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Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*

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SUMMARY

We present evidence that the embryo of the ascidian, *Ciona intestinalis*, is an easily manipulated system for investigating the establishment of basic chordate tissues and organs. *Ciona* has a small genome, and simple, well-defined embryonic lineages. Here, we examine the regulatory mechanisms underlying the differentiation of the notochord. Particular efforts center on the regulation of a notochord-specific *Ciona Brachyury* gene (*Ci-Bra*). An electroporation method was devised for the efficient incorporation of transgenic DNA into *Ciona* embryos. This method permitted the identification of a minimal, 434 bp enhancer from the *Ci-Bra* promoter region that mediates the notochord-restricted expression of both GFP and *lacZ* reporter genes. This enhancer contains a negative control region that excludes *Ci-Bra* expression from inappropriate embryonic lineages, including the trunk mesenchyme and tail muscles. Evidence is presented that the enhancer is activated by a regulatory element which is closely related to the recognition sequence of the *Suppressor of Hairless* transcription factor, thereby raising the possibility that the *Notch* signaling pathway plays a role in notochord differentiation. We discuss the implications of this analysis with regard to the evolutionary conservation of integrative enhancers, and the subdivision of the axial and paraxial mesoderm in vertebrates.

Key words: *Ciona intestinalis*, *Brachyury*, cell lineage, *Suppressor of Hairless*, transcription factor, notochord

INTRODUCTION

Enhancers integrate both positive and negative regulatory information to direct localized patterns of gene expression in the *Drosophila* embryo (reviewed by Gray and Levine, 1996). Integrative enhancers are typically 300-500 bp in length and contain tightly clustered binding sites for both transcriptional activators and repressors (e.g., Small et al., 1992; Hoch et al., 1992; Ip et al., 1992). In many instances, enhancers convert crude gradients of positional information into sharp, on/off patterns of expression. For example, the 500 bp stripe 2 enhancer from the promoter region of the *even-skipped* (*eve*) gene directs a sharp segmentation stripe of expression in response to the maternal *bicoid* gradient (Small et al., 1991; 1992). In principle, the stripe 2 enhancer can be activated in nearly the entire anterior half of the *Drosophila* embryo by multiple *bicoid* activator sites. However, spatially localized repressors establish both the anterior and posterior borders of the stripe (Stanojevic et al., 1991; Small et al., 1992). Studies of this kind have demonstrated the importance of integrative enhancers and spatially localized repressors in the specification of cell fate.

We have attempted to determine whether similar principles apply to chordate embryos. Although transgenic mice offer the possibility of detailed dissections of gene regulatory regions, the large investment of time and resources required for such studies has resulted in relatively few detailed characterizations of embryonic enhancers (e.g., Rhodes et al., 1994; Popperl et al., 1995). Transient expression assays have been used in both zebrafish and *Xenopus* embryos, whereby fusion genes are introduced into early cleavage-stage embryos via microinjection (e.g., Watabe et al., 1995). However, these studies have been hampered by mosaic incorporation of transgenic DNA on account of the numerous rounds of cleavage that intervene between fertilization and the establishment of basic embryonic tissues. In the present study we present evidence that the tunicate, *Ciona intestinalis*, provides a simple system for the analysis of embryonic enhancers.

There are several arguments that favor the use of *Ciona* for this type of analysis (e.g., Swalla, 1993; Nakatani and Nishida, 1994; Satoh and Jeffery, 1995; Kusakabe et al., 1995). First, *Ciona* is a chordate and so possesses a notochord and a dorsal hollow neural tube. Indeed, some believe that the ascidian tadpole represents a prototypical chordate (Garstang, 1928; Berrill, 1955). Second, *Ciona* has a small, compact genome. At 1.8×10^8 bp/haploid genome it is comparable in size to the genome of *Drosophila* (Lambert and Laird, 1971; Satoh, 1994). Consequently, it is relatively easy to isolate specific genes and associated regulatory regions. Third, *Ciona* has simple, well-defined embryonic lineages (Satoh, 1994). In fact, there are only 36 tail muscle cells and 40 notochord cells in the mature tadpole making it possible to follow individual blas-

tomeres throughout development. Finally, *Ciona* development is quite rapid, and the mature tadpole hatches from the chorion 18 hours after fertilization.

In the present study we investigate the *cis*-regulatory mechanisms underlying the differentiation of the notochord. Previous studies have identified a number of transcription factors important for chordogenesis in vertebrates. These include a mouse *forkhead* homolog, HNF-3β, the zebrafish *floating head* homeobox gene, and the mouse *Brachyury* gene (Herrmann et al., 1990; Ang and Rossant, 1994; Weinstein et al., 1994; Talbot et al., 1995). Both *Brachyury* and HNF-3β are known to be sequence-specific transcription factors (Kispert and Herrmann, 1993; Clark et al., 1993) and *floating head* is also thought to bind DNA on account of its homeodomain. Mutations in these genes cause severe disruptions in the specification or terminal differentiation of the notochord. Studies in *Xenopus* have implicated a number of signaling molecules in notochord induction or maintenance, including FGF and activin (Slack et al., 1987; Amaya et al., 1991; Isaacs et al., 1994). Recent studies suggest that FGF may also participate in the specification of the notochord lineage in the ascidian, *Halocynthia roretzi* (Nakatani et al., 1996).

Despite these numerous efforts, there is very little information concerning the nature of the *cis*-regulatory elements that mediate notochord-specific patterns of gene expression in any embryo. For example, *Brachyury* homologs have been implicated in notochord differentiation in a variety of vertebrates, including zebrafish, *Xenopus*, chick and mouse (Herrmann et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1994; Kispert et al., 1995). Surprisingly, notochord-specific regulatory elements have not been identified for any of these genes. In fact, the only notochord-specific enhancer that has been reported is associated with the mouse HNF-3β gene, which is expressed in the notochord, floor plate, and endoderm of mouse embryos (Sasaki and Hogan, 1993). The minimal notochord-specific enhancer is 520 bp in length and maps ~15 kb upstream of the HNF-3β transcription start site (Sasaki and Hogan, 1996).

Here we provide a detailed analysis of a notochord-specific enhancer from the *Brachyury* promoter region of *Ciona intestinalis*. On account of the rapidity of *Ciona* development and the ease of introducing transgenes into embryos via electroporation, simple DNA cloning procedures are the rate-limiting step in enhancer analysis. The characterization of a number of different *Brachyury-lacZ* fusion genes resulted in the identification of a 434 bp enhancer that mediates notochord-specific expression of *Ciona Brachyury* (*Ci-Bra*). Truncations of this minimal enhancer lead to ectopic expression in muscle and other tissues. These abnormal sites of expression correspond to sister lineages of the notochord that normally lack *Ci-Bra* expression. Thus, it would appear that one or more repressors are required to restrict *Ci-Bra* expression to the developing notochord. In addition, a potential activator element, containing a close match to a *Notch* response element, was also identified. We discuss these results with regard to integrative enhancers and the role of tissue-specific repressors in the subdivision of vertebrate mesoderm lineages.

MATERIALS AND METHODS

Collection of animals

Adult *Ciona intestinalis* were collected from several locations in San Diego and Los Angeles counties. Adults were maintained in a natural sea water aquarium at 18°C under constant light to prevent the spawning of eggs. To fertilize eggs, gametes from two or more individuals were mixed for 1.5-2 minutes, then excess sperm was removed. Embryos were cultured at 18°C and tadpole larvae began to hatch about 18 hours after fertilization (Whittaker, 1973).

In situ hybridization

Embryos were fixed in fresh 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS pH 7.5 at room temperature for 30 minutes then transferred to ethanol (100%) and washed 8 times before storage at 4°C. Upon beginning the procedure the embryos were washed twice more with ethanol, once with xylene/ethanol (50:50; vol:vol) then once in xylene/ethanol (75:25). They were then soaked in xylene/ethanol (75:25) for 1 hour with occasional rocking. Next, they were washed once with xylene/ethanol (50:50), five times with ethanol, and twice with methanol. The embryos were incubated with rocking for 5 minutes in methanol/PBT-2% paraformaldehyde (50:50; vol:vol) (PBT-2% paraformaldehyde contains 50% of the 4% paraformaldehyde fixation solution in 1× PBS and 1% Tween-80), then they were incubated with rocking for 25 minutes in PBT-2% paraformaldehyde. Following this they were then washed five times with PBT (1% Tween-80 in 1× PBS). Next the embryos were incubated for 5 minutes with rocking in PBT + 4 μg/ml non-predigested proteinase K, then washed twice quickly with PBT and four more times slowly. The embryos were then post-fixed again in PBT-2% paraformaldehyde for 25 minutes followed by five washes with PBT. Next, the embryos were rocked for 5 minutes in PBT/hybridization solution (hyb solution; 50:50; vol:vol) (hybridization solution: 50% formamide, 5× SSC, 100 μg/ml salmon sperm DNA, 50 μg/ml heparin, 0.1 % Tween-80), then washed for 5 minutes in hyb solution. They were then prehybridized for 1.5 hours in hyb solution at 55°C in a volume of 1 ml being inverted three times during this period. Next, the RNA antisense probe was prepared by adding 0.5 μl of probe to 50 μl of hyb solution, heating it to 80°C for 3 minutes, then placing it on ice. (RNA antisense probe was prepared essentially according to the instructions accompanying the Boehringer-Mannheim RNA labelling kit. In brief, the *Ci-Bra* cDNA was linearized at the 5′ end, and transcribed with T7 RNA polymerase. The probe was partially degraded by alkaline hydrolysis, precipitated, and resuspended in 50 μl of hyb solution). Next, as much prehybridization solution was removed as possible. Then the hyb solution containing the RNA probe was added. The tube was flicked to mix the embryos completely with the probe and then allowed to hybridize at least 18 hours at 55°C with occasional flicking of the tube to mix the probe. Next, the embryos were washed ten times for 15 minutes each in hyb solution at 55°C. They were then washed in hyb solution/PBT (75:25), (50:50), and (25:75) for 15 minutes each with rocking at room temperature. After this, they were washed five times for 10 minutes each in PBT. Next, the embryos were incubated in 0.5 ml of PBT + anti-digoxigenin antibody at a final dilution of 1:2000 (from Boehringer-Mannheim stock solution) with rocking overnight at 4°C. The next day, the embryos were washed four times 20 minutes each in PBT. Then they were rinsed once in AP staining buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5; 0.1% Tween-80), and then washed twice for 5 minutes each in AP staining buffer. Immediately before staining, 9 μl of NBT (75 mg/ml in 70% DMF) and 7 μl of BCIP (50 mg/ml in DMF) were added to 1 ml of AP staining buffer and mixed well. The last wash was drained from the embryos and 400 μl of AP/NBT/BCIP solution was added. The embryos were then transferred in this solution to a staining dish. The staining reaction was stopped after 2 hours by transferring embryos to an eppendorf tube containing 800 μl of PBT. They were then washed once in PBT/ethanol (50:50), and subsequently dehydrated by fourteen 5-minute washes in 100% ethanol. After one quick rinse in 100% xylene the embryos were mounted on glass slides with permount and photographed.

