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Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo

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SUMMARY

We have investigated the role of Bone Morphogenetic Protein 4 (BMP-4) and a BMP antagonist, chordin, in primitive streak formation and neural induction in amniote embryos. We show that both *BMP-4* and *chordin* are expressed before primitive streak formation, and that *BMP-4* expression is downregulated as the streak starts to form. When BMP-4 is misexpressed in the posterior area pellucida, primitive streak formation is inhibited. Misexpression of BMP-4 also arrests further development of Hensen's node and axial structures. In contrast, misexpression of chordin in the anterior area pellucida generates an ectopic primitive streak that expresses mesoderm and organizer markers.

We also provide evidence that chordin is not sufficient to induce neural tissue in the chick. Misexpression of chordin in regions outside the future neural plate does not induce the early neural markers L5, *Sox-3* or *Sox-2*. Furthermore, neither BMP-4 nor BMP-7 interfere with neural induction when misexpressed in the presumptive neural plate before or after primitive streak formation. However, chordin can stabilise the expression of early neural markers in cells that have already received neural inducing signals. These results suggest that the regulation of BMP signalling by chordin plays a role in primitive streak formation and that chordin is not sufficient to induce neural tissue.

Key words: BMP-4, BMP-7, chordin, Axis formation, Gastrulation, Neural induction, Hensen's node, Primitive streak, Spemann organizer

INTRODUCTION

The molecular bases of gastrulation and neural induction remain poorly understood in amniote embryos, and much of what we know about these processes in vertebrates is derived from studies of anuran amphibians. One of the key events during amniote gastrulation is the formation of the primitive streak. In the chick embryo, primitive streak formation involves a number of steps: the establishment of a specialized region at the posterior edge of the blastodisc within which streak development begins, the initial ingression of epiblast cells and their condensation into a mesenchymal rod, the interaction of these cells with the overlying epiblast to induce involution and the establishment of the organizer at the anterior tip of the streak. Two members of the TGFβ superfamily, activin (Mitrani et al., 1990; Cooke et al., 1994) and cVg1 (Seleiro et al., 1996; Shah et al., 1997) are capable of initiating the formation of an ectopic primitive streak. Vg1 is expressed in the posterior marginal zone, a region between area opaca and area pellucida that itself can induce the formation of an ectopic streak (Khaner and Eyal-Giladi, 1989; Eyal-Giladi and Khaner, 1989). These observations suggest that Vg1 may be an endogenous mediator of primitive streak induction.

The induction and early patterning of the nervous system in avian embryos involve the acquisition and maintenance of competence to respond to neural inducing signals (Streit et al., 1997), the reaction to the inducing signals themselves (evocation; Waddington, 1940; see also Streit et al., 1997), the stabilisation of this response, and the anterior/posterior and dorsal/ventral patterning of the neural plate (see Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996).

In *Xenopus*, a balance between the TGFβ superfamily members BMP-4 and BMP-7 and three secreted BMP antagonists, noggin, chordin and follistatin, has been implicated in both early axis determination and neural induction (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994, 1995; Re'em-Kalma et al., 1995; Piccolo et al., 1996; Zimmermann et al., 1996; Fainsod et al., 1997; for review Sasai and DeRobertis, 1997; Weinstein and Hemmati-Brivanlou, 1997). At the blastula stage, BMP-4 ventralizes mesoderm (Dale et al., 1992; Fainsod et al., 1994; Jones et al., 1992, 1996; Suzuki et al., 1994; Steinbeisser et al., 1995), whereas the three BMP antagonists have dorsalising activity

when injected into early embryos. At the late blastula/early gastrula stages, BMPs act as epidermal inducers and neural inhibitors (Wilson and Hemmati-Brivanlou, 1995; Hawley et al., 1995; Xu et al., 1995; reviewed in Weinstein and Hemmati-Brivanlou, 1997; Wilson and Hemmati-Brivanlou, 1997), whereas chordin, noggin and follistatin can suppress epidermal fates and promote neural differentiation.

The pattern of expression of *BMP-4* and *BMP-7* in the chick embryo at late primitive streak stages has recently been described (Watanabe and Le Douarin, 1996; Schultheiss et al., 1997; Tonegawa et al., 1997). Both genes are transcribed in a pattern complementary to that of the L5 epitope, a marker for cells that are competent to respond to neural inducing signals. This finding raised the possibility that BMPs might control the region of the early epiblast that is competent to receive inducing signals from the organizer (Streit et al., 1997). Since L5 is also expressed prior to primitive streak formation (Streit et al., 1997), we considered whether BMPs might have a role in patterning the avian embryo at these early stages of development.

We show here that *BMP-4* and *BMP-7* are expressed at pre-primitive streak stages and that misexpression of BMP-4 adjacent to cells that will contribute to the organizer prevents primitive streak formation, suggesting that inhibition of BMP-4 signalling is important for normal initiation of the primitive streak. To test this possibility, we examined the expression of the chick *chordin* gene, and found that it is initially expressed in cells adjacent to the site of primitive streak formation. Misexpression of chordin at the anterior edge of the prestreak embryo causes the formation of an ectopic primitive streak that expresses mesoderm as well as organizer markers. These findings suggest that chordin plays a role in primitive streak formation. However, we have found that chordin is not sufficient to induce neural tissue in the chick, and that BMP-4 and BMP-7 do not suppress the formation of the neural plate. Rather, chordin appears to act downstream of, or in conjunction with, other neuralising signals.

MATERIALS AND METHODS

Fertile hens' eggs (White Leghorn; Spafas, MA) and quails' eggs (Karasoulas, CA) were incubated at 38°C for 2-30 hours to give embryos between stages XII (Eyal-Giladi and Kochav, 1976) and 9 (Hamburger and Hamilton, 1951).

Cloning of chick *chordin*

A fragment of chick *chordin* (CR3) was isolated by RT-PCR from stage 10 notochord cDNA using degenerate primers designed to amplify DNA encoding the sequences WHPFL/VPP and CCKQCPV (primer 1: TGG CAC/T CCI TTC/T C/GTI CCI CC ; primer 2: AC IGG G/ACA T/CTG T/CTT G/ACA G/ACA). This fragment was used to isolate *chordin* cDNA clones from a stage 4 Hensen's node library in the expression vector pMT21 (provided by C. Hume and J. Dodd). None of these clones were full length and additional N-terminal coding sequence was determined by sequencing chick genomic DNA clones. A fragment of the *chordin* coding region encompassing the first 106 amino acids was obtained by PCR from stage 8 whole embryo cDNA and was cloned in frame to the longest cDNA in pMT21 (the full-length sequence has Genbank accession number AF031230). An epitope tagged version of chick *chordin* was generated by PCR-based addition of the HA epitope (Wilson et al., 1984) to the C terminus of the *chordin* coding sequence.

cDNA clones

BMP-4, BMP-7 and truncated dorsalin-1 (Liem et al., 1995) were cloned into pMT-23 to allow their transfection and expression in COS cells and contained sequences encoding the myc-epitope. Sox-2 and Sox-3 plasmids were provided by Drs R. Lovell-Badge and P. Scotting (Uwanogho et al., 1995; Collignon et al., 1996); chick brachyury was provided by Dr J. Smith and cNot-1 by Michael Kessel.

Cells and transfection

COS-1 cells were grown in DMEM containing 10% new born calf serum. Transfection with BMP-4, BMP-7, truncated dorsalin-1 and chordin was performed using lipofectamine (Gibco BRL). 24 hours after transfection, pellets containing 1000 cells were generated by setting up hanging drop cultures. The pellets were transplanted into embryos 48 hours after transfection. The presence of BMP-4, BMP-7 and chordin in conditioned medium collected from COS cells was confirmed in western blots using anti-myc or anti-HA antibodies.

Dissection and grafting techniques

Chick host embryos were explanted and maintained in New culture (New, 1955), modified as described by Stern and Ireland (1981), for 10-24 hours. Quail donor embryos were immersed in Pannett-Compton saline (Pannett and Compton, 1924), Hensen's node was excised and transplanted into the inner margin of the area opaca of a chick host as described previously (Storey et al., 1992). Pellets of transfected cells (500-1000 cells per pellet) were grafted into different regions of chick host embryos as indicated in the individual experiments.

DiI labelling

To determine the movements of host epiblast cells adjacent to grafts of transfected and control pellets, the carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl indocarbocyanine perchlorate (DiI, Molecular Probes, Inc.) was used. This was made up as an ethanol stock at 0.5% w/v and diluted 1:10 in 0.3 M sucrose at 50°C just prior to use (Stern, 1990). This solution was delivered to the desired epiblast cells from the apical surface of the epiblast using air pressure applied to a fine micropipette made from 50 μl borosilicate capillary glass (Sigma) in a vertical puller (Kopf).

