

Mouse *Crossveinless-2* is the vertebrate homolog of a *Drosophila* extracellular regulator of BMP signaling

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Abstract

The Dpp/BMP signaling pathway is highly conserved between vertebrates and invertebrates. The recent molecular characterization of the *Drosophila crossveinless-2* (*cv-2*) mutation by Conley and colleagues introduced a novel regulatory step in the Dpp/BMP pathway (Development 127 (2000) 3945). The CV-2 protein is secreted and contains five cysteine-rich (CR) domains similar to those observed in the BMP antagonist Short gastrulation (Sog) of *Drosophila* and Chordin (Chd) of vertebrates. The mutant phenotype in *Drosophila* suggests that CV-2 is required for the differentiation of crossvein structures in the wing which require high Dpp levels. Here we present the mouse and human homologs of the *Drosophila cv-2* protein. The mouse gene is located on chromosome 9A3 while the human locus maps on chromosome 7p14. CV-2 is expressed dynamically during mouse development, in particular in regions of high BMP signaling such as the posterior primitive streak, ventral tail bud and prevertebral cartilages. We conclude that CV-2 is an evolutionarily conserved extracellular regulator of the Dpp/BMP signaling pathway. © 2003 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Crossveinless-2; Chordin; Twisted gastrulation; Short gastrulation; BMP; Dpp; Tolloid; CR domain; von Willebrand factor type D domain; TIL domain; Extracellular matrix; Organogenesis; Somite; Sclerotome; Notochord; Neural crest; Dorsal root ganglion; Branchial arch; Cartilage; Vertebra; Intervertebral disc; Lung

1. Results

The Dpp/BMP (Decapentaplegic/Bone Morphogenetic Protein) signaling pathway is one of the best examples of conservation of mechanisms of development between vertebrates and invertebrates. In addition to the signal transduction machinery, a network of extracellular signaling regulators is conserved. Binding of BMP to the antagonist Sog/Chordin blocks Dpp/BMP activity. This inhibition is itself regulated by the co-factor Twisted gastrulation (Tsg) and by cleavage by the metalloproteinase Tolloid (reviewed by De Robertis et al., 2000). The recent molecular characterization of the *crossveinless-2* (*cv-2*) mutation, which affects *Drosophila* wing patterning, introduced a novel regulator of Dpp signaling (Conley et al., 2000). The *cv-2* fruitfly mutation results in the loss of the posterior crossvein and of the tip of longitudinal veins. Similar phenotypes are observed in mild *dpp* loss of function mutations, in mutants of the *tolloid* homolog *tolkin*, and by overexpression of the BMP antagonist *Sog* (de Celis, 1997; Conley et al., 2000; Yu et al., 2000). Thus, the phenotype of *cv-2* suggests that this gene promotes

high BMP signaling. Another *Drosophila* mutation with an identical phenotype in wing venation is *crossveinless* (*cv* or *cv-1*), which was first identified by Calvin Bridges many years ago (Bridges, 1920). Interestingly, the *cv-1* mutation maps to a gene encoding a second *Drosophila* Twisted gastrulation homolog, *dTsg-2* (L. Marsh, communication to FlyBase FBgn0000394). The CV-2 protein contains five cysteine-rich domains (CRs) with sequence similarity to the BMP-binding domains of Chordin (Conley et al., 2000).