DNA sequence analysis

Nested deletions of both strands of the minimal 434 bp *Ci-Bra* enhancer element shown in Fig. 5B were generated by exonuclease III deletion and sequenced by standard dideoxy chain termination methods (Sambrook et al., 1989). Computer analysis of the sequence was conducted using the DNA strider and MacVector programs.

Injection constructs

The basic injection vector was derived from pPD1.27 (Fire et al., 1990). This vector was linearized with *Not*I, end filled with the Klenow fragment of DNA polymerase and then cut with *Hin*dIII. This *Hin*dIII, blunt-ended fragment was then ligated to pSP72 (Promega) at the *Hin*dIII/*Eco*RV sites to make 72-1.27. This vector, 72-1.27, contains, from 5′ to 3′, a small polylinker, a nuclear localization sequence (NLS), the coding region for *lacZ* and the SV40 polyadenylation sequence. The SV40 NLS localizes *lacZ* to the nucleus as described by Fire et al. (1990). All injection constructs were further derivatives of this parent vector. The *Ci-Bra* constructs were made in the following manner: the 3.5 kb *Ci-Bra* genomic fragment was cloned into bluescript SK+ II (Stratagene) as a *Hin*dIII-*Mun*I fragment (into *Hin*dIII, *Eco*RI) (this *Mun*I site is at codon 17, see Fig. 5B). It was then removed as a *Hin*dIII-*Pst*I fragment and cloned into a derivative of 72-1.27 containing a blunt *Sal*I site to give the –3.5 kb construct. The –790 bp construct was made by digesting the 3.5 kb construct with *Eco*RI and *Pst*I, sub-cloning into an intermediate vector, then sub-cloning as an *Xho*I-*Pst*I fragment into 72-1.27. Most other *Ci-Bra* constructs were made from successive 5′ deletions of the 790 bp fragment generated by exonuclease III digestion (according to manufacturer's instructions; Promega). The exceptions were the –299 bp and –250 bp constructs which were cloned as *Ase*I-*Pst*I and *Ssp*I-*Pst*I fragments,respectively, into 72-1.27 with blunt *Sal*I. The –434 bp w/o triple repeat and the –250 bp w/o *Su(H)* constructs were made by site-directed mutagenesis on the constructs –434 bp and –250 bp using the following oligonucleotides, respectively: 5′-TTAAAATTCATAATTACAAAACGACAAACCTTATCTG-3′, 5′-GGAAACCAAGTTTCAAGTAACACGTCACAATACA-3′ (underlining indicates the nucleotides between which the deletion was made). The muscle-specific actin enhancer used in Fig. 3 was cloned by PCR from genomic DNA isolated from body-wall muscle of a *Halocynthia* sp. purchased at a local Japanese fish market using the following oligonucleotides: 5′-CGGGATCCTACAGACAT-GAAAATCATTATCCG-3′ and 5′-CGGGATCCGTATCTTCTTCAC-CATCAGACAT-3′. the PCR primers were based on sequences published in Hikosaka et al. (1993) and generated a product containing approximately 560 bp upstream of the transcriptional start site and the first eight amino acids of the coding sequence. It was cloned as a *Bam*HI fragment into 72-1.72. The *Ci-Bra* –790 bp on the actin basal enhancer (bottom construct in Fig. 5A) was made as follows: an actin basal enhancer was generated by PCR on *Halocynthia* sp. genomic DNA using the primers: 5′-CGGGATCCCATTTTTTCCGCTTTC-CTCCGTTTC-3′ and the second primer listed above for the full-length actin enhancer. This product was cloned as a *Bam*HI fragment into 72-1.72. A PCR product including –790 bp to –1 bp upstream of the TATA box from *Ci-Bra* was generated using the T7 primer from the polylinker of the bluescript SK+ II vector and the primer, 5′-GGC-CAAGCTTCAGGATTGTGACGTCATAACTTC-3′. This product was then cloned as a *Hin*dIII fragment upstream of the actin basal promoter in 72-1.72.

GFP mutants

The coding regions for wild-type green fluorescent protein (wtGFP) and P4-3 GFP (a blue light emitting mutant) were obtained from Roger Tsien at UCSD. 5′ *Kpn*I and 3′ *Eco*RI sites were added to all GFPs by PCR and exchanged for the *Kpn*I-*Eco*RI fragment containing the β-galactosidase coding sequence in 72-1.72 to generate the GFP injection constructs. The wtGFP coding region was randomly mutagenized by PCR (Heim et al., 1994) to make a single point mutation (Q81R) which we found to fluoresce more brightly in ascidian embryos than wtGFP. This Q81R mutant was used in Fig. 6. A second GFP mutant, eGFP (Clonetech) was used in the actin enhancer fusion shown in Fig. 3. eGFP has a double mutation (F64L and S65T) and is about 35-fold brighter than wtGFP (Cormack et al., 1996). A brighter blue emitting GFP was generated from P4-3 by the method described by Crameri and colleagues (Crameri et al., 1996). This was used in the *Ci-Bra* –3.5 kb fusion construct shown in Fig. 3.

Electroporation

The electroporation unit was a BioRad Gene Pulser with a capacitance extender. The settings for the machine were 125 V/cm, and capacitance setting of either 500 or 960 μF. The capacitance was adjusted so that the time constant of the pulse was about 20 mseconds. 0.4 cm cuvettes were used and held a total volume of 800 μl. The electroporation medium, 0.77 M mannitol, was first used by Nishida (1992) for egg fragment fusions. The brief protocol is as follows. Circular plasmid DNA was dissolved in 0.77 M mannitol in a total volume of about 500 μl at a concentration of 20-100 μg/ml. Fertilized eggs were dechorionated with 1% sodium thioglycolate and 0.05% protease E as described by Mita-Miyazawa et al. (1985) and then thoroughly washed in clean sea water. About 200-300 μl of eggs in sea water were added to the 500 μl of DNA/mannitol solution in a small dish and mixed. The DNA/eggs were immediately transferred to a cuvette. After the current pulse, the eggs were returned to clean sea water and reared at 18°C until the desired time point. A typical round of electroporation yielded hundreds of embryos expressing the transgene. Multiple rounds of electroporation were carried out on each transgene used in this work.

Microinjection apparatus

Linearized plasmid DNA was microinjected into fertilized eggs. The injection solution contained 20% glycerol, 100 mM KCl and DNA (100-300 μg/ml). A small chamber was made out of coverslip pieces held together by doublestick tape and fixed to an aluminum microscope slide holder (Kiehart, 1982). Fertilized eggs were placed within these chambers and then microinjected. Injected embryos were reared at 18°C.

RESULTS

The complete *Ciona* notochord lineage is summarized in Fig. 1. By the early tailbud stage, about 9 hours postfertilization, the notochord is composed of 40 cells that are arrayed in a single column spanning the length of the tail (Fig. 1H). These cells arise from two different lineages (Nishida, 1987). The primary lineage (in dark blue) is derived from the A4.1 blastomeres at the 8 cell stage (Fig. 1A) (Satoh, 1994); these specify the anterior-most 32 cells of the notochord. The remaining 8 cells, constituting the secondary lineage (in light blue), arise from the B4.1 blastomeres (Satoh, 1994). Recent studies suggest that an inductive interaction between presumptive endoderm cells and presumptive notochord cells occurs at the 32-cell stage (Fig. 1C) which triggers the differentiation of the primary lineage of the notochord (Nakatani and Nishida, 1994).