Immunocytochemistry and in situ hybridisation

Whole-mount immunostaining with monoclonal L5 antibody was performed as previously described (Streit et al., 1995). To visualise quail tissue, we used the monoclonal antibody QCPN (Developmental Studies Hybridoma Bank, maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City 52242, under contract N01-HD-2-3144 from NICHD). The staining was performed as described before (Streit et al., 1997).

Whole-mount in situ hybridisation using DIG-labelled RNA-probes and histology were performed as described by Théry et al. (1995) and Streit et al. (1997).

RESULTS

Expression of BMP-4 and BMP-7

The expression of *BMP-4* and *BMP-7* after the full primitive streak stage in the chick has been described (Liem et al., 1995; Watanabe and Le Douarin, 1996; Schultheiss et al., 1997). We have analysed the expression of both genes at earlier stages. Before primitive streak formation (stage XI-XIII; Fig. 1A,F,K,M) BMP-4 transcripts are present at low level in the entire embryonic (area pellucida) and extraembryonic (area

opaca) epiblast (Fig. 1A,K), while BMP-7 is expressed most strongly in the area opaca epiblast (Fig. 1F,M). At the onset of primitive streak formation at stage XIV-2, the expression of both transcripts is extinguished from the area pellucida (Fig. 1B,G,L,N). At stage 3/3^{+}, *BMP-7* expression reappears (Fig. 1H) in the posterior area pellucida epiblast, including the primitive streak, then expands until it surrounds the forming neural plate (stage 5, Fig. 1I) and expression persists throughout most of the non-neural ectoderm (Fig. 1J). By the full primitive streak stage (stage 4), *BMP-4* is detected in a ring of epiblast cells surrounding the future neural plate (Fig. 1C) and then condenses to a line of cells at the boundary between neural and non-neural ectoderm (Fig. 1D,E) (see also Liem et al., 1995; Schultheiss et al., 1997; Tonegawa et al., 1997). Thus, there are two phases of expression of *BMP-4* and *BMP-7* in the early embryo, separated by a period when neither gene is expressed in the area pellucida (stages XIV-3). These findings differ from *Xenopus*, where both genes are expressed in the prospective neural plate during the early phases of gastrulation (Dale et al., 1992; Fainsod et al., 1994; Hawley et al., 1995; Hemmati-Brivanlou and Thomsen 1995).

BMP-4 inhibits primitive streak development

Since *BMP-4* expression is downregulated from the posterior regions of the blastodisc as the primitive streak forms (stage XIV-2), we investigated whether extinction of *BMP-4* is required for the formation of the primitive streak. To test this, we implanted pellets of BMP-4-transfected COS cells (or mock-transfected cells as control) into the posterior edge of the area pellucida (Fig. 2A). Even after 16-36 hours, 66% (23/35) of experimental embryos had not formed a primitive streak. The majority of these embryos (8/12) did not express *brachyury*, a marker of streak mesoderm (Fig. 2C). The remaining embryos showed a strong reduction in *brachyury* expression, but the labelled cells were not organised in a recognizable streak. In 20% (7/35) of embryos the primitive streak was directed away from, or circumvented BMP-4-expressing cells and, in each of these embryos, the expression of the organizer marker *cNot1* was greatly reduced when compared with normal embryos. Only 5/35 embryos developed a normal streak with normal expression of *brachyury*. In all control embryos grafted with mock-transfected COS cells, the primitive streak developed normally and expressed *brachyury* (12/12; Fig. 2D).

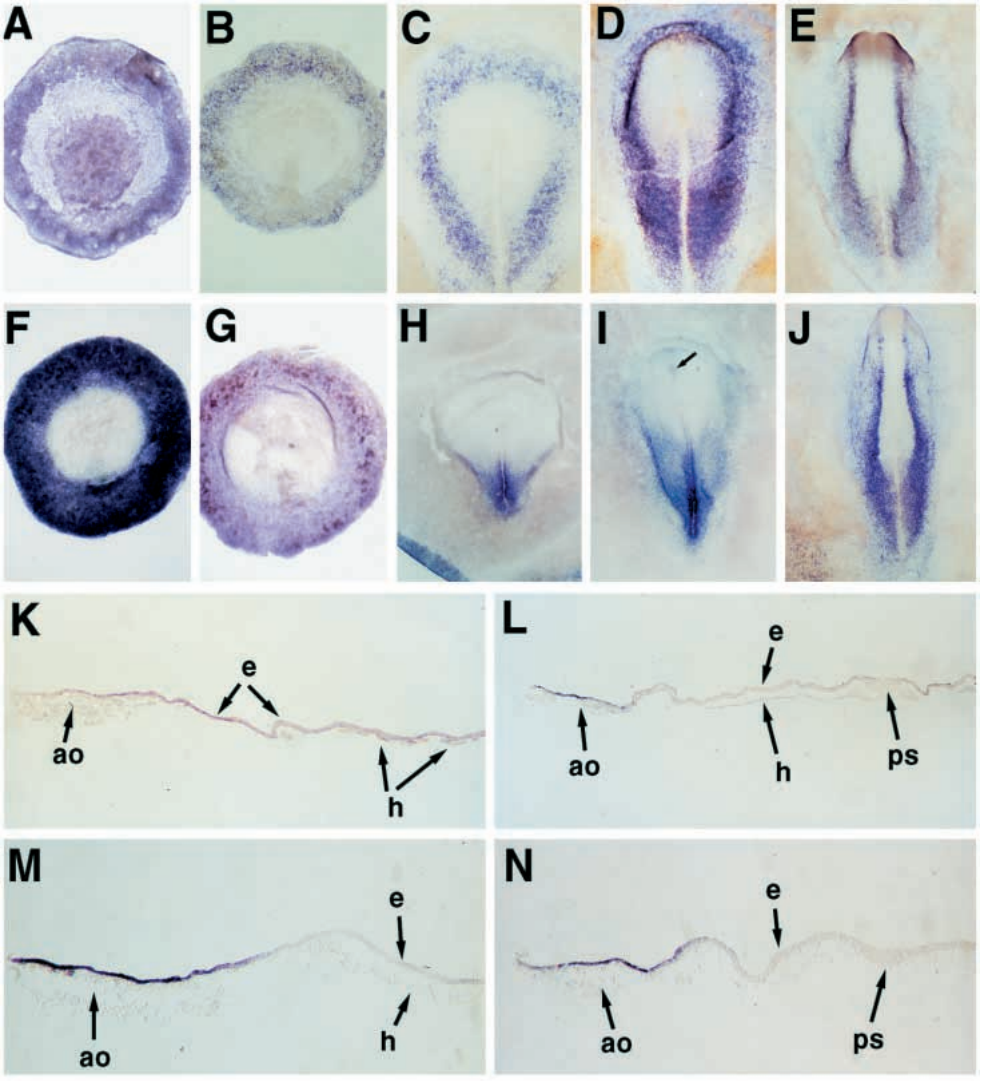

Fig. 1. Expression of *BMP-4* and *BMP-7* in the normal embryo. Whole-mount in situ hybridisation was performed with DIG-labelled probes for *BMP-4* (A-E) and *BMP-7* (F-J). At stage XIII (A), *BMP-4* is expressed throughout the epiblast of the embryo (see also K). By stage 2^{+} (B), expression has cleared from the central area pellucida. At stage 4 (C) and 5 (D), *BMP-4* reappears in the area pellucida as a ring encircling the future neural plate and extending further posteriorly to the primitive streak. (E). At stage 7-8, the expression at the edges of the developing neural plate intensifies. *BMP-7* expression begins before primitive streak formation and is strongest in the area opaca epiblast (F; stage XIII) (see also M). By stage 2-3 (G), it is cleared from the area pellucida. At stage 4 (H) it reappears over the posterior part of the primitive streak and adjacent epiblast, and this expression expands anteriorly to surround the presumptive neural plate by stage 5 (I). At this stage, *BMP-7* is also expressed in the prechordal region (arrow). (J) By stage 8, expression is concentrated at the edges of the neural plate and, at a lower level, in the non-neural ectoderm. (K) Sagittal section through the embryo in A, showing expression of *BMP-4* confined to the epiblast layer (e). (L) Transverse section through the embryo in B above, at the level of the primitive streak (ps). Expression is now restricted to the area opaca epiblast. (M,N) Sagittal (M) and transverse (N) sections through the embryos in F and G respectively, showing strong expression of *BMP-7* in the area opaca epiblast. ao, area opaca; e, epiblast; h, hypoblast; ps, primitive streak.

