INTRODUCTION

A variety of embryological experiments has led to a model to explain how the vertebrate neural tube is patterned along its dorsal-ventral axis. The fate of cells is thought to be determined via an interplay of signals that exert either a ventralizing or dorsalizing influence on the neurepithelium. The structures that differentiate ventrally (floor plate and motor neurons) do so in response to a signal from the underlying notochord: removal of the notochord results in a failure of these cells to differentiate (Smith and Schoenwolf, 1989; Clarke et al., 1991; Hirano et al., 1991; Yamada et al., 1991), while grafting a notochord adjacent to the lateral or dorsal neural tube causes ectopic formation of a floor plate and motor neurons (van Straaten et al., 1988; Placzek et al., 1990; Yamada et al., 1991), as does in vitro recombination of notochord with naive neural plate cells (Placzek et al., 1993; Yamada et al., 1993). The discovery that Sonic hedgehog (Shh), a vertebrate homologue of the Drosophila segment polarity gene hedgehog, encodes a secreted molecule expressed in the notochord and floor plate (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994) led to an investigation of the role of Shh in this process. Shh can substitute for the notochord in induction assays in vitro (Roelink et al., 1994) and ectopic expression of Shh causes activation of floor plate markers in inappropriate regions of the neurectoderm (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994). These experiments provide good evidence that Shh mediates the endogenous, notochord-derived, floor plate-inducing signal (for reviews see Ingham, 1995; Placzek, 1995).

As well as positively regulating certain genes that are normally expressed in the ventral region of the neural tube, the notochord can repress the expression of some genes which are normally restricted to the lateral or dorsal aspect of the neural tube (Darnell et al., 1992; Basler et al., 1993; Goulding et al., 1993; Liem et al., 1995) and again Shh reproduces this effect (Liem et al., 1995). While this provides evidence that signals from the notochord may be able to regulate gene expression at all dorsal-ventral levels, the realisation that grafting a notochord adjacent to the dorsal neural tube does not prevent the differentiation of dorsal cell types (neural crest cells and commissural neurons) led to the suggestion that a positive dorsalizing signal may also exist (Artinger and Bronner-Fraser, 1992). There is now evidence that during embryonic development such a signal is provided by the surface ectoderm, which abuts the dorsal neurectoderm: juxtaposition of these two tissues is sufficient to produce neural crest cells either in grafting experiments (Moury and Jacobson, 1989, 1990; Selleck and Bronner-Fraser, 1995) or in recombination assays (Dickinson et al., 1995; Liem et al., 1995). In recombination

SUMMARY

The expression pattern of bone morphogenetic protein-7 (BMP-7) in the hindbrain region of the headfold and early somite stage developing mouse embryo suggests a role for BMP-7 in the patterning of this part of the cranial CNS. In chick embryos it is thought that BMP-7 is one of the secreted molecules which mediates the dorsalizing influence of surface ectoderm on the neural tube, and mouse surface ectoderm has been shown to have a similar dorsalizing effect. While we confirm that BMP-7 is expressed in the surface ectoderm of mouse embryos at the appropriate time to dorsalize the neural tube, we also show that at early stages of hindbrain development BMP-7 transcripts are present in paraxial and ventral tissues, within and surrounding the hindbrain neurectoderm, and only later does expression become restricted to a dorsal domain. To determine more directly the effect that BMP-7 may have on the developing hindbrain we have grafted COS cell grafts

Key words: hindbrain, mouse embryo, BMP-7, dorsoventral pattern, COS cell grafts
assays members of the TGF-β superfamily of secreted proteins can mimic the effect of the surface ectoderm (Basler et al., 1993; Liem et al., 1995) and it is proposed that in vivo these dorsally located proteins counteract a long-range ventralizing influence of the notochord (Liem et al., 1995).

Few of the experiments that have led to this model have been performed in the mouse embryo, although ectopic expression of Shh in mouse embryos (Echelard et al., 1993) and analysis of mutant mouse embryos in which additional neurectoderm comes in contact with the surface ectoderm (Takada et al., 1994) suggest that the tissue interactions identified in other vertebrates also pattern the mammalian neural plate. Here we report that the TGF-β superfamily molecule, BMP-7, is expressed in the surface ectoderm of the developing mouse embryo from the late gastrulation stage of development. Expression of BMP-7 adjacent to the dorsal aspect of the neural tube is initiated some 12 hours before neural crest emigration (5-somite stage; Serbedzija et al., 1992), which provides the first overt sign of dorsal differentiation. Thus BMP-7 is expressed in the temporospatial manner expected of a dorsal signalling molecule. Paradoxically, at the same stage of development, BMP-7 transcripts in the developing hindbrain are found in all of the paraxial mesoderm, the axial mesendoderm and the midline neurectoderm itself. Subsequently, all but the surface ectoderm expression of BMP-7 recedes from the hindbrain region. To assess whether BMP-7 participates in patterning the hindbrain, COS cells were used to extend the duration of this ventrolateral BMP-7 expression. Such ectopic expression interferes with endogenous ventral signals and elicits expression of a subset of dorsally restricted genes in ventrolateral neurectoderm. Additionally, BMP-7 causes expansion of the neurectoderm due to a localised increase in cell number.

MATERIALS AND METHODS

cDNA clones

A full-length BMP-7 cDNA was isolated from a 7.5 dpc mouse primitive streak library (Harrison et al., 1995) via low stringency hybridisation to a probe corresponding to the inhibin βa mature region (Albano et al., 1993). An antisense riboprobe was generated to the full-length CDNA by Sall digest of the library clone (pSport vector) and transcription with SP6 polymerase. Mxs1 and Pax-3 riboprobes were as described in Rashbass et al. (1994). The Shh riboprobe was provided by A. McMahon (Echelard et al., 1993) and the AP-2 riboprobe was provided by P. Mitchell and corresponds to amino acids 275-434 of the AP-2 protein. To generate the AP-2 antisense riboprobe, pBH500 was linearised with BamHI and transcribed by T7 RNA polymerase.