The primary, A-lineage becomes clonally restricted at the 64-cell stage (Fig. 1D), whereby all of the descendants of the A7.3 and A7.7 blastomeres form only notochord. In contrast, the secondary, B-lineage is not yet restricted at this stage. The B7.3 progenitor undergoes one cleavage prior to clonal restriction; one of its descendants corresponds to a notochord

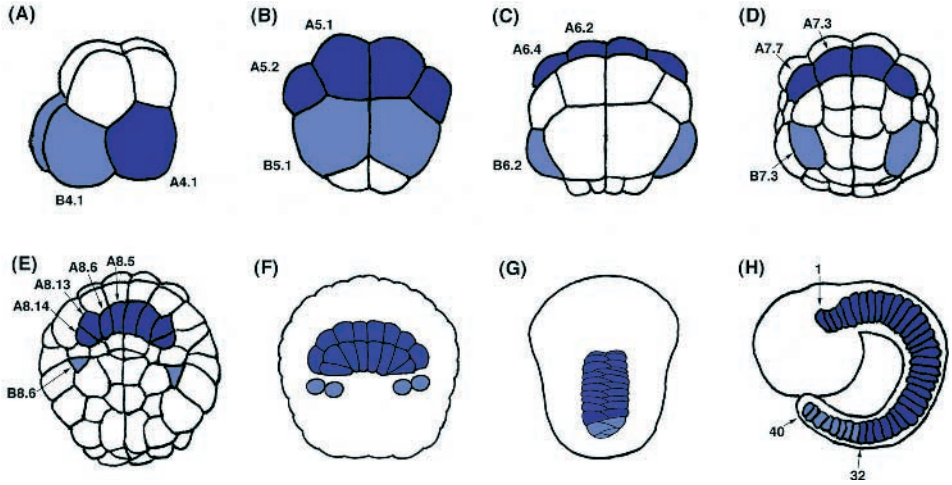

Fig. 1. Summary of notochord lineages. The drawings represent various stages of embryogenesis, beginning with an 8-cell embryo (A). The primary notochord lineage is established by the A4.1 blastomere (dark blue shading), while the secondary lineage is generated by B4.1 (light blue). The number immediately following the letter (in this case '4') refers to the cleavage cycle starting with the one-cell, fertilized egg. The drawing in B represents a 16-cell embryo, while that in C is at the 32-cell stage. The primary lineage notochord cells are first clonally restricted at the 64-cell stage (D). However, at this time the secondary lineage precursor, B7.3, is not yet clonally restricted and gives rise to both mesenchyme and notochord cells. Clonal restriction of the secondary lineage occurs at the 110-cell stage (E). F and G are simplified drawings of gastrulating and neurulating embryos, respectively. Intercalation of the notochord cells occurs during neurulation and culminates in the formation of a notochord composed of a single column of cells (H). These drawings were adapted from Satoh (1994).

precursor cell (B8.6), while the daughter cell, B8.5, is a progenitor of the mesenchyme (Satoh, 1994). The primary lineage undergoes a round of cleavage at the onset of gastrulation (Fig. 1E), and both lineages divide toward the end of gastrulation (Fig. 1F). The last division occurs during neurulation to give a total of 40 cells (Fig. 1G). During late gastrulation and neurulation the notochord cells undergo medial convergence, intercalation, and extension in the anterior-posterior axis ultimately giving rise to a single-file column of cells in the early tail-bud embryo (Fig. 1E-H) (van Beneden and Julin, 1886; Castle,

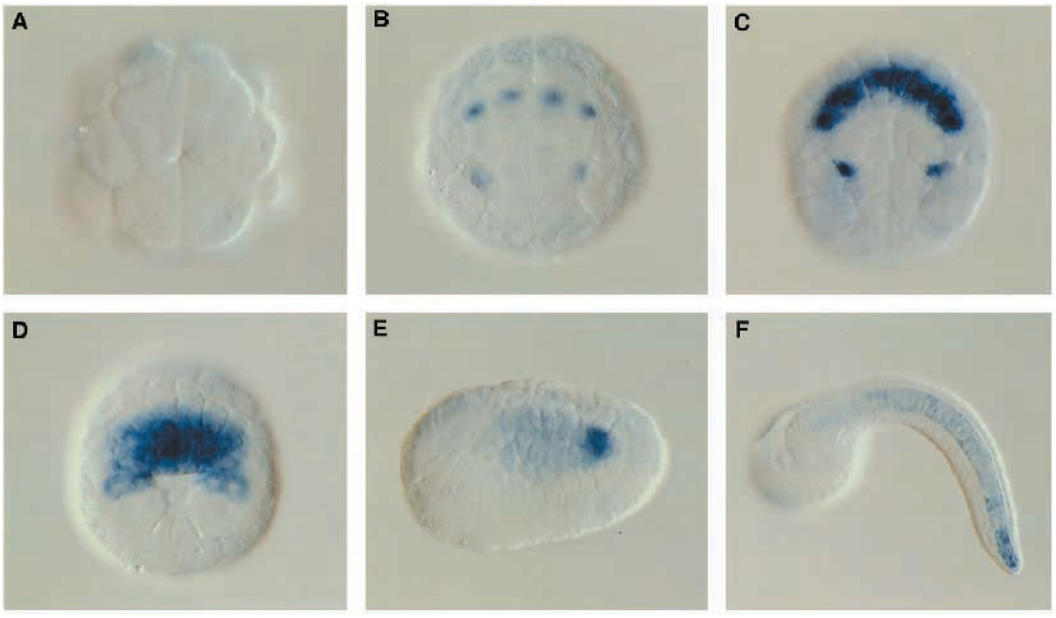

Fig. 2. In situ localization of *Ci-Bra* transcripts during embryogenesis. Whole-mount preparations of staged *Ciona* embryos were hybridized with a digoxigenin-labeled *Ci-Bra* antisense RNA probe, and stained with an alkaline phosphatase conjugated anti-digoxigenin antibody. The embryos in A-D are oriented to display the vegetal blastomeres, and the dorsal surface is up. (A) 32-cell embryo. No staining is detected above background levels. (B) 64-cell embryo. Staining is detected in the nuclei of primary lineage notochord precursor cells, and in the B7.3 blastomere. This is the time when the primary lineage cells are first clonally restricted to form the notochord. The hybridization signals are primarily concentrated in the nuclei of the stained cells. (C) 110-cell embryo. Staining is detected in the primary lineage (A8.5, A8.6, A8.13, and A8.14), as well as the B8.6 secondary lineage precursor cell, which is now clonally restricted to form notochord. The bulk of the hybridization signals persists in the nuclei of the stained cells. (D) Late gastrula (approx. 6 hours postfertilization). Staining persists in the notochord precursor cells in both the primary and secondary lineages. By this time, both groups of cells have divided, so that there are 16 primary lineage cells and four secondary lineage cells. The blastopore can be seen just beneath the stained primary lineage cells. By this time the hybridization signals are detected primarily in the cytoplasm of the stained cells. (E) Neurula (approx. 8 hours postfertilization). The invaginated notochord cells begin to align along the anteroposterior axis. Staining is diminished in the primary lineage cells, but persists at relatively high levels in the secondary lineage cells which are now located near the posterior pole. The 20 notochord cells undergo their final division at about this time. (F) Mid-tailbud embryo (approx. 12 hours postfertilization). The notochord cells have completed intercalation, and are arrayed as a single column of cells extending along the length of the tail. Staining persists in the cytoplasmic regions of the individual notochord cells, but the level of expression continues to remain at higher levels in the posterior, secondary lineage cells.

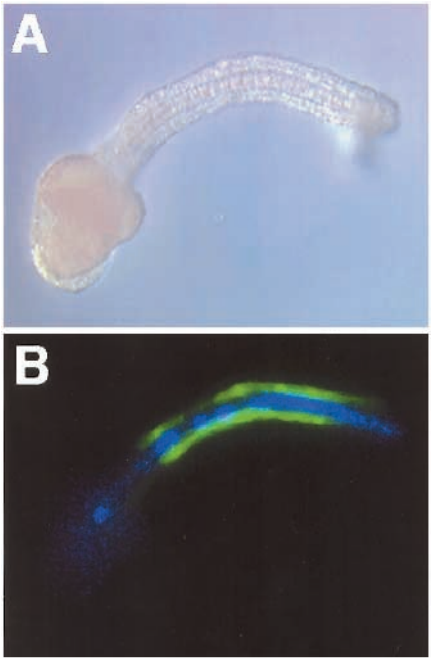

Fig. 3. Co-electroporation of transgenic DNA constructs. A one-cell embryo was electroporated in a solution containing two different transgenic DNA constructs, and then grown for about 12 hours (mid-tailbud stage) prior to photography. One of these constructs contains a 3.5 kb fragment from the *Ci-Bra* promoter region attached to a mutant form of GFP, which emits blue light. This *Ci-Bra* transgene is specifically expressed in the central notochord cells of the tail. The other transgenic DNA contains a 560 bp fragment from a *Halocynthia* muscle-specific actin gene attached to a GFP reporter that emits green light. This actin-GFP transgene is specifically expressed in the tail muscles. The transgenes appear to be incorporated in about 25-50% of the embryonic blastomeres. (A) Nomarksi photograph of the embryo. The notochord can be seen as a central, column of cells that run along the length of the tail. (B) Fluorescence photomicrograph of the embryo in A. The image is a fusion of the blue channel from a photograph taken with a DAPI filter set and the green channel from a photograph taken with a fluorescein filter set.

1896; Conklin, 1905; Cloney, 1964; Mancuso and Dolcemascolo, 1977; Miyamoto and Crowther, 1985).

Ci-Bra expression pattern

A 640 bp DNA fragment from the zebrafish *Brachyury* homolog, *no tail*, which contains most of the coding sequence of the conserved DNA binding domain, was used to screen a *Ciona* genomic DNA library (Schulte-Merker et al., 1994). Both *Ciona* genomic clones and the zebrafish probe were used to isolate a 1.5 kb cDNA from a gastrula-stage *Ciona intestinalis* cDNA library (kindly provided by Drs Tom Meedel and Jamie Lee). The cDNA appears to be full-length, based on size estimation from northern blot analysis (data not shown). Sequence analysis indicates that the *Ciona* cDNA represents a *bona fide Brachyury* homolog; the DNA binding domain (~200 amino acids in length) shares about 70% amino acid identity with vertebrate *Brachyury* genes and approximately the same with the *Halocynthia* homolog (R. W. Zeller, J. C. Corbo, and M. Levine, unpublished data).

