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The Snail repressor establishes a muscle/notochord boundary in the *Ciona* embryo

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SUMMARY

Previous studies have identified a minimal 434 bp enhancer from the promoter region of the *Ciona Brachyury* gene (*Ci-Bra*), which is sufficient to direct a notochord-specific pattern of gene expression. Here we present evidence that a *Ciona* homolog of *snail* (*Ci-sna*) encodes a repressor of the *Ci-Bra* enhancer in the tail muscles. DNA-binding assays identified four Ci-Sna-binding sites in the *Ci-Bra* enhancer, and mutations in these sites cause otherwise normal *Ci-Bra/lacZ* transgenes to be misexpressed in ectopic tissues, particularly the tail muscles. Selective misexpression of *Ci-sna* using a heterologous promoter results in the repression of *Ci-Bra/lacZ* transgenes in the notochord. Moreover, the conversion of the Ci-Sna repressor into an activator results in the ectopic induction of *Ci-Bra/lacZ* transgenes in the muscles, and also causes an intermixing of notochord and muscle cells during tail morphogenesis. These results suggest that Ci-Sna functions as a boundary repressor, which subdivides the mesoderm into separate notochord and tail muscle lineages.

Key words: Snail, *Ciona*, Repressor, Muscle, Notochord, *Ci-Bra* enhancer

INTRODUCTION

Brachyury encodes a sequence-specific transcriptional activator that is essential for notochord differentiation in a variety of chordates, including ascidians, zebrafish, *Xenopus*, chickens and mice (e.g. Hermann and Kispert, 1994; Kispert et al., 1995). In an effort to determine the mechanisms underlying the specification of the notochord, we have conducted a detailed analysis of *Brachyury* gene regulation in the ascidian, *Ciona intestinalis*.

Ciona offers a number of advantages for this type of analysis. First, it is possible to obtain efficient expression of transgenic DNA in electroporated embryos (Corbo et al., 1997a). Second, the *Ciona Brachyury* gene (*Ci-Bra*) is expressed exclusively in prospective notochord cells, beginning with their clonal restriction at the 64-cell stage of embryogenesis (Yasuo and Satoh, 1994; Corbo et al., 1997a). In contrast, vertebrate *Brachyury* genes exhibit two distinct expression patterns, including ubiquitous expression throughout the presumptive mesoderm followed by a more restricted pattern in the notochord (e.g. Schulte-Merker and Smith, 1995). Although there has been considerable progress in identifying the *cis* regulatory elements responsible for the early induction of *Xbra2* expression (Latinkic et al., 1997), comparatively little is known about the late, notochord-restricted pattern (e.g. Clements et al., 1996).

The only notochord-specific *cis* regulatory element known for any chordate *Brachyury* gene was identified in *Ciona* (Corbo et al., 1997a). The minimal *Ci-Bra* enhancer is 434 bp in length and appears to be activated by a combination of Suppressor of Hairless {Su(H)} and basic helix-loop-helix (bHLH) proteins (Corbo, 1997). These factors can activate the *Ci-Bra* enhancer in all of the mesoderm lineages, including the notochord, tail muscles and trunk mesenchyme. However, one or more repressors appear to restrict the *Ci-Bra* expression pattern to the notochord. Here, we present evidence that the *Ciona snail* gene (*Ci-sna*) encodes a transcriptional repressor, which subdivides the embryonic mesoderm into separate notochord and muscle lineages.

Previous studies have shown that Snail functions as a repressor in the early *Drosophila* embryo to subdivide the presumptive mesoderm and neurogenic ectoderm (Kosman et al., 1991; Leptin, 1991; Ip et al., 1992). *snail* is initially expressed in the prospective mesoderm of precellular embryos (Kosman et al., 1991; Leptin, 1991; Alberga et al., 1991). The Snail protein excludes the expression of neuroectodermal specification genes, such as *rhomboid* (Ip et al., 1992) and *single minded* (Kasai et al., 1992), in the mesoderm and restricts their expression to the presumptive neurogenic ectoderm. Thus, in *Drosophila*, mesoderm specification is achieved through a combination of transcriptional activators and repressors, which promote mesoderm differentiation and exclude alternative cell fates, respectively.

snail homologs have been described in a variety of vertebrates, including zebrafish, *Xenopus*, chickens and mice (e.g. Hammerschmidt and Nüsslein-Volhard, 1993; Ros et al.,

1997; Jiang et al., 1997). In zebrafish embryos, *sna-1* expression is gradually excluded from the axial mesoderm and restricted to paraxial lineages, which give rise to the somites (Hammerschmidt and Nüsslein-Volhard, 1993). This expression pattern is consistent with the possibility that *sna-1* helps establish a boundary between the axial and paraxial mesoderm.

We recently reported the isolation and characterization of a *Ciona snail* gene, *Ci-sna* (Corbo et al., 1997b). The gene is expressed in the tail muscles of the tadpole, as well as the trunk mesenchyme, the progenitors of the adult body wall muscles, the cerebral vesicle and the lateral ependymal cells of the spinal cord. This complex *Ci-sna* expression pattern is complementary to the *Ci-Bra* pattern. Moreover, truncated *Ci-Bra/lacZ* transgenes exhibit ectopic expression in most or all of these *Ci-sna* lineages (Corbo et al., 1997a), thereby raising the possibility that Ci-Sna functions as a repressor of the *Ci-Bra* expression pattern.

Here we present evidence that Ci-Sna directly represses *Ci-Bra* expression. Gel shift assays identified four different Ci-Sna protein-binding sites within the 434 bp notochord-specific *Ci-Bra* enhancer. Mutations in either one of two of these sites, sna1 or sna2, cause an otherwise normal *Ci-Bra/lacZ* transgene to be derepressed in the tail muscles. Repression is restored in these ectopic lineages upon insertion of a synthetic Snail protein-binding site, s2, from *Drosophila*. Additional evidence for Ci-Sna/*Ci-Bra* regulatory interactions was obtained by misexpressing *Ci-sna* in the notochord. This was achieved by attaching the *Ci-sna*-coding region to *Ci-Bra* regulatory sequences. Ectopic expression of *Ci-sna* results in the down-regulation of *Ci-Bra/lacZ* transgenes in the notochord. Finally, a Ci-SnaVP16 fusion protein induces ectopic expression of *Ci-Bra/lacZ* transgenes in the tail muscles and spinal cord. The fusion protein generates a mutant phenotype, whereby notochord cells and tail muscles fail to separate during the intercalary movements that occur during tail morphogenesis. Taken together, these results suggest that Ci-Sna directly represses *Ci-Bra* expression to subdivide the embryonic mesoderm into dorsal (axial) and lateral (paraxial) lineages.

MATERIALS AND METHODS

Animal husbandry

Adult *Ciona intestinalis* were collected at Half Moon Bay, the Oakland Marina and Bodega Bay in California. Animals were also purchased from the Marine Biological Laboratory collecting facility at Woods Hole, Massachusetts. Some of the animals used in the most recent experiments were kindly provided by Dr Nori Satoh in Japan. Mature eggs and sperm were collected from gravid adults and insemination was done in filtered sea water. Embryos were cultured at 15°C.

