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BRIEF COMMUNICATIONS

Cloning and Expression Analysis of the Chick DAN Gene, an Antagonist of the BMP Family of Growth Factors

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ABSTRACT **Differential screening-selected gene aberrative in neuroblastoma (DAN) is a member of a cystine knot protein family that includes Cerberus and Gremlin. First isolated in a screen to identify genes down-regulated in transformed rat fibroblasts, DAN has subsequently been cloned in *Xenopus*, mouse, and human. Overexpression of DAN suppresses the transformed phenotype and retards the cell's entry into S phase. Biochemical analyses have demonstrated DAN's ability to bind bone morphogenetic proteins and antagonize their signaling activity. In this study, chick DAN was cloned and sequenced, revealing a conserved cystine knot region as well as an N-glycosylation site. A riboprobe was designed from the 3′ chick DAN coding sequence and used for analysis of DAN in the developing chick embryo by in situ hybridization. Chick DAN was expressed beginning at stage 10 in the developing somites and the medial otic epithelium. Expression in the neural layer of the eye became apparent at stage 14. By stage 17, expression had expanded to the base of the hindbrain. Limb bud labeling began at stage 20, whereas expression in the branchial arches appeared at stage 25. Chick DAN expression generally corresponded to that of mouse DAN expression as shown by comparative in situ hybridization. However, chick DAN was found in the otic epithelium and notochord, whereas mouse DAN was restricted to the overlying otic ectomesenchyme and was absent from the notochord. This observation suggests that DAN may play different roles in chick and mouse otic and notochord development.**
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Key words: DAN; ear development; otic epithelium; inner ear; mouse; chick; somites

INTRODUCTION

Differential screening-selected gene aberrative in neuroblastoma (DAN) is a member of a recently described cystine knot protein family that includes Cerberus and Gremlin (Ozaki and Sakiyama, 1993, 1994; Topol et al., 1997; Hsu et al., 1998; Stanley et al., 1998). Also known as NO3, DAN was first identified through a differential screen for genes down-regulated in transformed rat fibroblasts (Ozaki and Sakiyama, 1993). Overexpression of DAN in transformed cell lines suppressed the transformed phenotype and reduced the growth rate, causing a retardation of the cell's entry into S phase (Ozaki and Sakiyama, 1994; Ozaki et al., 1995). In the mouse, DAN exhibits weak bone morphogenetic protein (BMP) antagonist activity in vitro and in vivo (Hsu et al., 1998; Stanley et al., 1998; Dionne et al., 2001). Ectopic DAN protein induces anterior neural tissue and endoderm in *Xenopus* animal cap assays, suggesting a block in the BMP signaling cascade. Biochemical analyses have demonstrated that DAN binds directly to BMP2 (Hsu et al., 1998) and interferes with BMP4 activity in animal cap assays, although not as strongly as the BMP antagonist, Cerberus (Hsu et al., 1998; Stanley et al., 1998).

Subsequently, the mouse, human, and *Xenopus* homologues have been cloned (Ozaki et al., 1996, 1997; Stanley et al., 1998; Hsu et al., 1998). The mouse, rat, and human proteins share greater than 90% identity and a C-terminal proline-rich region possibly responsible for protein-protein interactions. DAN is secreted, probably as a homodimer (Hsu et al., 1998; Stanley et al., 1998). All DAN homologues share an N-glycosylation site and 10 conserved cystines, 6 of which may be involved with a conserved glycine to form a putative cystine knot (Ozaki et al., 1996; Stanley et al., 1998).

DAN's importance for development is underscored by its tumor suppressor and BMP antagonist functions. Human DAN has been mapped to a region of chromosome 1p associated with deletions found in neuroblas-

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tomas (Fong et al., 1989; Weith et al., 1989; Enomoto et al., 1994; Cheng et al., 1995). The high degree of homology among the rat, mouse, human, and *Xenopus* DAN proteins also suggests that DAN may play a significant role in developmental regulation and cell growth. As a first step in addressing the role of DAN during normal embryonic development, the chick DAN homologue was isolated and its mRNA expression described in the developing embryo. Chick DAN expression was compared with that of mouse DAN, which had been previously described (Stanley et al., 1998). Expression patterns were similar in all tissues except the developing otocyst and notochord, suggesting unique roles of DAN in the differentiation of the mammalian and avian inner ears.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of Chick DAN

