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Suppressor of Hairless Activates *Brachyury* Expression in the *Ciona* Embryo

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The *Ciona Brachyury* gene (*Ci-Bra*) is regulated, in part, by a 434-bp enhancer that mediates restricted expression in the notochord. Here we present evidence that a *Ciona* Suppressor of Hairless {Ci-Su(H)} protein functions as an activator of this enhancer. Point mutations that reduce the binding of a GST/Ci-Su(H) fusion protein *in vitro* diminish the expression of mutagenized *Ci-Bra/lacZ* transgenes in electroporated embryos. Overexpression of a Ci-Su(H) fusion protein containing the *Drosophila* Hairy repression domain interferes with notochord differentiation, producing mutant tadpoles with shortened tails. Expression of a constitutively activated Xotch receptor in the notochord, endoderm, and CNS also alters tail morphogenesis. These results suggest that a Notch–Su(H) pathway might participate in notochord differentiation in *Ciona*.

***Key Words: Ciona intestinalis; Suppressor of Hairless;* notochord; *Brachyury*.**

INTRODUCTION

Brachyury encodes a sequence-specific transcriptional activator required for notochord specification in a wide variety of chordates, including zebrafish, frogs, chickens, and mice (e.g., Stott *et al.*, 1993; Schulte-Merker *et al.*, 1994; Cunliffe and Smith, 1994; Kispert *et al.*, 1995a,b; Muller and Hermann, 1997). In vertebrates, *Brachyury* is expressed throughout the presumptive mesoderm of gastrulating embryos (Kispert and Hermann, 1994; Cunliffe and Smith, 1994). At later stages, the *Brachyury* pattern becomes progressively restricted to the axial mesoderm and presumptive notochord (Clements *et al.*, 1996). Experimental studies in ascidians (*Halocynthia*) and *Xenopus* suggest that FGF induces *Brachyury* expression (Schulte-Merker and Smith, 1995; Nakatani *et al.*, 1996); FGF might work synergistically with activin in *Xenopus* (e.g., Latinkic *et al.*, 1997). Genetic studies in zebrafish and mice have identified two regulatory genes, *floating head* and *HNF-3β,* that, together with *Brachyury,* appear to be essential for notochord specification (Weinstein *et al.*, 1994; Talbot *et al.*, 1995). *floating head* encodes a homeodomain protein that is related to Xnot in *Xenopus* (Melby *et al.*, 1997), while *HNF-3β* encodes a winged-helix regulatory protein that is related to Pintallavis in *Xenopus* (O'Reilly *et al.*, 1995).

It is currently unclear how these different regulatory factors and signaling molecules regulate *Brachyury* expression and specify notochord. One complication of the vertebrate studies is that it has been difficult to uncouple the early, pan-mesodermal *Brachyury* pattern from the late, notochord-specific pattern (e.g., Clements *et al.*, 1996). For example, *Brachyury* 5′ regulatory elements have been identified in both *Xenopus* and mice that mediate the early pattern, but thus far no notochord-specific enhancer has been identified for any vertebrate *Brachyury* gene. In contrast, *Brachyury* is solely expressed in the presumptive notochord of ascidian embryos and does not exhibit the initial pan-mesodermal pattern seen in vertebrates (Yasuo and Satoh, 1994; Corbo *et al.*, 1997a).

Recent studies have identified a minimal 434-bp enhancer from the *Ciona Brachyury* (*Ci-Bra*) promoter region that directs notochord-specific expression of a *lacZ* reporter gene in electroporated embryos (Corbo *et al.*, 1997a). In principle, this enhancer can be activated in most mesodermal lineages, including the notochord, tail muscles, and trunk mesenchyme. However, a Snail repressor (Ci-Sna) is important for excluding expression from the tail muscles

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and restricting the pattern to the notochord (Fujiwara *et al.*, 1998). Mutations in critical Ci-Sna binding sites cause otherwise normal *Ci-Bra/lacZ* transgenes to be misexpressed in electroporated embryos. The previous studies did not conclusively identify *Ci-Bra* activators, although the minimal 434-bp enhancer was shown to contain two closely linked sequence motifs that are related to the optimal Suppressor of Hairless [Su(H)] binding site, RTGGGAA (Corbo *et al.*, 1997a; Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). The Su(H) activator often functions downstream of the Notch receptor (reviewed by Lecourtois and Schweisguth, 1997), thereby raising the possibility that Notch plays a role in notochord specification or differentiation in ascidians.

In the present study we provide evidence that a homolog of the *Drosophila* Suppressor of Hairless protein [Su(H)] functions as an activator of *Ci-Bra* expression in the notochord. A *Ciona Su(H)* gene, *Ci-Su(H),* was isolated by cross-homology with a *Xenopus* probe (Wettstein *et al.*, 1997), and a GST/Ci-Su(H) fusion protein was found to bind the two sequence motifs identified by sequence inspection. Nucleotide substitutions that reduce *in vitro* binding diminish the expression of *Ci-Bra/lacZ* transgenes. The overexpression of modified forms of the Ci-Su(H) protein, including a truncated protein and fusion proteins containing heterologous repression domains, results in attenuated expression of a *Ci-Bra/lacZ* transgene. One of the fusion proteins produces a mutant phenotype whereby tailbud-stage embryos possess shortened tails. Finally, a constitutively activated Notch receptor from *Xenopus,* Xotch* (Coffman *et al.*, 1993), causes an apparent expansion of the notochord when expressed in notochord, endoderm, and CNS lineages. These results suggest that a Notch–Su(H) signaling pathway might be important for notochord differentiation in *Ciona.*

MATERIALS AND METHODS

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

Transgenes and electroporations. The wild-type −434-bp and the truncated −251-bp *Ci-Bra/lacZ* transgenes used in Figs. 4A, 4C, 5A, and 6A are described by Corbo *et al.* (1997a). Site-directed mutagenesis was done using the 434-bp enhancer cloned into a pBluescript II plasmid (Sambrook *et al.*, 1989). A single nucleotide change (underlined) was created in the $Su(H)_1$ site using the following mutagenic oligonucleotide: 5′-GGAAACCAAGTTTCAACTTGCCACGCAAGACAATGGG-3′.

The construct described as *Ci-Bra* −434 bp opt. Su(H) (Fig. 4D) was designed in order to avoid possible binding of Dorsal/Rel-type proteins to the Su(H) binding sites. Potential palindromic sequences outside of the Su(H) cores were disrupted using the following oligonucleotides: 5′-AAAATCGGAAACCAAATTTCAACTTCCCACG-3′ for $Su(H)_1$ and 5′-ACAATGGGAAAGTAATATGTCACAATACACTTG-3′ for $Su(H)_2$.

