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Development of chick axial mesoderm: specification of prechordal mesoderm by anterior endoderm-derived TGFβ family signalling

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SUMMARY

Two populations of axial mesoderm cells can be recognised in the chick embryo, posterior notochord and anterior prechordal mesoderm. We have examined the cellular and molecular events that govern the specification of prechordal mesoderm. We report that notochord and prechordal mesoderm cells are intermingled and share expression of many markers as they initially extend out of Hensen's node. In vitro culture studies, together with in vivo grafting experiments, reveal that early extending axial mesoderm cells are labile and that their character may be defined subsequently through signals that derive from anterior endodermal tissues. Anterior endoderm elicits aspects of prechordal mesoderm identity in extending axial mesoderm by repressing notochord characteristics, briefly maintaining *gsc* expression and inducing *BMP7* expression. Together these experiments suggest that, in vivo, signalling by anterior endoderm may determine the extent of prechordal mesoderm. The transforming growth factor β (TGFβ) superfamily members BMP2, BMP4, BMP7 and activin, all of which are transiently expressed in anterior endoderm mimic distinct aspects of its patterning actions. Together our results suggest that anterior endoderm-derived TGFβs may specify prechordal mesoderm character in chick axial mesoderm.

Key words: Prechordal mesoderm, Notochord, Anterior endoderm, Hypoblast, BMP, Activin, Chick

INTRODUCTION

In the embryonic chick, precursor cells of the notochord and prechordal mesoderm originate in, or pass through, Hensen's node (the equivalent of the amphibian organiser), and then migrate anteriorly through a process of convergent extension to form a rod of axial mesoderm (Bellairs, 1986; Joubin and Stern, 1999; Psychoyos and Stern, 1996; Rosenquist, 1966; Schoenwolf et al., 1992; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991; Spratt, 1955). Previous studies have shown that, after extension, axial mesoderm cells play a critical role in patterning the overlying neural tube, inducing the differentiation of specific cells within the ventral neural tube (Dale et al., 1997; Foley et al., 1997; Pera and Kessel, 1997; Tanabe and Jessell, 1996). In particular, axial mesoderm cells induce distinct types of ventral midline cells along the rostrocaudal axis of the neural tube. Caudally, notochord induces floor plate differentiation (Dodd et al., 1998; Placzek et al., 2000) while, rostrally, the prechordal mesoderm can induce ventral midline cells of the rostral diencephalon (RDVM cells) (Dale et al., 1997, 1999). The distinct inducing activities of different regions of axial mesoderm arise due to their different signalling potential. Notochord expression of Sonic hedgehog (Shh) mediates its ability to induce floor plate cells (Chiang et al., 1996; Dale et al., 1997; Dodd et al., 1998; Ericson et al., 1996) whereas the co-expression of bone morphogenetic protein 7 (BMP7) and Shh appears to mediate the ability of prechordal mesoderm to induce RDVM cells (Dale et al., 1997, 1999). The mechanism that controls expression of BMP7 in the prechordal mesoderm of the chick is unclear.

Studies in chick embryos have suggested that axial mesoderm cells that initially migrate out of Hensen's node are a mixed population, in which notochord and prechordal mesoderm cells are intermingled (Foley et al., 1997). How these intermingled cells resolve into two spatially discrete populations remains unclear. Furthermore, although expression of *gsc* on early extending prechordal mesoderm cells suggests that these cells are already specified in the node, it is unknown if the same is true for other characteristics of prechordal mesoderm, including expression of BMP7.

The factors that govern specific gene expression in axial mesoderm cells of the prechordal mesoderm and notochord in a variety of species have begun to be elucidated. Many