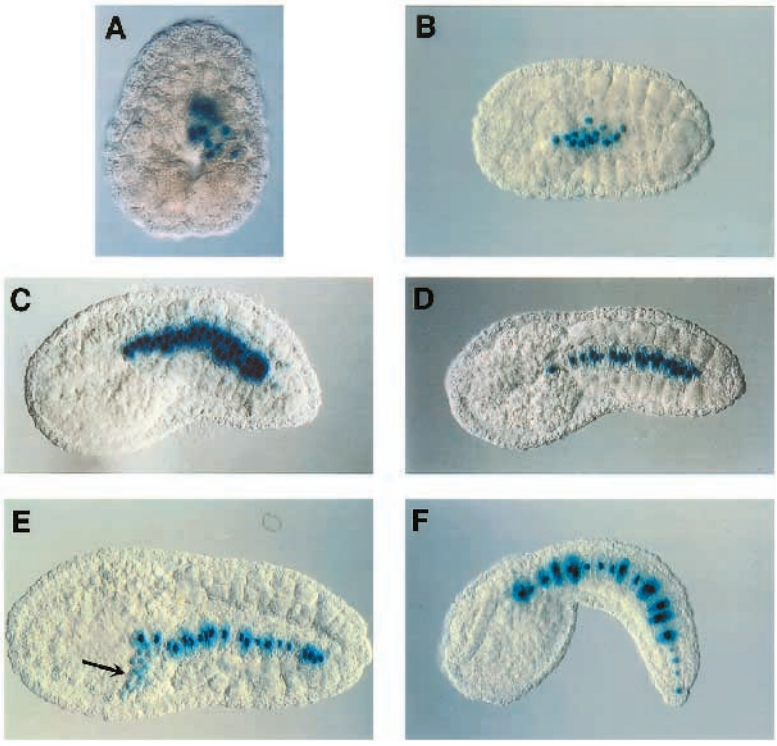

Fig. 4. Notochord differentiation in electroporated embryos. Embryos were electroporated at the one-cell stage with a transgenic DNA construct containing the 3.5 kb *Ci-Bra* promoter driving the expression of a *lac*Z reporter gene containing a nuclear localization signal. Embryos were collected at different times following electroporation and subsequently stained with X-gal to visualize the sites of expression. (A) Vegetal view of late gastrula (approx. 6 hours postfertilization). Both primary and secondary lineage notochord cells are stained in the right half of the embryo (a total of 10 stained cells). The stained cells are beginning to invaginate into the blastocoel. (B) Horizontal view of a neurula (approx. 7-8 hours postfertilization). Most of the staining is detected in the primary lineage cells derived from one side of the embryo. The central cluster of notochord cells is surrounded by a horseshoe-shaped array of muscle precursor cells. (C) Sagittal view of an early tail bud stage embryo (approx. 10 hours postfertilization). The notochord cells are undergoing the process of intercalation, which appears to proceed in an anteroposterior wave. The anterior cells are already arrayed in a single column of cells, while those in more posterior regions have not completed intercalation. (D) Sagittal view of a mid-tail bud stage embryo (approx. 11 hours). Intercalation is now complete, so that the stained notochord cells are arrayed in a single column of cells that run along the length of the tail. (E) Similar to D, except that this embryo is somewhat more flattened. The arrow indicates 'ectopic' staining in the mesenchyme. (F) Sagittal view of a 12 hour tadpole. Half of the primary lineage and secondary lineage notochord cells are stained, although the posterior secondary lineage cells are more lightly stained than the primary lineage cells. Note that stained cells are interspersed with unstained cells.

The 1.5 kb *Ci-Bra* cDNA was used for in situ hybridization assays to identify the spatial and temporal pattern of expression during *Ciona* embryogenesis (Fig. 2). Whole-mount preparations of staged embryos were hybridized with a digoxigenin-labeled *Ci-Bra* antisense RNA probe, as described in Materials and Methods. Specific hybridization signals are not detected prior to the 64-cell stage (e.g., Fig. 2A), which is consistent with northern blot analysis (data not shown). Staining is first detected in the clonally restricted notochord precursors, A7.3 and A7.7, at the 64-cell stage (Fig. 2B). Expression is also observed in a precursor of the secondary lineage, B7.3 (see below).

Staining persists in the notochord precursor cells during gastrulation (Fig. 2C,D). During this time there is a transition in the sub-cellular localization of *Ci-Bra* transcripts. The initial expression is restricted to nuclei (Fig. 2B), and the bulk of the staining persists in nuclei during the next cleavage cycle (Fig. 2C). However, staining is primarily restricted to cytoplasmic regions by late gastrulation (Fig. 2D), suggesting a significant lag between the onset of *Ci-Bra* transcription and the time when *Ci-Bra* is fully active.

Ci-Bra expression peaks during gastrulation, and is significantly reduced by neurulation (compare Fig. 2D,E). Relatively strong staining persists, however, in the posterior-most notochord cells, which arise from the secondary lineage. This asymmetric staining pattern persists in the early tadpole stages (Fig. 2F). The terminal differentiation of secondary lineage notochord cells is somewhat delayed relative to the primary lineage cells (data not shown); it is conceivable that this delay arises from sustained expression of *Ci-Bra* expression in the secondary lineage.

Ci-Bra enhancer analysis in electroporated embryos

Microinjection methods have been used to introduce reporter DNA constructs into *Ciona*, *Halocynthia*, and *Molgula* species (Hikosaka et al., 1992, 1993, 1994; Kusakabe et al., 1995, 1996). However, microinjection is laborious and time-consuming, particularly in the smaller *Ciona* embryo, and often causes gross disruptions in development (data not shown). Consequently, it has been difficult to conduct detailed analyses of enhancer elements. We have attempted to circumvent these problems by using an electroporation protocol to introduce DNA constructs into developing *Ciona* embryos. This involves proteolytic dechorionation of hundreds of fertilized eggs, followed by a brief electrical pulse in a solution containing the DNA construct of interest (see Materials and Methods). The method is extremely efficient and permits the analysis of large numbers of embryos in a short period of time. Moreover, it is substantially less invasive than microinjection. Typically a given round of electroporation will result in more than 50% of the embryos developing normally. Greater than 80% of these embryos express the transgene in 25% or more of their blastomeres.

An example of the usefulness of this method is presented in Fig. 3. In this experiment two different fusions genes were simultaneously electroporated into fertilized, single-cell embryos. The embryos were then allowed to develop to the mid-tail bud stage, mounted live, and photographed. The first construct is a fusion between a 3.5 kb genomic DNA fragment from the region immediately upstream of the *Ci-Bra* coding sequence and a mutant green fluorescent protein which emits blue light (see Materials and Methods). Notice the expression specifically in the mid-line notochord cells. The second construct is a fusion between a previously characterized muscle-specific, actin enhancer element from *Halocynthia* (Hikosaka et al., 1993; see Materials and Methods) and an enhanced green fluorescent protein that emits green light. The two bands of green fluorescence on either side of the notochord represent the tail muscles of the larva. This electroporation protocol allows facile introduction of multiple DNA constructs into embryos thus permitting visualization of several tissue types simultaneously in live embryos.

The 3.5 kb *Ci-Bra* genomic DNA fragment used in the preceding experiment was fused in-frame to a *lac*Z reporter containing a nuclear localization signal (summarized in Fig. 5A). This *Ci-Bra-lac*Z fusion gene was then introduced into embryos via electroporation. Embryos were collected, fixed and stained with X-gal after various periods of growth at 18°C. β-galactosidase activity is first detected in gastrulating embryos, approximately 6 hours after fertilization (Fig. 4A). The embryo in Fig. 4A shows expression in 10 of the 20 notochord precursors present at this stage suggesting that the transgene was retained in one of the two blastomeres at the two-cell stage. With this electroporation protocol, incorporation into 50% or more of the blastomeres is common (data not shown).

Expression of the reporter gene is first detected just two cleavage cycles (approximately 90 minutes) after the endogenous *Ci-Bra* gene is first expressed at the 64-cell stage (Fig. 2B). Given the lag between the beginning of *lac*Z transcription and the synthesis of detectable levels of β-galactosidase, it is likely that the transgene is activated at about the time when the endogenous *Ci-Bra* is first expressed. Thus, it would appear that the 3.5 kb *Ci-Bra* promoter region used in these experiments contains all of the *cis*-regulatory information required for authentic temporal and spatial expression. However, 'ectopic' expression is occasionally seen in mesenchyme cells in the trunk (arrow, Fig. 4E). As discussed below, this expression might reflect perdurance of *Ci-Bra-lac*Z products activated in the B7.3 secondary lineage precursor cell, which specifies both notochord and mesenchyme cells.

Analysis of *Ci-Bra-lac*Z transgene activity in older embryos permits visualization of the convergence and intercalation of the notochord cells which, in part, drive the extension of the tail (Miyamoto and Crowther, 1985). By neurulation (Fig. 4B), the notochord precursor cells have completed their final divisions and have converged toward the mid-line. About 1 hour later, at the early tailbud stage, the notochord cells begin to intercalate (Fig. 4C). It would appear that this process proceeds in an anterior-to-posterior wave such that the more anterior cells complete intercalation before those that lie more posterior. This is reminiscent of the anterior-posterior wave seen during vertebrate axial development and somitogenesis (Gilbert, 1994).

After intercalation is complete, all 40 notochord cells are organized in a single column along the length of the developing tail (Fig. 4D). The cells are tightly packed and columnar in appearance. During the next several hours each of these cells undergoes a subtle shape change, and becomes slightly more cuboidal (Fig. 4D-F). This process may, in part, account for the continued extension of the tail after intercalation is complete (see Miyamoto and Crowther, 1985).

Careful inspection of the embryo shown in Fig. 4E reveals that 20 of the 40 notochord cells stain positive for the *Ci-Bra-lac*Z transgene. Stained cells are often found next to unstained cells, which is consistent with previous lineage tracing experiments in *Halocynthia* (Nishida, 1987; Satoh, 1994). The earlier studies suggested that intercalation of cells from opposite sides of the embryo does not proceed as a regular process, but is somewhat random. However, in no case was intermixing observed among primary and secondary lineage notochord cells in posterior regions of the embryo (data not shown).