Site-directed mutagenesis

*Xho*I-*Pst*I genomic DNA fragments that contain either the full-length 434 bp *Ci-Bra* enhancer, or the truncated 251 bp enhancer were used as templates for mutagenesis (Corbo et al., 1997a). Mutagenized forms of the *Ci-sna*-coding sequence were prepared with a *Not*I-*Not*I DNA fragment containing a full-length *Ci-sna* cDNA (Corbo et al., 1997b). Both DNAs were cloned into the pBluescript II SK+ vector (Stratagene, La Jolla, CA). Site-directed mutagenesis was done essentially as described by Kunkel (1985). Uracil-containing single-stranded DNA was prepared using the CJ236 strain of *E. coli* and the VCSM13 helper phage (also purchased from Stratagene). The following mutagenic oligonucleotides were used to delete the sna1 and sna2 sites, respectively:

AATAGCGACAAACCTACGTCACAATACAAA (sna1)

TAACACGTCACAATAGTGACGTCATATC (sna2)

The following nucleotides were deleted between the underlined TA in the sna1 region and the underlined AG in the sna2 region:

TATCTGGTGTT (sna1) and CACTTG (sna2)

The following mutagenic oligonucleotides were used to insert the *Drosophila* s2 Snail-binding site in the truncated 251 bp *Ci-Bra* enhancer and the mutagenized enhancer lacking the sna2 site, respectively:

TTTTGACATGTCAATCACCTTGCTGGGCAAAATCGGAAACCA (251 bp–s2)

CACGTCACAATACACCTTGGTGACGTCATAT (sna2–s2)

The following mutagenic oligonucleotides were used to disrupt the central three zinc fingers (ZF) in the C-terminal region of the *Ci-sna*-coding sequence (Corbo et al., 1997b). The two Cys residues within each ZF were converted into Phe residues (indicated by the underlined residues):

GTAGCAAGTTCAATTTCAAAATATTCAAGAAAGAGTGCAG (ZF 2)

CGCTGCCATGCGAATTCCATATTTTTGGTAAAGCGTTCTC (ZF 3)

AGAAACCATACCAATTCACCGTGTTCTCCAGAGCTTTCGC (ZF 4)

Spacer sequences were inserted between sna1 and the Su(H) sites, and between sna2 and Su(H). This was accomplished by creating synthetic polylinker sequences using the following mutagenic oligonucleotides:

TTGACATGTCAATCAGTCGACGCATGCCCGCGGACTAGTAAATCGGAAACCAAG (sna1)

ATGGGAAAGTAACACGTCGACGCATGCCCGCGGACTAGTGTCACAATACACTTG (sna2)

A 233 bp *Cla*I-*Nco*I cDNA fragment from the *Drosophila knirps*-coding region (Nauber et al., 1988) was cloned in the pGEM-T-Easy vector (Promega, Madison, WI) and a 309 bp *Sph*I-*Spe*I DNA fragment containing both *knirps* and vector sequences was used as the final spacer.

Gel shift assays

Binding assays were done with synthetic DNA fragments that contain the sna2-, sna3- or sna4-binding sites from the *Ci-Bra* enhancer. These fragments were prepared by annealing the following complementary oligonucleotides:

sna2: ACGTCACAATACACTTGGTGACGT and TATGACGTCACCAAGTGTATTGTG

sna3: AAAACAAACACAAGGTGTTCGAT and CTGGATCGAACACCTTGTGTTTG

sna4: CTGGATGCCACCACCTACGGCGC and AAGTGCGCCGTAGGTGGTGGCAT

The resulting double-stranded DNA fragments were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Sambrook et al., 1989). Various cold competitor DNAs were used in the binding assays. These included the wild-type sna2, sna3 and sna4 sequences, a mutant form of sna2 (sna2MUT), sna 1 and sna1MUT, and the *Drosophila* s2 sequence. The following oligonucleotides were used to prepare these DNA fragments:

sna2MUT: AACACGTCACAATAGTGACGTCAT + TGATATGACGTCACTATTGTGACG

sna1: CAAACCTTATCTGGTGTTACGTCA + ATTGTGACGTAACACCAGATAAGG

sna1MUT: AATAGCGACAAACCTACGTCACAA + TGTATTGTGACGTAGGTTTGTCGC

s2: GATCTGCGGCACCTTGCTGGGCAG + GATCCTGCCCAGCAAGGTGCCGCA

Binding assays were done with a glutathione-S-transferase-Ci-Sna

(GST-Ci-Sna) fusion protein that contains the C-terminal 159 amino acids, which includes all five zinc fingers (Corbo et al., 1997b). This fusion protein was prepared with the pGEX-KG expression vector using the HB101 strain of *E. coli*. The GST-Ci-Sna fusion protein was purified from crude bacterial extracts using glutathione-agarose beads (Smith and Johnson, 1988).

The fusion protein was preincubated with 10 pmol/μl of cold competitor DNAs in a solution containing 10 mM Hepes (pH 7.9), 50 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 2 μg/ml leupeptin, 2.5 μg/ml pepstatin A, 0.5 μg/ml BSA, 0.1 μg/ml poly[dI-dC] at room temperature for 5 minutes. Aliquots containing 0.2 pmol/μl of the labeled sna2 DNA were added and incubated at room temperature for 15 minutes. Afterwards, the reaction mixtures were fractionated on a 5% polyacrylamide gel and subsequently prepared for autoradiography (Ip et al., 1991).

Construction of transgenic DNAs

The 3.5 kb, −434 bp, and −251 bp*Ci-Bra/lacZ* transgenes are described in Corbo et al. (1997a). Mutagenized *Ci-Bra* enhancer sequences (see above) were excised from the pBluescript vector using *Xho*I and *Pst*I. These DNA fragments were replaced with the corresponding region of the wild-type −251 bp *Ci-Bra/lacZ* transgene.

Ci-sna expression plasmids were prepared as follows. Wild-type or mutagenized *Ci-sna* cDNAs were excised from the pBluescript vector using *Kpn*I and *Sac*I. The resulting fragment was used to replace the *lacZ* reporter gene present in a truncated −251 bp *Ci-Bra/lacZ* transgene (see Corbo et al., 1997a). The truncated *Ci-Bra* enhancer directs *Ci-sna* expression in both the notochord and tail muscles.

The preparation of the *Ci-snaVP16* recombinant-coding sequence involved the use of the VP16 activation domain (see Rusch and Levine, 1997). A synthetic *Bam*HI site was introduced in the C terminus of the *Ci-sna*-coding region via site-directed mutagenesis. The VP16 activation domain was ligated to the C-terminal *Ci-sna*-coding region contained within an *Eco*RV-*Bam*HI fragment. This fragment contains the five zinc fingers, but lacks the putative repression domain located in more N-terminal regions of the protein. The resulting *Ci-snaVP16* recombinant was then placed downstream of a *Cla*I site, approximately 1 kb downstream of the translation start site of the *lacZ* open reading frame. *The lacZ-Ci-snaVP16* fusion gene contains a nuclear localization sequence (NLS) present in the modified *lacZ* reporter gene.

Electroporation and in situ hybridization assays

In situ hybridization assays were done with digoxigenin-labeled antisense RNA probes exactly as described by Corbo et al. (1997a).

Transgenic DNAs were electroporated into *Ciona* embryos as described previously (Corbo et al., 1997a). Briefly, aliquots containing ~50 μg of each *lacZ* reporter DNA was dissolved in 500 μl of 0.77 M mannitol. Co-electroporations involved the use of 50 μg of either the *Ci-Bra* or *Ci-sna* expression vectors. The DNAs were electroporated into embryos ~25-35 minutes after fertilization and the embryos were allowed to develop until tailbud stages. The embryos were fixed in formaldehyde and stained for β-galactosidase activity, as described by Corbo et al. (1997a). A typical experiment involves the electroporation of at least 100-200 stained embryos and each of the results presented in this study was obtained in at least three separate experiments.

RESULTS

In situ hybridization assays reveal *Ci-sna* expression in the progenitors of the primary and secondary tail muscles by the onset of gastrulation (Corbo et al., 1997b). It is also expressed in the primordia of the cerebral vesicle and lateral ependymal cells of the spinal cord. *Ci-sna* expression is lost in the central nervous system (CNS) during neurulation, but staining persists in the primary and secondary tail muscles during intercalation of the notochord and extension of the tail (arrowheads, Fig. 1A,B). A transverse, dorsal view of stained embryos clearly reveals the subdivision of the mesoderm into dorsal and lateral lineages. The dorsal-most, or axial, mesoderm gives rise to the notochord (Fig. 1B, arrow), while the lateral mesoderm differentiates into definitive tail muscles (Fig. 1B, arrowheads). Subsequent experiments were conducted to determine whether *Ci-sna* helps specify this boundary between the notochord and tail muscles.