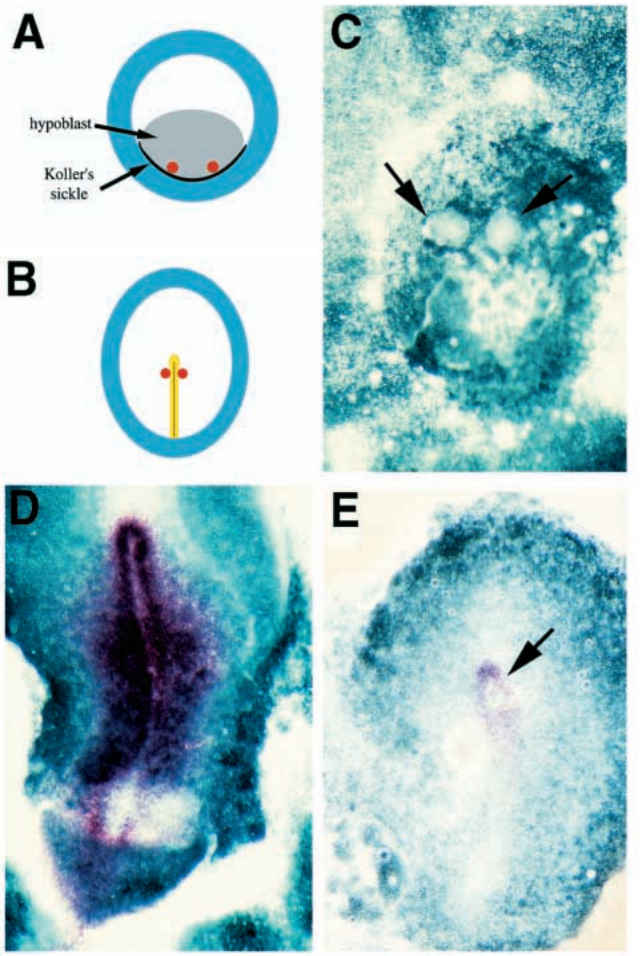

Fig. 2. BMP-4 inhibits primitive streak formation and antagonizes organizer function. (A) Diagram showing the site at which grafts were placed adjacent to the sickle of prestreak embryos; grafted cells are shown in red, area opaca in blue, hypoblast in gray and Koller's sickle in black. (B) Diagram showing the position of grafts placed adjacent to the node in stage 3^+ embryos; the primitive streak and the node are shown in yellow. (C) Embryo that had received two grafts of BMP-4 (arrows): no axial structures have formed and no *brachyury* expression is seen after 24 hours. (D) Control embryo that received two grafts of mock-transfected cells at the same position, showing normal development and normal *brachyury* expression. (E) After a graft of BMP-4, development is arrested and there is a reduction in the size of the domain expressing *chordin* in the node (arrow).

To investigate whether cells maintain the competence to respond to BMP-4 after the embryo has initiated primitive streak formation, transfected COS cells were grafted next to the anterior primitive streak at stage 3^+ (Fig. 2B). Of 15 embryos, 7 failed to form a morphological node, notochord, somites and neural plate after 24 hours and all showed substantial reduction of *chordin* expression in the anterior primitive streak (Fig. 2E; compare to Fig. 3E). In the remaining 8 embryos, the grafts had moved away from the vicinity of the node and were found under the developing neural plate; none of these showed any obvious abnormality or developmental delay.

Taken together, these results show that BMP-4 inhibits the formation of the primitive streak and the subsequent development of the axial structures that derive from it.

Chordin is sufficient to initiate primitive streak formation

The findings described above raise the possibility that the extinction of BMP expression is a prerequisite for primitive streak formation. In both *Drosophila* and vertebrates, BMPs have been shown to activate their own expression (Jones et al., 1992; Biehs et al., 1996; Schmidt et al., 1996) and, in *Drosophila*, this activity is inhibited by the product of the *short gastrulation* (*sog*) gene (Biehs et al., 1996). *Xenopus* and zebrafish homologues of *sog*, termed *chordin*, have been identified (Holley et al., 1995; Sasai et al., 1995; Schulte-Merker et al., 1997). We have cloned the chick homologue of *chordin*, and report here its pattern of expression in early avian development and the analysis of its potential role in primitive streak initiation.

Cloning and expression of chick *chordin*

A chicken *chordin* homologue was cloned using degenerate PCR primers designed to amplify sequences conserved between *Drosophila sog* (François et al., 1994) and *Xenopus chordin* (Sasai et al., 1994). The chick *chordin* gene is predicted to encode a protein of 940 amino acids (Fig. 3A) and, like *Drosophila* sog, *Xenopus* chordin and zebrafish chordino (Schulte-Merker et al., 1997), chick chordin contains a hydrophobic signal sequence and 4 cysteine-rich repeats (CR1-CR4). Overall amino acid identity between chick chordin and *Xenopus* chordin is 60% and between chick chordin and *Drosophila* sog 27%.

To investigate whether chick chordin has the biological activity exhibited by *Xenopus* chordin, synthetic mRNA encoding chick chordin was injected into *Xenopus* embryos at the 2-cell stage, animal caps were isolated, cultured until sibling embryos had reached neurula stage and analysed by RT-PCR (data not shown). chordin induced strong expression of the pan-neural marker *NCAM* in the absence of induction of the dorsal mesodermal markers *Xbra* and *α-actin*. Identical results were obtained with injection of native and HA-epitope tagged chick *chordin* mRNA. Induction of *NCAM* expression was also observed in isolated animal caps cultured in medium conditioned by COS cells transfected with chick chordin, but not in medium conditioned by control mock-transfected cells (data not shown). Western blot analysis of supernatant conditioned by COS cells transfected with the chordin-HA expression construct revealed a prominent band of ~105 kDa, a size similar to the predicted molecular weight of full-length chordin protein (Fig. 3B). Thus, chick *chordin* encodes a secreted protein with activity comparable to that described for its *Xenopus* counterpart.

We next examined the early expression pattern of chick *chordin* by whole-mount in situ hybridisation. At pre-primitive streak stages (XI-XII; Fig. 3C,D) *chordin* mRNA is found in the epiblast just anterior to Koller's sickle and in underlying middle layer cells. Both of these cell populations contribute to the organizer (Izpisúa-Belmonte et al., 1993; Hatada and Stern, 1994). As soon as the primitive streak forms (and concomitant with the downregulation of *BMP-4* and *BMP-7* in the area pellucida) *chordin* is strongly expressed at its anterior tip and subsequently appears in Hensen's node (Fig. 3E-G), where it