observations have suggested that members of the transforming growth factor β (TGFβ) superfamily, including Vg1, nodal and activin, play a key role in early steps in the induction of organiser properties (Joubin and Stern, 1999; Kodjabachian and Lemaire, 1998) and the subsequent development of axial mesoderm. In zebrafish, genetic analyses have revealed that nodal-related proteins may similarly affect development of the prechordal mesoderm and notochord. Embryos mutant for the *nodal*-related genes, *cyclops* and *squint*, or the CFC-EGF family member *one eyed pinhead* (required for the action of *cyclops*) show defects in prechordal mesoderm and notochord development (Schier and Shen, 2000). The precise manner in which members of the TGFβ family may control the development of axial mesoderm precursors, so that prechordal mesoderm and notochord are generated, remains unclear. In *Xenopus*, expression of *Xbra* and *Xgsc* appear in response to different threshold concentrations of activin (Green et al., 1992, 1997; Gurdon et al., 1996), while, in zebrafish, expression of *gsc* and *Floating head* appear to be controlled by differential exposure to nodal signalling (Gritsman et al., 2000); it is uncertain, however, whether a similar principle operates in the chick. Moreover, in no species is it yet clear which tissues act as the source of TGFβ signals and hence may contribute to fate specification of the prechordal mesoderm and notochord. In zebrafish, expression of *nodal* and *squint* are detected in the organizer, in axial mesoderm cells and in the yolk syncytial layer (Feldman et al., 1998; Sampath et al., 1998), either or all of which could contribute to the normal development of the axial mesoderm. In the chick, expression of TGFβs has been observed in the hypoblast, around Hensen's node and in anterior endoderm (Andree et al., 1998; Joubin and Stern, 1999; Mitrani et al., 1990; Schultheiss et al., 1997; Seleiro et al., 1996; Shah et al., 1997; Sugi and Lough, 1995; Yatskievych et al., 1997), raising the possibility that each of these tissues could contribute to the fate of axial mesoderm.

Here we report that, in the embryonic chick, TGFβ signalling by anterior endoderm may play a critical role in the specification of prechordal mesoderm. We show that axial mesoderm cells that will give rise to the notochord and prechordal mesoderm share many characteristics as they initially extend forwards from Hensen's node, including expression of *gsc* and *chordin*. We perform experiments in vivo and in vitro that suggest that signalling by anterior endoderm may specify the prechordal mesoderm and define the interface between prechordal mesoderm and notochord. Our experiments show that TGFβs that are transiently expressed within the anterior endoderm mimic its ability to elicit prechordal mesoderm character in axial mesoderm, acting to suppress notochord identity, to transiently maintain expression of the early expressed prechordal mesoderm marker, *gsc*, and to induce expression of the later prechordal mesoderm marker, *BMP7*. Together these studies suggest that, in chick embryos, anterior endoderm controls the character of axial mesoderm that has migrated away from Hensen's node, specifying prechordal mesoderm identity.

MATERIALS AND METHODS

In situ hybridization

Embryos and explants (n=4 minimum, respectively, for each marker and time point) were examined using standard techniques (Schaeren-Wiemers and Gerfin-Moser, 1993), with the omission of the proteinase K step. Digoxigenin-labelled antisense RNA probes were prepared by in vitro transcription from linearised template DNAs. pcvhh1 plasmid encoding SHH was linearised with *Sal*I and transcribed with SP6 polymerase (Ericson et al., 1995); pMT23/gsc plasmid encoding chick Gsc was linearised with *Eco*RI and transcribed with SP6 polymerase; pcBMP2 encoding chick BMP2 was linearised with *Hin*dIII and transcribed with T3 polymerase (Francis et al., 1994); mpcBMP4 encoding chick BMP4 was linearised with *Bam*HI and transcribed with T3 polymerase (Francis et al., 1994). pBH2 plasmid encoding chick BMP7 was linearised with *Xho*I and transcribed with T3 polymerase (Dale et al., 1999); pMT23 encoding Chordin was linearised with *Eco*RI and transcribed with T7 RNA polymerase (Streit et al., 1998); the 3′ UTR Hex (Prh) riboprobe was synthesised from a pBluescript SKII+ plasmid containing full-length *Hex* cDNA digested with *Sma*I and transcribed with T3 RNA polymerase (Compton et al., 1992). pcm21 encoding Netrin-1 (Serafini et al., 1994) was linearised with *Eco*RI and transcribed with T7 polymerase; pcbra4/9 encoding Brachyury (gift of V. Cunliffe) was linearised with *Xba*I and transcribed with T3 polymerase. Following antisense analysis, embryos and explants were either serially sectioned or examined as whole mounts.

Immunocytochemistry

Embryos and explants (n=4-6 minimum, respectively, for each marker and time point) were examined using standard techniques (Placzek et al., 1993). The following antibodies (dilutions in parentheses) were used: 15.3B9 (1:1) (Placzek et al., 1990); 4C7, anti-HNF3β mAb (1:40) (Ruiz i Altaba et al., 1995); 68.5E1, anti-Shh (1:50) (Ericson et al., 1996); QCPN (1:40); anti-Nkx2.1 (1:5000) (Lazzaro et al., 1991); 6G3 anti-FP3 mAb (1:10) (Placzek et al., 1993). Second antibodies (TAGO and Boehringer-Mannheim) were conjugated to fluorescein isothiocyanate (FITC) or Cy-3. For double-labelling with QCPN, sections were incubated in 1% Tween and 20% sheep serum.