The hatched, mature tadpole is presented in Fig. 6C. Terminal differentiation of the ascidian notochord results in a capillary-like morphology in which the notochord cells form a continuous, flattened sheet surrounding a fluid-filled lumen (Cloney, 1964, 1990; Mancuso and Dolcemascolo, 1977). For this reason the individual notochord cells have a flattened, irregular appearance. There appears to be a cryptic nuclear localization signal within the *Ci-Bra*-GFP transgene which results in higher levels of fluorescence in nuclei relative to cytoplasm.

Identification of a minimal *Ci-Bra* enhancer

A number of *Ci-Bra-lac*Z transgenes were analyzed in an effort to identify the minimal *cis*-regulatory elements that mediate notochord-specific expression (summarized in Fig. 5A). As discussed in the preceding experiments, a 3.5 kb DNA fragment from the *Ci-Bra* promoter region mediates an authentic, notochord-specific expression pattern in transgenic embryos. Similar results were obtained with truncated transgenes containing only 790 bp or 434 bp of *Ci-Bra* 5′ flanking sequences (see Fig. 5A). Both of these transgenes direct notochord-specific patterns of expression, although there appears to be a progressive loss in the level of expression as compared with the ‘full-length’ 3.5 kb promoter fragment. The smaller promoter fragments also occasionally show expression in the mesenchyme (see below).

The smallest *Ci-Bra* promoter fragment that directs an authentic expression pattern is 557 bp in length. It contains 434 bp 5′ of the putative TATA element, as well as the first 17 codons of the protein coding region (Fig. 5A,B). Our initial characterization of this promoter fragment involved the use of green fluorescent protein (GFP) as a reporter (Fig. 6). All of the panels presented in this figure correspond to a single microinjected embryo. GFP fluorescence is detected in both primary lineage (Fig. 6A) and secondary lineage (Fig. 6B) cells of the notochord at the mid-tail bud stage. In Fig. 6A and B the embryo lies within the chorion (not visible) with the trunk of the tadpole in the center and the tail wrapped around the trunk. Half of the notochord cells are stained, again suggesting the stabilization of the transgene after the first, left-right cleavage.

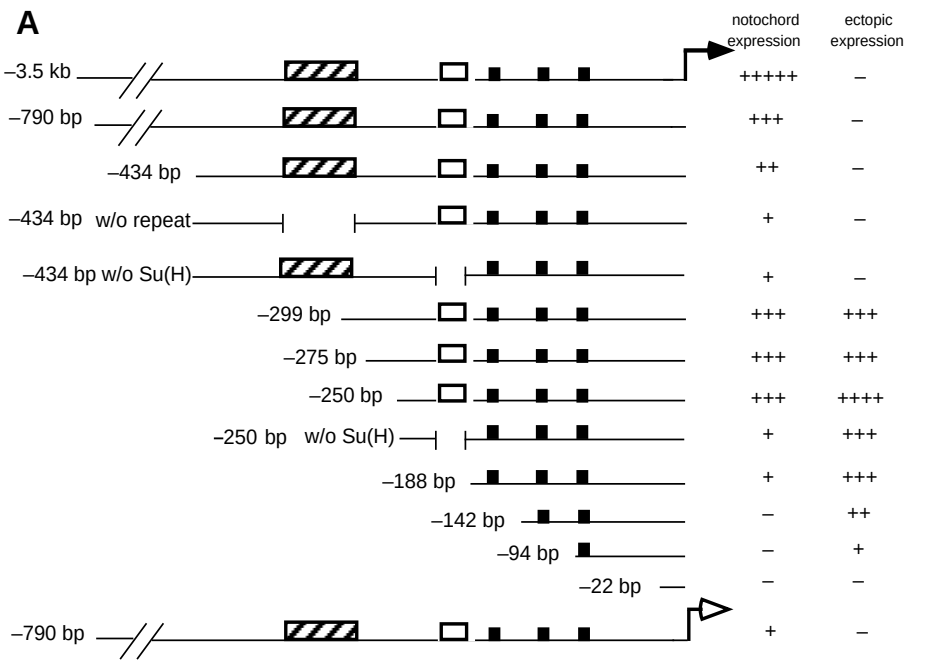

Fig. 5. Summary of *Ci-Bra* transgene constructs. (A) The diagrams show the different 5′ *Ci-Bra* promoter sequences that were analyzed in electroporated embryos. The boxes and rectangles represent different potential *cis*-regulatory elements. The hatched rectangle corresponds to three tandem repeats of a 15 bp sequence. This region is contained in or near the 5′ region that is important for repressing ectopic expression of *Ci-Bra* transgenes in trunk mesenchyme and tail muscles. The unfilled box corresponds to putative *Su(H)* binding sites. This region is important for expression in the notochord. The three filled, small boxes are E-box sequences, which may be important for expression in the muscles and mesenchyme. The last transgene corresponds to a 790 bp fragment from the *Ci-Bra* promoter region, extending from –1 bp to –790 bp upstream of the TATA element. This was attached to a minimal *Halocynthia* proximal promoter sequence containing a heterologous TATA sequence. (B) Nucleotide sequence of the minimal *Ci-Bra* promoter region that is sufficient to direct the expression of reporter genes in the notochord. The three 15 bp sequences are underlined, potential *Su(H)* binding sites and E-box sequences are boxed. The arrow corresponds to the 5′ end of the longest *Ci-Bra* cDNA that was isolated. It appears to map slightly downstream of the putative transcription start site. The minimal notochord-specific transgene includes the first 17 codons of the protein coding region.

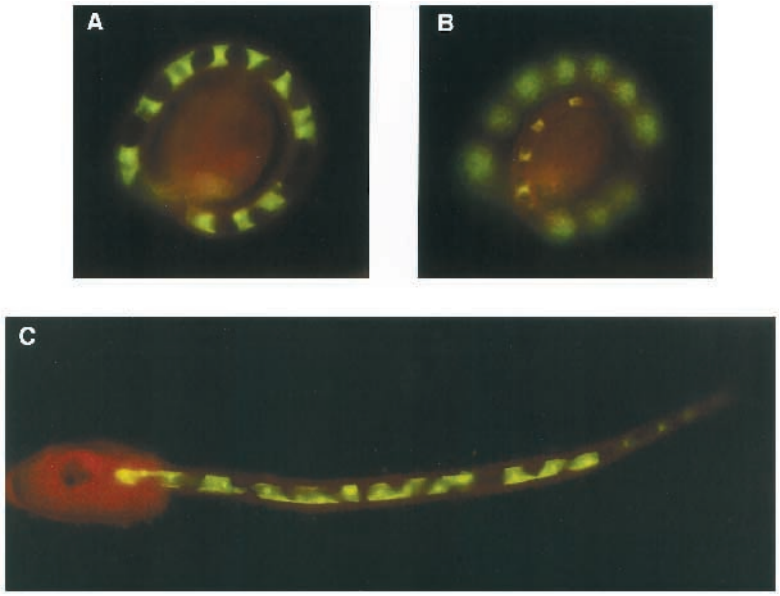

Fig. 6. Notochord-specific expression of the minimal *Ci-Bra* transgene. All of the photographs were obtained from a single, living embryo. A transgene containing the 434 bp element attached to a GFP reporter gene was microinjected into a one-cell embryo. (A) GFP expression at a mid-tailbud stage (approx. 12 hours postfertilization). The tail wraps around the central trunk since this living embryo is contained within an intact chorion. Staining is observed in half of the primary lineage notochord cells. (B) Same as A, except that the embryo was photographed in a different plane of focus to show GFP staining in 4 of the 8 secondary lineage notochord cells. (C) Same embryo as A and B after hatching from the chorion (approx. 18 hours postfertilization). Staining persists in half the notochord cells. By this time the individual notochord cells have become irregular in shape and flattened against the surrounding notochordal sheath.

These studies identified a minimal region of the *Ci-Bra* promoter that is sufficient for notochord-specific expression. Evidence that this region functions as a *bona fide* enhancer was obtained by attaching 5′ flanking sequences to a heterologous promoter (the construct with an open arrow at the bottom of Fig. 5A). A DNA fragment spanning sequences between −790 bp and −1 bp upstream of the putative TATA element was placed upstream of a heterologous, minimal promoter element from a *Halocynthia* muscle actin gene (see Fig. 3 and Materials and Methods). This composite *Ci-Bra*-actin-*lac*Z promoter was found to direct a notochord-specific staining pattern in transgenic *Ciona* embryos (data not shown).