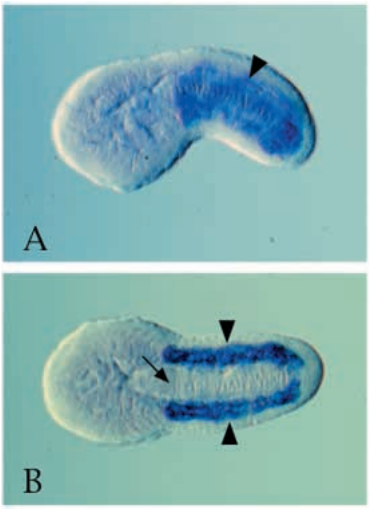

Fig. 1. Localization of *Ci-sna* RNAs. Fertilized eggs were dechorionated and allowed to develop to tailbud stages prior to fixation. The embryos were hybridized with a digoxigenin-labeled *Ci-sna* antisense RNA probe and stained with alkaline phosphatase. (A) Lateral view of a stained embryo showing *Ci-sna* expression in tail muscles (arrowhead). (B) Dorsal view of a stained embryo. Strong *Ci-sna* expression is observed in the lateral tail muscles (arrowhead), but there is no detectable staining in the notochord (arrow).

Ci-Sna binds to specific sites within the minimal *Ci-Bra* enhancer

Previous studies identified a minimal 434 bp enhancer from the *Ci-Bra* promoter region that mediates notochord-restricted expression of green fluorescent protein and *lacZ* transgenes in electroporated embryos (Corbo et al., 1997a). The enhancer appears to be activated by a combination of bHLH proteins and a *Ciona* homolog of the *Drosophila* Suppressor of Hairless {Su(H)} protein (Corbo, 1997; Corbo et al., unpublished data). A truncated *Ci-Bra* enhancer mediates ectopic expression in the trunk mesenchyme and tail muscles. These results suggest that bHLH-Su(H) synergy permits expression of the *Ci-Bra* enhancer in most mesodermal lineages, including the tail muscles, notochord and trunk mesenchyme, while one or more spatially localized repressors restricts *Ci-Bra* expression to the notochord.

As a first step towards assessing the role of Ci-Sna in *Ci-Bra* regulation, gel shift assays were conducted using a GST-Ci-Sna fusion protein that contains the carboxy-terminal 159 amino acid residues of Ci-Sna, and includes all five zinc fingers