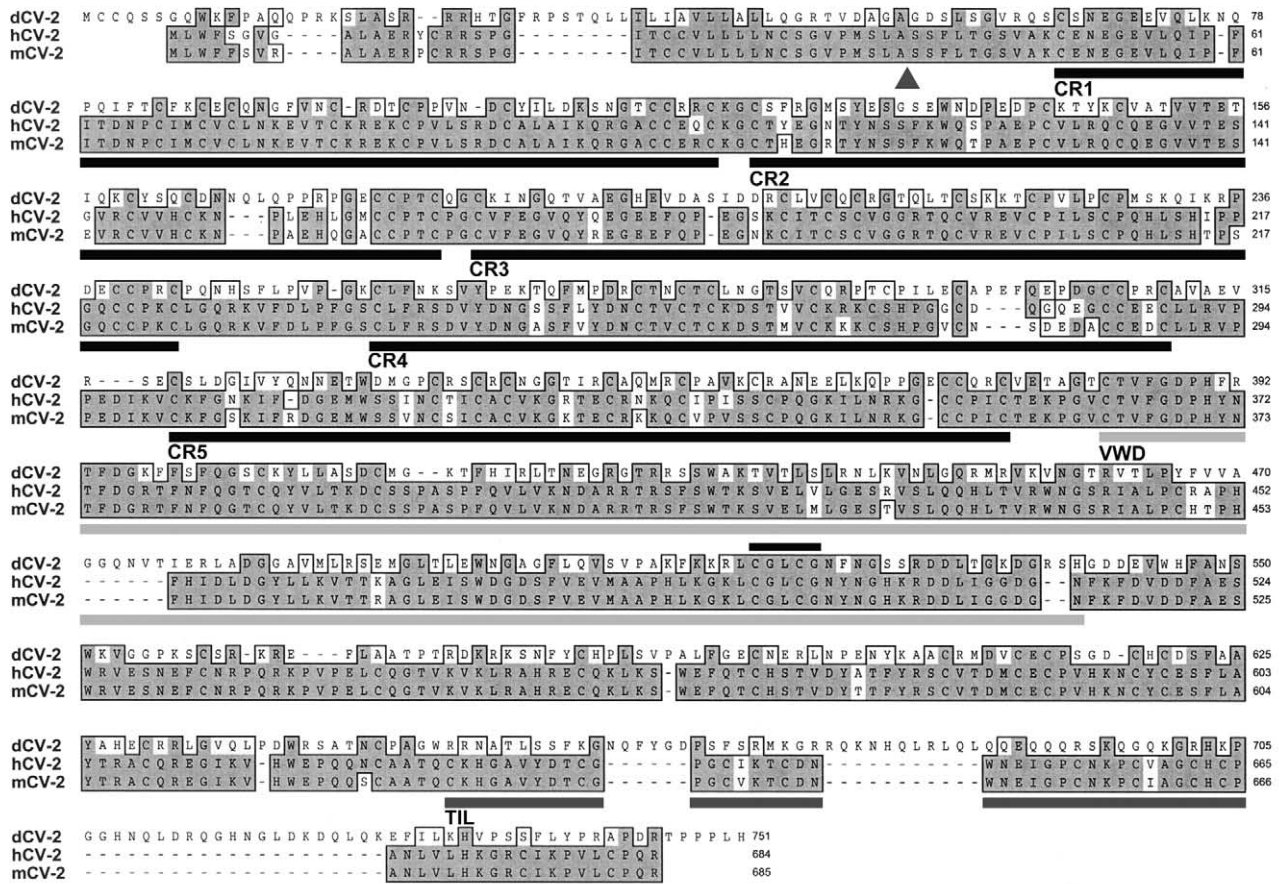
In this study we present the cloning of the first vertebrate homolog of the *Drosophila cv-2* gene. Mouse CV-2 is expressed dynamically during mouse embryogenesis, in particular at multiple sites of high BMP signaling during development.

1.1. Cloning of the mouse CV-2 homolog

We identified a mouse EST (GenBank AK014221) as a candidate for the homolog of the *Drosophila crossveinless-2* gene. BLAST search of the GenBank database at NCBI (<http://www.ncbi.nlm.nih.gov/blast/>) uncovered another partial mouse EST (BE289531), which was used to screen a mouse embryonic day 8.5 cDNA library. We cloned a 3.8 kb full-length cDNA containing a 570 bp 5' untranslated