The chick DAN sequence was obtained by 5′ and 3′ rapid amplification of cDNA ends (RACE) analysis by using primers designed from the ChickGBASE sequence database at the Roslin Institute (UDELPCO1CPK0001B22). The complete 662-bp cDNA sequence is deposited in GenBank as accession no. AF421881. Multiple sequence alignment analysis of chick DAN with the *Xenopus*, mouse, and human DAN protein sequences demonstrated a conserved cystine knot region and N-glycosylation site that were previously described in the mouse and are shared with other members of the DAN family (Ozaki et al., 1996; Biben et al., 1998; Stanley et al., 1998). Conservation of these features suggests chick DAN is a secreted protein with a highly conserved role in the development of most of the regions that express it. However, chick DAN lacks the proline-rich C-terminal sequence reported for the mouse, rat, and human DAN protein sequences. The chick DAN 166 amino acid coding region has 76% identity with the mouse DAN protein.

Embryonic DAN Expression in the Chick

DAN expression in the chick embryo was determined by whole-mount in situ hybridization of stage 8-35 embryos (n = 30). No labeling was apparent in embryos hybridized with the sense control probe (data not shown). DAN antisense labeling was first observed at stage 10 throughout the newly formed somites, the entire notochord, and the otic epithelium. Between stages 11 and 15, labeling was seen in both new and maturing somites and persisted in the notochord and the medial otic epithelium (Fig. 1A). Ocular expression began at stage 14 and continued throughout all stages examined. Stage 17 chick embryos expressed DAN in the same regions as stage 14 embryos (Fig. 1B,C), except that expression expanded to include the base of the hindbrain as seen in tissue sections (data not shown). However, the mesenchyme ventral to the hindbrain was not labeled as it is in the mouse (Stanley et al., 1998). At stage 19, expression extended to the ventral and dorsal regions of the limb buds and the intermediate mesoderm of the nephritic duct (Fig. 1H). Chick DAN expression in the branchial arches and maxillary process is faint at stage 22 (Fig. 1G,H), with strong expression apparent at stage 23. Ocular expression was restricted to the neural cell layer (Fig. 1F). The subtypes of neural cells that express chick DAN in the mature retina were not determined. At subsequent stages, expression remained strong in the facial primordia, eye, somites, medial inner ear, and the dorsal and ventral regions of the developing limbs. All labeling in the chick, except for that described in the otic tissue and the notochord, was similar to that previously described in the mouse (Stanley et al., 1998). This finding suggests different roles for DAN in mouse otic and notochord development than it has in the chick.

Chick DAN Expression in Somites

At early embryonic stages 10–12, expression was found throughout the newly formed somites (data not shown). In more mature somites with developed dermatome and sclerotome, DAN expression was restricted to the dorsal lip of the dermomyotome, the dorsal region of the sclerotome, and the myotome (Fig. 1G,H). Chick DAN expression eventually became restricted to the myotome. Expression levels within the somites did not appear to down-regulate and then reappear as the nascent somites matured, as was described in the mouse (Stanley et al., 1998).

Expression in the Developing Chick and Mouse Inner Ear

In situ hybridization of tissue sections (n = 26) was performed on chick embryos between stages 8 and 35 by using the chick DAN probe to characterize expression of DAN more precisely in the developing inner ear. Expression was first seen at stage 10 but was restricted to the medial otic pit epithelium, including the dorsal area where the endolymphatic sac and duct will emerge (data not shown). The expression was maintained in the entire elongating endolymphatic duct and sac as well as the medial otic epithelium between stages 15 and 23 (Fig. 2A–C). At stage 27, as the inner ear begins to form the superior plate (the pouch from which the posterior and superior semicircular canals develop) and the lateral plate (the pouch that forms the lateral semicircular canal), chick DAN expression was restricted to the medial side of the utricle, the saccule, and the emerging cochlea (Fig. 2D–F). DAN labeling was present throughout the endolymphatic duct and sac as well as their junction with the utricle. Expression was also restricted to the medial epithelium at the cochlear-saccular-utricular junction as well as the anterior side of the cochlea and the lagena. No expression was present in the lateral epithelium of the ampullae or any surface (interior or exterior) of the emerging semicircular canals of the stage 30 or older embryo. With the exception of the cochlea, this labeling was consistent through the rest of the stages studied, the latest being stage 35. By stage 29, cochlear expression

