhancer.

To determine whether the Ci-Bra binding sites are important for *Ci-trop* expression in vivo, we prepared a series of *Ci-trop/lacZ* truncations that extend into the 114 bp enhancer (Fig. 5E,F). A *lacZ* fusion gene containing 1,384 bp of *Ci-trop* 5′-flanking sequence exhibits strong expression in the notochord (Fig. 5E). However, notochord staining is markedly reduced upon removal of just 22 bp of 5′-flanking sequence (Fig. 5F). This 5′ deletion removes the two tandem Ci-Bra half sites contained in the distal portion of the 114 bp enhancer (the deleted sequence is boxed in Fig. 7A). The loss of expression is consistent with the possibility that Ci-Bra binding sites are important for *Ci-trop* regulation. However, we cannot rule out the possibility that expression also depends on additional regulatory factors (see below).

DISCUSSION

We have described a novel member of the tropomyosin superfamily, *Ci-trop*. The gene is specifically expressed in the developing notochord of *Ciona* embryos beginning with gastrulation and extending through tadpole stages. It is conceivable that *Ci-trop* plays a role in some of the cytoskeletal changes that occur in the notochord during intercalation and extension (e.g., Fig. 1). The early onset of *Ci-trop* expression identified it as a potential target of the Ci-Bra activator. Additional support for this possibility was obtained by analyzing *Ci-trop* expression in mutant *Ciona* tadpoles that contain excess notochord tissue due to the misexpression of *Ci-Bra*. Characterization of the first 3 kb of the *Ci-trop* 5′-flanking sequence led to the identification of a minimal, 114 bp enhancer that directs the expression of a heterologous *Ci-fkh/lacZ* reporter gene in the notochord. This enhancer contains