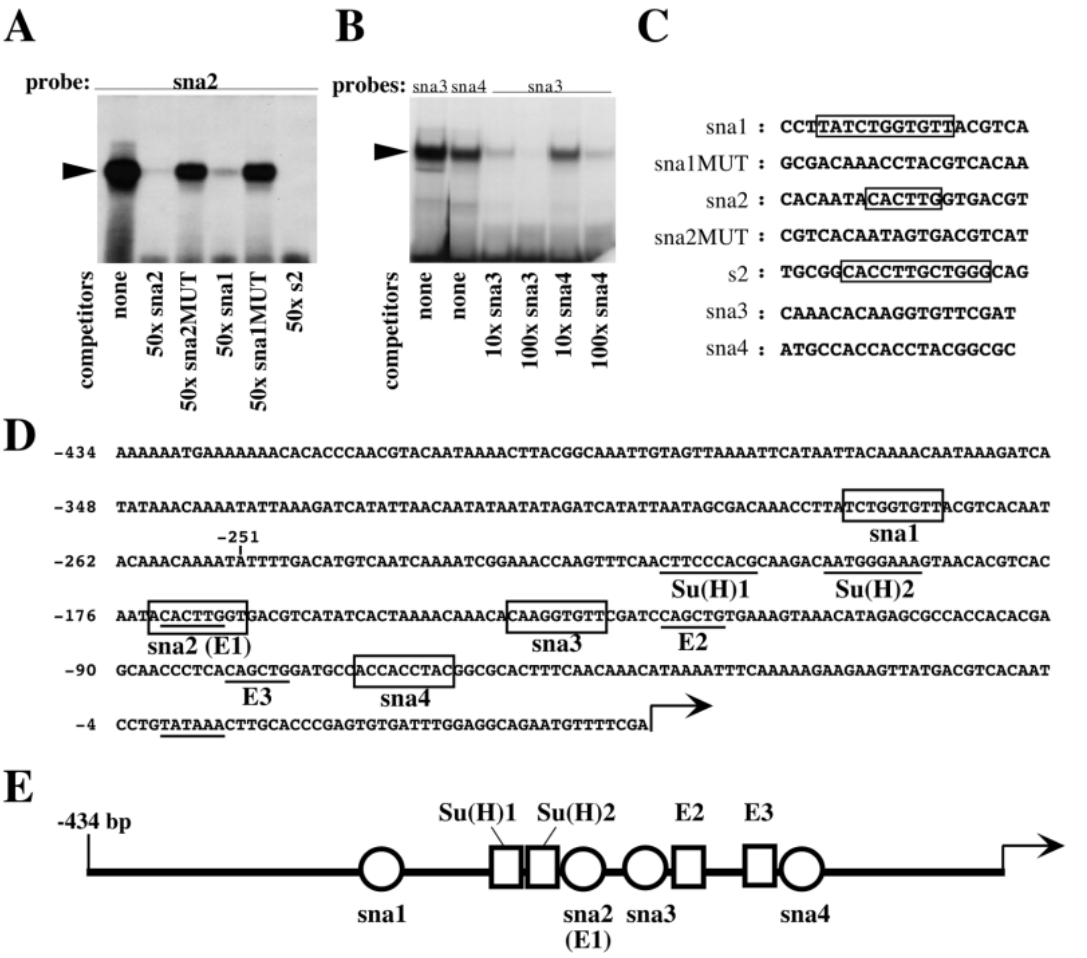

Fig. 2. Gel shift assays. (A) A truncated GST-Ci-Sna fusion protein was incubated with a ^{32}P-labeled sna2 synthetic DNA fragment. Protein-DNA complexes were fractionated on a polyacrylamide gel and visualized by autoradiography. Lane 1. No cold competitor DNA was added. A strong protein-DNA complex is observed (arrowhead). Lane 2. Same as lane 1 except that the GST-Ci-Sna fusion protein was preincubated with a 50 molar excess of unlabeled sna2 competitor DNA. There is a substantial reduction in the levels of specific protein-DNA complexes. Lane 3. Same as lane 2 except that the competitor DNA corresponds to a mutant form of sna2 (sna2MUT). The mutant DNA causes little or no inhibition of sna2/Ci-Sna complexes. Lane 4. The GST-Ci-Sna fusion protein was preincubated with a 50 molar excess of a DNA fragment containing the sna1 sequence. There is a substantial inhibition in the formation of sna2/Ci-Sna complexes, although the sna1 sequence might have a slightly lower binding affinity than sna2 (compare with lane 2). Lane 5. Same as lane 4 except that a mutant sna1 DNA was used as the competitor. There is no discernible block in the formation of sna2/Ci-Sna complexes. Lane 6. Same as lanes 4 and 5 except that the competitor DNA corresponds to the *Drosophila* Snail s2-binding site. The s2 sequence leads to a complete inhibition in the formation of sna2/Ci-Sna complexes, suggesting that it has a higher affinity for the GST-Ci-Sna fusion protein than either sna1 or sna2. (B) Gel shift assays with labeled sna3 and sna4 probes. The GST-Ci-Sna fusion protein forms a shifted complex (arrowhead) with these sequences in the absence of competitor DNA (lanes 1 and 2). Increasing amounts of unlabeled sna3 and sna4 competitor DNA reduces GST-Ci-Sna/sna3 complexes. (C) Sequences of the DNA fragments used for the binding assays. The boxed regions in the sna1 and sna2 fragments correspond to the sequences that were deleted in sna1MUT and sna2MUT, respectively. These sequences were also deleted in *Ci-Bra/lacZ* transgenes used for the experiments shown in Fig. 3B,C. The boxed region of the s2 DNA fragment corresponds to the sequence that was used in Fig. 3F. (D) DNA sequence of the minimal 434 bp *Ci-Bra* enhancer (see Corbo et al., 1997a). The enhancer appears to be activated by a combination of three E box elements, E1, E2 and E3 (E1 overlaps with the sna2 site), and two Su(H)-binding sites {Su(H)1 and Su(H)2}. Point mutations in these putative activator sites reduce or eliminate staining mediated by *Ci-Bra/lacZ* transgenes (Corbo, 1997; Corbo et al., unpublished data). Gel shift assays identified four Ci-Sna-binding sites, sna1, sna2, sna3 and sna4. As seen for the *Drosophila* Snail protein, the Ci-Sna-binding sites are related to E box sequences. (E) Summary of factor-binding sites. The rectangles correspond to putative activator sites, while the circles represent potential repressor elements (Ci-Sna sites). As mentioned above, the sna2 site overlaps the E1 E box sequence. The organization of the *Ci-Bra* enhancer is very similar to that observed for the *rho* neuroectoderm enhancer (NEE; Ip et al., 1992).

present in the full-length protein (Fig. 2). These experiments identified four different Ci-Sna-binding sites in the *Ci-Bra* enhancer, sna1, sna2, sna3 and sna4. The distal-most site, sna1, is located in the interval between –434 bp and –251 bp, which was previously shown to be important for restricted expression in the notochord (Corbo et al., 1997a).

DNA-protein complexes obtained with sna2 and the GST-Ci-Sna fusion protein are specifically inhibited with a 50-fold molar excess of unlabeled sna1 or sna2 DNA (Fig. 2A). However, mutant forms of sna1 (sna1MUT) or sna2 (sna2MUT) do not significantly block the shifted complexes (Fig. 2A). A high-affinity binding site for the *Drosophila* Snail protein, s2 (Ip et al., 1992), also mediates efficient competition in these assays (Fig. 2A).

Similar assays were used to identify two additional Ci-Sna-binding sites in the 434 bp *Ci-Bra* enhancer, sna3 and sna4 (Fig. 2B). Ci-Sna/sna3 protein-DNA complexes are blocked by the addition of increasing amounts of unlabeled sna3 and sna4 DNAs (Fig. 2B). The sequences of the wild-type and mutant Ci-Sna-binding sites used in these assays are shown in Fig. 2C.

The *Ci-Bra* enhancer sequence (Fig. 2D) contains two Ci-Su(H)-binding sites and three putative bHLH-binding sites (E boxes). The E1 E box overlaps with the sna2-binding site, which is similar to the situation observed for the *Drosophila* Snail protein whereby half the binding sites also include an E box consensus sequence (CANNTG). The sna1 and sna2 sites map quite close to Ci-Su(H) activator sites (Fig. 2E), which are essential for the expression of *Ci-Bra* transgenes in the notochord. A number of experiments were conducted to determine whether the sna1 and sna2 sites function as repressor elements that restrict the *Ci-Bra* pattern to the notochord.

Expression of mutagenized *Ci-Bra* transgenes in electroporated embryos

As shown previously (Corbo et al., 1997a), the 434 bp *Ci-Bra* enhancer directs robust expression of a *lacZ* transgene in the notochords of electroporated embryos (Fig. 3A, arrow). Staining is detected in both the primary notochord cells that arise from the A4.1 blastomere, as well as the secondary, B4.1-derived notochord cells located in the posterior regions of the tail. The wild-type enhancer also exhibits variable expression in the trunk mesenchyme (Fig. 3A, unfilled arrow).

Mutations in the sna1 site cause an otherwise normal *Ci-Bra/lacZ* transgene to be misexpressed in the tail muscles (Fig. 3B, arrowheads). This disruption in sna1 does not affect staining in the notochord (Fig. 3B, arrow). A similar expression pattern is obtained with a truncated *Ci-Bra* enhancer that lacks the sna1 site (Fig. 3E; see Corbo et al., 1997a). These results are consistent with the notion that sna1 corresponds to the negative response element located in the distal region of the *Ci-Bra* enhancer.

Additional mutagenesis experiments suggest that the sna2 site is also important for the normal, notochord-restricted staining pattern. Mutations in sna2 cause a derepression of the *Ci-Bra/lacZ* staining pattern that is virtually indistinguishable from that obtained with mutations in the sna1 site (Fig. 3C; compare with 3B). Again, staining is no longer restricted to the notochord, but is also detected in the tail muscles. These results suggest that both sna1 and sna2 are required for efficient repression. In contrast, a single Snail site appears to be sufficient to repress the *rhomboid* lateral stripe enhancer in *Drosophila* (Gray et al., 1994; see Discussion).

Mutations in the sna1 and sna2 sites result in the derepression of *Ci-Bra/lacZ* transgenes in the tail muscles, and possibly trunk mesenchyme. However, as noted above, the normal *Ci-Bra* enhancer exhibits variable expression in the mesenchyme (e.g. Fig. 3A) and, consequently, we will focus our attention on the repression of *Ci-Bra* in the tail muscles. Moreover, mutations in the sna3 and sna4 sites do not cause an obvious derepression of the *Ci-Bra/lacZ* staining pattern in tail muscles (data not shown), so subsequent experiments were restricted to the manipulation of the critical sna1 and sna2 sites.

Further evidence that Ci-Sna directly represses the *Ci-Bra* enhancer stems from *cis* complementation experiments, whereby a synthetic Snail-binding site was inserted in defective *Ci-Bra* enhancers lacking either the native sna1 or sna2 sites. The insertion of the *Drosophila* s2 sequence (Ip et al., 1992) in a defective *Ci-Bra* enhancer lacking the sna2 site restores an essentially normal staining pattern, whereby the modified *Ci-Bra/lacZ* transgene exhibits restricted expression in the notochord (Fig. 3D; compare with 3C). Similar results were obtained with a truncated *Ci-Bra* enhancer lacking the sna1 site (Fig. 3F). A synthetic s2 site at the 5′ end of the truncated enhancer restores the normal, notochord-restricted staining pattern (Fig. 3F, arrow; compare with 3E).

Spacer sequences were inserted between sna1 and Su(H)1, and Su(H)2 and sna2 (Fig. 3G, H) to determine whether Ci-Sna functions as a short-range repressor, as seen in *Drosophila* (Gray et al., 1994; Gray and Levine, 1996). Increasing the distance between sna1 and Su(H)1 results in a severe derepression of the *Ci-sna/lacZ* staining pattern (Fig. 3G). A similar derepression is observed when the same spacer sequence was placed between the Su(H)2 and sna2 sites (Fig. 3H). Staining is also detected in the cerebral vesicle (unfilled arrowheads, Fig. 3G, H), which is a normal site of *Ci-sna* expression (Corbo et al., 1997b; Erives et al., 1998). These results suggest that the critical sna1 and sna2 sites must map close to the Su(H) activator sites in order for Ci-Sna to repress *Ci-Bra* expression in the tail muscles.