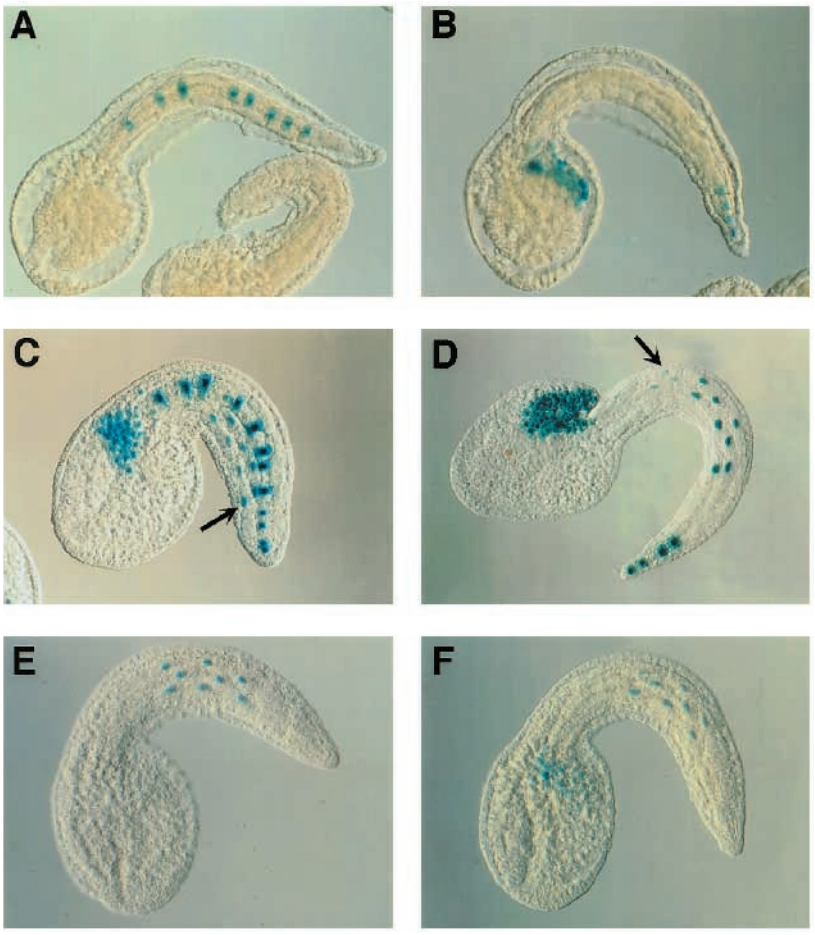

Fig. 7. Characterization of the minimal notochord-specific enhancer. Early tadpole embryos (approx. 12 hours postfertilization) were electroporated with various *Ci-Bra-lac*Z fusion genes at the one-cell stage. (A) Staining pattern obtained with the intact, minimal 434 bp *Ci-Bra* enhancer. Staining is restricted to 25% of the primary lineage notochord cells. (B) The same transgene as that used in A sometimes shows ectopic staining in the mesenchyme. This staining was determined to be in mesenchyme, based on its pattern, location, and the size of the cells showing expression. In addition, this ectopic staining is often seen when the transgene is expressed in secondary lineage notochord cells. Since the mesenchyme and secondary notochord lineages arise from a common precursor at the 64-cell stage (B7.3) and transgenes tend to be stabilized in a given lineage, this ectopic staining is likely to correspond to trunk mesenchyme. (C) Staining pattern obtained with a *lac*Z transgene containing the −250 bp *Ci-Bra* promoter sequence (see Fig. 5A). Strong staining is observed in the notochord (central cells along the length of the tail). In addition, ectopic staining is observed in tail muscles (arrow). (D) The same transgene as the one used in C sometimes shows ectopic staining in the mesenchyme and tail muscles. The most common region of ectopic staining is observed in a group of 8 muscle cells (strongly stained). This embryo shows a rare case of weak staining in more anterior muscle cells (arrow) derived from B7.8. (E) Staining pattern obtained with the −142 bp *Ci-Bra* promoter sequence (see Fig. 5A). Staining is essentially lost in the notochord, but ectopic expression continues to be observed in some tissues. In this embryo, the central group of 8 muscle cells expresses the transgene. (F) Same transgene as that used in E. In this tadpole ectopic staining is detected in both the tail muscles and mesenchyme.

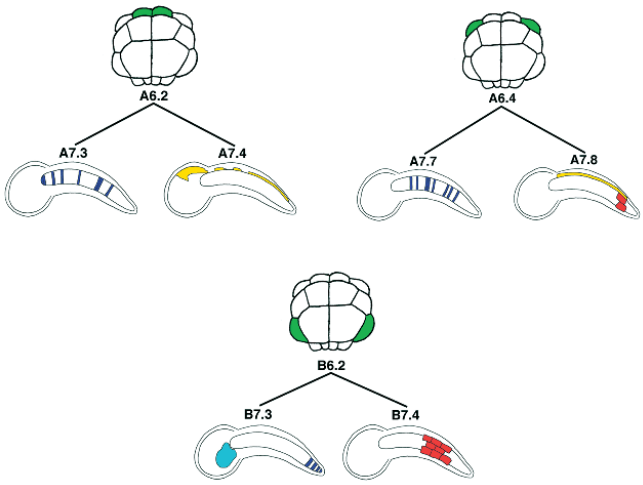

Fig. 8. Sites of ectopic expression correspond to notochord sister lineages. The upper diagrams represent 32-cell embryos that are labeled to identify selected blastomeres. The upper left diagram highlights the A6.2 blastomere. It gives rise to both primary lineage notochord cells (A7.3 descendants), as indicated in the diagram of the 12-hr tadpole below and to the left. This blastomere also gives rise to derivatives of the CNS, including portions of the brain vesicle and spinal cord (these all arise from A7.4, which is one of the daughter cells of A6.2). Truncated *Ci-Bra* fusion genes occassionally show ectopic staining in the CNS, presumbably due to derepression in A6.2 or its daughter cells. The diagrams in the upper right of the figure highlight the A6.4 blastomere and its descendants. It gives rise to primary lineage notochord cells (A7.7 descendants) as well as to spinal cord cells (yellow) and two muscle cells in a posterior region of the tail (red). Ectopic staining in these two muscle cells is often seen with truncated *Ci-Bra* transgenes lacking the distal repression elements (see Fig. 7C). The bottom diagram shows the derivatives produced by the B6.2 blastomere. This is the lineage that corresponds to the most common sites of ectopic expression mediated by truncated *Ci-Bra* transgenes. This blastomere gives rise to both mesenchyme (light blue) as well as secondary lineage notochord cells (dark blue). It also generates a group of 8 muscle cells in central regions of the tail. The mesenchyme and central muscles represent the most common sites of ectopic staining (see Fig. 7D,E).

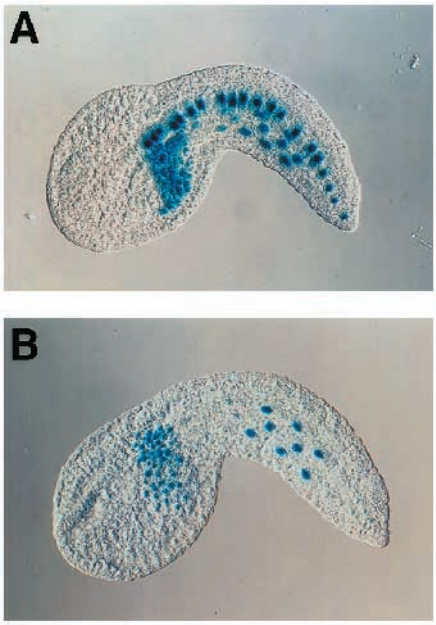

Fig. 9. *Su(H)* sequences may be important for *Ci-Bra* expression in the notochord. Sagittal views of early tadpole stage embryos (approx. 12 hours postfertilization) that were electroporated with *Ci-Bra* transgene constructs at the one-cell stage. (A) Staining pattern obtained with the truncated, –250 bp *Ci-Bra* fusion gene (see Fig. 6A). This *Ci-Bra* promoter fragment lacks the distal repression element, so ectopic staining is observed in the muscles and trunk mesenchyme. Strong staining is oberved in both the notochord and ectopic mesodermal tissues. (B) Staining pattern obtained with the –250 bp *Ci-Bra* promoter fragment containing an internal 24 bp deletion that removes the putative *Su(H)* binding sites. Staining is selectively lost in the notochord, but ectopic expression persists in the other mesodermal lineages, the tail muscles and mesenchyme.

Subsequent analyses focused on the minimal 434 bp *Ci-Bra* promoter fragment that directs an apparently normal expression pattern. The sequence of this fragment is presented in Fig. 5B. It includes several potential factor binding sites. There are three 15 bp repeats (underlined in the sequence presented in Fig. 5B, and indicated as a hatched rectangle in the summary in Fig. 5A), as well as two potential *Suppressor of Hairless* {*Su(H)*} binding sites (unfilled rectangles). Finally, the proximal-most region, located within the first 175 bp of the putative transcription start site, includes three potential E-box sequences (filled boxes). A number of experiments were performed to determine whether any of these putative *cis*-regulatory elements are required for the normal *Ci-Bra* expression pattern.

Negative regulation of the *Ci-Bra* enhancer

The –434 bp *Ci-Bra* promoter fragment was fused in-frame with the *lac*Z coding region containing a nuclear localization sequence. This minimal *Ci-Bra-lac*Z transgene usually directs a notochord-specific pattern of expression (Fig. 7A). However, strong staining in secondary lineage notochord cells is often associated with ‘ectopic’ expression in the mesenchyme (Fig. 7B). This embryo provides an example of mosaic expression, probably resulting from the relatively late stabilization of the transgene. It would appear that the transgene became stably incorporated in just one of the two B4.1 blastomeres at the 8-cell stage. Mesenchyme staining appears somewhat more frequently with the minimal *Ci-Bra* promoter fragment as compared with the full-length 3.5 kb promoter (data not shown). It is conceivable that the truncated fragment lacks a negative regulatory element which normally excludes *Ci-Bra* expression from the mesenchyme (see below).

The occurrence of repressor elements within the minimal *Ci-Bra* enhancer is suggested by the analysis of additional truncated *Ci-Bra-lac*Z transgenes (summarized in Fig. 5A). For example, removal of the distal-most ~180 bp from the enhancer results in consistent ectopic staining in the tail muscles (construct ‘–250 bp’ in Fig. 5; Fig. 7C,D). This deletion does not, however, compromise expression in the notochord. In fact, the smaller, –250 bp *Ci-Bra-lac*Z transgene directs consistently stronger staining in the notochord than does the –434 bp transgene (see Summary, Fig. 5A).