Embryo recovery and culture

Embryos were collected from C57BL/6 x DBA matings maintained on a light cycle of 14 hours light:10 hours dark, the midpoint of the dark period being 12.00 am. Noon on the day of appearance of the vaginal plug is considered 0.5 dpc. All embryos were dissected in M2 medium (Hogan et al., 1994) plus 10% fetal calf serum. Embryos for whole-mount in situ hybridisation were dissected from maternal tissue and Reichert’s membrane removed. The amnion was removed from 8.5 and 9.5 dpc embryos and holes made in regions likely to trap probe. Embryos were rinsed in PBS and transferred to 4% paraformaldehyde in PBS. Embryos for culture were dissected from maternal tissue and Reichert’s membrane removed. Only those in which the ectoplacental cone and yolk sac were fully intact were used for culture. All embryos were cultured using the conditions described by Beddington (1987).

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was carried out according to Wilkinson (1992), using the hybridisation conditions of Rosen and Beddington (1993). The length of proteinase K treatment varied depending on the stage of the embryos: 6.5 dpc embryos were incubated for 5 minutes, 7.0-8.5 dpc embryos for 10 minutes and 9.0 dpc and older embryos for 15 minutes. After completion of the in situ procedure, embryos were destained in PBT (PBS with 0.1% Tween 20: polyoxyethylene sorbitan monolaurate; Sigma) for 48 hours and post-fixed in 4% paraformaldehyde, 0.1% glutaraldehyde in PBS for 1 hour at room temperature. At this stage some embryos were placed in a drop of Aquamount (BDH) on a glass microscope slide and the surface ectoderm, endoderm and paraxial and lateral mesoderm removed with tungsten needles using the 40x objective of a dissecting microscope. The neurectoderm was bisected along the dorsal aspect of the neural tube and flattened under a glass coverslip.

Sectioning of embryos

Embryos were processed for paraffin sectioning by dehydration through an ethanol series, clearing in Histoclear (National Diagnostics) and embedding in paraffin wax (Histoplast, melting point 56°C). 7 μm sections were cut using a Bright microtome. Sections were dewaxed in Histoclear and mounted under coverslips in DPX mountant (BDH). Embryos were prepared for cryosectioning by equilibration in OCT cryoembedding solution (BDH) for 15 minutes at room temperature. The specimens were then frozen in fresh OCT and stored at −70°C. 10 μm frozen sections were cut using a Bright cryostat. Sections were mounted under coverslips in Mowial (Heimer and Taylor, 1974), except those cut from fluorescent specimens, which were mounted in glycerol: 4% PFA in PBS (1:1).

COS cell culture

COS cells were maintained in culture by incubation at 37°C in 5% CO2 and humidified air. The cells were grown on plasticware (COSTAR) in DMEM (HyClone), supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were detached from the plastic surface with 0.25% trypsin, 0.5% EDTA in PBS. COS cells for transfection were grown to 70% confluency in a 75 cm2 flask, then trypsinized and washed with PBS. Cells were resuspended in 500 μl of PBS and 5 μg of plasmid DNA (human BMP-7 in pcDNA1/Amp, provided by A. Furley and M. Jones) was added to the cells and incubated for 10 minutes at 4°C. The cells were electroshocked in a 0.5 cm chamber (Bio-Rad) using a Bio-Rad gene pulser under the following conditions: 0.45 kV, 250 μF. Cells were incubated at 4°C for another 10 minutes before plating on a 75 cm2 flask. Fresh COS cell medium was added 24 hours after the transfection. The transfection efficiency was estimated to be 25%, based on parallel, control transfections introducing TAG-1 (Furley et al., 1990) in the same expression vector and detection of TAG-1 protein with monoclonal antibody 4D7 (Dodd et al., 1988; both pcTAG-1 and mAb 4D7 were provided by A. Furley).

COS cell grafts

In independent assays, conditioned medium removed from COS cells transfected with BMP-7 or other expression constructs, is most active 72 hours after transfection (M. Placzek, personal communication). Therefore, 48 hours after transfection, cells from a 75 cm2 flask were detached using enzyme-free cell dissociation buffer (GIBCO BRL) and collected by centrifugation. The cells were resuspended in 600 μl of COS cell medium and placed in 10 μl hanging drops. The drops were incubated at 37°C in 6% CO2 and humidified air for 2 hours, or until they had formed an adherent sheet of cells. A sheet of COS cells was labelled with the fluorescent dye DiI (1,1'-dioctadecyl-3,3',3'
tetramethyl indocarboxyline perchlorate; Molecular Probes) by placing it in 10 μl of a 0.3 M sucrose solution containing 0.05% DiI in ethanol (Serbedzija et al., 1990) for 30 seconds. The labelled COS cells were transferred to M2 medium to remove excess DiI. They were again transferred to fresh M2 medium and broken into clumps of a suitable size for grafting by pipetting up and down with a hand-pulled, glass pipette of internal diameter 50-100 μm. A glass micropipette of internal diameter 30-40 μm, made as described in Beddington (1987), was used to graft COS cells. Recipient embryos were gently held by the ectoplacental cone using a pair of watchmakers’ forceps. The pipette containing COS cells was placed into the mesoderm adjacent to the prospective hindbrain neurectoderm on the left side of the embryo. The COS cells were expelled into the mesoderm by expiration. Typically 10-20 clumps, of 2-5 COS cells each, were expelled into the embryo.

Analysis of grafted embryos

At the end of the culture period embryos were dissected from the yolk sac and amnion and fixed in 4% PFA in PBS, overnight at 4°C. Embryos were placed under a coverslip in a shallow cavity slide (Fisons) and the location of the COS cells was visualised with rhodamine filter epifluorescence optics (Zeiss filter set 48 79 15) in a compound microscope (Axioskop, Zeiss), and recorded on film. Those embryos found to have COS cells in the mesoderm adjacent to the hindbrain neurectoderm were numbered and stored individually in 4% PFA in PBS at 4°C in the dark for further analysis. The fluorescent label used to mark the COS cells is unstable through in situ hybridisation, therefore after photographically recording the location of the COS cells the embryos were processed individually.

