Cloning of Full-Length Zebrafish $dcc$ and Expression Analysis During Embryonic and Early Larval Development

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Members of the DCC family play key roles in axon guidance in vertebrates as well as invertebrates. In zebrafish, only a short partial sequence of the $dcc$ gene has been reported to date. Here, we report the cloning of full-length zebrafish $dcc$. Zebrafish DCC shares the typical structure of the DCC subgroup of the immunoglobulin superfamily, consisting of four immunoglobulin and six fibronectin-type III repeats in the extracellular domain, a single transmembrane domain, and an intracellular domain with three conserved motifs. As a first step toward studying the function of $dcc$, we analyzed its sequence and characterized its expression pattern during embryonic and larval development. $dcc$ is expressed highly in brain and spinal cord, but distinct staining was also observed in developing pectoral fins, pancreas, intestine, and heart. Thus, $dcc$ may play roles not only in axon guidance, but in morphogenesis and functioning of these organs as well. Developmental Dynamics 234:732–739, 2005. © 2005 Wiley-Liss, Inc.

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INTRODUCTION

The DCC (deleted in colorectal cancer) family is a subgroup of the immunoglobulin superfamily, consisting of DCC and Neogenin in vertebrates, Frazzled in Drosophila, and UNC-40 in Caenorhabditis elegans. First described to function in axon guidance in the nematode C. elegans (Hedgecock et al., 1990; Ishii et al., 1992), members of the DCC family thereafter were also identified to act as axon guidance molecules in insects and vertebrates (Keino-Masu et al., 1996; Kolodziej et al., 1996; de la Torre et al., 1997; Fazeli et al., 1997). They have been shown to play roles in axon guidance both in vitro and in vivo by acting as receptors for ligands of the Netrin/UNC-6 family.

The function of DCC has been studied in detail in cultured vertebrate neurons and in invertebrates in vivo. DCC can bind Netrin directly (Stein et al., 2001). While the best-characterized role for DCC is to mediate attraction to Netrin, its function depends on the state of the neuron in which it is expressed. For instance, DCC can mediate repulsion from Netrin when co-expressed with UNC-5 (Hong et al., 1999). Moreover, it was observed in C. elegans that UNC-40 also has a Netrin-independent function, acting together with the SAX-3 receptor to mediate repulsion from Slit (Yu et al., 2002). In vertebrates, however, the in vivo function of DCC has only been studied in a few systems (Deiner et al., 1997; Fazeli et al., 1997; Deiner and Sretavan, 1999; Finger et al., 2002). Therefore, we set out to identify the full-length zebrafish $dcc$ gene and characterize its expression pattern, as a first step toward understanding the function of DCC in zebrafish.

The developing zebrafish nervous system lends itself to studying the molecular basis of axon guidance, both for finding new molecules and for studying the in vivo function of known...
molecules (Hutson and Chien, 2002b). The transparency of the larvae allows for observation of axon pathfinding in living zebrafish larvae by combining time-lapse video microscopy with transgenic fish expressing fluorescent proteins in axons or with dye-labeling of axons (Higashijima et al., 2000; Hutson and Chien, 2002a). Targeted and specific knockdown of genes using antisense morpholinos has added to the possibilities for manipulating the development of zebrafish (Nasevicius and Ekker, 2000). Designing targeted morpholinos requires knowledge of the full-length mRNA sequence.

A short fragment of zebrafish dcc has been cloned previously by Key and colleagues (Hjorth et al., 2001), but this ~200-amino acid fragment of the cytoplasmic domain did not allow a full sequence comparison with other DCC/Neogenin family members. In situ analysis by these authors revealed expression of dcc in the developing nervous system, including the first neuronal nuclei in the brain. Here, we report the cloning of full-length dcc, analysis of its sequence, and characterization of its expression pattern in the embryonic and early larval stages, revealing previously unreported domains of expression.

RESULTS AND DISCUSSION

Cloning of Full-Length Zebrafish dcc

Degenerate primers were designed against conserved sequences in the extracellular domains of known members of the DCC family. Nested polymerase chain reaction (PCR) with these primers on first-strand cDNA of wild-type (WT) zebrafish embryos at 36 hours postfertilization (hpf) yielded a 257-bp fragment of zebrafish dcc. A λ-cDNA library from 22–26 hpf embryos (kindly provided by M. Aoki and H. Okamoto) was then screened with this PCR fragment, yielding most of the cDNA sequence of zebrafish dcc. Missing 5′ and 3′ ends of the coding region were subsequently cloned by nested rapid amplification of cDNA ends (RACE) from double-stranded, adaptor ligated cDNA of 36-hpf WT zebrafish embryos.