Progressive truncations of the minimal enhancer result in the

loss of the notochord staining pattern and ultimately eliminate ectopic expression in the tail muscles and mesenchyme (summarized in Fig. 5A). For example, the −142 bp *Ci-Bra-lac*Z transgene shows no staining in either primary or secondary lineage notochord cells, but continues to exhibit ectopic patterns of expression (Fig. 7E,F). These results suggest that the minimal *Ci-Bra* enhancer contains three essential regulatory elements, a distal repressor(s) element that excludes expression in mesenchyme and muscles, a central element required for notochord expression, and proximal elements which mediate expression in ectopic mesodermal lineages.

Lineage analysis of ectopic expression

The mesenchyme represents the most consistent site of ectopic expression of *Ci-Bra-lac*Z transgenes (e.g., Fig. 7B,D,F). Mesenchyme staining is often associated with strong expression in secondary lineage notochord cells (e.g., Fig. 7B). Lineage maps indicate a direct connection between these two groups of cells (Satoh, 1994). At the 64-cell stage, the B7.3 blastomere is not yet clonally restricted to form notochord. Instead, just one of its descendants, B8.6, gives rise to secondary lineage notochord cells, while the other, B8.5, forms mesenchyme (Satoh, 1994).

Careful inspection of early embryos hybridized with a digoxigenin-labeled *Ci-Bra* antisense RNA probe reveals that *Ci-Bra* expression is activated in the B7.3 blastomere (Fig. 2B and data not shown). This observation raises the possibility that endogenous *Ci-Bra* transcripts are asymmetrically partitioned between the B8.5 and B8.6 daughter cells. Mesenchyme staining mediated by the transgenes might reflect the perdurance of *Ci-Bra-lac*Z transcripts that are first expressed in the B7.3 blastomere. Perhaps the *lac*Z reporter mRNA lacks the signal sequences required for asymmetric localization. Thus, it is possible that ectopic expression of *Ci-Bra-lac*Z transgenes in the mesenchyme is due to a failure of proper RNA localization (see Discussion).

Ectopic expression in tail muscles cannot be explained by an aberrant RNA localization process. Instead, it would appear that muscle-specific repressors normally restrict *Ci-Bra* expression to notochord precursor cells. The most frequent site of ectopic muscle staining is a group of 8 cells in central regions of the tail (e.g., Fig. 7D-F). Once again, lineage maps indicate a direct connection between these muscle cells and secondary lineage notochord cells (see Fig. 8). In particular, both groups of cells arise from a common progenitor: the B6.2 blastomere of 32-cell embryos (Fig. 8). This blastomere divides to give the B7.3 and B7.4 blastomeres at the 64-cell stage. As discussed above, the B7.3 cell is not yet restricted to form notochord. In contrast, the B7.4 cell is clonally restricted to form the 8 central tail muscles (Nishida, 1987). Perhaps the relatively precocious determination of this muscle-specific blastomere is associated with a transcriptional repressor, which excludes the expression of *Ci-Bra* and other notochord-specific genes. Truncated *Ci-Bra-lac*Z transgenes might exhibit ectopic expression in these cells, and their descendants, due to the removal of repressor binding site(s).

The preceding discussion raises the possibility that the repressor(s) responsible for restricting *Ci-Bra* expression to the notochord is somehow connected with the B6.2 lineage (see Fig. 8). However, additional observations suggest that the repressor(s) is more likely to be expressed in most or all muscles, as well as other tissues. Intense expression of truncated *Ci-Bra-lac*Z transgenes in primary lineage notochord cells is often associated with the staining of two muscle cells in posterior regions of the tail (arrow in Fig. 7C). Both groups of cells arise from the A6.4 blastomere of 32-cell stage embryos (see Fig. 8). This blastomere divides to give the clonally restricted A7.7 notochord precursor cell. The other daughter cell forms the two muscle cells mentioned above, as well as several cells in the 'spinal cord' (Satoh, 1994). Ectopic staining is sometimes seen in these latter cells as well (data not shown). Similarly, the A6.2 blastomere generates both primary lineage notochord cells as well as cells in the brain stem and spinal cord (see Fig. 8; Satoh, 1994). Ectopic expression has also been observed in these cells but at lower frequency than in muscle (data not shown).

In summary, ectopic expression of truncated *Ci-Bra-lac*Z transgenes corresponds to the sister lineages associated with both primary and secondary lineage notochord cells (summarized in Fig. 8). These results suggest that the ectopic expression is not due to 'position effects' associated with random sites of integration of the transgenes within the *Ciona* genome. Rather, it would appear that tissue-specific repressors are essential for restricting *Ci-Bra* expression to the presumptive notochord (see Discussion). Furthermore, the ectopic expression coincides with lineages that derive from notochord precursors at the 32-cell stage, which corresponds to the time of notochord induction (see Nakatani and Nishida, 1994 and the Discussion).

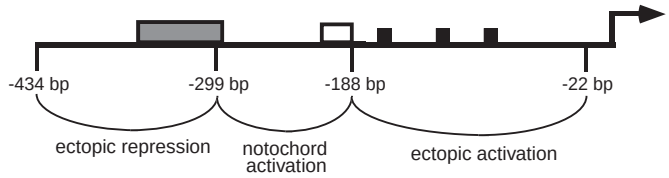

Fig. 10. Summary of the cis-regulatory elements in the minimal notochord-specific enhancer. The *Ci-Bra* enhancer contains three essential *cis*-regulatory elements. Distal sequences, located between −434 and −299 bp contains a negative response element that keeps the enhancer off in ectopic mesodermal lineages (muscles and mesenchyme). This region contains three copies of a conserved 15 bp sequence motif. It is currently unclear whether this repeat is important for repression. The second essential *cis*-regulatory element is located between −299 bp and −188 bp. It contains one, or possibly two, *Su(H)* binding sites. Removal of these sites significantly decreases expression in the notochord. Finally, proximal sequences, located between −188 bp and −22 bp upstream of the *Ci-Bra* TATA element are important for activation in the mesenchyme and muscles. This region contains 3 E-boxes; progressive truncations that remove these sequences lead to a sequential loss of expression in ectopic mesodermal lineages.

Activator elements in the minimal *Ci-Bra* enhancer

The preceding results suggest that a distal region of the *Ci-Bra* enhancer, between −434 bp and −299 bp (see Fig. 5A), contains one or more repressor elements which exclude expression from muscles and other tissues. Additional experiments were done to identify potential activator elements. Particular efforts were centered on the *Su(H)*-like binding sites between −213 bp and −188 bp (boxed in Fig. 5B) upstream of the TATA element, and

the three E-boxes located in a 100 bp interval between −173 bp and −74 bp upstream of TATA (see Fig. 5A summary).

A critical activator element appears to map between −250 bp and −188 bp upstream of the *Ci-Bra* TATA element (Fig. 5A). There is a precipitous reduction in the levels of notochord staining, but not ectopic expression, when comparing the −250 bp *Ci-Bra-lac*Z transgene with the −188 bp transgene. This interval of DNA contains a highly conserved copy of a *cis*-regulatory element that mediates transcriptional activation by the *Notch* signaling pathway (Tun et al., 1994; Bailey and Posakony, 1995). Activation of the *Notch* receptor is thought to result in the translocation of a transcriptional activator, *Su(H)*, from the cytoplasm to the nucleus (reviewed by Artavanis-Tsakonas et al., 1995). Once in the nucleus, *Su(H)* binds to a conserved recognition sequence (Tun et al., 1994; Bailey and Posakony, 1995; Jarriault et al., 1995). There is a close match to this binding site within the interval essential for notochord-specific expression between −250 bp and −188 bp (first box in Fig. 5B). There is a second sub-optimal *Su(H)* binding site six nucleotides downstream of the first (second box in Fig. 5B). The deletion of these two sites in the context of an otherwise normal −434 bp *Ci-Bra-lac*Z transgene results in a severe reduction in the expression of the transgene (data not shown).

The putative *Su(H)* binding sites were also removed from a −250 bp *Ci-Bra-lac*Z transgene, which normally directs strong expression in both the notochord and ectopic tissues (Fig. 9A). A 24 bp internal deletion (from −213 bp to −188 bp) within this transgene results in a significant reduction of staining in the notochord (Fig. 9B). However, ectopic staining in the mesenchyme and tail muscles is less severely affected. This observation suggests that separate activator elements are responsible for expression in the notochord and muscles.

It is conceivable that E-boxes are responsible for expression in muscles. Progressive truncations that sequentially remove the three E-boxes cause a corresponding reduction in muscle expression (see Fig. 5A summary).

DISCUSSION

We have presented a detailed characterization of a notochord-specific enhancer from the promoter region of the *Ciona Brachyury* gene. The *Ciona* embryo provides a rapid and useful system for the analysis of complex regulatory elements. Transgenic DNA can be efficiently introduced into developing embryos using a simple electroporation protocol. The small and compact genome permits the rapid isolation of potential regulatory factors, and the detailed lineage information permits assessment of ectopic patterns of gene expression with regard to the occurrence of localized transcriptional repressors.

The minimal *Ci-Bra* enhancer contains at least three discrete regulatory elements, as summarized in Fig. 10. One or more distal repression elements exclude *Ci-Bra* expression from muscles and possibly the mesenchyme and CNS. Separate activator elements mediate expression in the notochord and muscles. The notochord activator may be related to the *Drosophila* Suppressor of Hairless protein, thereby raising the possibility that Notch signaling participates in the notochord-restricted expression of the *Ci-Bra* gene. We discuss the parallels between the subdivision of the notochord and muscle lineages in *Ciona* and the specification of axial and paraxial mesoderm in vertebrates.