Calculation of relative mitotic frequency

Measurement of mitotic rate using BrdU labelling is hindered by the extremely inefficient uptake of BrdU during culture of intact mammalian embryos (Copp, 1990). Instead the nuclear stain DAPI was used to score the proportion of neurectoderm cells in mitosis. Paraffin sections (7 μm) were prepared from embryos. The sections were dewaxed in Histoclear and rehydrated through an ethanol series and washed twice in PBS. The sections were incubated in 2 μg ml⁻¹ DAPI (4’,6-diamidin-2-phenylindol-dihydrochloride; Boehringer Mannheim) in PBS for 5 minutes and washed twice in PBS (15 minutes each), before mounting under coverslips in Vectashield (Vector Laboratories). The sections were examined with epifluorescence optics as described above and scored using a 40× objective. The cell density was estimated by counting nuclei in a 2000 μm² area. The number of mitotic cells in the left and right sides of the neurectoderm in a given section was counted. An image of each scored section was captured onto computer (using a video camera (SONY) and Impprovision software), and used to determine the area of the neurectoderm. The relative mitotic frequency was calculated as the number of mitotic cells per volume of neurectoderm.

Photography

Low power (up to 7.5×) bright field and dark field photographs were taken in a dissecting microscope (Nikon) using tungsten film (Kodak 64T). Higher power (10-40×) photographs were taken in a compound microscope (Zeiss Axioskop) using tungsten film for bright field and Normaski photographs, or Kodak P1600 film for fluorescence and fluorescence/Normaski double exposures.

RESULTS

BMP-7 mRNA localisation during neural development

Whole-mount in situ hybridisation, followed by sectioning or dissection of the embryos, was used to examine the expression pattern of BMP-7. This analysis revealed that during germ layer formation BMP-7 transcripts are confined to mesoderm tissues, while during axis elongation and early organogenesis transcripts are found in derivatives of all three germ layers. These results confirm and extend the sectioned analysis of BMP-7 expression reported by Lyons et al. (1995). Here we focus on the subset of BMP-7 expression, which may be relevant to patterning of the neuroectoderm: expression in the node, notochord, mesoderm underlyings the developing neural folds, and in the neurctoderm and surface ectoderm.

BMP-7 mRNA localisation in mesoderm

As shown in Fig. 1, shortly after the onset of gastrulation (early streak stage; Downs and Davies, 1993) BMP-7 transcripts are detected at the anterior of the primitive streak. By the late streak stage BMP-7 mRNA is localised to the rostral third of the primitive streak, the node and to the axial mesoderm emanating from the node. As the allantoic bud forms, mRNA accumulates within the allantoids, and cells in the lateral wings of mesoderm in the distal two-thirds of the embryonic region show hybridisation signal. By the early headfold stage it is apparent that the non-axial mesoderm, which contains BMP-7 transcripts, corresponds to the prospective heart and cranial paraxial mesoderm (Fig. 1D). No further expression of BMP-7 is seen in the primitive streak and transcripts never accumulate in the trunk paraxial mesoderm. At this early headfold stage, BMP-7 is still detected in the node and in the emerging notochord. While expression in the most anterior and posterior (node) axial mesoderm persists throughout all stages examined (up to the 18-somite stage), expression in most of the intervening notochord declines as embryogenesis progresses.

In transverse sections of a 6-somite embryo, BMP-7 expression is only detected in sections up to 105 μm behind the rostral extent of the axial mesoderm and, in a 12-somite embryo, in sections up to 140 μm behind the rostral extent of the axial mesoderm. Expression in this region of the axial mesoderm is illustrated in transverse section (Fig. 1H) and in wholemount (Fig. 1K). During the early stages of somite formation the expression of BMP-7 in the cranial paraxial mesoderm declines and by the 6-somite stage BMP-7 transcripts are no longer seen in the mesoderm adjacent to the hindbrain region.

BMP-7 mRNA localisation in ectoderm

In embryos with a foregut pocket but no somites, BMP-7 mRNA is detected not only in mesoderm but also in endoderm and ectoderm. As the foregut and hindgut pockets form, mRNA becomes distributed throughout all of the gut endoderm and this expression persists through all stages examined. Sections of pre-somite stage embryos showed that expression is detected in both types of differentiated ectoderm but not in the undifferentiated ectoderm over the primitive streak. BMP-7 mRNA is localised to the ventral neuroectoderm in a region which does not extend the full anterior-posterior length of the neural groove. In dissected neural plates transcripts are detected in the ventral neuroectoderm of the cranial region but not at appreciable levels in ventral trunk neurctoderm. In 6-somite embryos transcripts are found in the midline of the neural plate in two regions (Fig. 1J): a region of higher signal extends from the anterior limit of the midbrain to the preotic sulcus, which corresponds to the future posterior limit of rhombomere 2 (Trainor and Tam, 1995); a lower level of signal extends from the preotic sulcus to the otic sulcus, which cor-
responds to the future posterior limit of rhombomere 4 (Trainor and Tam, 1995). In embryos with 10 somites (Fig. 1J) BMP-7 mRNA is only detected in the ventral midline of the midbrain but, in a 16-somite embryo, transcripts are found ventrally in the rostral diencephalon. This expression is transient; by the 18-somite stage the diencephalic expression disappears and ventral transcription is again seen only in the midbrain.

At the late headfold stage of development, transcripts accumulate in the surface ectoderm of the anterior (Fig. 1G) and posterior regions of the embryo. At this stage, the BMP-7 mRNA is not detected in the dorsal neurectoderm. Strong expression of BMP-7 throughout the surface ectoderm is evident at all stages examined. After neural tube closure expression commences in the dorsalmost neural tissue: sections of the anterior region of a 14-somite embryo revealing hybridisation to the prospective roof plate of the neural tube. This expression progresses caudally so that in embryos with 30 somites, BMP-7 mRNA is localised to the roof plate along the entire antero-posterior length of the neural tube.