Trans-complementation assays

Ci-Sna was misexpressed in the notochord by attaching the *Ci-sna*-coding sequence to a truncated 251 bp *Ci-Bra* enhancer (*Ci-Bra**) that mediates expression in all mesodermal lineages, including the notochord, tail muscles and trunk mesenchyme (see Fig. 3E). Fertilized eggs were co-electroporated with a mixture of transgenic DNAs. One of the transgenes corresponds to the truncated *Ci-Bra* enhancer driving the *Ci-sna* gene (*Ci-Bra**/*Ci-sna*). The other is a reporter gene that contains the 434 bp *Ci-Bra* enhancer attached to the *lacZ*-coding sequence (*Ci-Bra*/*lacZ*). Co-electroporation of these transgenes results in the repression of the reporter gene and the loss of *lacZ* staining (Fig. 4B). In contrast, there is no repression of the *Ci-Bra/lacZ* reporter gene when it is co-electroporated with an expression vector containing the truncated *Ci-Bra** enhancer driving a mutagenized *Ci-sna*-coding sequence (*sna****) (Fig. 4A). The mutagenized Ci-Sna protein contains point mutations in three of the five C-terminal zinc fingers.

These results suggest that ectopic expression of Ci-Sna in the notochord (and perhaps mesenchyme) leads to the repression of the *Ci-Bra/lacZ* transgene (see below). An additional control experiment was done to strengthen this conclusion. Embryos were co-electroporated with the *Ci-Bra**/*Ci-sna* expression vector (which encodes the normal Ci-Sna protein) and a mutagenized *Ci-Bra*/*lacZ* reporter gene lacking the sna2 site. Intense staining is observed in the notochord, tail muscles and trunk mesenchyme (Fig. 4C), similar to the pattern obtained with the same reporter gene in the absence of the *Ci-Bra**/*Ci-sna* expression vector (e.g. Fig. 3C). Thus, loss of the sna2 site renders an otherwise normal *Ci-Bra*/*lacZ* transgene immune to repression by ectopic Ci-Sna products in the notochord.

The misexpression of Ci-Sna does not cause an obvious mutant phenotype (e.g. Fig. 4B), suggesting that the endogenous *Ci-Bra* gene is not repressed. It is conceivable that the minimal 434 bp *Ci-Bra/lacZ* reporter gene, which was used in the preceding experiments (Fig. 4A,B), is sensitized for repression by Ci-Sna. Perhaps repression of the ‘full-length’ *Ci-Bra* promoter and endogenous gene depends on higher threshold levels of the Ci-Sna protein than those produced by the truncated 251 *Ci-Bra**/*Ci-sna* expression vector. To test

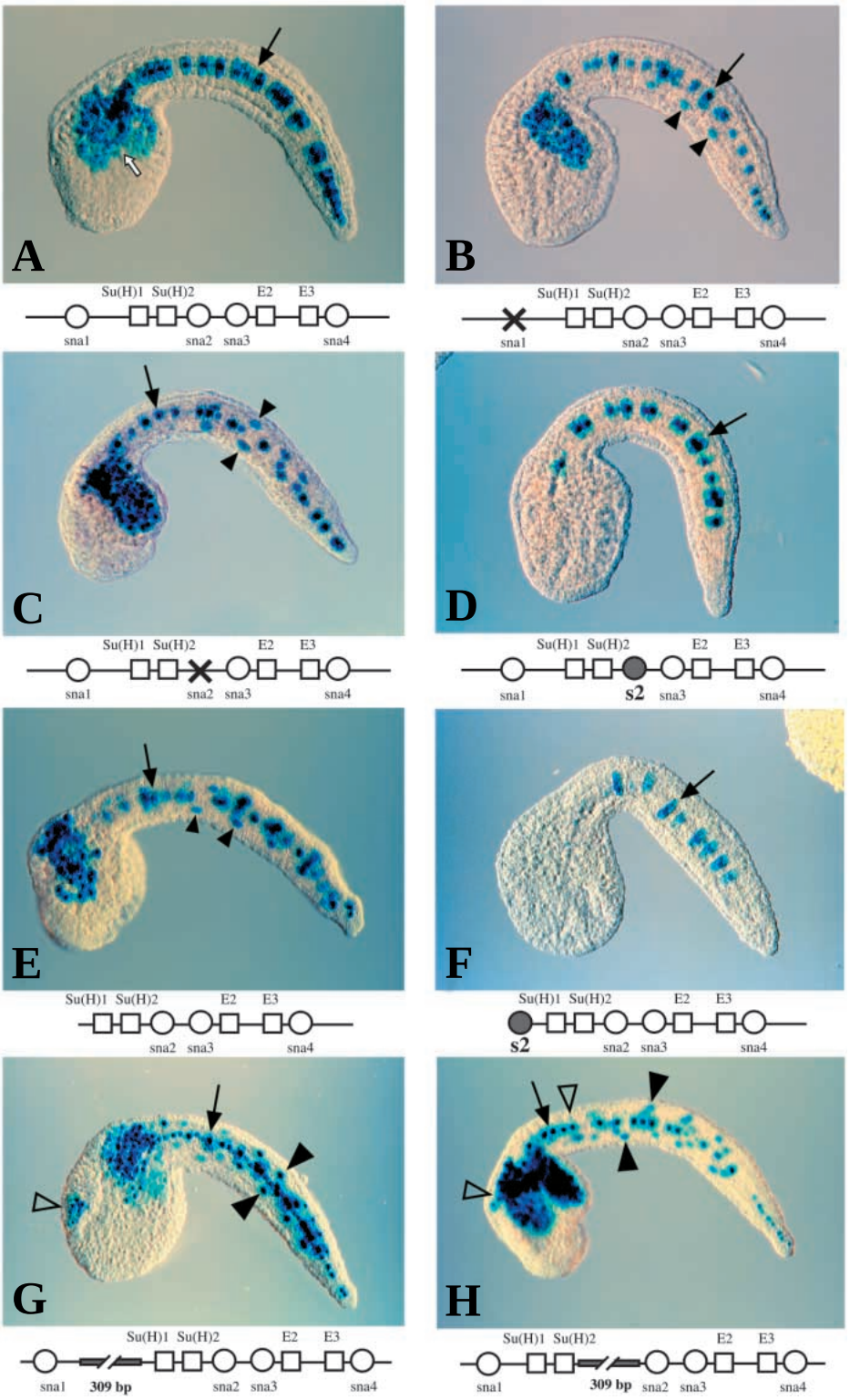

Fig. 3. Ci-Sna-binding sites are essential for the normal *Ci-Bra* expression pattern. Embryos were grown to mid-tailbud stages after electroporation with various *Ci-Bra/lacZ* transgenes (see diagrams below each panel) and the expression patterns were visualized by enzymatic staining. Black arrows indicate staining in the notochord; black arrowheads, staining in the tail muscles. Embryos are oriented with anterior to the left. (A) Expression pattern obtained with the wild-type 434 bp *Ci-Bra/lacZ* transgene. Staining is detected in the notochord and trunk mesenchyme (white arrow). (B) Expression pattern obtained with a mutant form of the 434 bp *Ci-Bra* enhancer, which contains a deletion within the sna1-binding site (note 'X' in the sna 1 site in the diagram). Staining is no longer restricted to the notochord, but is also detected in the tail muscles. (C) Expression pattern obtained with a mutant 434 bp *Ci-Bra* enhancer that contains a deletion within the sna2 site. Staining is detected in both the notochord and tail muscles. (D) Same as C except that the mutagenized *Ci-Bra* enhancer was modified to include a synthetic *Drosophila* s2 site in place of the native sna2 sequence. A normal *lacZ* staining pattern is observed. (E) Expression pattern obtained with a truncated –251 bp *Ci-Bra/lacZ* transgene. Staining is observed in both the notochord and tail muscles (see Corbo et al., 1997a). (F) Same as E except that the synthetic *Drosophila* s2 sequence was inserted 5′ of the Su(H)1 site. This restores a normal staining pattern. (G) Staining pattern obtained with a modified 434 bp *Ci-Bra*/*lacZ* transgene containing a 309 bp spacer sequence between the sna1 and Su(H)1 sites. Staining is detected in all mesodermal lineages, including the notochord and tail muscles. Staining is also detected in the cerebral vesicle of the CNS (open arrowhead). (H) Same as (G) except that the spacer was inserted between the Su(H)2 and sna2 sites. The reporter gene is expressed in all mesodermal lineages, as well as some of the ependymal cells of the spinal cord (open arrowhead).