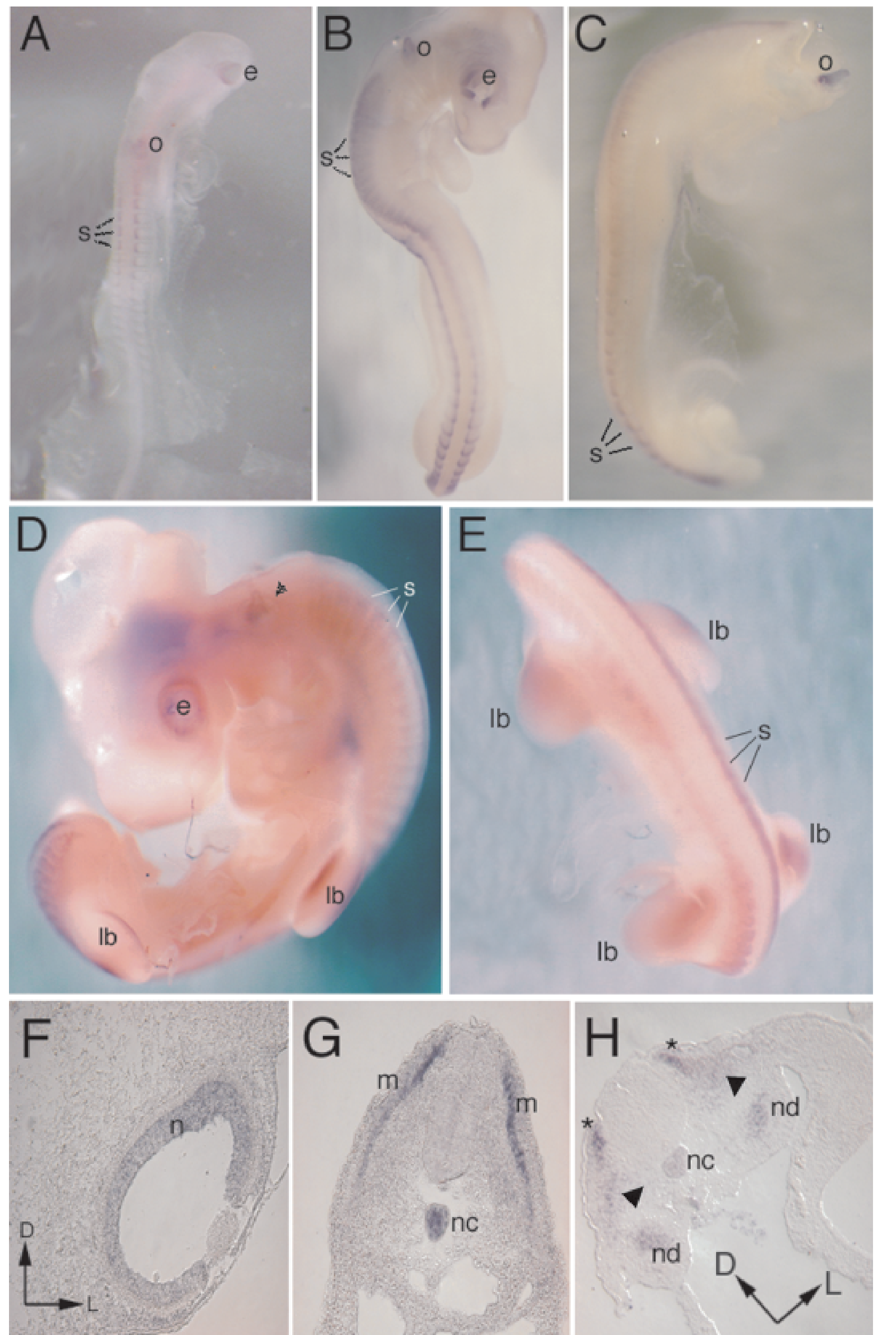

Fig. 1. In situ hybridization analysis of differential screening-selected gene aberrative in neuroblastoma (DAN) expression during chick embryo development. **A:** A 14-somite, stage 12 chick embryo. Labeling is restricted to the somites (s), medial inner ear (o), and eye (e). **B:** A stage 17 chick embryo. DAN is expressed in nascent and mature somites, the developing otocyst, and the eye. **C:** A stage 17 embryo with the head and right ear removed. DAN is restricted to the medial otic epithelium. **D:** A stage 22 embryo. DAN is expressed in the limb buds (lb), somites, eye, floorplate of brain, and inner ear (arrowhead). Limb bud staining is restricted to the ventral and dorsal surfaces as seen in the mouse (Stanley et al., 1998). **E:** Dorsal view of a stage 22 embryo with head and arches removed. Limb buds and all somites express DAN. **F:** Section through the eye of a stage 22 embryo. Staining is restricted to the neural layer (n). **G:** Section through posterior nascent somites at stage 22 demonstrating DAN expression in the myotome (m) and notochord (nc). **H:** Section through mature anterior somites in a stage 19 embryo. DAN is found in the dorsal lip of the dermomyotome (asterisks), the dorsal regions of sclerotome (arrowheads), the notochord (nc), and the intermediate mesoderm of the nephritic duct (nd). Axis in F applies to G. D, dorsal; L, lateral. Original magnifications = 15× in A,C, 12× in B, 10× in D,E,G,H, 5× in F.

was restricted to the region joining the saccule and to the lagena. DAN labeling was down-regulated in the apex and base of the cochlea (data not shown).

Such a medially restricted expression pattern suggests a role for DAN in establishing the polarity of the avian inner ear. Chick DAN may designate inner ear epithelium as medial tissue. We have found that Pax 2 is also restricted to medial otic epithelium and structures, specifically the endolymphatic duct and sac (Hutson et al., 1999). No known molecular interactions between Pax 2 and DAN have been reported at this time. DAN may perform this patterning role as an inhibitor of Wnt or of BMP4 in the ear or in an unique manner as yet undescribed.

Expression of DAN in the medial otic epithelium of the chick embryo had no parallel in mouse DAN expression patterns, suggesting different roles for DAN in chick and mouse organogenesis. Figure 2G–I shows the results of mouse DAN in situ hybridization in whole-mount embryos and in tissue sections. In whole-mounts, labeling was first observed in a faint region over the dorsal 1/3–1/2 portion of the developing oto-