**Experimental strategy for misexpression of BMP-7 in the 8.5 dpc mouse hindbrain**

Whole-mount in situ hybridisation revealed that during the initial stages of cranial neural development BMP-7 mRNA is transiently present in the ventral midline of the neurectoderm, and in mesoderm underlying the lateral neurectoderm. Only as neurulation proceeds does expression of BMP-7 occur exclusively in the dorsal aspect of the neurectoderm and adjoining

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**Fig. 1.** BMP-7 mRNA localisation. In all wholemount photographs the anterior of the embryo is to the left. (A) Lateral view of an early streak stage embryo: BMP-7 transcripts are at the anterior of the primitive streak. (B) Lateral view of a late streak embryo. Expression continues in the anterior of the primitive streak and transcripts are now in the anterior axial mesoderm and head process and in the extraembryonic mesoderm. (C) Lateral view of an early allantoic bud stage embryo. BMP-7 expression in the primitive streak has declined but transcripts are now detected in the lateral mesoderm of the distal two thirds of the embryonic region. The highest transcript levels are seen in the node and notochord. The expression in the extraembryonic mesoderm is now clearly seen in the allantois. The plane of the section shown in F is marked. (D) Lateral view of an early headfold embryo. BMP-7 transcripts are absent from the primitive streak, but the expression in node and notochord persists. Transcripts are seen in the anterior paraxial and lateral mesoderm and in the allantois. (E) Lateral view of two headfold, presomite embryos. In this view expression can be seen in the cranial paraxial mesoderm and heart mesoderm, the node and notochord and the allantois. The plane of the section shown in (G) is marked on the right embryo. (F) Transverse section through the distal embryonic region of an early allantoic bud stage embryo, as shown in C. Transcripts can be seen in both the lateral and axial mesoderm. (G) Transverse section through the embryo shown in E. As well as the mesodermal expression seen clearly in the wholemount embryos, BMP-7 transcripts are seen in the ventral neurectoderm (arrow) and the surface ectoderm (arrowhead). (H) Section through the forebrain and hindbrain of a 14-somite embryo showing transcripts in the most rostral axial mesoderm. (I) Lateral view of anterior neurectoderm isolated from a 6-somite embryo. The ventral expression of BMP-7 is most obvious in neurectoderm extending from the anterior of the midbrain to the preotic sulcus and is present up to the otic sulcus. (J) Lateral view of anterior neurectoderm isolated from a 10-somite embryo (some mesoderm remains around the forebrain region). The ventral neurectoderm expression is now essentially confined to the midbrain. (K) Lateral view of anterior neurectoderm isolated from a 16-somite embryo (some mesoderm remains around the telencephalon). The ventral neurectoderm of the midbrain and rostral diencephalon contains BMP-7 transcripts as does the dorsalmost neurectoderm in the anterior cranial region, am, axial mesoderm; ps, preotic sulcus; o, otic sulcus; p, primitive streak. Scale bar, 400 μm (E); 300 μm (A,B,C,D, I,J K); 100 μm (H); 75 μm (F,G).
5BMP-7 and hindbrain patterning

surface ectoderm. In order to prolong ventrolateral production of BMP-7 in the developing hindbrain, transiently transfected COS cells were implanted ventrolaterally in this region and recipient embryos allowed to continue development in vitro. Details of the experimental strategy are depicted in Fig. 2. The location of introduced COS cells was identified by virtue of labelling them with the lipophillic fluorescent dye, DiI. The efficacy of labelling and viability of labelled COS cells was assessed by growing labelled cells on a tissue culture surface for 24 hours using the same culture medium as for embryos. Examination of these cultured cells under phase contrast and fluorescent optics showed that the cells were living and that all were fluorescently labelled, demonstrating that the labelling procedure was both efficient and non-toxic.

In all of the experiments reported here COS cells were grafted into the cranial mesoderm of embryos with 3- to 8-somites. Since the region between the preotic and the otic sulcus corresponds to the future location of rhombomeres 3 and 4 (Trainor and Tam, 1995), these two indentations were used as a guide to ensure that grafts were placed in the vicinity of prospective hindbrain. Six embryos which had received grafts were fixed immediately and the location of the cells determined. As shown in Fig. 3, the COS cells could be reliably placed into the mesoderm adjacent to the neurectoderm of the hindbrain.

**Morphology of the grafted embryos**

In all experiments some embryos received grafts of COS cells that had been transfected with BMP-7 while others received nontransfected COS cells as control grafts. After 24 hours in vitro experimental and control embryos were dissected, fixed and examined for morphological abnormalities. In total, nontransfected COS cells were grafted into 51 embryos, which had between 3- and 8-somites at the time of grafting. These control embryos developed normally in culture as judged by axis elongation, somite number and the presence of a beating heart and other organ rudiments. None of the grafted embryos showed gross morphological abnormalities in the cranial region when viewed from either the lateral or dorsal aspect. When these embryos were viewed with fluorescent optics 38 were found to contain COS cells adjacent to the hindbrain neurectoderm. Two embryos were cryosectioned and the neurectoderm in the region of the COS cells found to be morphologically normal (Fig. 4).

When COS cells transfected with an expression construct for BMP-7 were grafted into the mesoderm underlying the developing hindbrain, changes in the neurectoderm occurred. While the general development of experimental embryos matched

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**Fig. 2.** Strategy for misexpression in the 8.5 dpc embryo. (A) COS cells were transiently transfected with an expression construct. (B) 48 hours after transfection the COS cells were collected and placed in hanging drop culture. (C) After 2 hours the COS cells had formed a coherent sheet of cells, which could be DiI-labelled and broken into suitable sized groups of cells for grafting. The labelled COS cells were grafted into mesoderm adjacent to the prospective hindbrain. (D) Embryos into which cells were grafted were allowed to develop in culture for 24 hours, dissected free of the extraembryonic membranes and fixed. Embryos were examined under fluorescent optics and the location of incorporated cells recorded. (E) Embryos in which cells were incorporated were examined further, for example by whole-mount in situ hybridisation (WMISH) to determine if changes in gene expression had occurred.