this possibility, co-electroporation assays were done with a *Ci-Bra*/*lacZ* reporter gene that contains 3.5 kb (rather than 434 bp) of the *Ci-Bra* 5′ regulatory region (Fig. 5C,D). Co-electroporation of this reporter gene with the wild-type *Ci-Bra**/*Ci-sna* expression vector results in only a slight reduction of *lacZ* staining in the notochord (Fig. 5D). Parallel experiments with the same Ci-Sna expression vector and the minimal 434 bp *Ci-Bra/lacZ* reporter gene results in the loss of *lacZ* staining (Fig. 5B), as seen previously (Fig. 4B). The mutagenized *Ci-Bra**/*Ci-sna**** expression vector fails to repress both the 434 bp and 3.5 kb *Ci-Bra*/*lacZ* reporter genes (Fig. 5A,C, respectively).

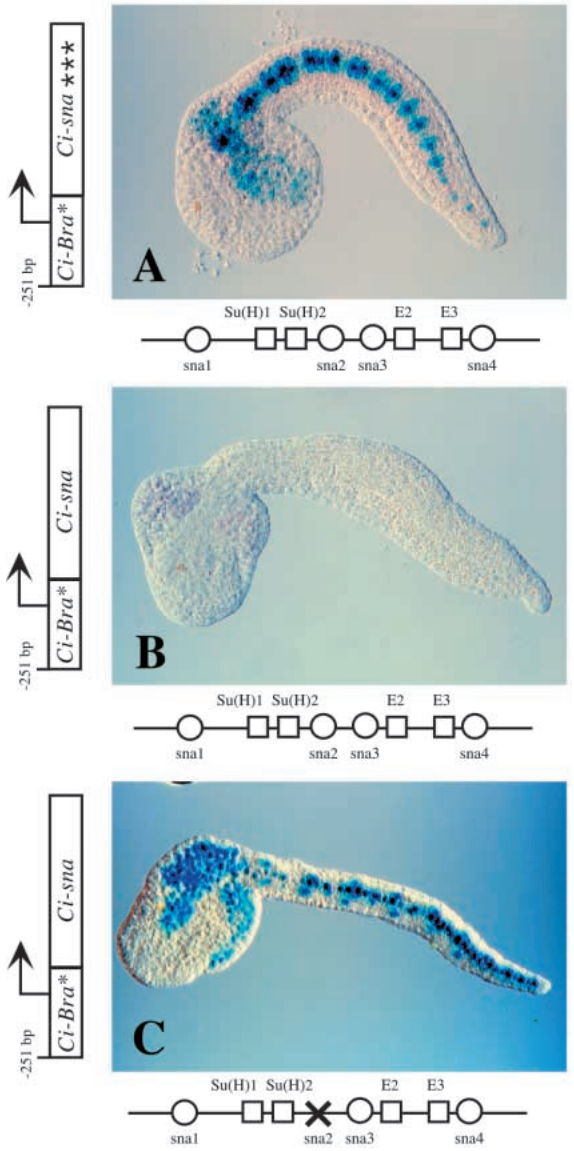

Fig. 4. Misexpression of Ci-Sna in the notochord. Tailbud stage embryos were co-electroporated with *Ci-Bra/lacZ* reporter genes and *Ci-Bra*/Ci-sna* expression vectors that misexpress Ci-Sna in the notochord. Embryos were stained to reveal β-galactosidase expression and are oriented with anterior to the left. (A) Tailbud stage embryo that was co-electroporated with the 434 bp *Ci-Bra*/*lacZ* reporter gene and a mutagenized expression vector (*Ci-Bra*/Ci-sna****) containing the truncated 251 bp *Ci-Bra* enhancer attached to a mutant *Ci-sna*-coding region lacking three of the C-terminal zinc fingers. The *lacZ* reporter gene exhibits normal expression in the notochord. (B) Same as A except that the reporter gene was co-electroporated with the normal *Ci-Bra*/Ci-sna* expression vector, which encodes a wild-type Ci-Sna protein. There is a complete loss of staining in the notochord. (C) Same as B except that the *Ci-Bra*/Ci-sna* expression vector was co-electroporated with a mutagenized *Ci-Bra/lacZ* reporter gene, which contains a deletion in the critical sna2 site. *lacZ* staining is observed in the notochord and tail muscles, suggesting that the loss of the sna2 site renders the *Ci-Bra* enhancer refractory to repression by Ci-Sna.

These results indicate that the *Ci-Bra*/Ci-sna* expression vector directs sufficient levels of the Ci-Sna protein to repress the 434 bp *Ci-Bra*/*lacZ* reporter gene, but not the 3.5 kb *Ci-Bra*/*lacZ* reporter. Perhaps the full-length *Ci-Bra* 5′ regulatory region contains multiple enhancers that mediate expression in the notochord, so that higher levels of Ci-Sna are required for repression. *In situ* hybridization assays suggest that the endogenous *Ci-Bra* gene is not repressed by the *Ci-Bra*/Ci-sna* expression vector in electroporated embryos (data not shown).

A Ci-SnaVP16 fusion protein disrupts the notochord/muscle boundary

Electroporation assays were conducted with a chimeric Ci-Sna protein that contains the VP16 activation domain in place of the Ci-Sna repression domain (see diagrams below the photomicrographs in Fig. 6). The Ci-SnaVP16-coding sequence was placed under the control of the 'full-length' *Ci-sna* promoter region, which includes the first 2.2 kb of 5′ flanking sequence and extends 3′ of the transcription start-site through the first intron of the transcribed region (Erives et al., 1998). This *Ci-snaVP16* fusion gene was co-electroporated with the 434 bp *Ci-Bra/lacZ* reporter gene (Fig. 6). Normally, the reporter gene is expressed only in the notochord and sporadically in the trunk mesenchyme (e.g. Figs 3A, 4A, 5A). However, the *Ci-snaVP16* transgene mediates ectopic activation of the *Ci-Bra* reporter in putative tail muscles (arrowheads, Fig. 6B,D). This ectopic expression is somewhat variable in intensity, but is nonetheless seen in many of the normal tail muscles. Moreover, the electroporated embryos exhibit a consistent mutant phenotype, whereby the tail is reduced in length and the tail muscles appear to be disorganized. These results suggest that the *Ci-snaVP16* transgene alters the expression of endogenous genes and causes a concomitant disruption in the separation of the notochord-muscle boundary.

DISCUSSION

We have presented evidence that Ci-Sna restricts the expression of a minimal 434 bp *Ci-Bra* enhancer to the notochord of *Ciona* embryos. Repression in tail muscles depends on paired Ci-Sna-binding sites located on either side of Su(H) activator sites. We propose that Ci-Sna functions as a boundary repressor that subdivides the mesoderm into dorsal and lateral lineages. This boundary appears to be disrupted in electroporated embryos that express a Ci-SnaVP16 fusion protein.