The original clone obtained in the library screen contained, embedded in surrounding dcc sequence, a 327-bp fragment that contained multiple termination codons and showed no homology to DCC genes from other species. This 327-bp region matched bp 565–724 and 889–1047 of the consensus sequence for Dr000392, a repetitive element estimated to represent approximately 1% of the zebrafish genome (Rick Waterman, personal communication). To test whether this repetitive element was truly present in dcc mRNA, we performed reverse transcriptase-PCR (RT-PCR) from WT embryos using primers flanking this region. Rather than the 704-bp fragment predicted from the library clone, this RT-PCR reaction yielded only a single band of 377 bp. Sequencing showed that this band lacked the 327-bp region, which was not homologous to other DCC genes, but otherwise matched the library clone perfectly. Thus, we conclude that this region is usually not present in dcc mRNA. Rather, we speculate that it represents a transposition event that occurred in the fish from which the library was made. Alignment of all sequences obtained without the repetitive element revealed a 4,266-bp open reading frame for zebrafish dcc predicted to encode for a protein of 1,421 amino acids. The start codon was predicted based on preceding termination codons in all frames and the presence of a Kozak consensus sequence. The dcc mRNA sequence has been submitted to GenBank (accession no. AY956314). To determine the genomic localization of dcc, we used radiation hybrid (RH) mapping (Hukriede et al., 1999). dcc mapped to linkage group 5 of the zebrafish genome, roughly 12cR from z13883.

Sequence Analysis of Zebrafish DCC

The predicted DCC protein shows the characteristic structure of DCC family members (Fig. 1A). It consists of four immunoglobulin (Ig) repeats followed by six fibronectin type III (FNIII) repeats in the extracellular domain, a single transmembrane domain, and an intracellular domain with three evolutionarily conserved motifs, named P1, P2, and P3 (Kolodziej et al., 1996).

The DCC family constitutes a subfamily of the Ig superfamily of axon guidance receptors. Two members of this family have been identified in vertebrates, DCC and Neogenin, and one in invertebrates, Frazzled in Drosophila melanogaster and UNC-40 in Caenorhabditis elegans. Phylogenetic comparison of the full-length coding sequence with other vertebrate DCC/Neogenin family members revealed that the gene cloned here is more closely related to the dcc genes than to the neogenin
genes of other vertebrate species (Fig. 1B). Furthermore, analysis of the individual Ig and FNIII domains showed an overall higher percentage of amino acid identity to vertebrate DCCs than to Neogenins (Fig. 1C). Thus, based on its sequence, we name this gene dcc. No cDNA sequence is available for the PCR fragment previously described as zebrafish dcc (Hjorth et al., 2001); however, given that 195 of 199 amino acids match the corresponding region in our full-length clone, we consider it to be a fragment of the same gene.

In the past few years, considerable light has been shed on the function of intracellular domains of many axon guidance receptors, which are often highly conserved across species. The DCC domain P1 is required for interaction of the DCC and UNC-5 cytoplasmic domains. In turn, this interaction can convert DCC-mediated attraction to UNC-5/DCC-mediated repulsion (Hong et al., 1999). The DCC domain P3 is required for self-association of DCC and for the function of DCC in chemotraction and outgrowth of neurons. It has been suggested that ligand binding can trigger self-association of the cytoplasmic domains of DCC and that this multimerization could then signal attraction and outgrowth-promotion (Stein and Tessier-Lavigne, 2001). The P3 domain is also required for interaction of the DCC and Robo1 cytoplasmic domains. This interaction appears to cause silencing of the attractive effect of Netrin-1 on cultured vertebrate neurons (Stein and Tessier-Lavigne, 2001). Thus, the P1 and P3 domains are likely to have important functions in axon guidance. We found that P1–P3 are all present in zebrafish DCC (Fig. 1D), with a particularly high percentage of amino acid identity between the P1 (88%) and P3 (100%) domains of zebrafish DCC and DCC from other vertebrates.

Expression Pattern of Zebrafish dcc During Embryonic and Early Larval Development

Expression pattern in the spinal cord.