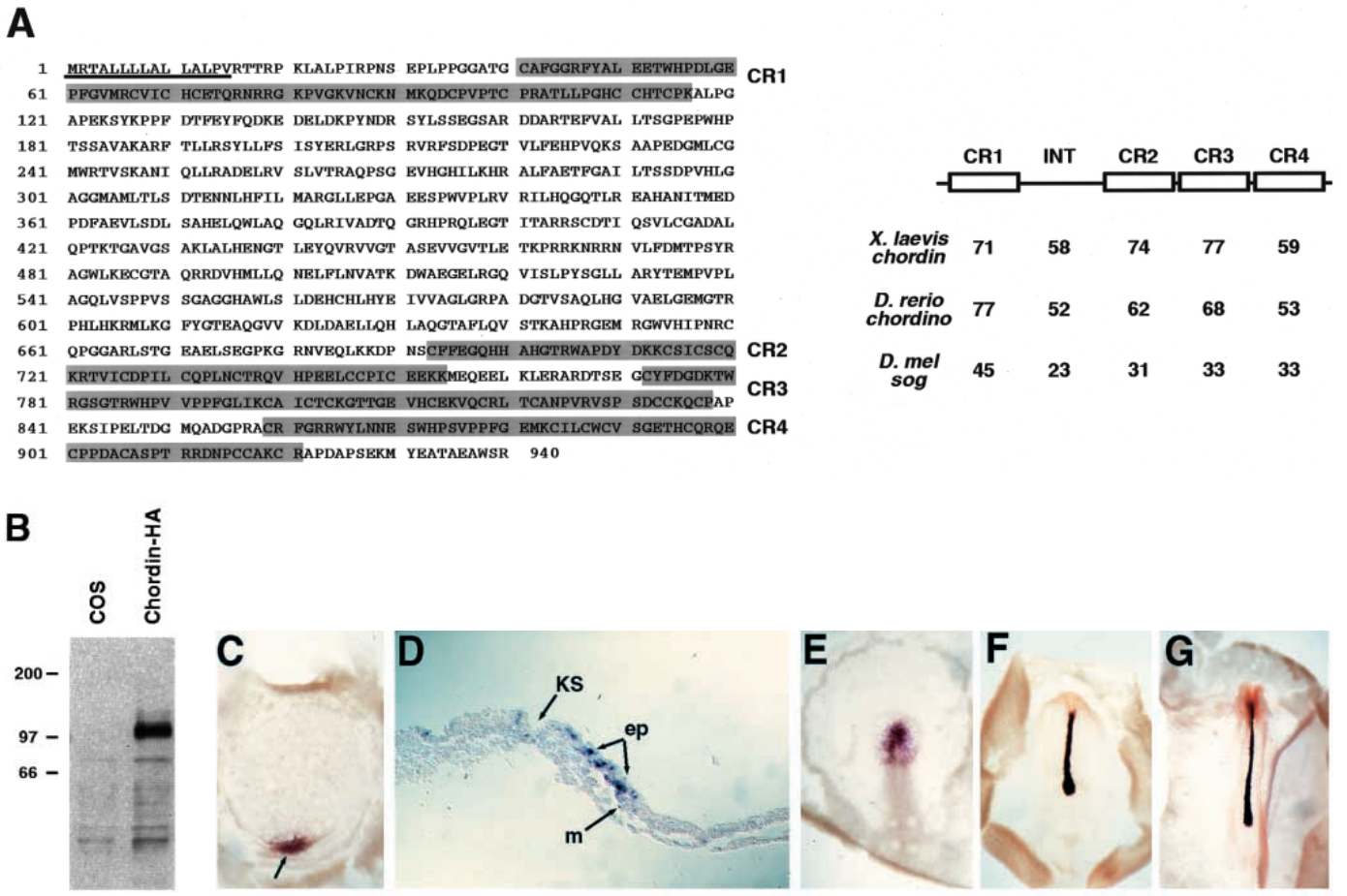

Fig. 3. Sequence and expression of chick *chordin.* (A) The predicted amino acid sequence of chick *chordin.* The proposed signal sequence is underlined and the cysteine-rich repeats CR1-CR4 are boxed in grey. At the right, a schematic representation of the proteins and the percentage of amino acid identity between the respective domains CR1-CR4 and the intervening region (INT) of chick and *Xenopus* chordin, zebrafish chordino and *Drosophila* sog. (B) Western blot using anti-HA epitope antibody 12CA5. Conditioned media were collected from mock- (lane 1) and chordin-HA-transfected (lane 2) COS cells and separated on a 7.5% polyacrylamide gel. Bars on the left indicate positions of molecular weight standards in kDa. (C) At stage XIII, *chordin* is expressed posteriorly, adjacent to Koller's sickle (arrow). (D) In sagittal section, expression is seen in the epiblast (ep) just anterior to Koller's sickle (KS) and in a few middle layer cells (m). Posterior is to the left. (E) By stage 3, transcripts have concentrated in the anterior tip of the primitive streak. At stage 6 (F), 8 (G) and thereafter, *chordin* is restricted to the entire length of the notochord and Hensen's node.

persists at least until stage 23. The head process and notochord express *chordin* at high levels as soon as the cells emerge from the node (Fig. 3F,G). Thus, *chordin* is expressed in organizer precursor cells, in the organizer itself and in one of its derivatives, the notochord.

Chordin can elicit the formation of an ectopic axis

To assess whether chordin is sufficient to initiate primitive streak formation, chordin- or mock-transfected COS cells were transplanted anteriorly (180° away from the normal expression domain of *chordin*) into a chick host embryo at stage XII-3 (Fig. 4A). Embryos were grown until stage 4-8 and the expression of markers for mesoderm (*brachyury*), organizer (*cNot-1*) and neural plate (*Sox-3*) assessed by whole-mount in situ hybridisation.

Misexpression of chordin at the anterior edge of the area pellucida (just inside the marginal zone) results in the formation of a structure resembling a short primitive streak, expressing *brachyury* (18/23; Fig. 4B). In about half the embryos (8/18), this terminated in a node-like structure which, in all cases analysed (n=4), expressed *cNot-1* (Fig. 4C). Ectopic *Sox-3* (4/4) was expressed in a horseshoe pattern around the ectopic streak reminiscent of the pattern in the early neural plate of the normal embryo (Fig. 4D,E). Strikingly, similar ectopic streak-like structures could still be generated in host embryos that had initiated primitive streak formation. In contrast, grafting of chordin-expressing cells to the anterior marginal zone (just outside the area pellucida), did not result in ectopic streak structures, nor in ectopic expression of *brachyury* (0/12) or *Sox-3* (0/15) (Fig. 4F).

These results show that BMP-4 inhibits primitive streak formation and that misexpression of chordin at the edge of the area pellucida is sufficient to initiate the formation of a secondary axis.

Chordin is not sufficient to induce neural tissue in competent epiblast

In *Xenopus*, chordin induces the expression of neural markers in animal caps and in caps excised from *chordin*-injected embryos (Sasai et al., 1995; Piccolo et al., 1997; Sasai and De

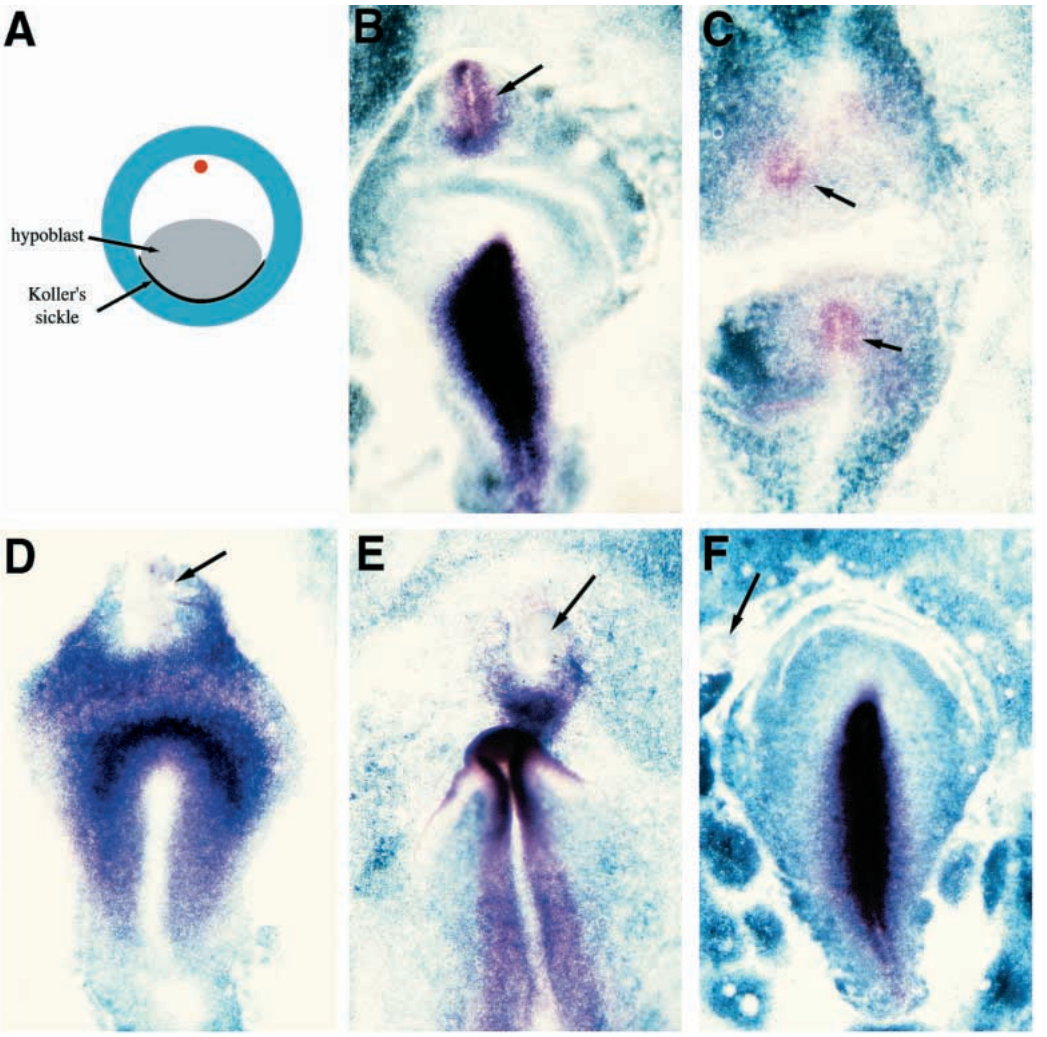

Fig. 4. Chordin generates an ectopic primitive streak. (A) Diagram showing the site (red) where transfected COS cells were grafted in a pre-primitive-streak-stage embryos. (B) An ectopic primitive streak has formed (arrow) and expresses *brachyury*. (C) Both the normal and the ectopic primitive streak contain a node expressing the homeobox gene *cNot1* (arrows). (D) The ectopic primitive streak (arrow) is surrounded by *Sox-3* expression, like the endogenous streak. (E) Even in embryos allowed to develop longer, a ring of *Sox-3* expression remains (arrow). (F) When grafted into the anterior/lateral marginal zone, chordin (arrow) does not generate an ectopic primitive streak or ectopic *brachyury* expression.