**Fig. 3.** Location of grafted cells. (A) A 4-somite embryo. Immediately after grafting the fluorescent COS cells the embryo was fixed, and yolk sac and amnion removed so that the position of the incorporated cells could be determined. The cells are located between the preotic sulcus and the otic sulcus. (B) A transverse section through a 6-somite embryo, which was fixed immediately after the grafting procedure. The fluorescent COS cells are located in the mesoderm underlying the hindbrain neurectoderm. pos: preotic sulcus, os: otic sulcus. Scale bar, 200 μm (A); 100 μm (B).
that of controls, it was apparent that a large proportion of them displayed distorted and often highly convoluted neurectoderm on the grafted side of the hindbrain (Fig. 4C). The 47 embryos that had received an experimental graft were viewed with fluorescent optics and all embryos that showed overt deformity (38) contained COS cells within the ventrolateral mesoderm. Two embryos were cryosectioned and the neurectoderm in the region of the COS cells found to be kinked in a manner consistent with an overproduction of neurectoderm (Fig. 4D).

Dorsal-ventral pattern in the neurectoderm adjacent to the COS cells

After establishing the location of grafted COS cells, perturbations in hindbrain dorsal-ventral pattern were assessed using whole-mount in situ hybridisation to a variety of genes that show restricted expression patterns in the developing neural plate.

Expression of dorsal markers in the ventral neurectoderm

BMP molecules have previously been implicated in differentiation of dorsal neurectoderm structures (Liem et al., 1995), therefore the expression pattern of Msx1 was examined. In wild-type embryos Msx1 is expressed in a narrow domain within the dorsal neural tube (Hill et al., 1989). Msx1 neurectoderm transcription is initiated in a narrow lateral domain during headfold stages of development, and upon neural tube closure Msx1 is seen in the roof plate. Therefore, during normal development Msx1 transcripts never cross the ventral midline of the developing cranial neurectoderm (Fig. 5J).

The localisation of Msx1 mRNA was examined in embryos which received a graft of nontransfected COS cells. Embryos in which the cranial neural folds were still open were examined in whole mount and flat mount to determine that the dorsal restriction of Msx1 was maintained. Sectioning of embryos in which closure of the cranial neural folds was more advanced was used to ascertain that Msx1 expression in the roof plate was maintained. In all 11 control embryos analysed, no differences were noted in any features of Msx1 expression (Fig. 5 and Table 1). In contrast, embryos that had received a graft of BMP-7-transfected COS cells showed an enlargement of the domain expressing Msx1 (Fig. 5C-E). In mildly affected embryos this was seen as a shift of the Msx1 hybridisation signal to a more ventral level. In embryos with very distorted neurectoderm this shift in expression was accompanied by the presence of additional neural tissue, which invariably expressed Msx1. In all of the embryos examined, ectopic expression of Msx1 was either confined to the neurectoderm on the grafted side or was markedly more extensive on this side. The variation in the extent of the phenotype presumably reflects differences in the exact size and/or location of the graft. Fig. 5D shows a flat mount preparation of an embryo in which the grafted side shows additional neural tissue, and in rhombomere 3 there is a ventral shift in the expression domain of Msx1 on both sides of the neurectoderm. In this case the COS cells were located ventrolateral to the neurectoderm in the region of rhombomere 3.

The analysis of Msx1 expression in the neurectoderm suggests that the ventrolateral neurectoderm has taken on characteristics normally associated with the dorsal aspect of the neurectoderm. Cells in the dorsal region of the neural tube give rise not only to dorsal neural tube cells but also to the neural crest population (Chan and Tam, 1988). We examined the pattern of mRNA accumulation of two genes expressed in different subsets of the neural crest. In wild-type embryos Msx1 expression is seen in the post-migratory neural crest cells that populate the branchial arches, and the dorsal limit of this expression is never more dorsal than the base of each arch. Grafts of nontransfected COS cells did not result in any difference in Msx1 transcript accumulation in the branchial arches. However, in five of the six embryos in which BMP-7-transfected COS cells were grafted the domain of Msx1 expression in the branchial arches extended more dorsally on the grafted side than on the non-grafted side, suggesting an

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COS cells were grafted into hindbrain mesoderm, and after recording the location of the grafted cells, mRNA localisation of the marker genes listed was examined.

*In general all embryos examined in whole mount were subsequently examined in either flat mount or section, however in the case of Msx1, all necessary information could be collected from whole mount specimens of younger embryos. Thus some embryos (six that had received nontransfected COS cells and two that had received BMP-7 transfected COS cells) were examined only in whole mount.

†The neural folds of this embryo were damaged slightly during whole mount in situ hybridisation; it is most likely that absence of Pax-3 expression in this region is due to physical damage to the tissue.
increased production of neural crest cells (Table 1). The sixth embryo was developmentally less advanced, having only 10 somites after culture, and no hybridisation signal was evident in any of the branchial arches.

To further examine neural crest production at the site of the graft, AP-2 expression was assessed. The AP-2 transcription factor is expressed in the surface ectoderm and in the dorsal neur ectoderm from early neural plate stages and when neural crest begins to emigrate from the hindbrain region (at the 5-somite stage) it is expressed in the migratory cells (Mitchell et al., 1991). In embryos which had received a graft of nontransfected COS cells no ventral shift in AP-2 expression was seen. In contrast, as shown in Table 1, of the four embryos that received a graft of BMP-7-transfected COS cells all showed ectopic expression of AP-2. In all cases AP-2 was expressed in a more ventral location in the neur ectoderm than in unoperated embryos. In the two embryos in which this was most pronounced there was also evidence of neural crest cells arising from the ventral neur ectoderm (Fig. 5). Taken together with the Msx1 expression it seems that the ventrolateral source of BMP-7 has caused the ventrolateral neur ectoderm to adopt a dorsal fate.