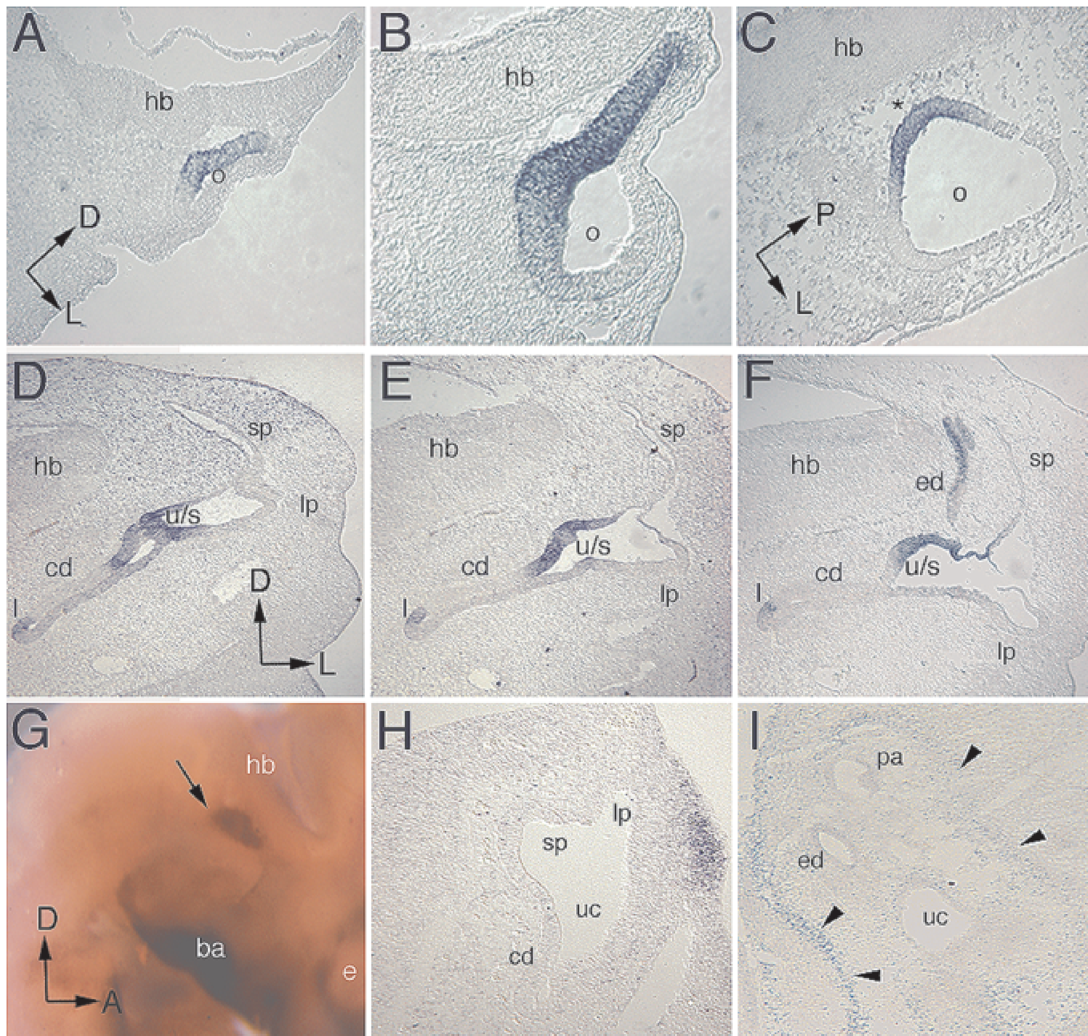

Fig. 2. In situ hybridization analysis of differential screening-selected gene aberrative in neuroblastoma (DAN) in the developing chick and mouse inner ear. **A:** DAN expression in the medial otic epithelium of a stage 15 chick embryo. **B:** Chick DAN expression in a section (14 μm) of a stage 19 inner ear. Labeling is restricted to the medial epithelium of the otocyst (o). **C:** DAN expression in the medial otic epithelium of a stage 23 chick embryo. No labeling was found in any lateral otic epithelium, but expression is apparent in the region of the emerging endolymphatic duct (asterisk).**D–F:** Sequential 12μm sections through a stage 27 chick inner ear progressing from anterior to posterior. DAN expression is observed throughout the cochlea (cd) in more anterior sections but becomes increasingly restricted to the lagena (l) and cochlear-utricular-saccular junction (u/s) in more posterior sections. The entire endolymphatic duct and sac (ed) are also labeled. The lateral plate (lp) and superior plate (sp) from which the semicircular canals form do not express DAN. G–I: DAN expression in the mouse inner ear. **G:** Embryonic day (E) 11.5 mouse embryo labeled with mouse DAN. Expression is present in the branchial arches (ba) and in the dorsal ectomesenchyme over the otocyst (arrow). **H:** Mouse DAN expression in an E12.5 embryo (12-μm section). Labeling is confined to the dorsal ectomesenchyme of the developing inner ear. **I:** Section through an E14.5 mouse inner ear (12 μm). Arrowheads indicate mesenchymal DAN labeling surrounding the condensing otic capsule. hb, hindbrain; uc, utricular-cochlear junction; pa, posterior ampulla. Axis in A applies to A,B. Axis in D applies to D–F and that in G applies to G–I. Original magnifications = 10× in A,C,H, 20× in B, 5× in D–G,I.

cyst at embryonic day (E) 9.5 (n = 3; data not shown). This expression increased and expanded slightly between E10.5 and E12 ($n_{10-10.5} = 17$, $n_{11} = 11$, $n_{12} = 3$, Fig. 2G), with strong labeling apparent in the branchial arches, the first and second of which contribute to middle ear formation. In situ hybridization of sections at these stages (n = 7) confirmed that DAN expression was restricted to the ectomesenchyme overlying the otocyst (Fig. 2H). At E13.5 and E14.5 (n = 4, Fig. 2I), labeling was confined to the mesenchyme surrounding the hindbrain as well as that surrounding the condensing tissue and cartilage of the inner ear. No labeling was observed in the otic epithelium at any stage of development in the mouse. In contrast, chick DAN expression was restricted to the medial surfaces of the inner ear epithelium, including the cochlea and entire endolymphatic duct and sac but excluded from the otic mesenchyme. This finding suggests that as yet undescribed notochord patterning mechanisms differ between chick and mouse development.