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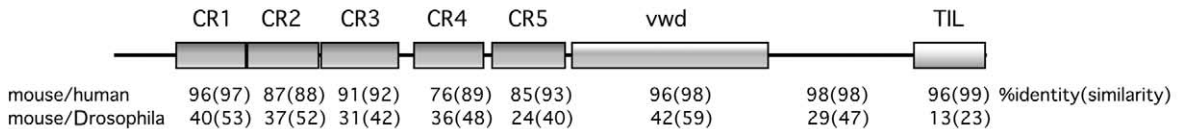


Fig. 1. Comparison of the mouse, human and *Drosophila* CV-2 proteins. (A) Alignment of the protein sequences using ClustalW. Amino acid identities are indicated by a dark shading, and similarities with a lighter shade. The arrowhead indicates the cleavage site for the predicted mammalian signal peptides. Bars underneath the sequences indicate the structural domains present in the mammalian proteins: CR 1–5, Chordin-like cysteine-rich domains; VWD, von Willebrand factor type D domain; TIL, Trypsin Inhibitor-like cysteine-rich domain. The black bar above the sequences indicates the disulfide isomerase motif, CGLCG, conserved in all three proteins. (B) Structure of the mouse protein (GenBank AF454954) and comparison of its amino acid sequence to the human predicted protein and to *Drosophila* CV-2 (NP_524809).

region (UTR), a 2 kb open reading frame and a 1.2 kb 3' UTR. This clone sequence was deposited in GenBank as AF454954.

BLAST searches of NCBI genomic databases showed that the mouse CV-2 locus is located on chromosome 9, band 9A3 (<http://www.ncbi.nlm.nih.gov/genome>). Additional searches identified several human ESTs (BF997956, BF999553) and a putative human genomic homolog located on chromosome 7p14 (Contig NW_000351.1). A recent report from NCBI indicates a predicted protein XP_166592 at this locus, which was annotated as ‘similar to crossveinless-2’ but lacked the two N-terminal CR domains and the signal peptide. We could deduce the

complete sequence of the putative human CV-2 transcript by aligning human genomic sequence with the mouse cDNA; the predicted human CV-2 amino acid sequence is shown in Fig. 1A.

1.2. Comparison of the mouse, human and *Drosophila* CV-2 proteins

The mouse *Crossveinless-2* cDNA encodes a 685 amino acid protein with a putative signal peptide (SignalP V1.1; Nielsen et al., 1997) (Fig. 1A). The mouse CV-2 protein is secreted; it was detected in the supernatant of 293T cells transiently transfected with a construct encoding the full-