Robertis, 1997). The expression of *chordin* in the node and notochord therefore led us to investigate whether chordin is sufficient to induce neural tissue in competent epiblast in the chick embryo. Grafts of chordin-expressing cells were placed into the area opaca or into the non-neural ectoderm of the area pellucida (Fig. 5A), regions that can respond to a graft of Hensen's node forming a complete neural axis (Storey et al., 1992; Streit et al., 1997).

In the area opaca, we did not detect the formation of a neural-plate-like structure or induction of the early pan-neural markers L5 (0/10; Fig. 5B), *Sox-2* (0/12) or *Sox-3* (0/21; Fig. 5C) in host tissue next to the chordin graft. Similarly, in the non-neural ectoderm of the area pellucida, ectopic expression of *Sox-2* (0/11) was not detected (Fig. 5D), and only one embryo (1/14) showed ectopic expression of *Sox-3*. In all cases analysed (14/14), the epiblast overlying the graft had the morphology of squamous, non-neural ectoderm (Fig. 5E,F) rather than a columnar, neural plate-like structure. These results suggest that chordin is not sufficient to impose neural character on naïve ectodermal cells, even though the organizer itself is able to elicit the formation of a complete neural axis in both regions.

The different outcomes of chordin misexpression in chick and *Xenopus* prompted us to investigate whether BMP-4 and/or BMP-7 can interfere with the early steps of neural induction in the chick embryo. To test this, COS cells transfected with BMP-4 or BMP-7 (experimental) or a truncated form of dorsalin-1 (control) were transplanted into the prospective neural plate at pre-primitive streak and primitive streak stages. In pre-primitive streak stage embryos, transfected cells were grafted lateral to the midline, about half-way between the anterior and posterior edges of the area pellucida at stage XI-XII (Fig. 6A). In primitive streak stage hosts, transfected cells were grafted to the prospective mid-/hindbrain region at stage 3^{+}. In both cases, embryos were grown until they reached stages 4-5 and the expression of L5 and *Sox-3* examined. BMP-4 and BMP-7 did not affect the expression of either marker in host embryos of any stage (Fig. 6B,C; Table 1).

Taken together, these results suggest that chordin is not sufficient to elicit neural differentiation from competent epiblast, and BMP-4 and BMP-7 are not able to prevent neural development in the future neural plate of the chick embryo. In addition, despite the complementary patterns of expression of L5 and BMPs, neither chordin nor BMP-4 or BMP-7 misexpression affected the distribution of L5.

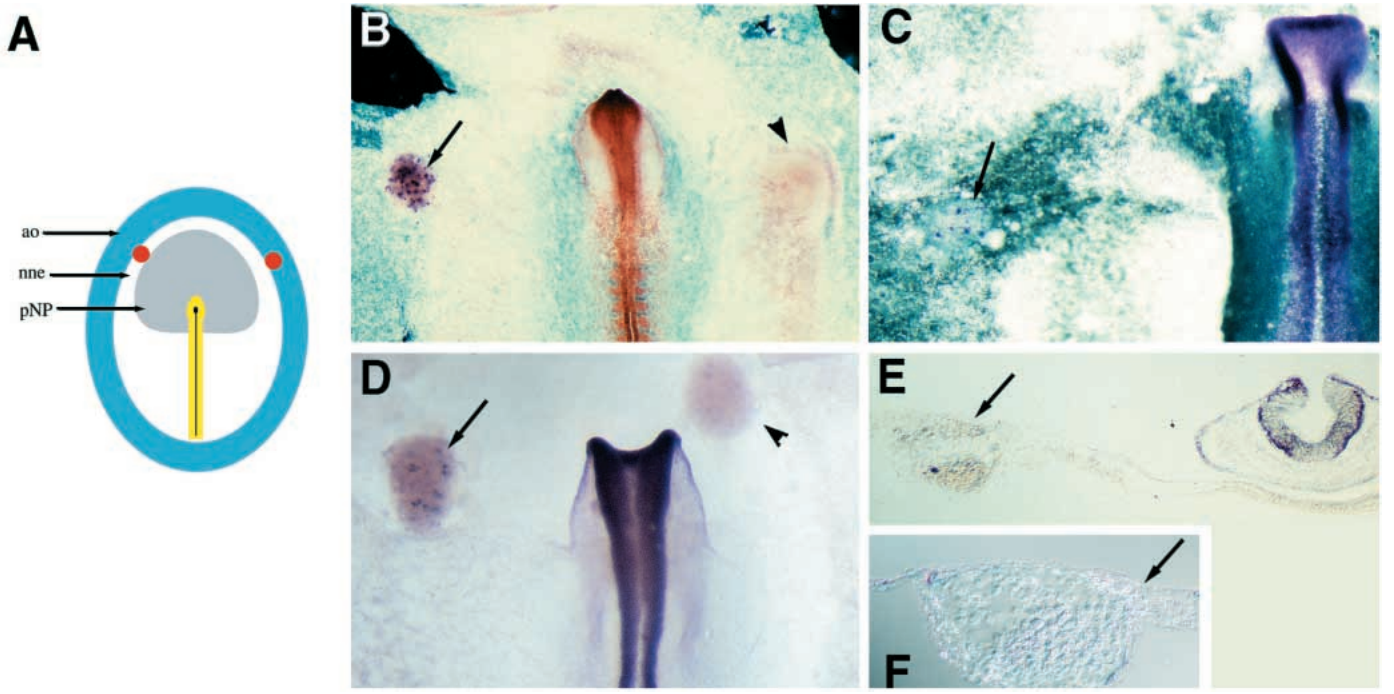

Fig. 5. Chordin does not induce neural tissue in extraembryonic or non-neural embryonic ectoderm. (A) Diagram of the sites (red) at which grafts of chordin-transfected and control cells were placed. nne, non-neural ectoderm; ao, area opaca; pNP, presumptive neural plate. (B,C) Chordin- (arrow) or mock- (arrowhead) transfected COS cells were transplanted into the area opaca. No ectopic expression of L5 (B; brown) is observed; chordin-transfected cells were visualized by an antibody against the HA-epitope (purple). (C) *Sox-3* expression is shown by in situ hybridisation; no ectopic expression is seen associated with chordin-expressing cells (arrow). (D) Chordin- (arrow) and mock- (arrowhead) transfected cells were implanted in the non-neural epiblast of the area pellucida: no expression of *Sox-2* is seen in host tissue above chordin-expressing cells. Note: light background staining is frequently seen within transfected cell pellets. (E,F) The epiblast overlying the chordin pellet (arrow) has a squamous, non-neural appearance and does not express *Sox-3*. (E) Bright-field view, while F is a higher magnification view of the same section with Nomarski DIC optics.

Chordin stabilises the neural state of prospective neural plate cells

Chordin acts downstream of, or in parallel with, other early neuralising signals

The finding that chordin is not sufficient for neural induction in embryonic or extraembryonic epiblast of the chick suggests that additional or alternative signals from the organizer are required. To investigate whether chordin acts in concert with node-derived signals, a quail Hensen's node (stage 3^+-4) was grafted into the area opaca of a chick host at stage 3^+-4. After 3 or 5 hours, the quail node was removed and replaced by chordin- or mock-transfected COS cells. Embryos were grown until stage 8-10 and the expression of *Sox-3* and *Sox-2* analysed. Complete removal of the node was assessed by subsequent staining with the quail-specific antibody QCPN. Exposure to a node for as little as 3 hours before a graft of chordin cells elicited ectopic expression of *Sox-3* (4/5, Fig 7A,B), a result not obtained with control cells. After a 5 hour exposure to the node (Fig. 7C,D), there is also a thickening of the epiblast overlying the graft (4/5). Ectopic *Sox-2* expression was never detected (n=15). These results are consistent with the idea that an additional signal is required upstream of, or in parallel with, chordin to initiate neural induction and that this signal can be provided by Hensen's node.