Tissue dissection and explant culture

Endodermal explants were dissected from HH stage 3+ to 4+ embryos, as follows: two parallel cuts was made in the embryo, immediately above Hensen's node and immediately above the germinal crescent. Lateral cuts were made at the embryonic-extraembryonic boundary. Endoderm was removed from overlying epiblast (either enzymatically in 1 mg/ml Dispase or mechanically), and dissected into four pieces (along the anteroposterior axis), and trimmed mediolaterally (Fig. 5A,B). Pieces 1 (closest to Hensen's node) and 3 were analysed (Fig. 5A,B). Fate-mapping analyses (Bachvarova et al., 1998, figure 2B) shows that piece 1 is fated to be embryonic endoderm and piece 3 is anterior endoderm.

Prechordal mesoderm was identified and dissected as previously described (Dale et al., 1999; Seifert et al., 1993). Prechordal mesoderm at stage 5– was identified by its morphology (broader than more posterior notochord), position and marker expression (*Shh* positive, *gsc* positive, see Fig. 2R,T). In the majority of in vitro experiments, nascent notochord (extending approximately 20-100 μm from the node, anteriorly) was dissected. In experiments designed to test the in vitro induction of *gsc*, nascent notochord from stage 7 embryos was used since, in this case, notochord could be dissected free of endoderm. Tissues were dissected using Dispase and cultured in collagen according to our established techniques (Placzek and Dale, 1998; Placzek et al., 1993).

Explants were examined after a defined culture period, the precise time dependent on that predicted for the explant to reach the equivalent of stage 8 (i.e. a time at which in vivo prechordal mesoderm co-expresses *Shh*, *BMP7* and *gsc*). In vivo, development of chicks from stage 5 to 8 takes approximately 7-9 hours. An additional period of 15 hours was added in vitro to allow for recovery of the explants,

as documented previously (Placzek et al., 1993). Endodermal explants cultured alone were examined at 16 and 20 hours after incubation. Endoderm-notochord recombinates were examined after 22-24 hours. Culture times for other experiments are indicated in the figure legends. After culture, explants were fixed and examined as whole mounts or after sectioning. In each experiment, a subset of explants was examined for co-expression of markers on serial adjacent sections. In addition, chick-quail chimaeric recombinants were examined by double labelling with QCPN (see below).

Chick strains

The experiments and in situ hybridisation analyses were performed on Hysex hybrid brown chick embryos. In agreement with previous publications (Schultheiss et al., 1997; Andree et al., 1998; Streit et al., 1998), we detect *BMP2*, but not *BMP4* or *BMP7*, in the anterior endoderm of White Leghorn chick embryos.

Chick-quail recombinates

In experiments to test the effect of anterior endoderm on prechordal mesoderm or notochord, reciprocal recombinations using chick and quail tissues were performed. After analysis by in situ hybridisation, recombinates were labelled with QCPN. The expression patterns documented here for chick (Hysex) were similar in quail, with the exception of *BMP7*. In quail, *BMP7* was detected in nascent notochord and prechordal mesoderm, then downregulated in notochord.

Chick-rat recombinates

In the two-step assay, chick notochord was first cultured with BMP2/7 for 22 hours, washed extensively, and recombined with an E9 rat lateral neural plate explant for a further period of 30 hours. E9 lateral neural plate explants were dissected as previously described (Placzek et al., 1993). Rat tissue was used in preference to chick tissue, due to our previous documentation of its ability to respond to prechordal mesoderm and generate Nkx2.1-positive cells (Dale et al., 1997).

In experiments in which notochord explants were treated with BMP7, the following additional experiment was performed to ensure that BMP7 was not simply 'sticking' to the notochord explant: notochord explants from stage 22 embryos, which are unable to induce floor plate (Placzek et al., 1993), were treated with BMP7, and assayed for their ability to induce cells characteristic of dorsal spinal cord (Liem et al., 1997). No induction of dorsal cell types was observed.