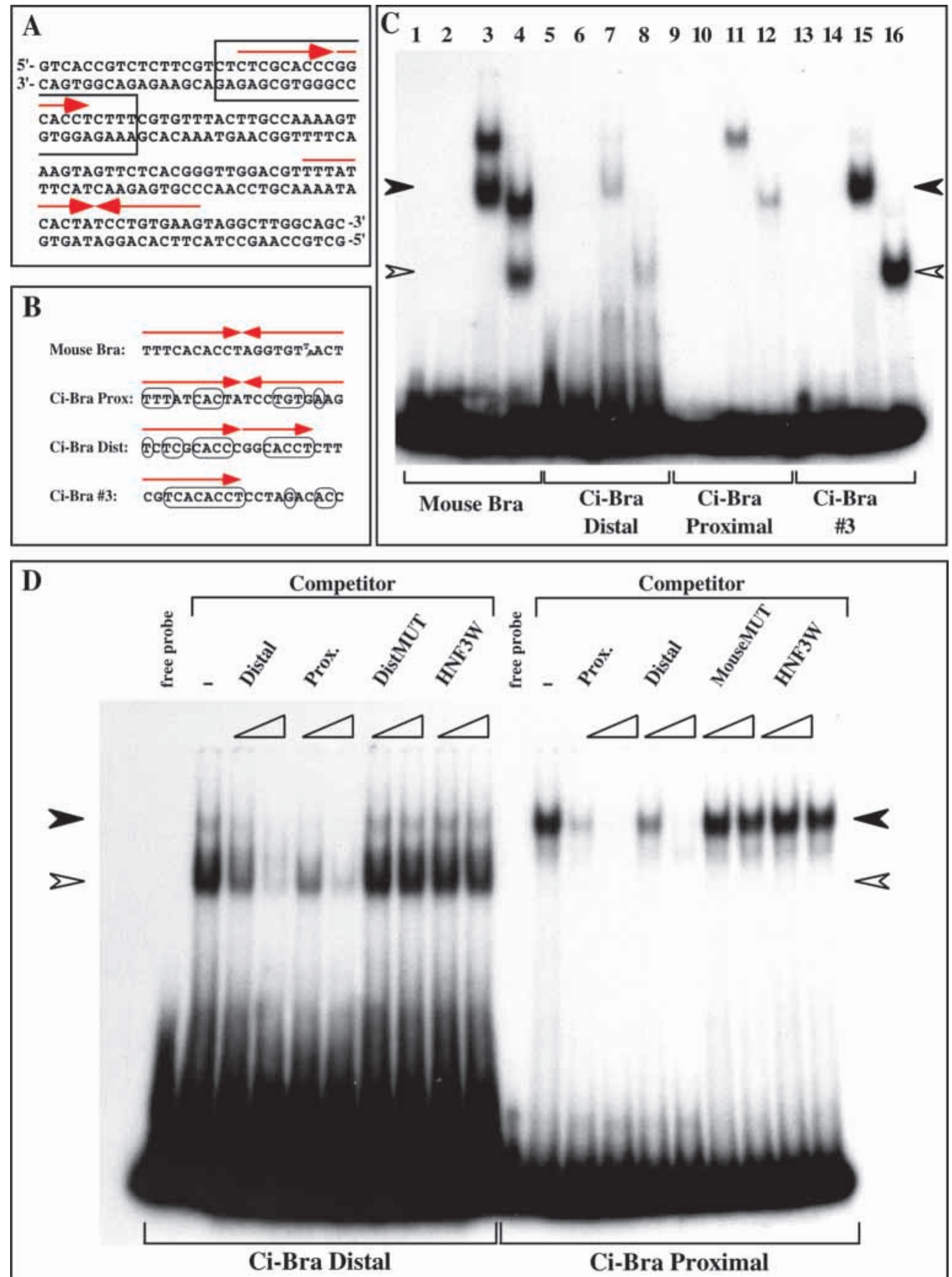

Fig. 7. DNA binding assays. Different DNA sequences from the *Ci-trop* 5′ regulatory region were tested for binding to a GST/Ci-Bra fusion protein that contains the entire T-box DNA binding domain. Particular efforts focused on the minimal, 114 bp enhancer that is sufficient to direct the expression of a *Ci-fkh/lacZ* fusion gene in the notochord of electroporated embryos. (A) DNA sequence of the 114 bp notochord-specific enhancer. There are five potential Ci-Bra half-sites scattered across the length of the enhancer; four are indicated by the horizontal arrows. Two of the half-sites are arranged in tandem (‘Ci-Bra Distal’) and two are arranged in inverted orientation (‘Ci-Bra Prox’). The boxed nucleotides indicate the 22 bp truncation that eliminates notochord-specific staining directed by a *Ci-trop/lacZ* fusion gene (see Fig. 5, E and F). This 22 bp truncation removes the distal recognition sequence (see below). (B) Comparison of the putative Ci-Bra binding sites with the mouse Brachyury recognition sequence (‘Mouse Bra’). The mouse consensus sequence contains two half-sites (arrows) arranged as inverted repeats (Kispert and Herrmann, 1993). The putative Ci-Bra recognition sequence located in the proximal portion of the *Ci-trop* enhancer (Ci-Bra Prox) exhibits a similar organization and shares 10/20 identities with the mouse sequence (the conserved residues are circled). The putative distal Ci-Bra half-sites are arranged in tandem and share 7/10 and 5/10 identities with the mouse half-sites. The half-site indicated as Ci-Bra #3 shares 8/10 identity with the mouse half-site and is located ‘1.1 kb upstream of the *Ci-trop* transcription unit. (C) Gel-shift assays were done with a GST/Ci-Bra fusion protein and different ^{32}P-labeled DNA sequences, including the optimal mouse palindromic binding site (‘Mouse Bra’; Kispert and Herrmann, 1993), the tandem distal half-sites (‘Ci-Bra Distal’), the inverted proximal half-sites (‘Ci-Bra Proximal’), and the Ci-Bra #3 half-site. Lanes 1, 5, 9 and 13 contain labeled DNA fragments lacking the fusion protein. Lanes 2, 6, 10 and 14 contain a nonfusion GST protein. DNA-protein complexes are detected for each of the four labeled DNA sequences after addition of the GST/Ci-Bra fusion protein (lanes 3, 7, 11 and 15). The GST/Ci-Bra fusion protein appears to form two distinct complexes with the mouse sequence. These might correspond to the binding of Ci-Bra monomers and dimers (unfilled and filled arrowheads to the right of the autoradiogram). Smaller complexes are observed upon addition of 0.5U of the thrombin protease, which removes the GST moiety from the fusion protein (lanes 4, 8, 12 and 16). Single, possibly monomeric, complexes were observed for the distal element in the 114 bp Ci-trop enhancer (lane 7) and Ci-Bra sequence #3 (lane 15). These complexes are reduced in size upon addition of thrombin (lanes 8 and 16). The proximal binding site appears to bind a dimeric form of the GST/Ci-Bra fusion protein (lane 11) and the cleaved Ci-Bra peptide (lane 12). (D) Gel-shift assays were done with a GST/Ci-Bra fusion protein and ^{32}P-labeled DNA fragments containing either the distal or proximal binding sites in the 114 bp enhancer. As seen in C, the distal DNA sequence forms a small, putative monomeric complex with the fusion protein (lane 2; see unfilled arrowhead to the left of the autoradiogram). A