One candidate for a factor that might cooperate with chordin is HGF/SF. Hensen's node expresses *HGF/SF* at the time of neural induction and the protein can prolong neural competence in the area opaca (Streit et al., 1997). To test this possibility, COS cells were transfected with *HGF/SF* and implanted together with chordin-transfected cells into the area opaca of a chick host at stage 3^+-4 (n=22). On the contralateral side, each embryo was grafted with either HGF/SF cells or chordin cells alone. After 14-20 hours, neither HGF/SF nor chordin, nor the combination of chordin and HGF/SF elicited expression of either marker in area opaca epiblast. This result shows that HGF/SF is not the node-derived factor that cooperates with chordin to initiate stable neural gene expression.

A role for chordin in the maintenance of the neuralised state

We next considered whether the role of chordin in neural induction might lie downstream of initial neuralising signals, perhaps by stabilising the neural state of induced neural cells. To test this, we made use of an observation (documented in detail below) that implanted COS cells adhere to overlying epiblast cells, and prevent them from becoming incorporated into the neural tube.

To define normal cell movements, groups of cells in the prospective mid/hindbrain region of the neuroepithelium at stage 3^+ were labelled with DiI, and analysed at different time intervals. All labelled cells became incorporated into the neural

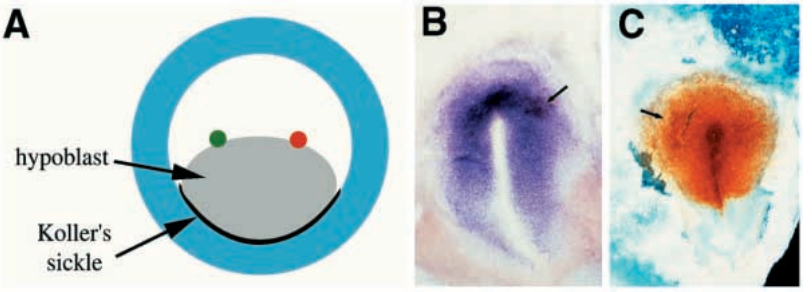

Fig. 6. BMP-4 and BMP-7 do not prevent neural induction. (A) Diagram showing the position of grafts placed into the presumptive neural plate of pre-primitive streak stage embryos. (B) After a graft of BMP-4 on the right (arrow), no change is seen in the pattern of *Sox-3* expression. (C) After a graft of BMP-7 on the left (arrow), no change is seen in the pattern of L5 expression.

plate (Foley et al., 1997 and Fig. 8A-D). In contrast, when mock-transfected COS cells were transplanted in contact with the labelled host cells, a group of labelled cells remained associated with each COS cell graft, and a trail of labelled cells connected this site with the elevating neural plate (Fig. 8F-H). Chordin-transfected cells similarly perturbed epiblast cell movements (Fig. 8F-H). However, *Sox-3* and *Sox-2* expression in these labelled cell groups was detected only in association with the chordin cells and not with control cells (Fig. 8I-L).

In a separate series of experiments conducted without DiI labelling, the expression of *Sox-3* was analysed at different intervals after grafting. In embryos grown until stage 6-7, ectopic expression of *Sox-3* was observed in 33/35 embryos and labelled cells were continuous with the neural plate of the host in most cases (23/35 [66%]; Fig. 8I). Moreover, the overlying epiblast showed a columnar morphology (Fig. 8M), which was not observed with control cells (Fig. 8N). As the period of culture increased, the ectopic *Sox-3* domain gradually separated from the neural plate of the host (Fig. 8J,O). By stage 8-9, 6/10 embryos exhibited residual *Sox-3* expression in association with transfected cells (Fig. 8K,P). By stage 10 (24 hours after grafting), no ectopic *Sox-3* expression was seen close to the graft (0/7) and the host neural plate appeared normal. In these embryos, the morphology of the epiblast above the graft resembled non-neural ectoderm. Similar results were obtained for *Sox-2* expression (*n*=8; Fig. 8L).

These experiments show that COS cells prevent adjacent prospective neuroepithelial cells from becoming incorporated into the neural plate. Under these circumstances, chordin transiently maintains the expression of neural markers in the adherent epiblast tissue.

Table 1. BMPs do not affect the expression of early neural markers

	Grafts into prestreak embryos			Grafts into stage 3-3$^+$ embryos		
	L5	Sox-2	Sox-3	L5	Sox-2	Sox-3
BMP-4	2 (17)	n.d.	0 (24)	n.d.	n.d.	0 (10)
BMP-7	0 (31)	0 (14)	n.d.	0 (19)	n.d.	0 (14)
tdsl-1	0 (30)	0 (10)	0 (22)	0 (6)	n.d.	0 (8)

BMP-4-, *BMP-7-* (experimental) or truncated *Dsl-1-* (control)-transfected COS cells were grafted into the prospective neural plate of pre-primitive-streak or streak-stage embryos. The expression of L5, *Sox-2* and *Sox-3* was assessed in whole mounts. Numbers show embryos in which the expression above the experimental graft was reduced when compared to the control graft on the contralateral side of the same embryo. Numbers in brackets represent the total number of embryos analysed; n.d.= not determined.

DISCUSSION

The present results suggest that the initiation and maintenance of the primitive streak requires the suppression of BMP

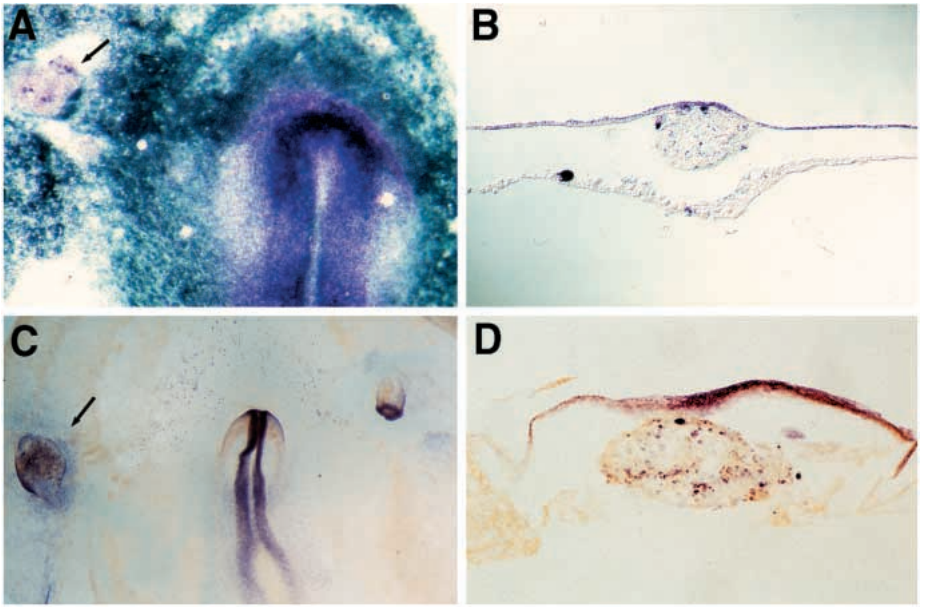

Fig. 7. Chordin acts downstream of signals from the organizer. Two stage 3$^+$ quail Hensen's nodes were grafted into the area opaca of a stage 3$^+$ chick host. After 3 hours (A,B) or 5 hours (C,D), the node on the left (arrow) was removed and replaced with a graft of chordin-expressing cells. Expression of *Sox-3* appears in blue, anti-quail antibody (QCPN) in brown. (A) After 3 hours' exposure to the node followed by chordin-expressing cells, a low level of Sox-3 expression is observed (arrow). (B) A section through the graft: the epiblast overlying the cells has the squamous morphology of non-neural epiblast and expresses *Sox-3*. (C) After 5 hours' exposure to the node before grafting chordin-secreting cells, strong ectopic *Sox-3* expression is seen. On the right, the node was allowed to remain, serving as a positive control. (D) The epiblast above the chordin-transfected cells has developed the columnar morphology of a neural plate.