After the mutations were confirmed by sequencing, the fragments were removed from the pBluescript II vector as *Ssp*I–*Pst*I fragments and cloned into the pSP1.72-27 *Ci-Bra/lacZ* expression vector (see Corbo *et al.*, 1997a, for details about the vector).

A putative dominant negative form of Ci-Su(H) was prepared via PCR amplification using two oligonucleotides flanking nt 331–1371 (347 amino acid residues). The 5′-primer has a linker sequence containing a *Pst*I site, while the 3′-primer contains a *Spe*I restriction site, an in-frame stop codon, and a *Sac*I site. After PCR amplification, the product was digested with *Pst*I and *Sac*I and cloned into the pSP1.72-27 injection vector containing a 3.5-kb fragment of the *Ci-Bra* promoter (*Ci-Bra* −3.5 kb/*lacZ*; see Corbo *et al.*, 1997a) cut with *Pst*I/*Sac*I (this digestion removes most of the *lacZ* sequence, leaving the polyadenylation signal intact). After being cloned into the injection vector, the entire PCR-amplified sequence was checked by sequencing on both strands with internal primers (Sambrook *et al.*, 1989) (see Fig. 5B and Table 1).

The *Ci-Bra* −3.5 kb/*Ci-Su(H)*^{DBD}–WRPW fusion gene (Fig. 5C and Table 1) was prepared by cloning the C-terminal repression domain of Hairy at the 3′ end of the *Ci-Bra* −3.5 kb/*Ci-Su(H)*^{DBD} sequence. The C-terminal repression domain was prepared by annealing the following two oligonucleotides: 5′-CTAGTCAGATCAAGGAAGAAGAACAACCATGGAGACCATGGTAAGAGCT-3′ and 5′-CTTACCATGGTCTCCATGGTTGTTCTTCTTCCTTGATCTGA-3′. These oligonucleotides encode the last 12 amino acids of the Hairy sequence, including the WRPW motif, followed by an in-frame stop codon, and *Spe*I and *Sac*I restriction sites. Both the Hairy repression domain and the newly formed junctions were checked by sequencing (Sambrook *et al.*, 1989).

The *Ci-Bra* −3.5 kb/*Ci-sna*RD–*Ci-Su(H)*^{DBD} fusion gene (Table 1) was prepared by cutting a *Mun*I site located in the *Ci-sna* cDNA upstream of the sequence encoding the Zn-finger DNA-binding domain. This site was then blunted with Klenow DNA polymerase (New England Biolabs) and the repression domain was ligated into the previously blunted *Pst*I site located upstream of the *Ci-Su(H)*^{DBD}.

The *Xotch* coding sequence that was used in Fig. 6 includes a 96-bp fragment from the N-terminal region of the *Xenopus* N-cadherin gene that contains an initiating ATG and a signal peptide. This sequence was fused to a truncated *Xotch* coding region spanning codons 1801 to 7914, which includes the transmembrane domain and the entire intracellular domain (Coffman *et al.*, 1993). The N-cadherin/*Xotch* fusion sequence was placed in-frame with a 2.7-kb *Eco*RI–*Not*I genomic DNA fragment from the 5′-flanking region of the *Ciona forkhead* gene (*Ci-fkh*). The *Ci-fkh* sequence includes ~2.6 kb of 5′ flanking sequence, a 75-bp 5′-UTR, and the initiating ATG codon (Corbo *et al.*, 1997b).

Aliquots containing 100 μg of a given *Ci-Bra/lacZ* fusion gene were electroporated as described by Corbo *et al.* (1997a). All the fusion genes were tested in parallel on several different batches of embryos. The results from a representative experiment are shown in Table 1.

Cloning and characterization of Ci-Su(H). A full-length cDNA for the *Xenopus XSu(H)1* gene (Wettstein *et al.*, 1997; kindly provided by Drs. Daniel Wettstein and Chris Kintner) was used to screen a gastrula-stage cDNA library (kindly provided by Drs. Jamie Lee and Tom Meedel; see Corbo *et al.*, 1997a). From a total of 500,000 recombinants, just one positive clone was isolated. This clone contains a 1-kb insert including the 3′ trailer sequence and a small portion of the 3′ coding region. The remainder of the coding region was isolated using a PCR-based RACE assay with the following primer: 5′-GTTGCCTGGGTCCAGGCTCAGGGG-3′. Details regarding the construction of the RACE cDNA library are

described in Corbo *et al.* (1997a). A 1.5-kb RACE product was isolated and cloned. The cDNA and RACE fragments were sequenced on both strands using standard methods (Sambrook *et al.*, 1989).

Expression and purification of a GST/Ci-Su(H) fusion protein. An *Nco*I site was created at the putative initiating ATG codon in the *Ci-Su(H)* RACE product using the following mutagenic oligonucleotide: 5′-TGGATAACTAGATTTGCCATGGATCACCCCCACCAC-3′. The entire RACE product (encompassing codons 1–478) was subsequently cloned into the pGEX-KG expression vector (Guan and Dixon, 1991) as an *Nco*I–*Sac*I fragment (the *Sac*I site derives from the polylinker of the pBluescript vector). The resulting pGEX-Ci-Su(H) plasmid was introduced into *Escherichia coli* (HB101), and the fusion protein was expressed and purified as described by Guan and Dixon (1991). The protein was recovered by dialysis in 20 mM Hepes, pH 7.5, 50 mM KCl, 3 mM EDTA, 0.5% NP-40, 10% glycerol, 1 mM $ZnCl_2$, and 1 mM DTT.

Gel shift assays. The following 32-bp double-stranded oligonucleotides were used in the binding assays (only the 5′–3′ strand is shown). These sequences encompass normal and mutant versions of the two Ci-Su(H) binding sites within the 434-bp enhancer: TCAACTTCCCACGCAAGACAATGGGAAAGTAA (wild-type), TCAACTTGCCACGCAAGACAATGGGAAAGTAA [single change in $Su(H)_1$], TCAACTTGCCACGCAAGACAATGGCAAAGTAA (single change, both sites), and TCAACTTAAAACGCAAGACAATTTTAAAGTAA (clustered changes). Oligonucleotides were annealed, radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP, and purified by chromatography using Biogel P-10 columns. The labeled probes were recovered at a concentration of ~1 μM in TE buffer.