In the spinal cord (SC), we detected specific expression of dcc from 15 hpf onward. Whereas initial expression was patchy or clustered, over time, SC expression became more uniform (Fig. 2). This tendency was also reflected along the rostrocaudal axis within individual embryos. Embryos at 19 and 24 hpf showed a patchier expression of dcc in the caudal SC than in the rostral SC (Fig. 2A,B). In older embryos, dcc was uniformly expressed along the rostrocaudal axis (48 hpf; Fig. 2C). Along the dorsoventral axis, dcc seemed to be expressed at 19 hpf in two stripes, one dorsal and one ventral (Fig. 2A). The dorsal stripe extended further posterior than the ventral stripe. At 24 hpf, cells expressing dcc were located throughout the entire dorsal–ventral extent of the SC (Fig. 2D), with exception of the caudal SC where expression was still restricted to dorsal and ventral stripes (not shown). SC expression faded later during development: while still weakly detectable at 72 hpf, it was not detectable at 120 hpf (not shown).

What cells express dcc in the SC? Along the ventrodorsal axis of the SC, motoneurons are the most ventral neurons, interneurons are located more dorsolateral than motoneurons, and sensory Rohon–Beard neurons are the most dorsal neurons (Bernhardt et al., 1990; Kuwada et al., 1990). Based on cross-sections (Fig. 2D,E), dcc seems to be expressed in motoneurons, interneurons, and most likely also in Rohon–Beard neurons. Hjorth and coworkers (2001) drew similar conclusions from whole-mount analysis, except that they concluded that Rohon–Beard neurons do not express dcc. Further analysis using double-labeling with markers of known cell types will be required to resolve this discrepancy.

Expression pattern in the hindbrain.

The zebrafish hindbrain contains seven rhombomeres, which harbor identified reticulospinal (RS) interneurons (Mendelson, 1986; Metcalfe et al., 1986). The time course and expression pattern of dcc in the hindbrain suggest that dcc is expressed in RS neurons. Weak expression of dcc in the hindbrain was detected by 11.5 hpf (not shown). At this developmental stage, some RS neurons, which are...
among the first cells of the nervous system to be generated, are already born (Mendelson, 1986). From 15 hpf onward, we detected bilateral patches of cells distributed along the rostro-caudal axis in a ladder-like manner in the lateral margins of the hindbrain (Fig. 3A; 22 hpf). This expression became more uniform during development (Fig. 3). However, even at later developmental stages, distinct regions of expression could be detected (Fig. 3B,C, stars). At later stages, there was prominent expression around both the upper and lower rhombic lip (Fig. 3C; see Koester and Fraser, 2001). Expression of dcc in the hindbrain was detected up to 120 hpf, the latest time point analyzed (not shown). To analyze whether early dcc expression was restricted to distinct rhombomeres, we performed double in situ hybridization at 17 hpf with krox-20 as a marker for the third and fifth rhombomeres. From this analysis, we concluded that dcc was expressed by at least one cell on each side of each rhombomere, starting with the first rhombomere (Fig. 3D). The early ladder-like repetitive expression pattern of dcc in the hindbrain is similar to the previously described spatial organization of the RS neurons (Mendelson, 1986). This finding is consistent with the hypothesis that the cells in the hindbrain that express dcc include the RS neurons.

Expression pattern in the tectum.

A dynamic expression pattern of dcc was also seen in the optic tectum. At 24 hpf, dcc was first detected in a region of the mesencephalon corresponding to the developing tectum (Fig. 4A, double arrow). At this stage, expression was also seen in the developing tegmentum and cerebellum. At 36 hpf, expression in the tectum had spread into a wider patch (Fig. 4B, double arrow). At 48 hpf, expression became restricted to the margins of the tectum, excluding the rostral sector (Fig. 4C, double arrow). This expression pattern persisted through 120 hpf (not shown). Because new cells are added at the dorsal, caudal, and ventral margins of the tectum (Marcus et al., 1999), this pattern reflects the pattern of neurogenesis in the developing tectum. Thus, dcc appears to be expressed during a restricted time window in neurogenesis of tectal neurons.

Expression in the retina.

dcc was also expressed in the retina but appeared quite late. We were unable to detect any retinal expression at 36 hpf. By 48 hpf, dcc was detected in the retinal ganglion cell (RGC) layer (Fig. 4D). By 72 hpf, dcc was also expressed in the inner nuclear layer (INL; Fig. 4E). Expression of dcc in the RGC layer and INL was still detected at 120 hpf (data not shown).