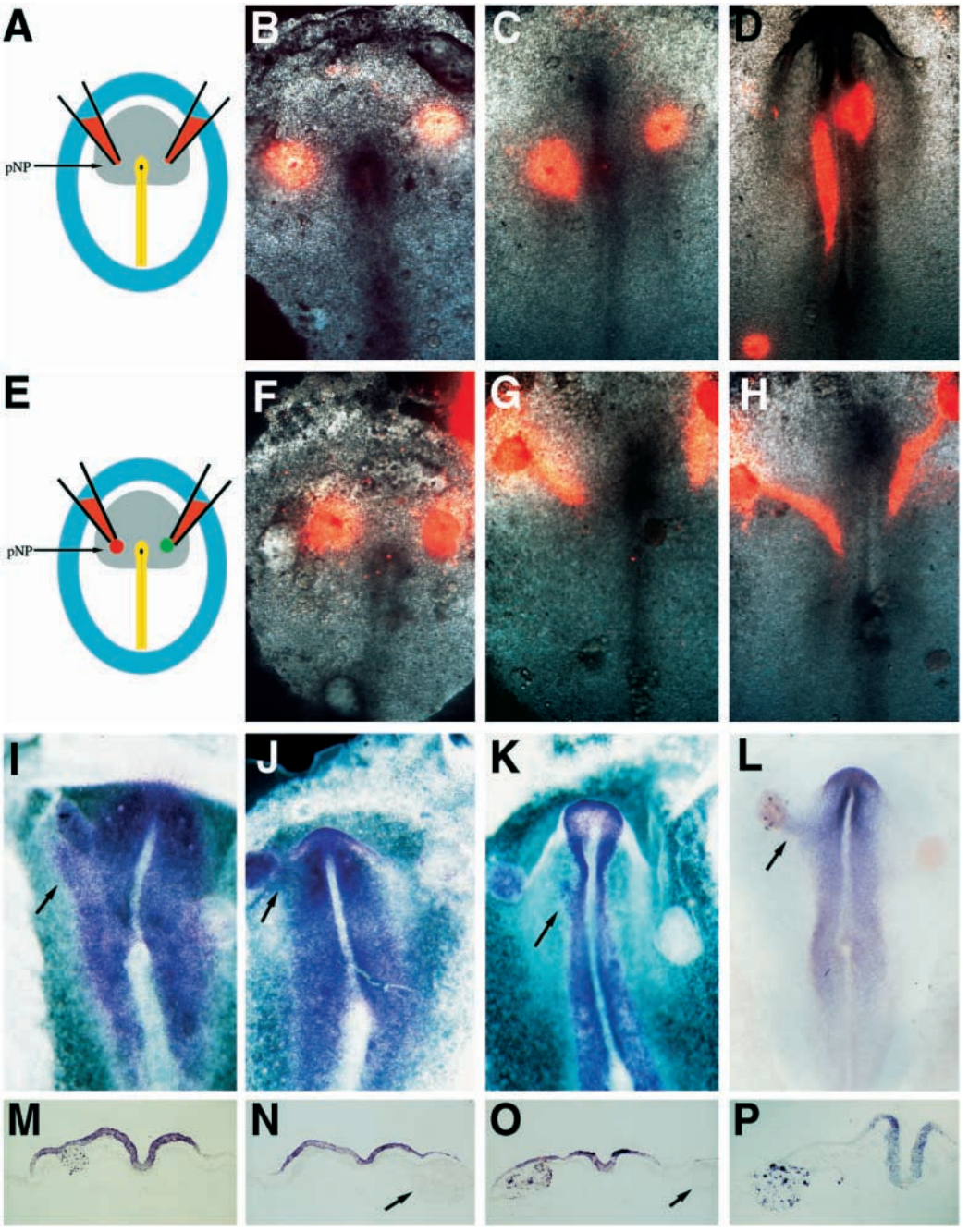

Fig. 8. Chordin maintains the neural state of prospective neural plate cells. (A) Diagram showing the site of DiI labelling (orange) in the presumptive mid/hindbrain region of a stage 3^{+}-4 chick embryo. (B-D) Control embryo, shown immediately after labelling (B), after 9 hours (C) and after 18 hours (D). The labelled cells have become incorporated into the appropriate regions of the neural tube. (E) Diagram: a spot of DiI was placed on each side of the embryo as above, and chordin- (red) and mock- (green) transfected COS cells grafted onto the labelled epiblast cells. The embryo is shown immediately after the operation (F), after 9 hours (G) and after 18 hours (H). A trail of labelled cells connects the forming neural tube of the host with the site of grafting and labelled epiblast cells in contact with the pellet are excluded from the neural tube. An expansion of the neural plate is seen by in situ hybridisation with *Sox-3* (I-K,M,O,P) and *Sox-2* (L). (I) After 10 hours, the ectopic *Sox-3* expression is continuous (arrow) with the host neural plate. In section, the epiblast above the chordin graft (M) has columnar morphology and expresses *Sox-3*, whereas epiblast above the mock-transfected control (N, arrow) is squamous. (J) By 14 hours, the expression of *Sox-3* near the graft and that in the host neural plate have started to separate (arrow); shown in section in O. Note the absence of expression and the squamous morphology of the epiblast over the control pellet (arrow). (K) After 20 hours, only a small remnant of the lateral expansion of the host neural plate remains (arrow). (P) The epiblast above the graft does not differ from non-neural ectoderm. (L) Embryo 14 hours after grafting chordin- and mock-transfected COS cells. A lateral expansion of the *Sox-2* domain can be seen (arrow).

signalling. Our results also suggest that chordin is not sufficient for neural induction in the chick, but can maintain the expression of early neural markers in cells that have previously been exposed to neural inducing signals. Below we discuss these findings in the context of current ideas on the mechanism of gastrulation and neurulation in different vertebrate organisms.

Chordin and BMPs can control primitive streak development

The misexpression of chordin at the anterior edge of the area pellucida induces the formation of an ectopic primitive streak. Of many factors investigated, only cVg1 (Seleiro et al., 1996; Shah et al., 1997) and activin (Cooke et al., 1994) can initiate the formation of an ectopic streak 180° from the normal site of streak formation. Whereas cVg1 must be applied to the marginal zone to induce an ectopic axis (Shah et al., 1997), chordin can initiate primitive streak formation only when applied to the area pellucida. In addition, unlike cVg1 (Shah et al., 1997) and the marginal zone itself (Eyal-Giladi and Khaner, 1989; Khaner and Eyal-Giladi, 1989), both of which lose the ability to initiate axis formation as the primitive streak of the host begins to form, chordin continues to be able to

generate an ectopic streak as late as the mid-primitive streak stage. Together with the findings that *chordin* is expressed close to Koller's sickle before primitive streak formation and that BMP-4 can inhibit formation of the normal streak, our results suggest that chordin is an endogenous mediator of primitive streak formation in the chick.

In the pre-primitive streak embryo, *chordin* is expressed in two separate populations of cells destined to contribute to the organizer. At stages X-XI, the bulk of the cells that will make up Hensen's node lie in the epiblast of the posterior midline and then move to the centre of the blastoderm, where they remain until they are met by the tip of the extending primitive streak at about stage 3 (Hatada and Stern, 1994). A second cell population that contributes to the organizer is located in the middle layer adjacent to Koller's sickle between stages XI and XIV, and is characterised by their expression of *goosecoid* (Izpisúa-Belmonte et al., 1993), *Otx-2* (Bally-Cuif et al., 1995) and *HNF-3β* (Ruiz i Altaba et al., 1995). These cells appear to move anteriorly within the middle layer during the formation of the primitive streak and contribute primarily to the endoderm (Izpisúa-Belmonte et al., 1993). *chordin* is expressed in both populations, as well as in the structure to which they give rise, Hensen's node. Like chordin, the middle layer cells can induce an ectopic primitive streak (Izpisúa-Belmonte et al., 1993; Callebaut and Van Nueten, 1994) that includes a node expressing organizer markers. Also like chordin, this ability extends to primitive streak stages. However, nothing is known about the inducing properties of the epiblast anterior to Koller's sickle – our results suggest that they too may be able to initiate primitive streak formation.

Which of the steps of primitive streak formation involves the action of chordin? In the first step, a middle layer of cells begins to form at around stage XI and is progressively joined by more ingressing cells from the epiblast, which coalesce to form the early primitive streak – a thickened rod of mesenchymal cells that starts to form posteriorly and gradually expands anteriorly along the midline of the blastoderm (see Stern, 1991). Until stage 3, the body of the primitive streak is restricted to this middle layer and there is no obvious morphological difference between the overlying epiblast and that of more lateral, non-streak regions (Vakaet, 1984). In the second step at stage 3^+, the epiblast above the mesenchymal streak develops a groove and involution of epiblast into the streak begins (Vakaet, 1984; Stern, 1991). A graft of any part of the primitive streak from a stage 3 donor (including the posterior end) can induce the formation of an ectopic streak (Vakaet, 1973). It was proposed that the mesenchyme of the early streak induces a 'secondary primitive streak' in the overlying epiblast, characterised by the appearance of a groove and culminating in the formation of a morphological Hensen's node (Stern, 1991). Since *chordin* is expressed in the middle layer, can induce an ectopic streak and organizer at stage 3, and can only act on cells within the area pellucida, it seems likely that this molecule is involved in the induction of the secondary primitive streak. Chordin may therefore lie downstream of cVg1 in the chain of molecular events accompanying primitive streak development. This conclusion is further strengthened by the finding that cVg1 protein can induce *chordin* expression in epiblast explants in vitro (Shah et al., 1997).