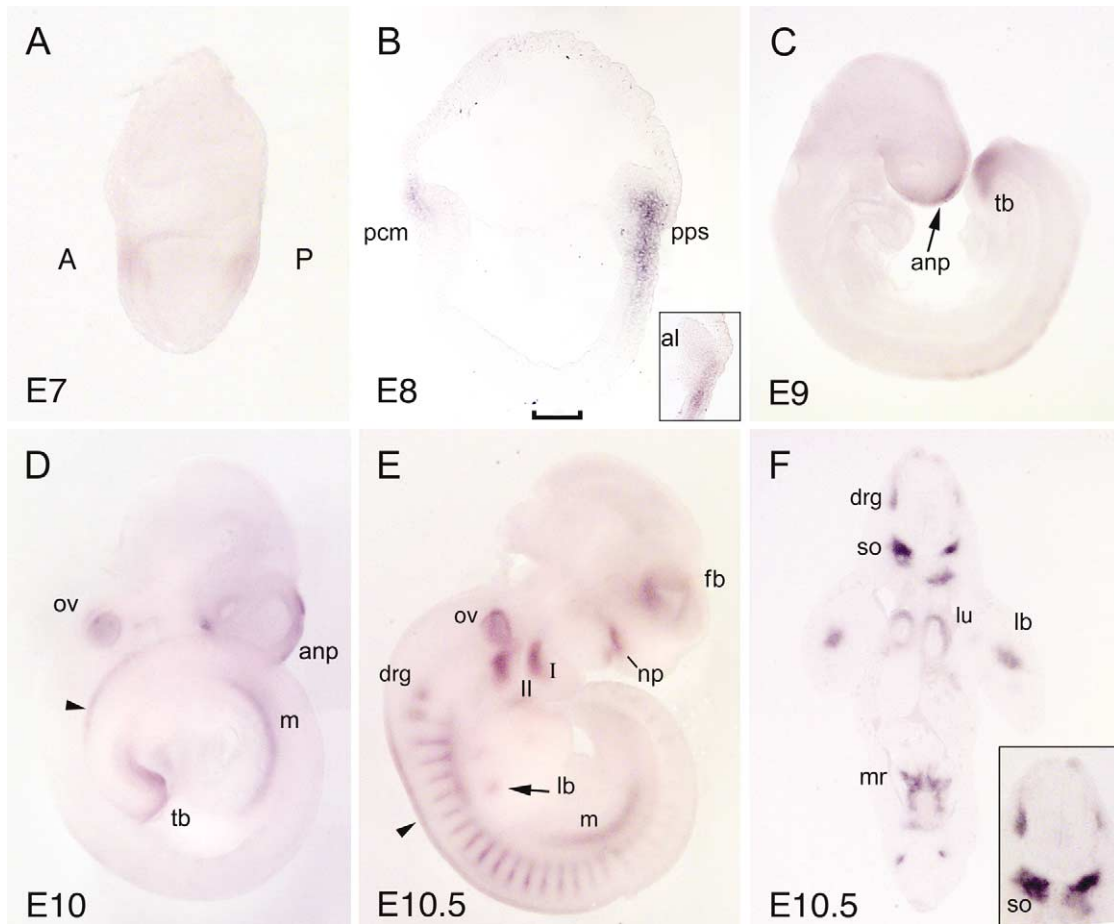


Fig. 2. Expression of CV-2 during early mouse development. In situ hybridizations on (A) mid-gastrula, (B) sagittal sections at head-fold stage (note the lack of expression in the allantois, as shown in the inset), (C) E9 embryo, (D) E10 embryo, (E) E10.5 embryo, and (F) transverse section of E10.5 embryo (inset: higher magnification of the neural tube and somites); note that, in histological section, strong staining in the sclerotome is seen, as well as weaker expression in the mesonephric ridge, lung and limb buds. A, anterior region; al, allantois; anp, anterior neuropore; drg, dorsal root ganglion; fb, forebrain; lb, limb bud; lu, lung; m, mesonephros; mr, mesonephric ridge; np, nasal process; ov, otic vesicle; P, posterior; pcm, precardiac mesoderm; pps, posterior primitive streak; so, somite; tb, tail bud; I and II, first and second branchial arches; bracket in (B) indicates Hensen's node; arrowheads in (D,E) indicate the neural tube roof.

length protein carrying a C-terminal epitope tag (data not shown). When synthetic mRNA encoding mouse CV-2 transcripts were injected into *Xenopus* embryos, secondary axes of the types seen for other regulators of BMP signaling were induced (our unpublished observations). The mouse CV-2 protein shares 30% overall identity (44% similarity by ClustalW analysis) with its fly homolog and 93% identity (96% similarity) with the predicted human CV-2 protein (Fig. 1B). The mouse protein contains five Chordin-like cysteine-rich domains and one von Willebrand factor type D domain (vwd), as described for *Drosophila* CV-2 (Conley et al., 2000). The CR domains of CV-2 share sequence similarities with the cysteine-rich domains (CR) of Chordin, which bind BMP (reviewed in Garcia Abreu et al., 2002). In addition, a search of the Pfam database revealed the existence of a carboxy-terminal Trypsin Inhibitor-like cysteine-rich domain (TIL; Fig. 1A) which was absent from the *Drosophila* CV-2 protein (<http://www.sanger.ac.uk/Software/Pfam/>; Bateman et al., 2002). The TIL domain and the von Willebrand factor D domain could mediate the

oligomerization of CV-2 or its interaction with other proteins. A motif noted by Conley et al. (2000), the CGLCG sequence observed in disulfide isomerases could regulate the formation of disulfide bonds; it is conserved in the mouse, human and *Drosophila* CV-2 proteins (Fig. 1A).