One puzzle is why dcc was not expressed in RGCs at 36 hpf, which is several hours after the first RGC axons have exited the eye at 32–34 hpf (Burrill and Easter, 1995). This finding is in contrast to observations in mice, where DCC acts as an axon guidance receptor for RGC axons at the optic disc (Deiner et al., 1997). The following considerations could explain this discrepancy. On the one hand, dcc mRNA might be expressed in RGCs at 36 hpf at levels too low for us to detect. On the other hand, dcc might indeed not be expressed in RGCs at this time. This would be different than in mice, where DCC protein is present on some of the earliest RGC axons leaving the eye (Deiner et al., 1997). If this is indeed the case, the zebrafish dcc cloned here cannot act to guide the earliest growing retinal axons out of the eye (although it might have this role for axons of later-born RGCs). One explanation might be that another member of the DCC/Neogenin family guides the earliest growing RGCs. We have been unable to find evidence for a second dcc gene in available zebrafish genomic sequence (A. Suli and C.-B. Chien, data not shown).

Expression in the developing pectoral fins.

dcc was expressed early in the developing pectoral fins (Fig. 5A–D, arrowheads). At 24 hpf, dcc was expressed in the mesenchymal condensation of the forming fin buds (Fig. 5A, arrowheads). At 36 hpf, dcc was still uniformly expressed in the fin bud mesenchyme (Fig. 5B, arrowheads). However, at 48 hpf, the expression became proximally restricted. At the same time, dcc was more strongly expressed in the dorsal (medial) and ventral (lateral) region of the fin bud, apparently around the inner chondrogenic condensation (Fig. 5C, 48 hpf, long arrows; Fig. 5D, 55 hpf, long arrows). This expression pattern is consistent with the histological observation that the growing chondrogenic condensation divides the mesenchyme of the bud into a dorsal and a ventral half (Grandel and Schulte-Merker, 1998). These are believed to differentiate into the dorsal and ventral muscle masses of the pectoral fin (Grandel and Schulte-Merker, 1998). At 120 hpf, expression of dcc in the pectoral fin was still restricted to a ventral and a dorsal spot, both located in the proximal region of the fin (not shown).

Expression in heart, pancreas, and intestine.

dcc is expressed in the heart at 36 hpf (Fig. 5E), and at 120 hpf, in the pancreas and weakly in the intestine (Fig. 5F,G). Expression in the pancreas was seen in regions where exocrine pancreatic tissue is found. Without analysis in sections, we cannot determine if it is also expressed in the central head region where the endocrine pancreatic tissue is located (Biemar et al., 2001). In mice, antibody staining revealed DCC expression in neural crest derived cells in the stomach and midgut at embryonic day (E) 16 (Seaman et al., 1997; Strähle et al., 1997), similar to the expression of mouse Netrin-1 (Deiner et al., 1997). As both DCC and Neogenin can bind to Netrin-1 (Keino-Masu et al., 1996), one of the zebrafish Neogenins might have the function performed by mouse DCC in guiding retinal axons out of the eye.
et al., 2001). Recently, it has been shown that the formation of submucosal and pancreatic ganglia of the enteric nervous system involves DCC function in neural crest-derived cells in mice (Jiang et al., 2003). Thus, based on its expression in intestine and pancreas, zebrafish dcc might have a similar function in the development of the enteric nervous system.

**Concluding Remarks**

Here, we have cloned full-length zebrafish dcc and compared its sequence with other members of the vertebrate DCC family. We especially concentrated on the analysis of the conserved intracellular domains, because recent findings have implicated these domains in heterophilic and homophilic interactions. The zebrafish dcc gene shares these domains with other vertebrate dcc genes, consistent with the possibility that zebrafish DCC may also use these domains to interact with itself or other guidance receptors.

Expression analysis revealed that dcc is expressed in neurons of the forebrain, midbrain, hindbrain, and SC at developmental stages where some of these neurons are sending out axons. The zebrafish netrin1 homologs netrin1a and netrin1b are expressed at the ventral midline of the hindbrain and SC at these stages (Lauderdale et al., 1997; Strähle et al., 1997), consistent with a model in which DCC acts as a Netrin1 receptor to attract commissural neurons to the midline, as in mouse (Fazeli et al., 1997). Similarly, expression of dcc in the rhombic lip of the hindbrain is consistent with a model in which some of these cells migrate into the basilar pons by means of Netrin1-mediated attraction to the ventral midline, as in mouse (Yee et al., 1999). We have also recently found that the zebrafish homolog of netrin2 is expressed early in the notochord, and later in the roof of the midbrain and hindbrain, where it could potentially affect the development of dcc-expressing neurons (Park et al., 2005). Furthermore, expression was also detected outside the nervous system, implicating zebrafish dcc in functions other than neuronal guidance.