Protein–DNA complexes were formed at room temperature for 15 min in 20 μl of the following reaction mix: 5×10^5 cpm probe, 1 μl 10 mg/ml BSA, 2 μl 1 mg/ml poly(dI–dC), 10 μl 2× binding buffer [20 mM Hepes, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 20% (v/v) glycerol, 0.1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 2 μg/ml leupeptin, 1.25 μg/ml pepstatin], 5 μl of the GST/Ci-Su(H) fusion protein (at ~100 ng/μl). The reaction mixes were fractionated on 5% polyacrylamide/0.5× TBE gels and analyzed by autoradiography.

RESULTS

The *Ci-Bra* promoter region contains a 434-bp enhancer that mediates notochord-specific gene expression (Corbo *et al.*, 1997a; Fujiwara *et al.*, 1998). Previous studies have identified a number of potential *cis*-regulatory elements in this enhancer, including four Ci-Sna repressor sites that are critical for restricted expression in the notochord (summarized in Fig. 1A). The enhancer also contains three E-box motifs and two putative Su(H) recognition sequences [$Su(H)_1$ and $Su(H)_2$]. A 24-bp deletion that removes these latter sites causes a loss of *Ci-Bra/lacZ* expression in the notochord of electroporated embryos (Corbo *et al.*, 1997a). Subsequent studies were done to determine whether Su(H) is a bona fide activator of *Ci-Bra*.

Isolation and characterization of a Ciona Su(H) homolog. A DNA fragment containing a conserved portion of the *Xenopus* Su(H) coding region was used to screen a *Ciona* cDNA library. A single cDNA clone was isolated, and PCR-based RACE assays were done to obtain the 5′ end of the coding sequence (Corbo *et al.*, 1997a). The analysis of both the RACE and cDNA fragments yielded a sequence of 2523 bp containing an open reading frame coding for a protein composed of 554 amino acid (AA) residues. The putative Ci-Su(H) protein shares extensive homology with both *Drosophila* and vertebrate homologs (Fig. 2). For ex-

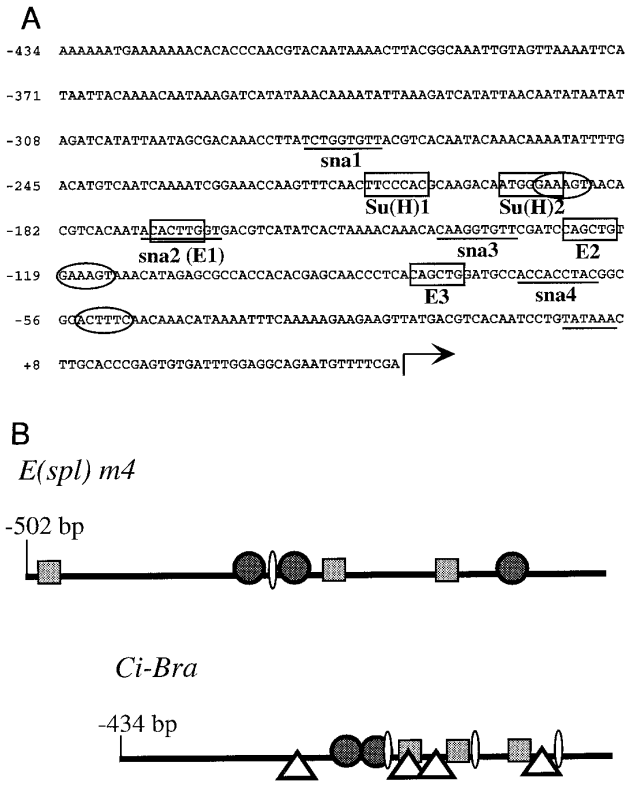

FIG. 1. Comparison of the *Ci-Bra* enhancer with the *E(spl) m4* enhancer. (A) Previous studies (Corbo *et al.*, 1997a; Fujiwara *et al.*, 1998) identified a 434-bp sequence from the *Ci-Bra* 5′ regulatory region that is sufficient to mediate notochord-specific expression of GFP and *lacZ* reporter genes in electroporated embryos. The putative TATA element is underlined (TATAAA), and the 5′ end of TATA corresponds to nucleotide +1. The *Ci-Bra* enhancer contains four binding sites for a *Ciona* Snail repressor (Ci-Sna), and the distal sna1 and sna2 sites are essential for notochord-restricted expression. The enhancer contains three E-box sequences (E1 overlaps the sna2 site) and two potential Su(H) recognition sequences. A 24-bp deletion that removes the $Su(H)_1$ and $Su(H)_2$ motifs inactivates *Ci-Bra/lacZ* expression in the notochord of electroporated embryos (Corbo *et al.*, 1997a). (B) The *E(spl)m4* enhancer in *Drosophila* and *Ci-Bra* show similar organizations. The *E(spl)* gene complex includes several genes that encode bHLH repressor proteins. One of these, *m4*, is regulated by a promoter region that contains three Su(H) protein binding sites (circles) and three E-boxes (squares), which interact with bHLH activator proteins. The enhancer also contains an evolutionarily conserved hexamer motif, GAAAGT (ovals). The *Ci-Bra* enhancer contains two Su(H) binding sites, three E-boxes, and three copies of the hexamer motif. The diagram of the *Ci-Bra* enhancer also includes the four Ci-Sna repressor sites (triangles).