weaker, larger complex is also observed (filled arrowhead); this latter complex might contain a dimeric form of the fusion protein. The shifted complexes are dminished upon addition of increasing amounts of unlabeled Ci-Bra Distal DNA (lanes 3 and 4) and unlabeled Ci-Bra Proximal DNA (lanes 5 and 6). However, the addition of either a mutant form of the distal sequence containing mutations in both half-sites (DistMUT, lanes 7 and 8), or a competitor sequence containing a putative HNF3β binding site (HNF3W, lanes 9 and 10) does not eliminate the complex. The Ci-Bra Proximal sequence exhibits a single shifted complex (lane 12) that appears to coincide with the upper portion of the Ci-Bra Distal band (lane 2, left panel). Addition of excess unlabeled proximal and distal sequences eliminates the Proximal/Ci-Bra shifted complex (lanes 3-6). However, neither a mutant form of the mouse palindromic sequence (MouseMUT, lanes 17 and 18) nor the HNF3W sequence (lanes 19 and 20) block complex formation when used as competitors. In all cases, the unlabeled competitor DNAs were added in 5x and 50x molar excess. The DistMUT point mutations do not impair the notochord-specific expression of an otherwise normal *Ci-trop/Ci-fkh* fusion promoter (see Fig. 6C). However, subsequent studies revealed a cryptic Ci-Bra site in the plasmid vector that was used in these experiments (data not shown).

at least two sets of low-affinity Ci-Bra half-sites. Deletion of the distal sites eliminates the notochord activity of an otherwise normal *Ci-trop/lacZ* fusion gene. These observations suggest that *Ci-trop* is a direct target gene of the Ci-Bra regulatory protein. An implication of this study is that Brachyury directly influences the cellular morphogenesis of the notochord through the regulation of cytoskeletal genes such as *Ci-trop*.

Regulation of the *Ci-trop* gene

Ci-trop expression is activated during gastrulation, shortly after the first detection of *Ci-Bra* gene products. High levels of expression persist throughout the period of notochord differentiation during neurulation and tailbud stages. DNA binding assays provide evidence for a direct regulatory interaction between Ci-Bra and *Ci-trop*. In particular, the 114 bp *Ci-trop* notochord enhancer contains at least two Ci-Bra recognition sequences (see Fig. 7). The distal sequence is composed of two low-affinity half-sites arranged in tandem, while the proximal sequence contains two half-sites in inverted orientation (see Fig. 7). A 22 bp deletion that removes the distal sequence eliminates the notochord-specific expression directed by an otherwise normal *Ci-trop/lacZ* fusion gene (Fig. 5). This result provides evidence that Ci-Bra directly activates *Ci-trop*. However, point mutations that eliminate binding to the GST/Ci-Bra fusion protein in vitro ('DistMUT', see Fig. 7D) do not impair the notochord-specific expression of the *lacZ* reporter gene in vivo (data not shown). The minimal enhancer appears to contain a large number of low-affinity half-sites, thereby complicating our efforts to mutagenize individual sites. Because a deletion of the distal Ci-Bra sites produces a more severe effect than point mutations it is conceivable that additional regulatory factors work in concert with Ci-Bra to regulate *Ci-trop* expression in the notochord. These factors might bind to sequences that overlap or are immediately adjacent to the distal Ci-Bra sites.