<table>
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<tr>
<th>Table 2. Neurectoderm mean nuclear density</th>
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<td>Location</td>
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<tr>
<td>Nontransfected</td>
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<td>BMP-7-transfected</td>
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<td>Distant from graft</td>
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Values are presented as the means of the nuclear density at each of the sites listed, ± s.e.m. Student’s t-test was performed to compare the mean nuclear density at sites adjacent to the graft, with that on the nongrafted side and distant from the graft.

Pax-3 expression is unaffected by ectopic expression of BMP-7

Pax-3 is expressed in the dorsal half of the neural tube in both the mouse and the chick (Goulding et al., 1991, 1993), and experiments in the chick have identified Pax-3 as a gene whose dorsal restriction may be primarily regulated by the notochord expression of Shh (Goulding et al., 1993; Liem et al., 1995). However, it has also been shown that BMP molecules can upregulate expression of Pax-3 in chick spinal cord that is already expressing low levels of Pax-3 (Liem et al., 1995). Unlike the chick, at no level of the mouse cranial or spinal neur ectoderm does Pax-3 expression cross the ventral midline between the onset of Pax-3 transcription and the 20-somite stage. This provides the opportunity to ask whether BMP-7 is capable of eliciting Pax-3 expression in neur ectoderm that otherwise would not express Pax-3, but that is competent to express other dorsal markers. In all embryos which had received grafts of either nontransfected or BMP-7-transfected COS cells, Pax-3 expression in the hindbrain region remained unaltered on the grafted side compared to the non-grafted side (Fig. 7 and Table 1). Interestingly, the whole-mount in situ hybridisation analysis of Pax-3 expression in the developing hindbrain region showed that Pax-3 is not expressed in the roof plate at all levels of the hindbrain (see Fig. 7), although it is detected throughout the roof plate at other rostro-caudal levels.

Increased area of the neur ectoderm

From analysis of the experimental embryos it was obvious that the volume of the neur epithelium is increased in the region of the graft. The increase in size may result from an increase in the volume of the individual cells, or from an increased number of cells. A nuclear stain was used to distinguish between these possibilities: if cell volume increases, the density of nuclei must decrease. The nuclear density was sampled at four sites in each embryo: in right and left neur ectoderm adjacent to the COS cells, and right and left neur ectoderm at a site distant from the graft. In each embryo 3-8 sections were scored at each site. The mean nuclear density of the neur ectoderm adjacent to the graft was compared to that at each of the three other sites. For each comparison the significance of difference was determined by Student’s t-test. Table 2 shows that the mean nuclear density adjacent to the graft was not significantly different from that at any of the three other sites. Thus the neur ectoderm

<table>
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<th>Table 3. Neurectoderm relative mitotic frequency</th>
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Values are presented as the ratio of the relative mitotic frequency on the grafted side of the neur ectoderm, to the relative mitotic frequency on the nongrafted side, ± s.e.m. Student’s t-test was performed to compare the relative mitotic frequency adjacent to the graft with the corresponding frequency distant from the graft.

*P<0.01.
expansion results from an increase in cell number. The nuclear stain used in these experiments (DAPI) also allows identification of both mitotic cells and apoptotic cells. From the analysis of the DAPI-stained sections, no alteration in the proportion of apoptotic cells was discernible. However in the region of BMP-7-transfected COS cells an abundance of mitotic figures was apparent on the ventricular surface.

To quantify this, the relative mitotic frequency was determined as described (Materials and Methods). When this was measured at 30 μm intervals along the hindbrain (data not shown) it was found to vary in a manner consistent with that reported for chick neurectoderm (Guthrie et al., 1991). This indicated that centres of increased proliferation, corresponding to the rhombomeres, are probably also found in the mouse. Despite this variation along the axis, at any one point the relative mitotic frequency on both sides of the neurectoderm should be the same. Therefore to determine whether there are more mitotic figures adjacent to the BMP-7-transfected COS cells.

Fig. 4. Morphology of grafted embryos. (A) Embryo that received a control graft, after 24 hours culture. (B) Transverse section through the region of grafted nontransfected COS cells. (C) Embryo that received a graft of BMP-7-transfected COS cells, after 24 hours culture. The deformity, adjacent to the COS cells, is marked with an arrow. (D) Transverse section through the region of grafted BMP-7-transfected COS cells. Scale bar, 400 μm (A,C); 100 μm (D); 75 μm (B).