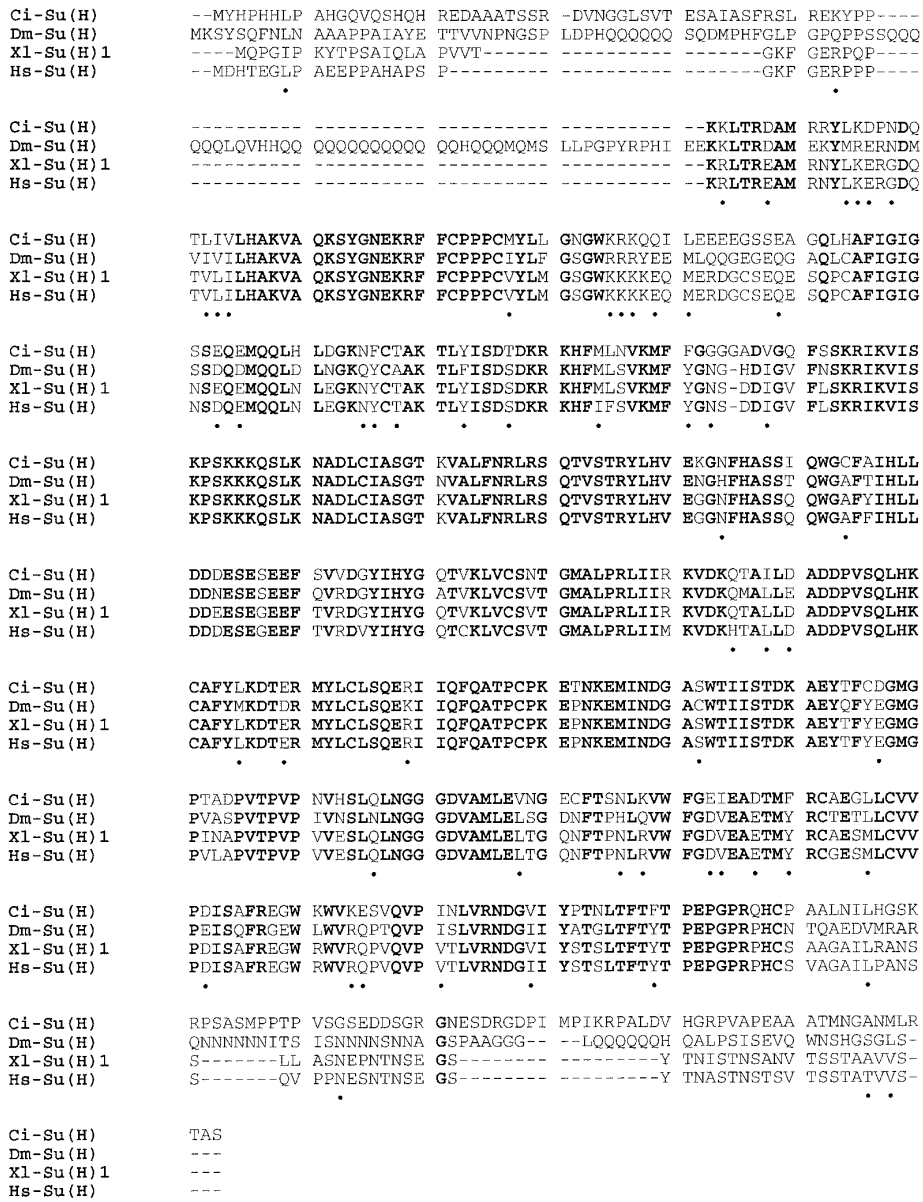

FIG. 2. Comparison of the *Ciona* Su(H) [Ci-Su(H)] protein sequence with the corresponding *Drosophila* (Dm), *Xenopus* (Xl), and human (Hs) sequences. The *Ci-Su(H)* coding region is 2523 bp in length and encodes a putative protein of 554 amino acid residues. Bold face indicates identical residues in all four proteins; the dots indicate conserved residues. The GenBank accession number for the *Ci-Su(H)* cDNA sequence is AF085173. Dm-Su(H) refers to the *Drosophila melanogaster* Su(H) protein (Schweisguth and Posakony, 1992); X-Su(H)1 refers to the *Xenopus laevis Su(H)* homolog number 1 (Wettstein *et al.*, 1997); and Hs-Su(H) refers to the human *Su(H)* homolog *RPB3* (Amakawa *et al.*, 1993).

ample, the most conserved region of the protein, extending from AA residues 54 to 480, shares 77 and 81% amino acid identity, respectively, with the corresponding regions of the *Drosophila* and *Xenopus* proteins. This high degree of homology leaves little doubt that the *Ciona* protein is a homolog of Suppressor of Hairless. Northern blots and *in situ* hybridization assays indicate that the *Ci-Su(H)* gene is broadly expressed during the time of notochord specification and differentiation (data not shown).

Gel-shift assays were carried out to determine whether the Ci-Su(H) protein binds the two putative recognition sequences in the 434-bp *Ci-Bra* enhancer (see Fig. 1A). These experiments involved the use of a GST/Ci-Su(H) fusion protein that contains AA residues 1–478, which

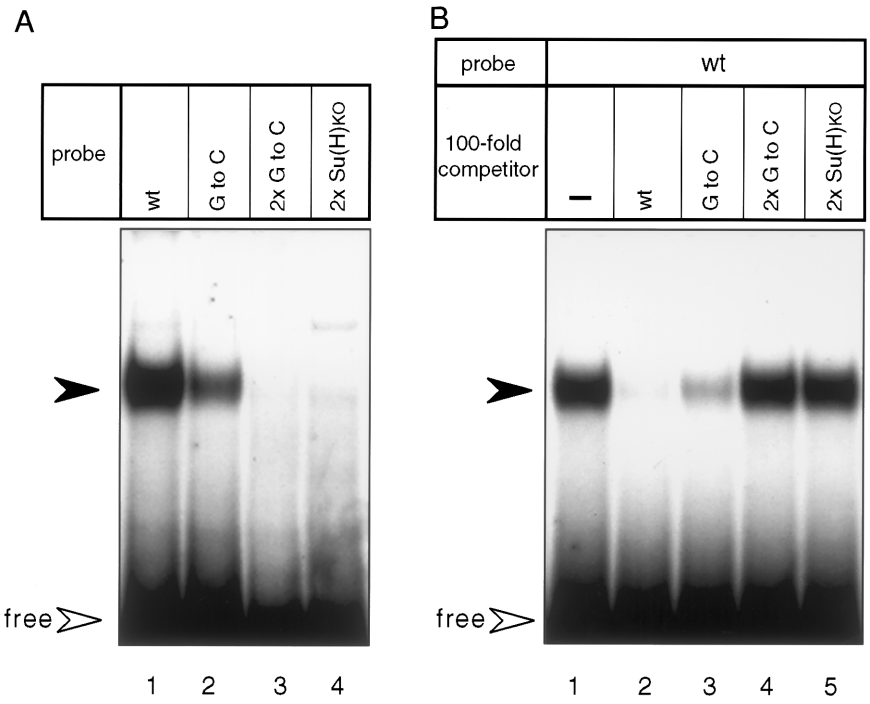

FIG. 3. Gel shift assays. (A) Wild-type or mutagenized 32-bp DNA fragments from the *Ci-Bra* enhancer, containing both putative Su(H) binding sites, were radiolabeled with ^{32}P, mixed with a GST/Ci-Su(H) fusion protein, and fractionated on a polyacrylamide gel. Lane 1, shifted complexes (black arrowhead) are formed when the fusion protein is mixed with the wild-type 32-bp fragment. Lane 2, same as lane 1 except that the distal Ci-Su(H)$_1$ site contains a single nucleotide substitution (G to C). There is at least a two- to threefold reduction in the levels of shifted complexes. Lanes 3 and 4, both lanes contain mutagenized oligonucleotides with either single nucleotide changes in both Su(H) motifs (lane 3) or clustered mutations in both sites (lane 4). The mutagenized templates fail to form shifted complexes. (B) Competition assay carried out with a 100-fold excess of unlabeled oligonucleotides. Lane 1, shifted complexes formed by mixing the wild-type template with the fusion protein (same as lane 1 in A). Lane 2, same as lane 1 except that a 100-fold excess of the unlabeled, wild-type DNA was added as competitor. This nearly abolishes the formation of shifted complexes with the radiolabeled probe. Lane 3, same as lane 2 except that the competitor DNA contains a single G to C substitution in the Ci-Su(H)$_1$ site. The mutant competitor impedes, but does not completely block, the formation of shifted complexes with the radiolabeled probe. Lanes 4 and 5, the cold competitor contains either single nucleotide changes (lane 4) or clustered mutations (lane 5) in both Su(H) binding sites. The mutant competitors have no effect on the formation of shifted complexes.