Mechanisms of repression

Systematic mutations in each of the four Ci-Sna-binding sites within the 434 bp *Ci-Bra* enhancer suggest that the sna1 and sna2 sites are critical for restricted expression in the notochord, while mutations in sna3 and sna4 result in only very weak derepression in the tail muscles (see Fig. 3 and data not shown). Repression appears to depend on tight linkage between the sna1 and sna2 sites and Su(H) activator sites. The insertion of spacer sequences between sna1 and Su(H)1 or between Su(H)2 and sna2 results in a severe derepression of *Ci-Bra/lacZ* transgenes in the tail muscles (see Fig. 3). However, it is conceivable that this derepression results from alterations in the overall organization of the *Ci-Bra* enhancer. The insertion

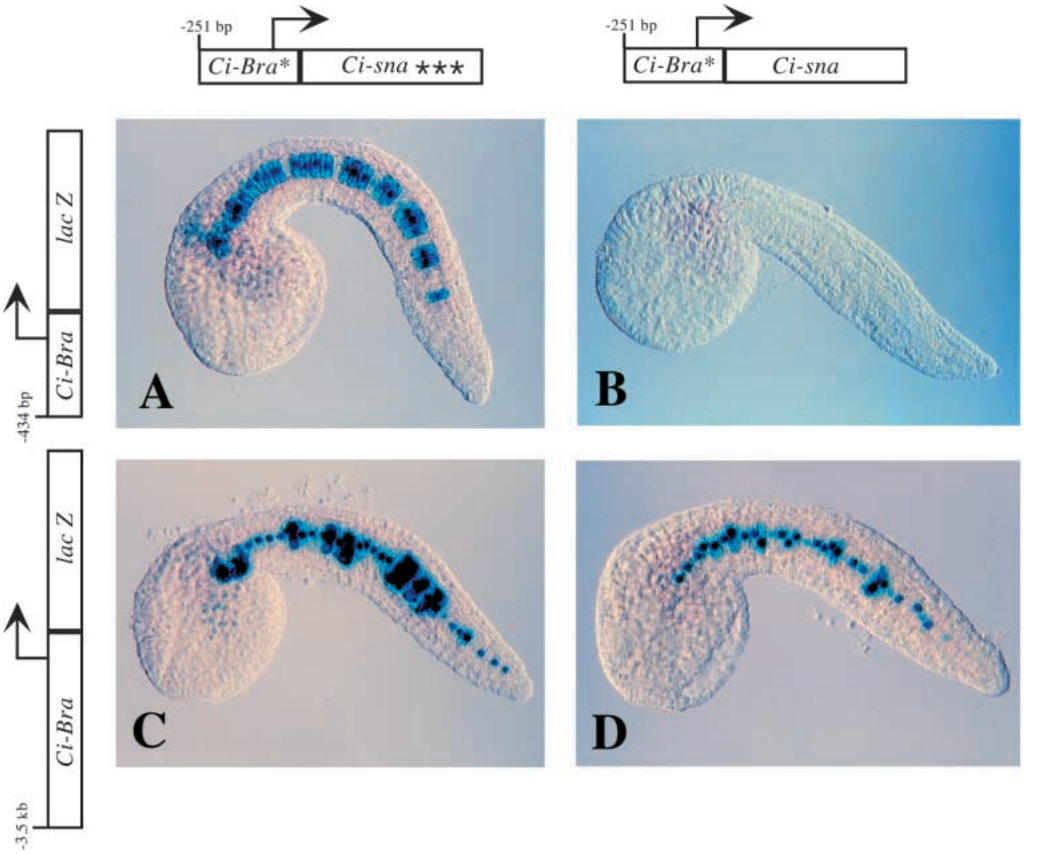

Fig. 5. Ectopic Ci-Sna products do not repress the full-length *Ci-Bra* promoter region. Embryos were co-electroporated with *Ci-Bra*/*Ci-sna* expression vectors and *Ci-Bra*/*lacZ* reporter genes. Embryos were stained and oriented as in Figs 3, 4. (A) The minimal 434 bp *Ci-Bra*/*lacZ* reporter gene was co-electroporated with a mutagenized *Ci-Bra**/*Ci-sna**** expression vector lacking three of the Ci-Sna zinc fingers. A normal *Ci-Bra* staining pattern is observed in the notochord. (B) Same as A except that the 434 bp *Ci-Bra*/*lacZ* reporter gene was co-electroporated with the normal *Ci-Bra**/*Ci-sna* expression vector, which encodes a wild-type Ci-Sna protein. There is complete repression of the reporter gene in the notochord. (C) A full-length 3.5 kb *Ci-Bra*/*lacZ* reporter gene was co-electroporated with the mutagenized *Ci-Bra**/*Ci-sna**** expression vector. Intense staining is detected in the notochord. (D) Same as C except that the normal *Ci-Bra**/*Ci-sna* expression vector was used. The wild-type Ci-Sna protein results in only a slight reduction in the staining directed by the 3.5 kb *Ci-Bra*/*lacZ* reporter gene (compare with the 434 bp reporter gene in B).

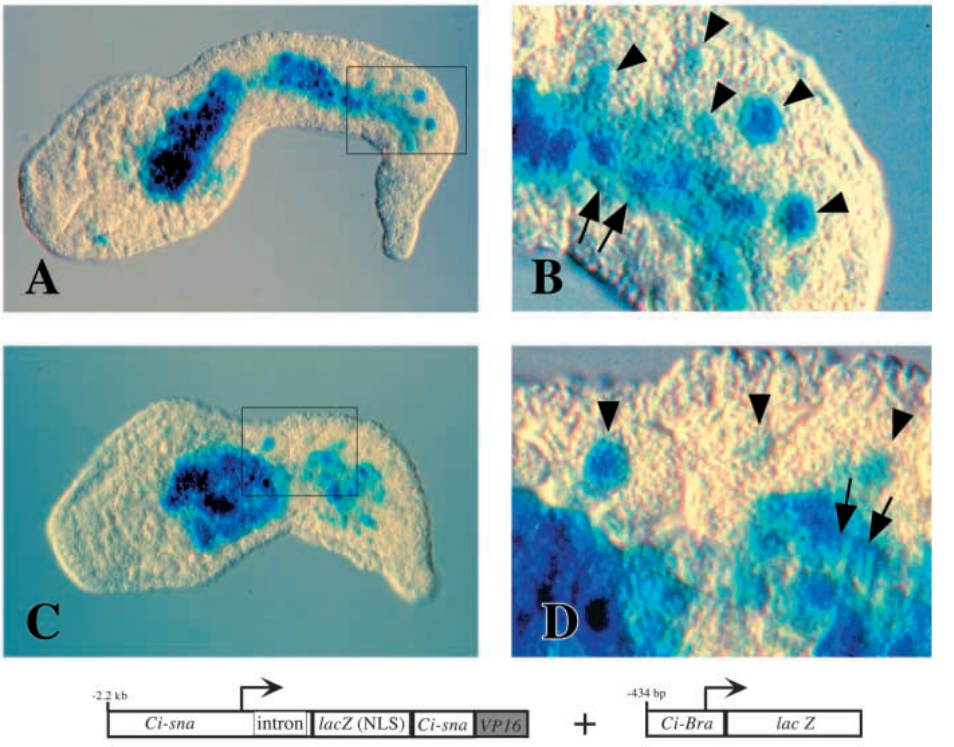

Fig. 6. A Ci-SnaVP16 fusion protein disrupts tail morphogenesis. Embryos were co-electroporated with the 434 bp *Ci-Bra/lacZ* reporter gene, and an expression vector that contains the Ci-SnaVP16 recombinant-coding region under the control of the normal *Ci-sna* promoter region. The fusion protein contains the Ci-Sna DNA-binding domain, and the VP16 activation domain in place of the normal repression domain. (A) This co-electroporated embryo exhibits a relatively mild phenotype, perhaps due to the incorporation of only small amounts of the *Ci-snaVP16* transgene. (B) High magnification view of the boxed region in A. The *Ci-Bra/lacZ* reporter gene exhibits ectopic expression in the tail muscles (arrowheads), in addition to normal expression in the notochord (arrows). (C) An embryo that exhibits a somewhat more severe mutant phenotype. There is a substantial failure of tail elongation. (D) High magnification view of the boxed region in C. The *Ci-Bra/lacZ* reporter gene is expressed in the notochord (arrows) and is also misexpressed in the tail muscles (arrowheads).

of the same spacer sequences in different regions of the *Ci-Bra* enhancer also result in ectopic expression (S. Fujiwara, unpublished results). Perhaps the precise organization of the *cis* regulatory elements within the *Ci-Bra* enhancer is essential for normal expression, similar to the situation seen for the TCRα enhancer in mammalian T cells (e.g. Bruhn et al., 1997).