The organization of the 114 bp enhancer might permit 'all or none' regulation of *Ci-trop*. We have presented evidence that the enhancer contains two different classes of Ci-Bra recognition sequences, which appear to bind monomeric (distal) and dimeric (proximal) forms of the protein. Perhaps low levels of Ci-Bra above a minimal threshold cooperatively interact with these recognition sequences, thereby ensuring high levels of *Ci-trop* expression in response to changing levels of the Ci-Bra regulatory protein. This situation appears to be distinct from the regulation of the *Xenopus eFGF* gene by *Xenopus* Brachyury. In this case, a single Brachyury half-site was identified as playing a critical role in *eFGF* regulation (Casey et al., 1998). Perhaps only high levels of X-Bra are sufficient to regulate *eFGF*, and there may be more of a linear dose response between the levels of X-Bra and *eFGF* expression.

In situ hybridization assays suggest that *Ci-trop* is expressed exclusively in the notochord (see Fig. 3). However, a number of the *Ci-trop/lacZ* fusion genes that were analyzed exhibit expression in epidermal cells of the tail. It is possible that the *Ci-trop* regulatory region includes repressor elements, which normally exclude expression in the epidermis. Perhaps these repressor elements are missing in the 3 kb 5′-regulatory region that was analyzed in this study. Alternatively, it is conceivable that *Ci-trop* is normally transcribed in the epidermis, but the RNAs may be unstable. *Ci-trop/lacZ* fusion genes might exhibit expression in the epidermis due to enhanced stability of *lacZ* mRNAs.

Positioning *Ci-trop* in the notochord gene network

Previous studies have shown that ectopic expression of *Ci-Bra* results in at least a partial transformation of endodermal and neuroectodermal tissues into notochord. This transformation correlates with the ectopic activation of *Ci-trop* and other putative Ci-Bra notochord-specific target genes in heterologous tissues (Takahashi et al., 1999). These observations, along with the analysis of the *Ci-trop* 5′-regulatory region, provide both *trans* and *cis* evidence for a tight correlation between *Ci-Bra* and *Ci-trop* expression in the notochord. An implication of these studies is that vertebrate Brachyury genes might influence the cellular morphogenesis of the notochord through the direct regulation of cytoskeletal genes. In contrast, a number of developmental transcription factors appear to function indirectly by influencing the expression of other regulatory genes or signaling pathways. For example, *Hox* genes indirectly influence the morphogenesis of the *Drosophila* midgut through the regulation of signaling molecules such as Dpp and Wingless (reviewed by Bienz, 1997), while the Dorsal gradient initiates the differentiation of the *Drosophila* mesoderm through the regulation of transcription factors such as Twist and Snail (reviewed by Rusch and Levine, 1996). Recent subtractive hybridization screens identified a number of 'cellular morphogenesis' genes as potential targets of Ci-Bra. These include *collagen 2A1* and *Ezrin* in addition to *Ci-trop*. It will be interesting to determine whether these are also directly regulated by Ci-Bra. The *Ciona Ezrin* (*Ci-ERM*) gene exhibits a late onset of expression as compared with *Ci-trop* (Takahashi et al., 1999). It is conceivable that these distinct temporal patterns depend on different arrangements or affinities of Ci-Bra binding sites in the *Ci-trop* and *Ci-ERM* promoter regions.

We thank Dr Russell Doolittle for the Ci-trop sequence analysis presented in Fig. 2, and Dr Tom Meedel for helpful suggestions. We thank Drs Yutaka Nibu and Dave Keys for help in preparing figures and Albert Erives for help in sequence analysis. This work was supported by a grant from the NSF (IBN-9514138).