1.3. Expression of the mouse CV-2 gene

CV-2 expression during mouse embryogenesis was investigated by in situ hybridization on whole mounts and sections. At mid-gastrulation, CV-2 transcripts were detected in the proximal part of the embryonic region (Fig. 2A). In histological sections, CV-2 is expressed in the mesoderm and can be detected both in the posterior primitive streak and in the precardiac mesoderm (Fig. 2B). By embryonic day 9 (E9.0), CV-2 expression was observed in the ventral tail bud (Fig. 2C,D). CV-2 was also expressed at the closing anterior neuropore and in the roof of the neural tube (Fig. 2C,D).

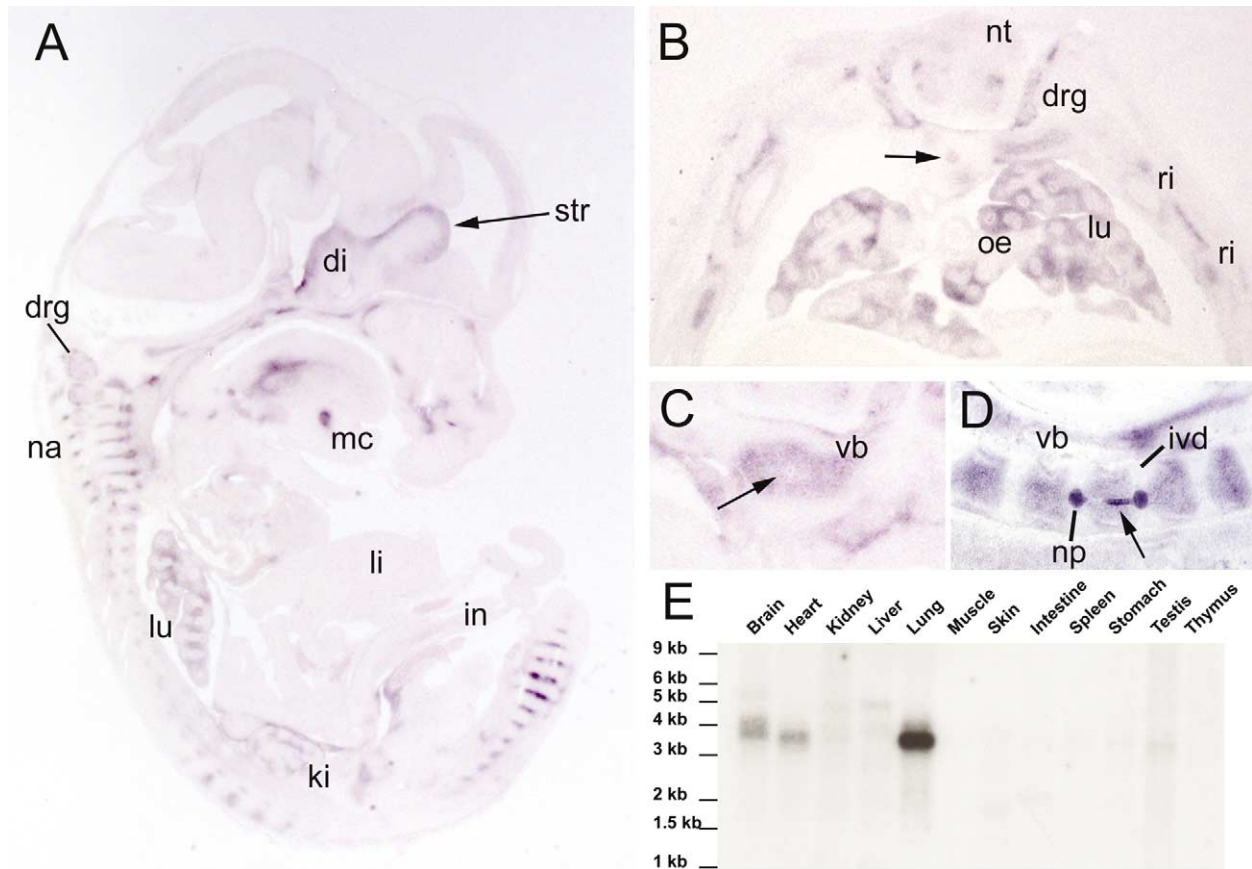


Fig. 3. *CV-2* expression during late organogenesis and in adult mouse tissues. In situ hybridization on (A) parasagittal section of E13.5 embryo, (B) transverse section of E14.5, (C) vertebral body at E14.5, (D) sagittal section through the spinal cord at E14.5; (E) Northern blot analysis of *CV-2* expression in adult mouse tissues. A major transcript of 3.8 kb was detected strongly in the lung and heart; two transcripts of 3.8 and 4 kb were detected in the brain, suggesting *CV-2* produces alternative transcripts. di, diencephalon; drg, dorsal root ganglion; in, intestine; ivd, intervertebral disc; ki, kidney; li, liver; lu, lung; mc, Meckel's cartilage; na, neural arches; np, nucleus pulposus deriving from the notochord in the intervertebral disc; nt, neural tube; oe, esophagus; ri, ribs; str, striatum of the basal ganglia; vb, cartilage of the vertebral bodies; arrows indicate the notochord in (B–D).

CV-2 expression is dynamic and evolved rapidly between E9 and E10.5 as additional domains of expression appeared, such as the mesonephric ridge (Fig. 2E,F). In the developing head, *CV-2* expression was limited to the telencephalon and several craniofacial structures: the tip of the nasal processes, the posterior part of the first branchial arch, the second branchial arch and the otic vesicle (Fig. 2E). The expression started in the central part of the anterior