CONCLUSIONS

The species-specific differences observed in DAN expression suggest that DAN performs unique roles in somitic and otic development in the two organisms, perhaps through interactions with BMPs. Overexpression of DAN in *Xenopus* animal cap assays results in the formation of cement gland-like structures and the induction of pan-neural markers and markers for the cement gland, cardiac and foregut tissues, hindbrain, and mesoderm. All of these structures are dorsoanteriorly derived, suggesting that these effects may be mediated through the inhibition of BMP or Wnt signaling pathways (Hemmati-Brivanlou and Melton, 1997). Biochemical assays have confirmed the ability of DAN to bind BMPs and inhibit their signaling, although this inhibition is much weaker than that of Cerberus or other BMP antagonists (Stanley et al., 1998; Hsu et al., 1998; Dionne et al., 2001). BMP4 is another highly conserved mRNA whose expression pattern differs in these two species. BMP4 is expressed in sensory hair cells in the mature avian inner ear but only in the supporting cells of the mouse inner ear (Morsli et al., 1998; Wu and Oh, 1996). For DAN to act as a BMP or Wnt inhibitor in vivo, it must be expressed in the appropriate tissues at appropriate times.

The significance of the differential expression patterns observed in these experiments is not yet known. Function-blocking studies must be performed on both chicken and mouse. However, a testable model for interactions between BMPs and DAN, along with other BMP antagonists found in and in tissues around the inner ear, can be proposed (Fig. 3). Such a model can serve as a starting point for additional functional experiments. BMP4 and BMP5 mRNAs are expressed in an anterior streak and a posterior focus at stage 16 of chick otic development (Oh et al., 1996; Wu and Oh, 1996). As the inner ear grows and acquires its three-dimensional structure, BMP4 expression is restricted to the presumptive sensory epithelium (Oh et al., 1996; Wu and Oh, 1996). BMP 7 is expressed throughout the otic epithelium at stage 16, except for a ventral region of the medial epithelium. Chick DAN expression appears to be restricted to the medial otic epithelium. More experiments must be performed to determine whether the anterior and posterior BMP4/5 mRNA foci also express DAN or whether DAN mRNA expression is restricted to the otic epithelial tissue located between the two foci, which, from our initial results, is more likely. Furthermore, noggin, another BMP antagonist (Smith and Harland, 1992; Wilson et al., 1995), is expressed in the periotic mesenchyme surrounding the BMP4/5 mRNA foci (Gerlach et al., 2000).

Studies have shown that BMP4 is a secreted protein, capable of acting over distances as a morphogen in amphibians and fish (Harland, 1994; Re'em-Kalma et al., 1995; Dosch et al., 1997; for reviews of BMP4 activity see Hogan, 1996; Hild et al., 2000). Experiments using noggin- and BMP4-expressing cells on beads implanted into chick periotic mesenchyme also demonstrate morphogenic activity in avians (Gerlach et al., 2000). The inner ear developmental abnormalities seen upon implantation of noggin protein-expressing cell beads were abrogated when BMP4 protein-expressing cells on beads were co-implanted in direct contact with the noggin protein-expressing cells on beads. However, the effect could be progressively titrated by implanting the beads farther apart (Gerlach et al., 2000), indicating that the distance between the two molecular sources was critical. A similar effect was seen when pellets of chordin protein-producing cells were used instead of noggin-producing cells (Gerlach-Bank and Barald, unpublished data). These experiments demonstrate that the abnormalities induced in the chordin experiments were BMP4 dependent. The effects were also distance-dependent. As proposed in the model (Fig. 3), DAN and noggin may act cooperatively in early otic development, sequestering the activity of secreted BMP4/5 protein to the area of the anterior and posterior cell foci in the otic epithelium. There are presently no available noggin or BMP4 antibodies that function in immunohistochemcial assays, preventing the direct analysis of these proteins in vivo. We have examined the inner ear and its surrounding mesenchyme for the presence of other BMP antagonists, including Gremlin, Cerberus, and Chordin. None of these is expressed in the periotic mesenchyme or the otic epithelium in our in situ hybridization studies (unpublished data). It is possible that sensory epithelia in the chick, which are capable of regeneration (Oesterle et al., 1993; reviewed in Tsue et al., 1994; Stone et al., 1998) are shaped and maintained differently from those of mammals, which have limited capacity for regeneration and repair (Forge et al., 1993; Zheng et al., 1999). By investigating and understanding critical molecular differences, we may be able to sort out these critical functional differences on a molecular level.