Previous studies on the *rhomboid* (*rho*) neuroectoderm enhancer (NEE) suggest that a single Snail repressor site is sufficient to block Dorsal-bHLH synergy and inhibit expression in the ventral mesoderm (Gray et al., 1994). The minimal 300 bp NEE contains four high-affinity Snail-binding sites and removal of all but one of the sites, s2, does not substantially alter the normal staining pattern in transgenic embryos (Ip et al., 1992; Gray et al., 1994). The s2 site is located near the middle of the enhancer and is closely linked to two of the four Dorsal activator sites. However, s2 continues to mediate repression even when positioned upstream of the distal-most Dorsal site. The only obvious constraint on s2 function is that it must map within ~50 bp of a Dorsal activator site (Gray et al., 1994).

There may be differences in the way that the *Drosophila* Snail and Ci-Sna proteins mediate repression. Intact sna1 and sna2 sites appear to be required for the repression of *Ci-Bra* in the tail muscles. Neither site is sufficient for repression, despite the tight linkage of each site with Su(H) activator sites. There are several potential explanations for these apparent differences in the *Drosophila* and *Ciona* Snail repressors. First, it is possible that the *rho* NEE contains cryptic low affinity sites (Ip et al., 1992). These sites may act in concert with s2 to surround Dorsal activator sites, similar to the situation observed for the *Ci-Bra* enhancer. Second, the *Drosophila* Snail repression domain contains two potential binding motifs for the putative corepressor protein, CtBP (Boyd et al., 1993; Schaeper et al., 1995; Sollerbrant et al., 1996; Nibu et al., 1998). In contrast, Ci-Sna contains just a single copy of this motif (P-DLS-K). Perhaps efficient repression by Snail depends on the recruitment of at least two CtBP corepressors to the template DNA. Finally, the *Ciona* Su(H) activator may be more robust than the *Drosophila* Dorsal activator and require multiple repressors for inhibition.

Mutant phenotypes

A Ci-SnaVP16 fusion protein appears to interfere with muscle differentiation when expressed under the control of the normal *Ci-sna* promoter region, which is activated prior to the onset of *Ci-Bra* expression. The VP16 moiety presumably converts the Ci-Sna repressor into an activator without altering the target specificity of the normal protein (e.g. Sze et al., 1997; Rusch and Levine, 1997). By analogy to the *Drosophila* Snail repressor, it is conceivable that Ci-Sna normally promotes muscle differentiation by blocking the expression of inappropriate tissue determinants in muscle lineages. Consistent with this view is the finding that the *Ci-sna/Ci-snaVP16* expression vector results in the ectopic induction of *Ci-Bra/lacZ* transgenes in the tail muscles, where they are normally never expressed. The simplest interpretation of the mutant phenotype is that there is a breakdown in the notochord/muscle boundary, and an intermixing of notochord and muscle cells during tail morphogenesis.

This mutant phenotype represents the first use of electroporation and gene fusion methods to assess gene function in an ascidian, although it has been reported that antisense oligonucleotides can produce specific mutant phenotypes in a distantly related ascidian, *Molgula* (Swalla and Jeffery, 1996). In addition, mutant phenotypes have been obtained in *Halocynthia* by RNA injection (e.g. Wada et al., 1997). In principle, the targeted expression of modified transgenes offers several advantages over these other methods. Most notably, RNA injection usually results in the ectopic expression of a given gene in a broad assortment of tissues, thereby obscuring issues of cell autonomy. As we characterize more tissue-specific enhancers in *Ciona*, it should be possible to misexpress regulatory genes in precise patterns.

Snail as a boundary repressor

Ectopic expression of Ci-Sna in the notochord does not produce an obvious mutant phenotype (Fig. 5) and *in situ* hybridization assays suggest that the endogenous *Ci-Bra* gene is not repressed (S. Fujiwara, unpublished results). Moreover, Ci-Sna fails to repress a *lacZ* transgene containing the full-length, 3.5 kb *Ci-Bra* promoter region. These observations raise the possibility that Ci-Sna might not be sufficient to establish or maintain the muscle-notochord boundary. Alternatively, it is conceivable that the expression vector used to misregulate *Ci-sna* in the notochord (the truncated 251 bp *Ci-Bra* enhancer) results in only low levels of Ci-Sna, which are sufficient to repress the minimal 434 bp *Ci-Bra* enhancer

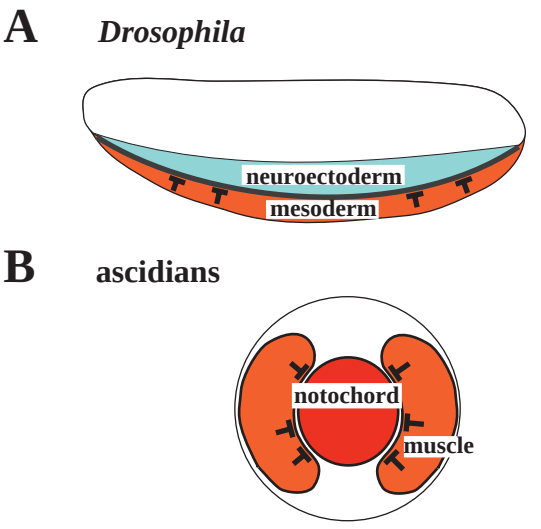

Fig. 7. Ci-Snail is a boundary repressor. We propose that Snail proteins function as evolutionarily conserved repressors to establish boundaries between adjoining tissues. (A) In *Drosophila*, *snail* is expressed in ventral regions of early embryos where it establishes the limits of the presumptive mesoderm. The Snail repressor excludes the expression of neuroectodermal specification genes, such as *rho* and *single minded*, in the ventral mesoderm, and restricts their expression to lateral regions that form the neurogenic ectoderm. (B) This study provides evidence that Ci-Sna functions as a repressor, which creates a boundary between the notochord and tail muscles. The conversion of Ci-Sna into an activator (Ci-SnaVP16) appears to cause a breakdown in this boundary and results in severe disruptions in tail morphogenesis. It is conceivable that vertebrate *snail* genes, such as the *sna-1* gene in zebrafish, also functions as boundary repressors to subdivide the embryonic mesoderm into axial (notochord) and paraxial (somites) lineages.

but are insufficient to repress the full-length gene. Evidence for this latter possibility stems from the observation that the misexpression of Ci-Sna using the full-length, 3.5 kb *Ci-Bra* promoter region causes a disruption in notochord intercalation (S. Fujiwara, unpublished results).

Despite these considerations, the preponderance of the evidence presented in this study suggest that Ci-Sna functions as a boundary repressor, which helps subdivide the embryonic mesoderm into distinct muscle and notochord lineages (Fig. 7B). Previous studies have shown that the *Drosophila* Snail repressor helps establish a boundary between the embryonic mesoderm and neurogenic ectoderm (Fig. 7A; see Leptin, 1991; Kosman et al., 1991). We propose that one or more members of a vertebrate *snail*-related gene family may also act as boundary repressors to help delineate the axial and paraxial mesoderm.

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