includes nearly the entire conserved domain (AA residues 54 to 480; see Fig. 2). The fusion protein was incubated with a 32-bp synthetic oligonucleotide that contains both putative Ci-Su(H) binding sites in the *Ci-Bra* enhancer. This fragment was labeled with ^{32}P, incubated with the fusion protein, and then fractionated on a polyacrylamide gel (lane 1, Fig. 3A). A single G-to-C point mutation in the Ci-Su(H)$_1$ binding site results in a several-fold reduction in binding (lane 2, Fig. 3A), while mutations in both sites eliminate the shifted complex (lanes 3 and 4, Fig. 3A). Competition assays indicate that a 100-fold excess of the unlabeled, wild-type 32-bp fragment nearly eliminates the shifted complex (lane 2, Fig. 3B). The mutagenized template containing the single G-to-C substitution diminishes, but does not completely block, the formation of the shifted complex (lane 3, Fig. 3B). However, mutations in both Su(H) binding sites abolish the ability of the 32-bp *Ci-Bra* fragment to inhibit the shifted complexes (lanes 4 and 5, Fig. 3B).

Correlation between in vitro binding and in vivo expression. To determine whether the Ci-Su(H) binding sites are important for *Ci-Bra* expression *in vivo*, *Ci-Bra/lacZ* fusion genes were mutagenized and electroporated into one-cell embryos. The initial experiments involved the use of a truncated, 251-bp *Ci-Bra* enhancer, which lacks the distal sna1 repressor site (see Fig. 1A) and mediates expression in both the notochord (red arrowhead; Fig. 4A) and tail muscles (orange arrowhead; Fig. 4A). A single point mutation in the Su(H)$_1$ site (G-to-C) diminishes binding *in vitro* (Fig. 3A, lane 2) and virtually abolishes expression in the notochord in electroporated embryos (Fig. 4B). In contrast, staining in the tail muscles is unaffected (orange arrowhead, Fig. 4B) and thereby serves as an internal control for the efficacy of the electroporation technique.

The optimal Su(H) recognition sequence, RTGGGAA, is related to the half-site of Rel-containing transcription factors (Israel *et al.*, 1989), thereby raising the possibility that