EXPERIMENTAL PROCEDURES

Cloning and Sequencing Chick DAN cDNA

Fertilized White Leghorn chicken eggs obtained from Bilbie Aviaries (Ann Arbor, MI) were incubated at 37 ± 1°C. Total RNA was isolated from stage 24 embryos (all staging is according to Hamburger and Hamilton, 1951) by using Trizol (GibcoBRL). The isolated RNA was reverse transcribed with Superscript II (GibcoBRL) to produce cDNA for the RACE reaction.

The published mouse DAN sequence (Ozaki et al., 1996; accession no. D50263) was used to search the ChickGBASE provided by Chickmap at the Roslin Institute (www.ri.bbsrc.ac.uk/chickmap/ChickMapHomePage.html). A 202-bp homologous sequence was identified (UDELPCO1CPK0001B22) and used to design gene-specific primers for use in 5′-RACE and 3′-RACE. The oligonucleotide primers (5′-RACE primer = 5′ AGG CAG GCC CTG TTC TGG ATG GAC T, 3′-RACE primer = 5′ CTC GCC CTT TTC CCG GAT AAG AGT G) were synthesized and used with the SmartRACE cDNA Amplification Kit from Clontech to obtain the complete chick DAN cDNA sequence. DNA sequence analysis and mul-