limb buds at E10.5 (Fig. 2E,F). In the lung, *CV-2* expression was limited to the mesenchyme from the onset of its formation and was maintained through embryonic development (Figs. 2F and 3A,B). In adult mice, the lung was the tissue with the strongest expression of *CV-2*, as determined by northern blot (Fig. 3E).

During somitogenesis, *CV-2* expression was detected in dorsal root ganglia and migrating neural crest cells (Fig. 2E,F). In histological sections at E10.5, intense *CV-2* expression was detected in the somite on each side of the notochord, in the presumptive sclerotome (Fig. 2F, see inset). The notochord was negative for *CV-2* expression at that stage but later, at E14.5, *CV-2* expression was detected in the caudal region of the notochord and in the nucleus

pulposus of the intervertebral disc (Fig. 3D). At E13 and E14.5, *CV-2* expression was observed in multiple cartilages of the developing skeleton (Fig. 3A–D). In the axial skeleton, *CV-2* expression in the cartilage of the developing vertebral bodies was complementary to that of *Chordin*, which is expressed in the intervertebral regions (Figs. 3C,D and 4). These mutually exclusive expression domains are reminiscent of those observed in *Drosophila* wing discs, in which *cv-2* is expressed in the veins and *short gastrulation*, the *Chordin* homolog, is expressed in the intervein domains (Conley et al., 2000; Yu et al., 1996).

We conclude that *CV-2* encodes a novel secreted protein containing CR domains similar to the BMP binding domains of *Chordin*. *CV-2* is expressed in regions of high BMP signaling, such as the posterior primitive streak, the ventral tail bud, the growing branchial arches and prevertebral cartilages (Hogan, 1996). These domains correlate with the expression of several BMP genes. For example, the ventral mesoderm of the tail bud strongly expresses BMP4 (Goldman et al., 2000), BMP5 and BMP7 are expressed in the midline of the forebrain (Solloway and Robertson, 1999), and BMP6 is expressed in a similar manner to *CV-2* on the