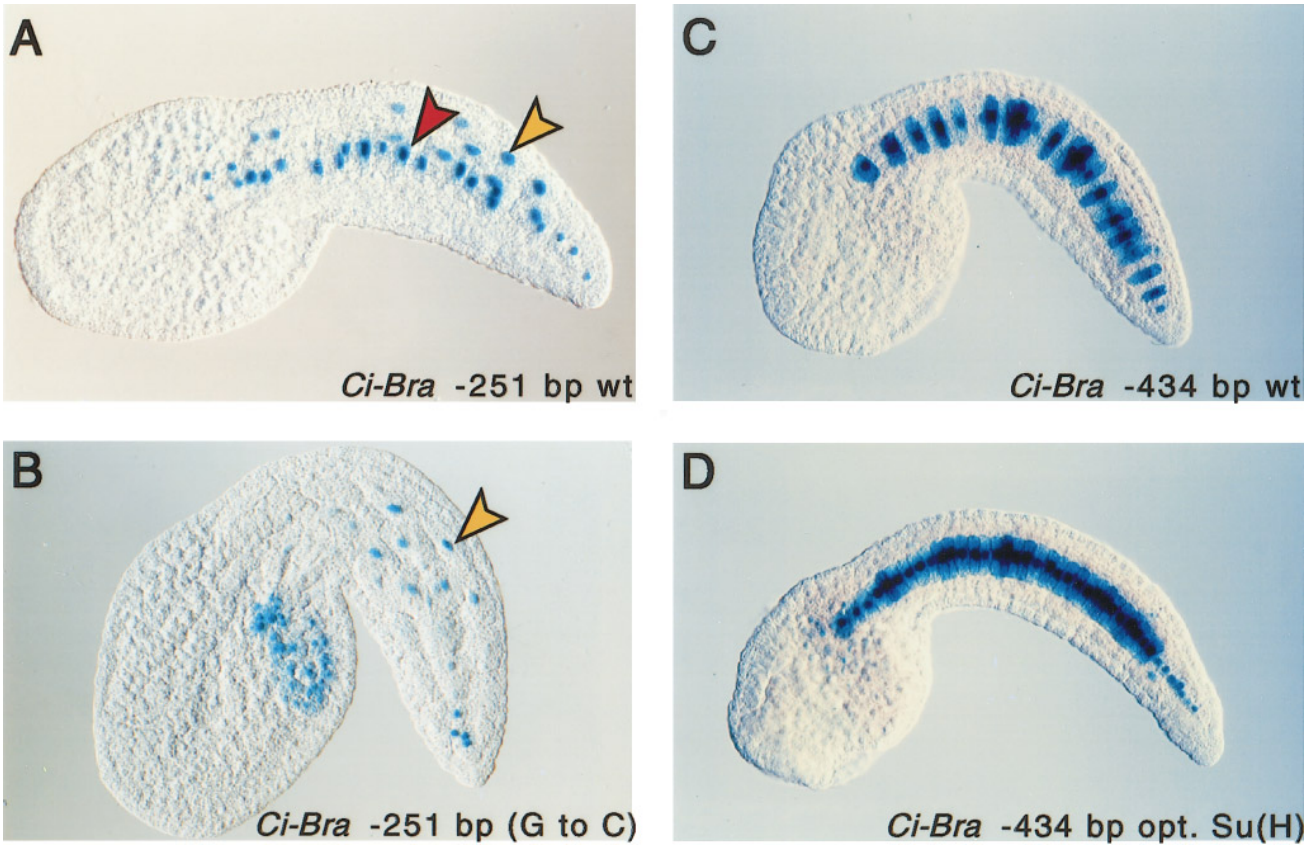

FIG. 4. Expression of mutagenized *Ci-Bra/lacZ* fusion genes in electroporated embryos. The embryos are at tailbud stage of development and are oriented with anterior to the left and dorsal up. The expression patterns were visualized by histochemical staining with X-gal. (A) Embryo electroporated with a truncated 251-bp *Ci-Bra/lacZ* transgene. The removal of the distal sna1 site (see Fig. 1A) results in the derepression of the staining pattern, so that expression is detected in both the notochord (red arrowhead) and tail muscles (orange arrowhead). (B) Same as A except that a single nucleotide substitution was created in the $Su(H)_1$ motif (GTGGGAA to GTGGCAA). This substitution results in reduced binding of a GST/Ci-Su(H) fusion protein (see Fig. 3A). The mutagenized transgene exhibits weak staining in the tail muscles and trunk mesenchyme, but is essentially inactive in the notochord. This pattern was observed in 80% of electroporated embryos. Most of the remaining embryos exhibited residual staining in the notochord. (C) Staining pattern obtained with the wild-type 434-bp *Ci-Bra/lacZ* enhancer. Expression is restricted to the notochord. (D) Same as C except that the *Ci-Bra* enhancer contains nucleotide changes just outside of each Ci-Su(H) recognition sequence, which eliminate weak dyad symmetry and potential Rel binding sites. A normal staining pattern is observed, with the levels of expression slightly higher than normal.

NF-κB or other Rel proteins participate in *Ci-Bra* regulation. Rel proteins bind DNA as obligate dimers and recognize a symmetric sequence, usually GGG-----CCC or GGG-----CCA (e.g., Ip *et al.*, 1991). To confirm the specificity of the Su(H) binding sites for Ci-Su(H) protein, we disrupted the potential dyad symmetry of this site. The $Ci\text{-}Su(H)_1$ site, which includes the sequence GGG-------AAC on the bottom strand, was changed to GGG-------AAT. In addition, the $Ci\text{-}Su(H)_2$ site, which includes the sequence GGG-------CAC, was changed to GGG-------TAT. These alterations do not influence the binding of the GST/Ci-Su(H) fusion protein *in vitro* (data not shown), but result in slightly stronger staining *in vivo* (Fig. 4D; compare with Fig. 4C). These results establish a positive correlation between the binding of Ci-Su(H) to the *Ci-Bra* enhancer and the expression of *lacZ* fusion genes in electroporated embryos.

Expression of dominant negative and repressor forms of Ci-Su(H). Modified forms of the *Ci-Su(H)* coding sequence were placed under the control of the full-length, 3.5-kb *Ci-Bra* promoter region, which directs robust expression in the notochord. A truncated coding sequence containing just the region encoding the conserved DNA-binding domain of Ci-Su(H) reduces the expression of a coelectroporated *Ci-Bra/lacZ* fusion gene (Fig. 5B; compare with Fig. 5A). Although there is a significant reduction in *lacZ* staining (Fig. 5B), the truncated Ci-Su(H) protein fails to produce a consistent mutant phenotype, suggesting that the endogenous *Ci-Bra* gene is not inhibited (see Table 1). The *Ci-Bra/lacZ* fusion gene contains the minimal, 434-bp notochord-

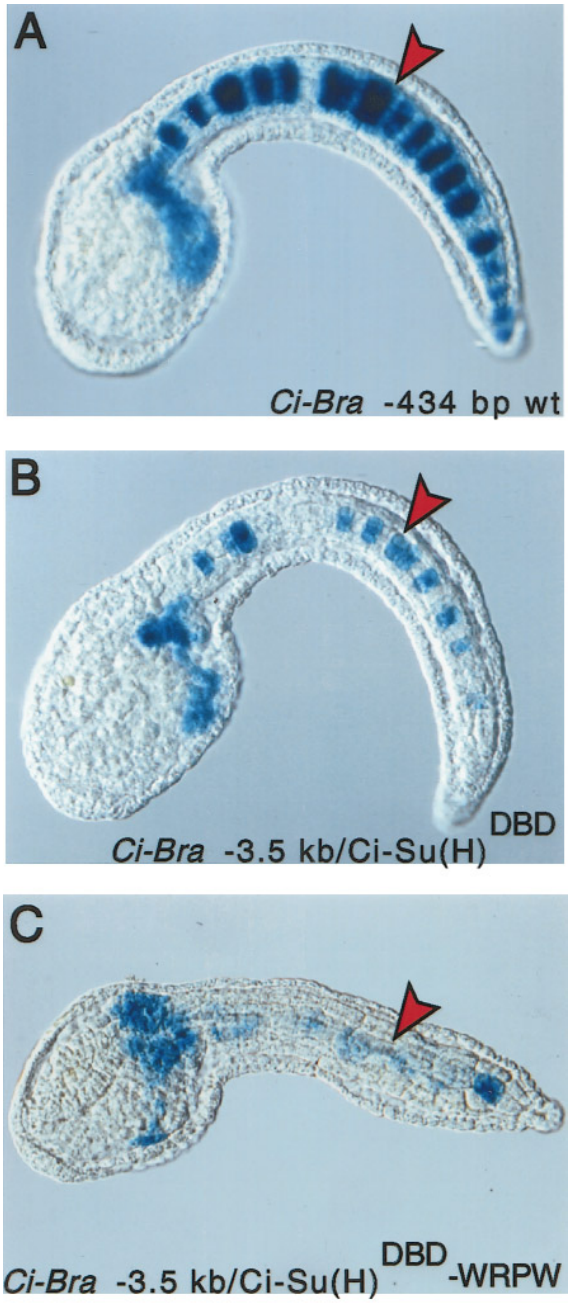

FIG. 5. Modified forms of Ci-Su(H) affect notochord differentiation. Embryos were electroporated at the one-cell stage and allowed to develop through mid-tailbud stages prior to X-gal staining. They are oriented with dorsal up and anterior to the left. (A) Embryo electroporated with the wild-type 434-bp *Ci-Bra/lacZ* reporter gene. Intense staining is observed in the notochord cells (red arrowhead). Weak expression is also detected in the trunk mesenchyme. (B) Same as A except that the embryo was coelectroporated with an expression vector containing the full-length 3.5-kb *Ci-Bra* promoter region attached to the *Ci-Su(H)* DNA-binding domain (DBD) coding region. Expression of the mutant protein in the notochord results in reduced expression of the 434-bp *Ci-Bra/lacZ* reporter gene (compare with A). (C) Same as B except that the embryo was coelectroporated with an expression vector encoding a Ci-Su(H)–WRPW fusion protein. The WRPW motif is essential for interacting with the Groucho corepressor and mediating transcriptional repression (e.g., Paroush *et al.*, 1994). Expression of this fusion protein in the notochord results in a severe reduction in the expression of the 434-bp *Ci-Bra/lacZ* reporter gene (compare with A). In addition, greater than 10% of the electroporated embryos exhibit this mutant phenotype, whereby the tail is stubby and shortened due to a failure in the extension of the notochord (see Table 1).

specific enhancer and may be sensitized to the effects of the truncated Ci-Su(H) protein (see Discussion).