**EXPERIMENTAL PROCEDURES**

**Cloning of Zebrafish dcc**

The programs Blockmaker (http://blocks.fhcrc.org/blockmrk/make_blocks.html) and Codehop (http://blocks.fhcrc.org/codehop.html) were used to design degenerate primers (DCC2, 3, and 4) against evolutionarily conserved regions of DCC using mouse, human, and X. laevis DCC and D. melanogaster Frazzled sequence. Total RNA was isolated from 36-hpf WT embryos and reverse transcribed into first-strand cDNA using the SUPERSCRIPT preamplification system (Gibco) and a reverse degenerate primer (DCC4; 5'-GATGGTCTCATCCATCTCATTGCGT-rtgdat-3'). In a first round of PCR, degenerate primers DCC2 (DCC2; 5'-GTCGGCCGCCTGacngtna-3') and DCC4 were used; in a second round of PCR, DCC2 and nested reverse primer DCC3 (DCC3; 5'-TTGAGCGCGTCACGATctrtg-3') were used. For both PCR reactions, Platinum TAQ (Gibco) was used to provide a hot start. Cycling parameters were as follows: 94°C for 2 min, then 40 cycles 94°C for 5 sec, 55°C for 30 sec, 72°C for 1 min, then 2 sec × 10 min.

The cloned PCR product was used to generate a probe labeled with α-32PdCTP using the Prime It II kit (Stratagene). With this probe, a longer dcc fragment was detected.

Subsequently, the 5' and 3' ends of the coding region were cloned by nested 5' and 3' RACE. Double-strand adaptor-ligated cDNA was generated using the Marathon cDNA amplification kit (Clontech). The Advantage cDNA PCR Kit (Clontech) was used for the first and second rounds of PCRs. The gene-specific primers used in the first round of PCRs were 5DCCRN (5DCCRN; 5'-TGCGCTGCTCCATACTGCAACTCAA-3') for 5'RACE and 3DCCFN (3DCCFN; 5'-GGATTGACTGGACACCCCAAA-ACT3') for 3' RACE, in combination with the supplied adaptor-specific, nested primer AP2. Cycling parameters for nested PCR were as follows: 94°C × 30 sec, then 5 cycles 94°C × 5 sec, 73°C × 4 min, then 25 cycles 94°C × 5 sec, 72°C × 4 min.

To span the repetitive element in the library clone, cDNA from 36-hpf WT embryos was generated using the SUPERSCRIPT preamplification system (Gibco). The PCR reaction used Pfu Turbo Polymerase (Stratagene). Primer sequences were as follows: DCC7F, 5'-CCCTCAATCTGGACCC-GCTC-3'; and DCC7R, 5'-CTGGTGTG-GTTGACGGCTTTCT-3'. Cycling parameters were as follows: 94°C × 1 min, then 30 cycles 94°C × 30 sec, 60°C × 30 sec, 72°C × 1 min, then 72°C × 5 min.

**Mapping of Zebrafish dcc**

dcc was mapped using the LN54 radiation hybrid panel (kind gift of M. Ekker; Hukriede et al., 1999) and sequence-specific primers (DCC8F, AATTGCATGGCTTTTCC; and DCC8R, TTACTAAGGCACCGTTTG). In brief, the in situ hybridization was performed as follows: 2× in PBST, proteinase K digestion (10 μg/ml) for a duration depending on the age of the embryos, 2× in PBST, 1× in H2O, followed by a treatment with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 7.0) for 1 hr, 2× in PBST. The next steps were performed at 65°C: 5 min incubation in Hyb−, 2–6 hr of incubation in Hyb− and subsequent ON hybridization with asRNA probe in Hyb+. The next day, the embryos were washed 2× 30
min in 50% formamide in 2× SSC, 15 min in 2× SSC, and 2× 30 min in 0.2× SSC, then incubated in 2% Blocking Reagent (Roche) in Blocking Solution at room temperature for 1–5 hr. They were then incubated ON at 4°C in anti–digoxigenin (DIG)–alkaline phosphatase (AP) antibody for DIG-labeled probes, diluted 1:5,000 in 2% Blocking Reagent, washed 30 min in 2% Blocking Reagent, 3× 30 min in Blocking Solution and 3× 5 min in freshly prepared staining buffer. After staining with BM Purple AP Substrate (Roche), embryos were washed several times in PBST, fixed in 4% PFA in PBS for 30 min at RT and stored in 100% MeOH at 4°C. Embryos were mounted in a benzyl benzoate/benzyl alcohol (2:1) mixture.

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