Fig. 5. Msx1 and AP-2 mRNA localisation. (A) Dorsal view of an embryo that received a graft of nontransfected COS cells, to the left side, showing Msx1 mRNA localisation. Msx1 transcripts are confined to the dorsal neurectoderm, and rhombomeres 3 and 5 exhibit increased transcript levels. (B) Lateral view of the embryo in C, showing the location of the BMP-7-transfected COS cells. (C) Dorsal view of the embryo in B, showing the expanded expression domain of Msx1 on the grafted (left) side. (D) Flat mount of hindbrain neurectoderm of an embryo that received a graft of BMP-7-transfected COS cells to the left side. The Msx1 expression domain expands ventrally in rhombomere 3 on both the grafted and non grafted sides. On the grafted side there is additional neural tissue, which also expresses Msx1 (arrow). (E) Transverse section through the hindbrain of an embryo that received a BMP-7 graft (left side) showing the ventral shift of the Msx1 expression domain and additional neural tissue expressing Msx1. (F) Lateral view of an embryo showing the location of nontransfected COS cells. (G) Transverse section through the grafted region of the embryo in F showing AP-2 expression. (H) Lateral view of an embryo showing the location of the BMP-7-transfected COS cells. (I) Transverse section through the grafted region of the embryo in H showing ventral AP-2 expression and AP-2-positive cells exiting the neurectoderm at two sites on the left side of the neural tube. (J) Dorsal view of a 2-somite embryo showing Msx1 mRNA localisation. Rostral of the node Msx1 transcripts are confined to the lateral edge of the neural plate; this expression does not extend to the anterior of the embryo. (K) Dorsal view of a 3-somite embryo showing AP-2 mRNA localisation. AP-2 is expressed in the surface ectoderm and rostral to the node transcripts are seen at the lateral edge of the neural plate; the neurectoderm staining does not extend to the anterior of the embryo. r, rhombomere. Scale bar, 450 μm (A,C); 300 μm (B,F,H, J, K); 150 μm (D); 100 μm (G,I); 75 μm (E).
cells, a ratio of the relative mitotic frequency on the grafted side to that on the nongrafted side was calculated. For each embryo this ratio was calculated for 8-10 sections corresponding to the region of the graft and for 8-10 sections at the level of the otic vesicle. For each set of data the significance of difference was determined by Student’s t-test. Table 3 shows that in embryos in which nontransfected COS cells were grafted there was no difference between the grafted and non-grafted side of the neurectoderm, either at the site of the graft or remote from the graft. In contrast to this, relative mitotic frequency increased by more than twofold in the vicinity of grafted BMP-7-transfected COS cells, whereas outside of the region of the graft the relative mitotic frequency did not increase. These data, taken together with the increase in cell number, are consistent with BMP-7 causing increased proliferation of the neurectoderm.

DISCUSSION

This study shows for the first time that the activity of secreted molecules can be examined in the early mouse embryo by the precision grafting of cells expressing the desired molecule. The ectopic expression study identifies three distinct activities of BMP-7 in the mammalian hindbrain. Firstly BMP-7 promotes dorsal cell fate, and in general this is consistent with the activity identified for BMP-7 using in vitro assays of chick spinal cord development (Liem et al., 1995). The two other previously unidentified activities are interference with accumulation of Shh transcripts in the floor plate and an ability to increase cell number in the neurectoderm. When these data are combined with the expression information presented in the first part of this paper, several functions may be suggested for BMP-7 during the early establishment of the central nervous system.

Dorsal differentiation of the cranial neurectoderm

In vertebrates the first differentiated cell population to arise from the lateral neurectoderm is the neural crest population (Nichols, 1981; Tosney, 1982; Sadaghiani and Thiébaud, 1987), which usually emerges shortly after closure of the neural tube. In the mouse embryo, the cranial neural crest is formed unusually early: the first crest emigrates from the hindbrain region in the 5-somite embryo, and all of the neural crest cells have left the hindbrain neurectoderm by the time the neural folds fuse to form the neural tube (Serbedzija et al., 1992). Thus the influences that promote dorsal differentiation in this region must operate in concert with or very soon after

Fig. 6. *Shh* mRNA localisation. (A) Lateral view of the embryo in B, showing the location of the nontransfected COS cells. (B) Lateral view of the embryo in A showing that the expression of *Shh* remains unaltered in the region of the graft. (C) Transverse section through the hindbrain region of an embryo with a control graft showing *Shh* expression in the notochord and floor plate. (D) Lateral view of the embryo in E, showing the location of the BMP-7-transfected COS cells. (E) Lateral view of the embryo in D. *Shh* expression is maintained in the hindbrain notochord but is depleted in the floor plate in the region of the graft (arrow). (F) Transverse section through the hindbrain of an embryo that received a graft of BMP-7-transfected COS cells to the left side showing diminished floor plate expression of *Shh*. Scale bar, 450 μm (B,E); 300 μm (A,D); 75 μm (C,F).

Fig. 7. *Pax-3* mRNA localisation. (A) Transverse section through the hindbrain of a wild-type embryo showing absence of *Pax-3* transcripts in the roof plate. (B) Dorsal view of an embryo that received a graft of nontransfected COS cells, to the left side, showing *Pax-3* expression. (C) Dorsal view of an embryo that received a graft of BMP-7-transfected COS cells to the left side showing *Pax-3* expression. The neurectoderm is expanded in the region of rhombomere 2. (D) Flat mount of the embryo shown in B; the expression domain of *Pax-3* is unaltered in the region of the graft (arrow) relative to the non-grafted side. r, rhombomere. Scale bar, 450 μm (B,C,D); 50 μm (A).
the signals that induce the anterior epiblast to adopt a neural fate. The acquisition of dorsal neurectoderm fate has previously been studied in the chick spinal cord. It is proposed that two mechanisms interact to generate dorsal restriction of gene products: the expression of early patterning genes is regulated primarily by ventral repression, mediated by notochord-derived Shh, and later, positive signals possibly mediated by surface ectoderm-derived BMP molecules promote the differentiation of definitive dorsal cell types such as neural crest cells (Liem et al., 1995).

During the earliest stages of mouse cranial neurectoderm development, ventral repression of dorsally restricted genes does not appear to be necessary. Msx1 and AP-2 transcripts are first localised to the lateral edge (future dorsal) of the cranial neural plate at the headfold stage of development. During the early stages of development neither gene is expressed in the midline neurectoderm above the node (see Fig. 5J,K) and their transcripts are never seen in the midline neurectoderm of the cranial neural plate. Here we show that ectopic expression of BMP-7 is sufficient to elicit the expression of both Msx1 and AP-2 in neural tissue, which otherwise would never express these genes. Furthermore, this occurs in the presence of a notochord expressing Shh. These data argue that in the mouse cranial neurectoderm, notochord-derived Shh is not the major factor regulating Msx1 expression as has been proposed for the chick spinal cord (Liem et al., 1995). Possibly, differentiation of the cranial neural crest population is initiated before ventral repression is in operation: both Msx1 and AP-2 expression begin either before, or concomitant with, the notochord expression of Shh (Échelard et al., 1993). The early acquisition of dorsal cell fate that occurs in the cranial region may be more dependent upon a BMP-mediated signal, and certainly the early onset of BMP-7 expression in the surface ectoderm is consistent with it playing such a role.