In *Xenopus* and *Drosophila*, chordin and sog exert their effects through antagonism of BMP-4 and dpp (Holley et al., 1995; Sasai et al., 1995; Piccolo et al., 1996; Biehs et al., 1996; François et al., 1994; Yu et al., 1996). This action of chordin can be mimicked by other BMP antagonists, such as noggin (Smith and Harland, 1992; Smith et al., 1993; Lamb et al., 1993; Re'em-Kalma et al., 1995; Holley et al., 1996; Jones et al., 1996; Zimmerman et al., 1996) and follistatin (Hemmati-Brivanlou et al., 1994; Fainsod et al., 1997). Consistent with this, we find that ectopic expression of BMP-4 adjacent to the site of primitive streak initiation and *chordin* expression, prevents the formation of a primitive streak. In addition, when BMP-4 is misexpressed adjacent to the node in a later (stage 3^+) embryo, axial development is arrested and embryos appear to have a reduced or absent node and lack structures normally generated by the organizer.

The distinct actions of BMP-4 at these two stages suggests that two different processes are being inhibited by BMPs: at early stages, BMP-4 interferes with the development of the primitive streak. Since chordin promotes primitive streak formation, and both chordin and BMP-4 are expressed in adjacent domains before the primitive streak is initiated, the interaction between chordin and BMPs is likely to play a role during normal primitive streak development in the chick. At later stages (stage 3^+), BMPs appear to interfere with the further development of the node, a step that may be more analogous to the dorsoventral patterning of mesoderm described in *Xenopus* (e.g. Dale et al., 1992; Jones et al., 1992, 1996; Fainsod et al., 1994; Schmidt et al., 1995; Steinbeisser et al., 1995). Consistent with this idea, both BMPs are expressed in the **posterior** portion of the primitive streak at this stage, which generates only 'ventral', i.e. extraembryonic and lateral, mesoderm.

Chordin is not sufficient for neural induction

Our results provide evidence that chordin is not sufficient to elicit neural induction in regions of the chick epiblast that are competent to be induced by the organizer, and that BMP-4 and BMP-7 do not inhibit neural induction by the organizer. These conclusions are based on the following results:

(1) neither *BMP-4* nor *BMP-7* is expressed in the prospective neural plate at the stages when neural induction takes place;

(2) *chordin* continues to be expressed in Hensen's node at stages after this structure has lost its neural inducing activity;

(3) chordin does not mimic a graft of the organizer either in the inner margin of the area opaca or in the non-neural epiblast of the area pellucida – it does not elicit the formation of a neural plate or the expression of neural markers in either region;

(4) misexpression of BMP-4 or BMP-7 within the prospective neural plate region at any stage of development (even before primitive streak formation) does not prevent neural induction.

The observations that noggin (Connolly et al., 1997) and follistatin (Connolly et al., 1995), two other inhibitors of BMP activity (Zimmerman et al., 1996; Holley et al., 1996; Fainsod et al., 1997), are also expressed in tissues lacking neural inducing activity also support these conclusions. Our results, however, do not rule out the possibility that the chick contains BMP family members other than BMP-4 or BMP-7, which are not inhibited by chordin but are sensitive to other BMP antagonists, or a combination of such molecules produced by the organizer. For example, the combination of noggin, chordin

and follistatin could be required to inhibit the full spectrum of avian BMP activities. However, this possibility does not easily account for the finding that both BMP-4 and BMP-7 are unable to prevent neural induction by the organizer in the normal embryo. A similar conclusion is emerging from studies of zebrafish mutants for the homologs of BMPs and their antagonists (Hammerschmidt et al., 1996). In the chordin mutation, *chordino* (Schulte Merker et al., 1997), the neural plate is reduced but not absent, whereas in *swirl* (BMP-2 mutation; Kishimoto et al., 1997) the neural plate is expanded but does not completely replace the epidermis. Moreover, *chordino* × *swirl* double-mutants display a phenotype identical to that of *swirl*, suggesting that no additional BMPs are present that overlap functionally with *swirl* (Hammerschmidt et al., 1996).

We find that chordin does not mimic the induction of neural tissue by Hensen's node, and that BMPs do not block neural induction. These observations suggest that neural induction by the organizer in the chick is not explicable merely as the release from negative, epidermal-inducing signals mediated by BMPs. Such a mechanism has been proposed in *Xenopus* (Hemmati-Brivanlou and Melton, 1997; Sasai and De Robertis, 1997; Wilson and Hemmati-Brivanlou, 1995). This conclusion is supported by the finding that dissociation of early chick epiblast explants at a variety of stages does not elicit neural differentiation, but rather causes an increase in the number of cells that differentiate into skeletal muscle (George-Weinstein et al., 1996; A. S. and C. D. S., unpublished observations).

Our results suggest instead that chordin acts **downstream** of, or in conjunction with, other neuralising factors secreted by the node. When a node is transplanted to the area opaca of a host embryo and removed after 5 hours, followed by a graft of chordin-secreting cells, ectopic *Sox-3* expression is induced in the host and a neural-plate-like epithelial morphology results. Furthermore, when COS cells (either control or chordin-transfected) are implanted into the prospective neural plate of stage 3^{+}-4 embryos, they adhere so well to the host epiblast that they prevent it from joining the host neural plate. When these cells secrete chordin, *Sox-2* and *Sox-3* are ectopically expressed, albeit transiently, adjacent to the grafted cells. Both findings suggest that chordin can either strengthen, stabilise or maintain the induced state of cells that have recently received neuralising signals.

Comparison with *Xenopus* and *Drosophila*

Our results provide evidence that BMPs and chordin are involved during early axial development of the chick, consistent with the conclusions reached in *Xenopus* (Dale et al., 1992; Sasai et al., 1994; Jones et al., 1996). However, unlike in *Xenopus* (Sasai et al., 1995; Piccolo et al., 1996; Wilson and Hemmati-Brivanlou, 1995; Hawley et al., 1995), we find no evidence for a neuralisation of non-neural epiblast by chordin or for an inhibition of neural induction by BMP-4 or BMP-7. Rather, we show that the chordin/BMP system appears to act either downstream of, or in conjunction with, other factors produced by the organizer. Similar conclusions are reached in *Drosophila*, where the *sog* mutant phenotype in the nervous system is detectable only during mid-gastrulation (François et al., 1994; Biehs et al., 1996). Thus, *sog* mutants have ventral neural progenitors despite some reduction in the size of the domains of *rhomboid, lethal of scute* and *thick veins*, which has led to the proposal that the major role of *sog* in the nervous system is to stabilise or maintain a subdivision of the primary ectoderm into neural and non-neural territories, established previously by other signals (Biehs et al., 1996).

The chordin/BMP (dpp/sog) antagonistic system of signalling molecules has been tightly conserved in evolution (for review see, DeRobertis and Sasai, 1996; Ferguson, 1996; Holley and Ferguson, 1997). In *Drosophila*, like in *Xenopus* and chick, it is used many times during development and one function is to control dorsoventral polarity of the embryo. It seems unlikely therefore that *Xenopus* has recruited this system for a new function (neural induction). We believe that a more likely explanation for these differences concerns the assays used for neural induction in *Xenopus* and chick. For example, animal caps excised from chordin-injected (and therefore dorsalised) embryos may contain part of an expanded prospective neural plate which arises from an increase in the size of the organizer. In addition, it is interesting to note that tissue dissociation is only effective as a means of eliciting neural differentiation in *Xenopus* during a brief time period (Knecht and Harland, 1997), indicating that mechanisms other than release from negative signals might be involved.

In the chick, a large area of extraembryonic epiblast (the area opaca) is competent to respond to signals from the organizer by generating a complete, patterned nervous system. While it might be argued that signals in addition to neural inducing molecules may be required in this extraembryonic region first to convert it into 'embryonic' epiblast and then to neuralise it, chordin fails to produce ectopic neural structures even in the **embryonic** non-neural epiblast (overlying the germinal crescent) at any stage in development unless an ectopic primitive streak is generated first. These results suggest that chordin can only elicit the formation of an ectopic neural plate through prior induction of tissue with organizer properties, or in conjunction with other neural inducing signals from the organizer.

Conclusion

When considered together, our experiments with BMPs and chordin provide further evidence in support of the idea that both the initiation of primitive streak formation and neural induction comprise a series of experimentally separable events. In the case of primitive streak formation, these two classes of factors appear to be involved at two distinct steps: in initiation of the axis and later in the maintenance of the organizer. During early neural development, the initial induction of the neural plate can be distinguished from its maintenance, and chordin only seems to play a direct role in the latter process.

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