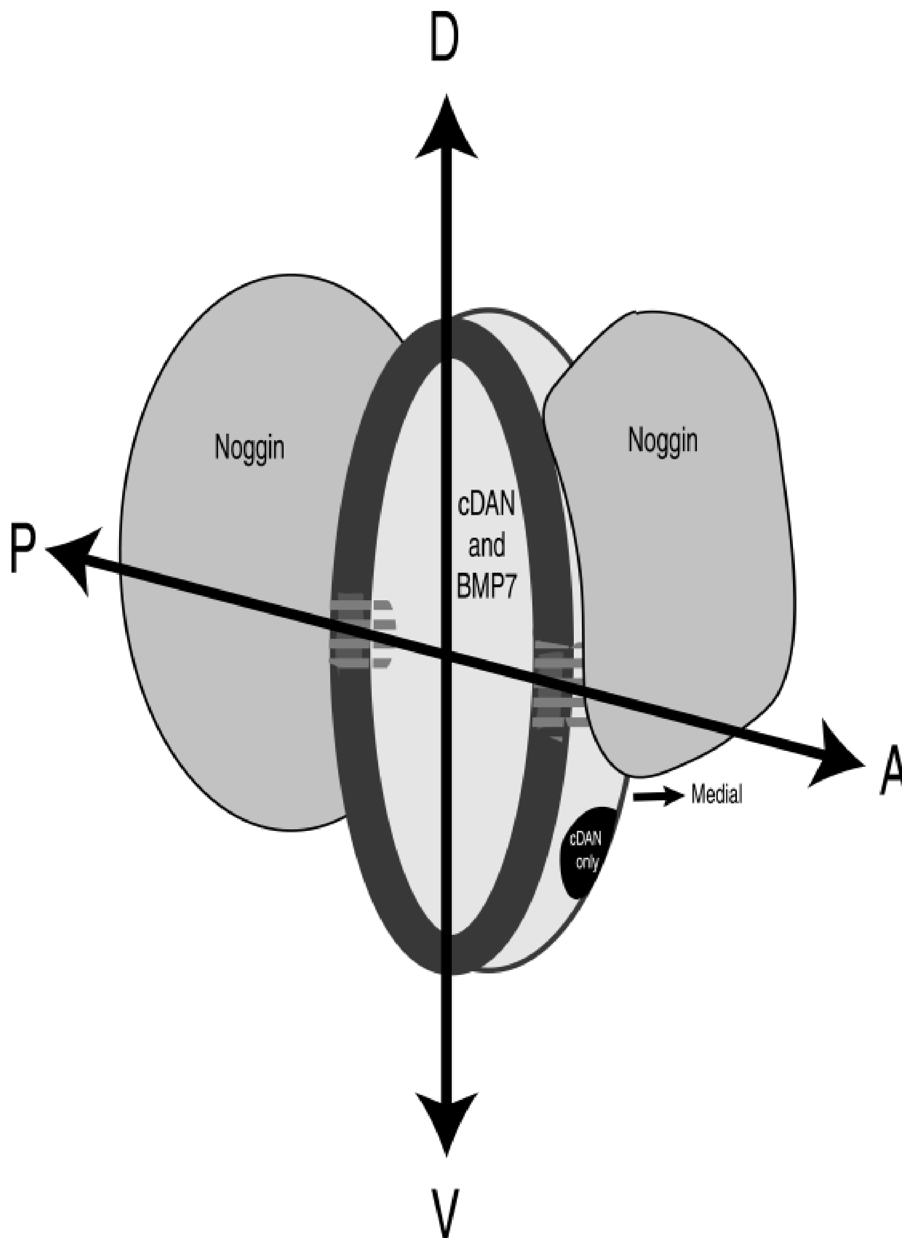

Fig. 3. Model of BMP4 and BMP antagonists in the stage 16 otocyst. The medial surface of the otocyst with the lateral or outer surface removed is represented by the center, light gray demi-ovoid. Differential screening-selected gene aberrative in neuroblastoma (DAN) expression is restricted to the entire medial otic epithelium during chick development (light gray). Noggin has been previously shown to be expressed in the anterior and posterior periotic mesenchyme (dark gray). BMP4 and BMP5 expression are restricted to an anterior and a posterior focus during this period (hatched regions). These BMP4/5 foci may also express DAN; however, this expression has not yet been confirmed experimentally. BMP7 is expressed throughout the otic epithelium (light gray oval) except for a ventral region (black region). The relative expression patterns suggest a mechanism by which Noggin and DAN could interact to restrict BMP4/5 protein activity to the anterior and posterior epithelial otic tissues in the avian ear at this stage. A, anterior; D, dorsal; P, posterior; V, ventral.

tiple sequence alignment was performed by using LaserGene (DNAStar, Madison, WI). Database searches were performed by using the BLAST program at NCBI. The chick DAN cDNA sequence has been submitted to GenBank and received the accession no. AF421881. Multiple sequence alignment was performed by using the Clustal method in the MegAlign program (LaserGene, DNASTAR; Madison, WI).