In an effort to disrupt endogenous gene activity, the Ci-Su(H) DNA binding domain was attached to the repression domains of Ci-Snail and the *Drosophila* Hairy repressor (see Table 1). The latter protein contains the C-terminal sequence motif WRPW which interacts with the evolutionarily conserved corepressor Groucho (reviewed by Fisher and Caudy, 1998). Coelectroporation of embryos with the *Ci-Bra/Ci-Su(H)*–WRPW fusion gene and the *Ci-Bra/lacZ* reporter gene results in both diminished *lacZ* staining and a mutant phenotype, whereby 12% of the embryos exhibit a conspicuous shortening of the tail (Fig. 5C; Table 1). This phenotype appears to result from a failure of the notochord cells to undergo a change in cell shape, whereby columnar cells become more cuboidal in shape.

A constitutively activated Xotch receptor causes an expansion of the notochord. The preceding experiments suggest that Ci-Su(H) is essential for the expression of the *Ci-Bra* gene. Su(H) often functions downstream of the Notch receptor (reviewed by Artavanis-Tsakonas *et al.*, 1995; Lecourtois and Schweisguth, 1997). To determine whether Notch might play a role in notochord differentiation we examined the consequences of expressing a constitutively activated form of the *Xenopus* Xotch receptor in electroporated *Ciona* embryos. This mutant form of Xotch has been previously shown to cause an expansion of muscle and neuronal tissues in injected *Xenopus* embryos (Coffman *et al.*, 1993). Activation of Xotch was achieved by deleting most of the extracellular sequences, including the EGF repeats. A signal sequence from N-cadherin was placed at the 5′ position of the modified *Xotch* coding sequence, which retains the transmembrane-spanning domain and all of the intracellular sequences including the cdc10 repeats (Coffman *et al.*, 1993). This region is highly conserved among all vertebrate Notch receptors (e.g., Wettstein *et al.*, 1997), so it seemed reasonable to suppose that it would function in the *Ciona* system.

This *Xotch* sequence was placed under the control of the *Ciona forkhead* (*Ci-fkh*) promoter region, which mediates expression in the notochord, endoderm, and CNS (Corbo *et al.*, 1997b; Di Gregorio *et al.*, in preparation). The *Ci-fkh/Xotch** fusion gene produces a mutant phenotype, whereby tailbud-stage embryos have a stubby tail and an apparent increase in the number of notochord cells (Fig. 6B). The notochord-specific 434-bp *Ci-Bra/lacZ* reporter transgene

TABLE 1
Effects of the Expression of Modified Forms of Ci-Su(H) and Xotch in *Ciona* Embryos

Fusion gene[a]	Scored embryos[b]	Positive embryos[c]	Qualitative expression	% expression	τ = RC (ms)[d]	% phenotypes
Ci-Bra −434 bp/lacZ	122	100	Strong	82	20.9	0
Ci-Bra −3.5 kb/Ci-Su(H)^{DBD}	444	205	Weak	46	20.8	1
Ci-Bra −3.5 kb/Ci-snaRD–Su(H)^{DBD}	356	101	Weak	28	19.2	8
Ci-Bra −3.5 kb/Ci-Su(H)^{DBD}–WRPW	293	106	Weak	36	17.8	12
Ci-fkh −2.6 kb/Xotch^{CA}	278	202	Strong	72	21.7	20

[a] All the fusion genes were coelectroporated with the Ci-Bra −434 bp/lacZ as a reporter.
[b] Only the fully developed tailbud-stage embryos were scored.
[c] Only the embryos with at least half of the notochord cells stained were considered positive.
[d] Electroporation settings were 50 V and 1 mF.

exhibits an expanded staining pattern when it is coelectroporated with the *Ci-fkh/Xotch** transgene. The simplest interpretation of this result is that misexpression of the activated Xotch receptor results in a partial transformation of the endoderm and/or CNS into notochord.

DISCUSSION

We have presented evidence that Su(H) is an essential activator of *Ci-Bra* expression in the *Ciona* notochord. A close correlation was established between the binding of a GST/Ci-Su(H) fusion protein *in vitro* and the expression of *Ci-Bra/lacZ* transgenes *in vivo*. Evidence was also presented that a dominant negative form of Ci-Su(H) attenuates the expression of a *Ci-Bra/lacZ* transgene in electroporated embryos. In addition, repressor forms of Ci-Su(H) produce mutant phenotypes, whereby tailbud-stage embryos possess shortened tails. The evidence that Su(H) activates *Ci-Bra* expression raises the possibility that a Notch signaling pathway participates in notochord specification. Ectopic expression of a constitutively activated form of the *Xenopus* Xotch receptor results in the overexpression of a coelectroporated *Ci-Bra/lacZ* transgene and an apparent increase in the number of notochord cells.

Similarity between Ci-Bra and Enhancer of split regulation. The overall configuration of the *Ci-Bra* enhancer is remarkably similar to the *cis*-regulatory regions of the *Enhancer of split* gene complex [*E(spl)-C*] in *Drosophila* (Bailey and Posakony, 1995; Furukawa *et al.*, 1995; Lecourtois and Schweisguth, 1995). The *E(spl)-C* includes seven transcription units that encode bHLH repressors, as well as several other genes involved in neurogenesis such as *groucho* (reviewed by Knust, 1994). Some of the bHLH genes are activated by signaling through the Notch pathway. For example, the first 502 bp of the *m4* 5′ flanking region contain three Su(H) binding sites intermixed with three E-boxes (see Fig. 1B). Previous studies have shown that a single G-to-C substitution in each of the three Su(H) sites results in a severe reduction in the activities of a *m4/lacZ* transgene in wing imaginal disks (Bailey and Posakony, 1995). There is a comparable loss in staining when the two proximal E-boxes are mutated. These studies prompted the suggestion that Notch stimulates *m4* expression through synergistic interactions between Su(H) and bHLH activators (Bailey and Posakony, 1995). In addition, a conserved hexamer motif, GAAAGT, was shown to be closely linked to the Su(H) sites in both the *Drosophila E(spl)-C* genes and the mouse homologs of these genes. It is not known whether *trans*-acting factors bind this putative *cis*-regulatory element.