Brachyury gene expression in lower chordates and vertebrates

The *Brachyury* gene has been isolated from zebrafish, *Xenopus*, chick, mouse, amphioxus, and ascidians (Herrmann et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1994; Yasuo and Satoh, 1994; Holland et al., 1995; Kispert et al., 1995). However, *cis*-regulatory elements responsible for notochord-restricted expression of *Brachyury* have not been identified in any of these organisms, despite attempts in mice (Clements et al., 1996). Transgenic mice containing *lac*Z fusion genes with as much as 8.3 kb of 5′ flanking sequence and 5 kb of 3′ sequence from the mouse *Brachyury* promoter region show expression in primitive streak but fail to exhibit expression in the node or notochord (Clements et al., 1996). The relative ease with which the *Ci-Bra* enhancer was identified probably reflects the compact organization of the *Ciona* genome, which is less than 1/10th the size of mammalian genomes.

The simple genomic organization of *Ciona* is also suggested by a preliminary analysis of a *forkhead*/HNF-3β homolog. As discussed earlier, the only vertebrate notochord-specific enhancer that has been identified is located about 15 kb upstream of the mouse HNF-3β transcription start site. A second enhancer, located more than 5 kb downstream of the gene, mediates expression in the floor plate (Sasaki and Hogan, 1996). In the case of the *Ciona forkhead* homolog, notochord-, CNS-, and gut-specific expression are mediated by the proximal 1.8 kb upstream of the transcription start site (J. C. Corbo, unpublished results). Future studies will determine whether vertebrate enhancers and composite *cis*-regulatory elements mediate authentic patterns of expression in transgenic *Ciona* embryos. If so, this system might provide a rapid means of identifying vertebrate regulatory elements that mediate conserved patterns of gene expression in basic embryonic tissues.

All of the vertebrate *Brachyury* homologs that have been examined are initially expressed throughout the early mesoderm (for review see Herrmann, 1995). Expression becomes progressively restricted to axial mesoderm that forms the notochord during later stages of embryogenesis. A similar pattern of expression has been observed in a cephalochordate, amphioxus (Holland et al., 1995). However, both ascidians that have been studied, *Halocynthia roretzi* and *Ciona intestinalis*, possess *Brachyury* genes that are expressed exclusively in the notochord (with the single exception of the transient expression in the B7.3 blastomere shown in this study; see Fig. 2B).

However, a potential evolutionary link between the ascidian and cephalochordate/vertebrate expression patterns is suggested by the analysis of truncated *Ci-Bra-lac*Z transgenes in *Ciona*. In particular, proximal regions of the *Ci-Bra* promoter mediate expression in the muscles and mesenchyme. For example, the *Ci-Bra-lac*Z transgene shown in Fig. 9A, which contains the first 250 bp of the 5′ flanking sequence, exhibits expression in the notochord, muscles, and mesenchyme. This expression pattern is reminiscent of the pan-mesodermal pattern of expression observed in higher chordates. Thus, it is possible that the apparent differences in the ascidian and vertebrate *Brachyury* expression patterns are

primarily due to temporal differences in the expression of specific repressors of *Brachyury* in the non-notochordal mesoderm lineages.

Ci-Bra repression in the mesenchyme and muscles

A key finding of the present study is that transcriptional repression is essential for the notochord-specific pattern of *Ci-Bra* expression. The *Ci-Bra* enhancer shares some similarities with *Drosophila* embryonic enhancers, including the *eve* stripe 2 and *rhomboid* lateral stripe enhancers, which direct localized stripes of gene expression (Stanojevic et al., 1991; Small et al., 1992; Ip et al., 1992). These enhancers can be activated in broad domains, but spatially localized repressors restrict the pattern and define the stripe borders. It would appear that the *Ci-Bra* enhancer functions in a similar way, and integrates both activators and repressors to define a notochord-specific pattern of expression. We have presented evidence that the enhancer can be activated throughout the embryonic mesoderm, in the muscles, mesenchyme, and notochord. Repressors are important for excluding expression from ectopic tissues, particularly the muscles (see below), and restricting the expression pattern to the developing notochord (see Fig. 10 summary).

Yamaguchi and colleagues (1994) have recently shown that the *Brachyury* gene may be subject to similar negative regulation in the paraxial mesoderm of mouse embryos. Fibroblast growth factor receptor 1 (FGFR-1) is normally expressed at high levels in the paraxial mesoderm. However, knock-out mice lacking the FGFR-1 gene exhibit a gross expansion of the *Brachyury* expression into the presumptive somitic mesoderm. This result suggests that a repressor of *Brachyury* normally present in the paraxial mesoderm is absent in this mutant, thus permitting ectopic expression.

The identity of the *Ci-Bra* repressor(s) in *Ciona* is not known. However, preliminary studies suggest that a *Ciona snail* homolog is expressed at the right time and place to function as a potential *Ci-Bra* repressor (J. C. Corbo and A. Erives, unpublished results). The *Drosophila snail* gene encodes a zinc finger repressor that is expressed throughout the presumptive mesoderm of early embryos (Alberga et al., 1991; Leptin, 1991; Kosman et al., 1991). This repressor is important for restricting the expression of neuroectodermal regulatory genes, such as *rhomboid*, to lateral regions of the early embryo and excluding expression from the presumptive mesoderm (Ip et al., 1992). Moreover, vertebrate *snail* homologs exist that are expressed at high levels in paraxial mesoderm but are excluded from axial mesoderm (e.g., Hammerschmidt and Nusslein-Volhard, 1993). These homologs seem to form a boundary around the axial mesoderm during early stages of development. We are presently investigating the possibility that the *Ciona snail* homolog forms a 'boundary' between notochord and muscle/mesenchyme.

Transcriptional repression might not be the only mechanism responsible for the notochord-specific pattern of *Ci-Bra* expression. As discussed previously, it is possible that ectopic expression in the mesenchyme stems from the normal expression of the endogenous *Ci-Bra* gene in the B7.3 blastomere of 64-cell stage embryos. Previous studies on the expression of the *Halocynthia* notochord-specific *Brachyury* homolog suggested expression solely within clonally restricted notochord precursor cells (Yasuo and Satoh, 1994). Expression was not observed in the B7.3 blastomere, but instead, secondary lineage staining was first detected at the 110-cell stage, after the division of B7.3 to form the clonally restricted B8.6 blastomere. It is possible that *Brachyury* regulation is slightly different in *Halocynthia* and *Ciona* since these ascidians are quite divergent, and represent different taxonomic orders within the class Ascidiacea (Pleurogona and Enterogona, respectively; Satoh, 1994). Indeed, the *Brachyury* coding region of *Ciona* is equally divergent from the *Halocynthia* and vertebrate *Brachyury* genes (R. W. Zeller and J. C. Corbo, unpublished results).

Ci-Bra transcripts synthesized in B7.3 are either rapidly degraded or are asymmetrically distributed between the B8.5 and B8.6 daughter cells. It is possible that post-transcriptional processing of B7.3 transcripts is mediated by 'signal' sequences contained within the *Ci-Bra* mRNA (such as the 3′ untranslated region). *Ci-Bra-lac*Z transgenes might lack this signal sequence, and consequently, *lac*Z transcripts synthesized in B7.3 might be maintained in both the B8.5 (mesenchyme) and B8.6 (notochord) lineages. We do not believe that this model provides a complete explanation for ectopic expression in the mesenchyme since truncated *Ci-Bra-lac*Z fusion genes exhibit a higher incidence of mesenchyme staining as compared with the full-length *Ci-Bra* enhancer. The simplest explanation for this observation is that the loss of distal repression elements results in ectopic transcription of the defective *Ci-Bra-lac*Z fusion genes in both the mesenchyme and muscles, as discussed above.

Activation of the *Ci-Bra* enhancer

We have presented evidence that the *Ci-Bra* enhancer may be activated by a combination of *Su(H)* binding sites and E-box sequences, which map between −213 bp and −74 bp upstream of the TATA element. The organization of these binding sites is reminiscent of the promoter regions of *Drosophila* genes expressed in proneural clusters, such as the genes of the *Enhancer of split* complex (ES-C) (Kramatschek and Campos-Ortega, 1994; Bailey and Posakony, 1995). These latter genes are activated by a combination *of achaete-scute* bHLH proteins and the *Su(H)* activator. Selected mutations in these binding sites suggest that the two proteins function synergistically in the activation of ES-C gene expression (Bailey and Posakony, 1995). This transcriptional synergy might also apply to the activation of the *Ci-Bra* gene in the notochord. Perhaps bHLH activators are expressed in all mesodermal lineages, including the notochord, muscles, and mesenchyme. These activators might be present at limiting concentrations in the notochord, so that expression depends on both the bHLH proteins and *Su(H).* In contrast, bHLH activators, such as *myo*D, might be present at sufficient concentrations in the muscles and mesenchyme to activate truncated *Ci-Bra-lac*Z fusion genes lacking the *Su(H)* binding site.

It is currently unclear whether the putative *Ciona Su(H)*-like activator is constitutively expressed in notochord cells, or regulated by a Notch signaling pathway. Of interest in this regard is a recent study by Nakatani and Nishida (1994) in which they demonstrate that a specific cell-cell interaction is required at the 32-cell stage in ascidians for induction of notochord fate. When they remove the notochord precursors A6.2 or A6.4 from a 32-cell stage embryo and culture them in isolation they do not express notochord markers or morphology. However, when either A6.2 or A6.4 is co-isolated with the

adjacent endodermal precursor A6.1 or A6.3, the descendants of A6.2 and A6.4 express both notochord markers and morphology. Nakatani and colleagues (1996) have shown further that this cell-cell interaction is required for the induction of *Brachyury* expression in the notochord lineage. It is conceivable that a Notch-like signaling pathway might participate in this induction. Interestingly, a recent study in zebrafish identified a *Notch* homolog that is specifically expressed in the developing notochord at the time of cell fate specification (Bierkamp and Campos-Ortega, 1993). Future studies will investigate the role of the Notch signaling pathway in *Ciona* notochord development.

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