BMP-7 is not sufficient to elicit the expression of all genes that show a dorsal restriction during normal development. The dorsal-ventral domain of Pax-3, a gene whose expression in the mouse embryo is initiated later than that of Msx1 and AP-2, is not altered in response to BMP-7. However the possibility that the level of Pax-3 expression may be altered by BMP-7, as it can be in the chick spinal cord (Liem et al., 1995), cannot be ruled out. The dorsal restriction of Pax-3 appears to be primarily regulated via negative signals, which derive from the notochord in both the chick (Goulding et al., 1993; Liem et al., 1995) and the mouse spinal cord (Ang and Rossant, 1994; Rashbass et al., 1994). It is likely that in the cranial region of the mouse embryo Pax-3 expression is also repressed by the notochord and that BMP-7 cannot interfere with this interaction. In the chick spinal cord, Shh can mimic the ability of the notochord to decrease the level of Pax-3 expression in dorsal neurectoderm explants (Liem et al., 1995). The assay presented here suggests that ventral repression of Pax-3 expression continues in the absence of high levels of floor plate expression of Shh.

Timing of cranial floor plate induction

BMP-7 is expressed in axial mesendoderm and the midline neurectoderm at a stage when the neurectoderm should still be labile with respect to dorsoventral pattern. What is the function of a midline ‘dorsalizing’ signal? One possible function is suggested by the overexpression of Shh and HNF3-β in Xenopus embryos, which demonstrates that in vivo mechanisms exist that restrict the timing of floor plate induction (Ruiz i Altaba et al., 1995a). It is possible that BMP-7 regulates the timing of floor plate induction in the hindbrain region. It is believed that the initiation of floor plate development occurs in response to a contact-dependent signal emanating from the notochord (Placzek et al., 1993), and that Shh mediates this (Échelard et al., 1993; Roelink et al., 1994). An immediate early response to induction is expression of the winged helix transcription factor HNF3-β (Ruiz i Altaba et al., 1995b) which, in turn, is sufficient for the activation of Shh floor plate expression (Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1995a). In the cranial region of the mouse embryo there is a considerable time-lag between the notochord expression of Shh and the onset of floor plate expression of Shh. For example, in the developing hindbrain region Shh first appears in the notochord some 15 hours before the onset of floor plate expression of Shh, and HNF3-β is present in the floor plate for some 12 hours before Shh is expressed (Échelard et al., 1993). Once Shh floor plate expression is initiated, it spreads quickly along the axis, so that in the posterior of the embryo there is much less delay between the notochord and floor plate expression of Shh. The data presented here show that the disappearance of BMP-7 from the ventral hindbrain precedes the appearance of Shh in this region, and that BMP-7 is capable of interfering with Shh expression in the floor plate. The midline expression of BMP-7 may delay initiation of cranial floor plate development until a sufficient proportion of the embryonic axis has been generated to allow relatively synchronous ventral differentiation.

Growth of the cranial neurectoderm

Cell culture studies carried out with recombinant BMP-7 have shown that this molecule inhibits proliferation of embryonal carcinoma cells (Andrews et al., 1994), but stimulates the proliferation of osteoblasts (Knutsen et al., 1993; Chen et al., 1995). However little is known about the ability of BMP molecules to regulate cell growth during embryogenesis. Mice lacking either functional BMP-4 or a BMP type I receptor fail to undergo gastrulation. In each case the primary defect may be decreased proliferation of the pre-gastrulation epiblast, suggesting that in vivo a BMP signalling pathway may mediate cell growth (Mishina et al., 1995; Winnier et al., 1995). At later stages of chick hindbrain development, BMP-4 has been shown to stimulate apoptosis in prospective neural crest cells in odd-numbered rhombomeres (Graham et al., 1994). Because BMP-4 is not expressed in the mouse hindbrain (Winnier et al., 1995), the relevance of this observation to the development of the mammalian cranial neurectoderm development is not clear. The assay reported here provides the first evidence that BMP molecules may stimulate growth of the neurectoderm, although from this assay it is impossible to distinguish whether this effect is specific to all neurectoderm or only to dorsal neurectoderm. However, the finding that BMP-7 can increase neurectoderm cell number, combined with its expression surrounding the cranial neural plate during the headfold stage of development, makes it tempting to speculate that BMP-7 could be involved in the differential growth of the headfold neurectoderm relative to the trunk neurectoderm during the early stages of neural development.

Recently two laboratories reported the production of mice...
that carry null alleles of BMP-7. While mice homozygous for the mutations appear to have defects consistent with BMP-7 participating in cell growth and/or survival during embryogenesis, the abnormalities observed are limited to limb, eye and kidney development. The mice do not have defects consistent with an absolute requirement for BMP-7 during establishment of the central neural system (Dudley et al., 1995; Luo et al., 1995). The conflict between these findings and the data presented here highlights the difficulties of experimental analysis of single members of multi gene families. It is known, for example, that BMP-2 is localised to the surface ectoderm and therefore overlaps with BMP-7 (Lyons et al., 1995; our unpublished data). It is possible that embryos lacking BMP-2 fail to develop because of a lack of amnion formation (cited in Hogan, 1995), such an effect will have to await the construction of a conditional BMP-2 mutation, unless a haploinsufficiency of BMP-2 can be observed in BMP-7−/− embryos.

The studies presented here identify several potential functions for the signalling molecule, BMP-7, during early neural development and highlight differences between the establishment of the cranial neur ectoderm and the spinal cord. The initial restriction of expression of BMP-7 to the anterior region of the developing embryo may be relevant to several features that are unique to cranial development. These features include the rapid growth of the headfield, the early generation of the neural crest from this region, and the delay in the induction of Shh expression in floorplate by the notochord. The establishment of an assay with which the function of secreted factors may be tested in different locations in the mouse embryo will contribute to the understanding of the roles of such molecules during mammalian development.

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