REFERENCES

Bienz, M. (1997). Endoderm induction in *Drosophila*: the nuclear targets of the inducing signals. *Curr. Opin. Genet. Dev.* **7**, 683-688.

Bikle, D. D., Munson, S., Morrison, N. and Eisman J. (1993). Zipper protein, a newly described tropomyosin-like protein of the intestinal brush border. *J. Biol. Chem.* **268**, 620-626.

Casey, E. S., O'Reilly, M. A., Conlon, F. L. and Smith, J. C. (1998). The T-box transcription factor Brachyury regulates expression of *eFGF* through binding to a non-palindromic response element. *Development* **125**, 3887-3894.

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

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

Cunliffe, V. and Smith, J. C. (1994). Specification of mesodermal pattern in *Xenopus laevis* by interactions between *Brachyury*, *noggin* and *Xwnt-8*. *EMBO J*. **13**, 349-359.

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

Goodwin, L. O., Lees-Miller, J. P., Leonard, M. A., Cheley, S. B. and Helfman, D. M. (1991). Four fibroblast tropomyosin isoforms are expressed from the rat alpha-tropomyosin gene via alternative RNA splicing and the use of two promoters. *J. Biol. Chem*. **266**, 8408-8415.

Guan, K. L. and Dixon, J. E. (1991). Eukaryotic proteins expressed in *Escherichia coli*: an improved thrombin cleavage and purification procedure of fusion proteins with glutathione *S*-transferase. *Anal. Biochem.* **192**, 262-267.

Kispert, A. and Herrmann, B. G. (1993). The *Brachyury* gene encodes a novel DNA binding protein. *EMBO J*. **12**, 3211-3220.

Kispert, A., Koschorz, B. and Herrmann B. G. (1995). The T protein encoded by *Brachyury* is a tissue-specific transcription factor. *EMBO J*. **14**, 4763-4772.

MacLeod, A. R., Talbot, K., Smillie, L. B. and Houlker, C. (1987). Characterization of a cDNA defining a gene family encoding *TM30p1*, a human fibroblast tropomyosin. *J. Mol. Biol*. **194**, 1-10.

Meedel, T. H. and Hastings, K. E. (1993). Striated muscle-type tropomyosin in a chordate smooth muscle, ascidian body-wall muscle. *J. Biol. Chem*. **268**, 6755-6764.

Overdier, D. G., Porcella, A. and Costa, R. H. (1994). The DNA-binding specificity of the hepatocyte nuclear factor 3/forkhead domain is influenced by amino acid residues adjacent to the recognition helix. *Mol. Cell Biol.* **14**, 2755-2766.

Pittenger, M. F., Kazzaz, J. A. and Helfman, D. M. (1994). Functional properties of non-muscle tropomyosin isoforms. *Curr. Opin. Cell Biol*. **6**, 96-104.

Rusch, J. and Levine, M. (1996). Threshold responses to the Dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. *Curr. Opin. Genet. Dev*. **6**, 416-423.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. New York, Plainview: Cold Spring Harbor Laboratory Press.

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

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

Stott, D., Kispert, A. and Herrmann, B. G. (1993). Rescue of the tail defect of *Brachyury* mice. *Genes Dev*. **7**, 197-203.

Tada, M., Casey, E. S., Fairclough, L. and Smith, J. C. (1998). *Bix1*, a direct target of *Xenopus* T-box genes, causes formation of ventral mesoderm and endoderm. *Development* **125**, 3997-4006.

Takahashi, H., Hotta, K., Erives, A., Di Gregorio, A., Zeller, R. W., Levine, M. and Satoh, N. (1999). *Brachyury* downstream notochord differentiation in the ascidian embryo. *Genes Dev*. **13**, 1519-1523.

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

Yasuo, H. and Satoh, N. (1998). Conservation of the developmental role of *Brachyury* in notochord formation in a urochordate, the ascidian *Halocynthia roretzi*. *Dev. Biol*. **15**, 158-170.