In Situ Hybridization

The 3′-RACE sequence from the chick DAN cDNA (from nucleotide [nt] 73-498) was subcloned into pGEM-T-Easy (Promega). The sense digoxigenin-labeled probe was synthesized by *Not*I linearization of the plasmid DNA and transcribed by using Sp6 RNA polymerase. The antisense-digoxigenin–labeled probe was synthesized by using *Pst*I to linearize the plasmid DNA and transcribed with T7 RNA polymerase (Roche Molecular Biochemicals). The mouse DAN probe provided by R. Harvey (Heart Research Institute, Sidney, AU) was synthesized as previously described (Stanley et al., 1998). Whole-mount in situ hybridization was performed on stage 8–24 chick embryos essentially as described in our previous work (Gerlach et al., 2000). The same procedure was also used on E8.5–E14 ICR strain mouse embryos (Taconic).

In situ hybridization was also performed on tissue sections (Jensen and Wallace, 1997). Stages 8–35 chicken embryos and E8.5–14.5 mouse embryos were collected and fixed as for whole-mount in situ hybrid-

izations. Fixed embryos were then cryoprotected in 20% sucrose, embedded in OCT (Miles), and cut on a Microm cryostat (Zeiss) at 12 μm. Sections were mounted on SuperFrost Plus slides (VWR) and hybridized. Staining was detected by using Alkaline phosphatase. The color reaction included 10% polyvinyl alcohol to enhance the signal (DeBlock and Debrouwer, 1993).

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