The *Ci-Bra* and *m4* enhancers appear to possess a similar organization (Fig. 1). The 434-bp *Ci-Bra* enhancer contains two Su(H) binding sites and three E-boxes. Both classes of binding sites are essential for the expression of *Ci-Bra/lacZ* transgenes in electroporated embryos (Fig. 4 and data not shown). The distal Ci-Su(H)$_1$ site contains a good match to the mouse Su(H) consensus sequence (Tun *et al.*, 1994). The proximal Ci-Su(H)$_2$ site is identical to the Su(H) recognition sequence contained in the β2-microglobulin enhancer that was used to purify the mouse *Su(H)* homolog (originally called *KBF2*; Israel *et al.*, 1989). Furthermore, the two proximal E-boxes in the *Ci-Bra* enhancer contain GC core sequences, which is typical of the E-boxes found in the enhancers of *E(spl)-C* genes. Finally, the *Ci-Bra* enhancer contains three copies of the hexamer motif seen in *Drosophila* and mouse *E(spl)-C* regulatory regions.

Recently, a *Notch* homologue has been isolated from a distantly related ascidian, *Halocynthia roretzi* (*HrNotch*). Maternal transcripts are distributed throughout the early embryo, but, during neurulation, *HrNotch* transcripts are detected primarily in neuroectodermal precursors (Hori *et al.*, 1997). In zebrafish and mouse, *Notch* homologues are expressed not only in the neural primordia, but also in various mesodermal derivatives, including the prospective notochord (Bierkamp and Campos-Ortega, 1993; Williams *et al.*, 1995). These results are consistent with the possibility that *Notch* homologue(s) could play a role in *Ciona* notochord development.

Mutant phenotype. Ci-Su(H) fusion proteins containing repression domains from either Ci-Snail or the *Dro-*

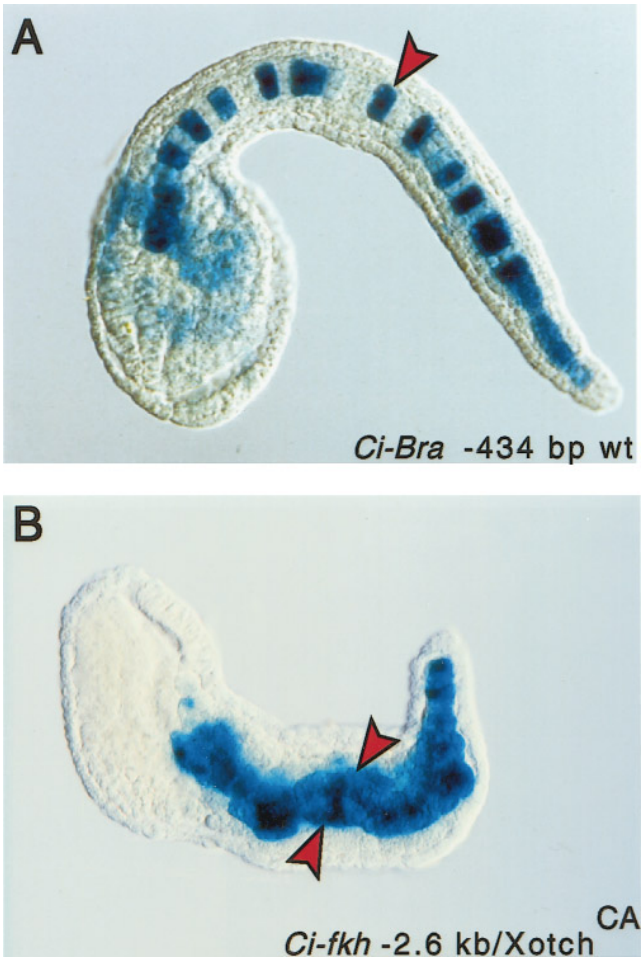

FIG. 6. Ectopic expression of a constitutively activated Xotch receptor. The *Ciona forkhead* (*Ci-fkh*) promoter region was attached to the highly conserved intracytoplasmic domain of the Xotch receptor (Coffman *et al.*, 1993). The *Ci-fkh* promoter region (2.6 kb of 5′ flanking sequence) mediates expression in the notochord, endoderm, and ventral ependymal cells of the spinal cord (Corbo *et al.*, 1997b; Di Gregorio *et al.*, in preparation). Expression of the activated Xotch receptor in these tissues results in a consistent mutant phenotype, including a stubby tail and enlarged notochord (red arrowheads). A shows a control embryo that was electroporated with the wild-type 434-bp *Ci-Bra/lacZ* transgene. The embryo in B was coelectroporated with the same reporter gene and with the *Ci-fkh/Xotch** transgene. The *Ci-Bra/lacZ* staining pattern is expanded, suggesting a transformation of endoderm and/or CNS into notochord.

sophila Hairy repressor affect notochord differentiation, whereas a truncated, dominant negative form of Ci-Su(H) does not (Fig. 5 and Table 1). Both sets of modified proteins attenuate the activity of the minimal, 434-bp *Ci-Bra* enhancer. One explanation for these observations is that the endogenous *Ci-Bra* promoter region contains multiple notochord-specific enhancers, so that the binding of the dominant negative Ci-Su(H) protein to the proximal, 434-bp enhancer does not interfere with the activities of more distal enhancers. Evidence for multiple enhancers stems from the observation that a full-length, 3.5-kb *Ci-Bra/lacZ* transgene containing an internal deletion of the 434-bp enhancer continues to direct notochord-specific expression in electroporated embryos (S. Fujiwara, unpublished results). *Brachyury* has been shown to be regulated by FGF signaling in another ascidian, *Halocynthia*. Perhaps FGF and Notch function in a partially redundant fashion through separate enhancers to direct notochord-specific expression of *Ci-Bra*.

Repressor forms of Ci-Su(H) interfere with notochord differentiation, which is consistent with the possibility that the endogenous *Ci-Bra* gene is inactivated, resulting in a failure to express target genes required for notochord cell shape changes. The early phases of the *Ci-Bra* expression pattern are presumably normal since the full-length *Ci-Bra* promoter region was used to express the modified Ci-Su(H) coding sequences. The ability of the Ci-Su(H)/Ci-Snail and the Ci-Su(H)–WRPW fusion proteins to interfere with endogenous gene activities (see Table 1) suggests that the most effective experimental strategy for assessing the function of a transcriptional activator is to convert it into a repressor, rather than simply removing the activation domain and relying on competition between the truncated and wild-type proteins. In the specific example described in this study, repressor forms of Ci-Su(H) bound to the proximal, 434-bp enhancer may be able to repress multiple notochord-specific enhancers in the *Ci-Bra* promoter region.

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