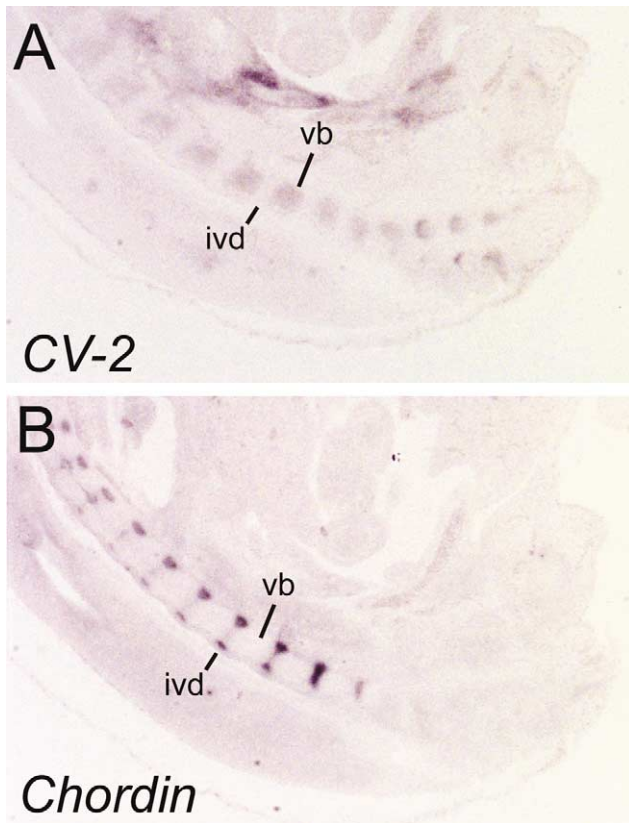


Fig. 4. *CV-2* and *Chordin* are expressed in a complementary pattern in the vertebral bodies and intervertebral discs at E14.5. Expression of (A) *CV-2* and (B) *Chordin* transcripts on parasagittal sections of a E14.5 embryo. vb, cartilage of the vertebral bodies; ivd, intervertebral discs.

dorsal side of the neural tube (Kim et al., 2001). The BMP antagonist *Chordin* is expressed in a complementary pattern in some of these tissues (Fig. 4). For example, *Chordin* is expressed in the dorsal mesoderm (notochord and Hensen's node; Bachiller et al., 2000) and intervertebral regions whereas *CV-2* is expressed in the ventral mesoderm and prevertebrae. In zebrafish, *Chordin* transcription is negatively regulated by BMP signaling (Schulte-Merker et al., 1997). The expression pattern of *CV-2* suggests that this gene is positively regulated by BMP signals. Thus, BMP homeostasis in the embryo may be regulated by two secreted molecules, *CV-2* and *Chordin*, that contain similar cysteine-rich BMP-binding modules and are under opposing transcriptional controls.

2. Methods

The full-length *CV-2* cDNA contained in pCMV-Sport 2 was isolated from a Superscript™ Mouse 8.5 day embryo cDNA library (Life Technologies). The probe used was a 600 bp *EcoRI-XbaI* fragment isolated from a partial mouse EST (GenBank BE289531) containing 400 bp of coding sequence and 200 bp of 3' UTR. This fragment was also used as a probe to hybridize a mouse Multiple Choice™

Northern Blot (OriGene Technologies, Inc.) that contained 2 μ g of polyA⁺ RNA in each lane. Digoxigenin-labeled anti-sense probes were synthesized using the *CV-2* EST plasmid linearized with *EcoRI*. For mouse *Chordin*, a pCS2-myc-*Chordin* plasmid was linearized with *HindIII*. Both probes were transcribed using the T7 RNA polymerase (Boehringer Mannheim). In situ hybridizations were realized as previously described (<http://www.hhmi.ucla.edu/derobertis/>; Belo et al., 1997; Henrique et al., 1995). Sequence alignments were produced using the ClustalW program (Mac Vector 6.5; Thompson et al., 1994).

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