Grafting experiments in vivo

Grafts were performed using standard techniques (Placzek et al., 1990). Briefly, notochord cells were displaced from underlying endoderm, and anterior endoderm explants were grafted between the notochord and the originally adjacent endoderm. After operating, embryos were resealed and allowed to develop for 16-24 hours to reach stage 12 or 13, and fixed, serially sectioned and processed for in situ hybridisation or immunohistochemistry.

Purified proteins

Human BMP7/Osteogenic Protein 1 (OP1) (Creative Biomolecules), human BMP2 (Genetics Institute) and activin (gift of V. Cunliffe) were added at the onset of culture. Proteins were used at the following concentrations: BMP7, 1-10 ng/ml; BMP2, 1-10 ng/ml; activin, 20 units, i.e. within the effective ranges used in other assays (Green et al., 1992; Sampath et al., 1992).

Blocking reagents

Anti-BMP7 IgG 1B12 was used as previously described (Dale et al., 1997). Concentrated noggin-conditioned CHO cell medium was a gift from R. Harland, and was used at a 1:20 dilution. Endodermal explants were preincubated in blocking antibody for 60 minutes on ice. Noggin was added to the culture medium.

RESULTS

Distinct properties of axial mesodermal cells

To identify properties of axial mesoderm cells that could serve as markers defining differentiated notochord and prechordal mesoderm, we examined the profile of marker expression in early neurula stage chick embryos (HH stages 6+ to 7; Hamburger and Hamilton, 1951). Prechordal mesoderm cells co-express the gene encoding the transcription factor *goosecoid* (*gsc*) and the signalling molecules *Shh* and *BMP7* but show only residual expression of the signalling molecule, *chordin*, and do not express the surface molecule 3B9 (Fig. 1A-E,K-O; see also Dale et al., 1999). In contrast, notochord cells express *chordin* at high levels, *Shh* and 3B9, but express neither *gsc* nor *BMP7* (Fig. 1A-E,P-Z; see Dale et al., 1999). In vivo, therefore, for a short period between stage 7 and 9, *gsc* and *BMP7* are co-expressed in prechordal mesoderm and are mutually exclusive of *chordin* and 3B9, which mark posterior notochord (Table 1).

Co-expression of markers on early extending notochord and prechordal mesoderm

To assess when prechordal mesoderm acquires its distinctive marker profile, we examined marker expression at earlier stages (HH stages 4 to 5). As previously reported (Izpisua Belmonte et al., 1993), *gsc* is expressed at stage 4 in Hensen's node (not shown), and then expressed in prechordal plate and prechordal mesoderm cells (Fig. 2A,E,H,K,O,R) that migrate anteriorly over the period stage 4+ to 5+ (Bellairs, 1986; Izpisua Belmonte et al., 1993; Selleck and Stern, 1991). In addition, weaker expression of *gsc* is also detected in more posterior notochord cells (Izpisua-Belmonte et al., 1993; Fig. 2U). *Shh* is co-expressed with *gsc* in prechordal mesoderm and notochord (Fig. 2J,T,X), but is not expressed in more anterior prechordal plate (Fig. 2G,Q and see Dale et al., 1999). No expression of *BMP7* is detected in prechordal mesoderm cells as they migrate forwards (Fig. 2I,S). Instead, prechordal mesoderm cells that are migrating anteriorly share with notochord, expression of the notochord markers *chordin* and 3B9 (Fig. 3E,H). Indeed, a panel of other notochord markers assayed at these early stages, including *netrin-1*, *brachyury* and *cnot-1* appear to be expressed by both notochord and prechordal mesoderm, becoming exclusive to notochord only at stage 6-7 (Fig. 3F,G; Dale et al., 1999 but see Kispert et al., 1995; Stein and Kessel, 1995).

Early extending axial mesoderm containing cells that will normally contribute to both notochord and prechordal

Table 1. Marker profile in prechordal mesoderm (PM) and notochord (NC) over the period stage 4⁺ to 9

	Stage 4⁺/5⁻		Stage 6		Stage 7/8		Stage 9	
	PM	NC	PM	NC	PM	NC	PM	NC
Shh	+	+	+	+	+	+	+	+
gsc	+	+	+	–	+	–	–‡	–
BMP7	–	–	+	–	+	–	+	–
chordin	+	+	+*	+	–	+	–	+
3B9	+	+	–	+	–	+	–	+

*Expression is very weak. See Dale et al., 1999.

‡Expression is no longer detected in prechordal mesoderm cells, but is detected in overlying RDVM cells (M. P., unpublished observations).

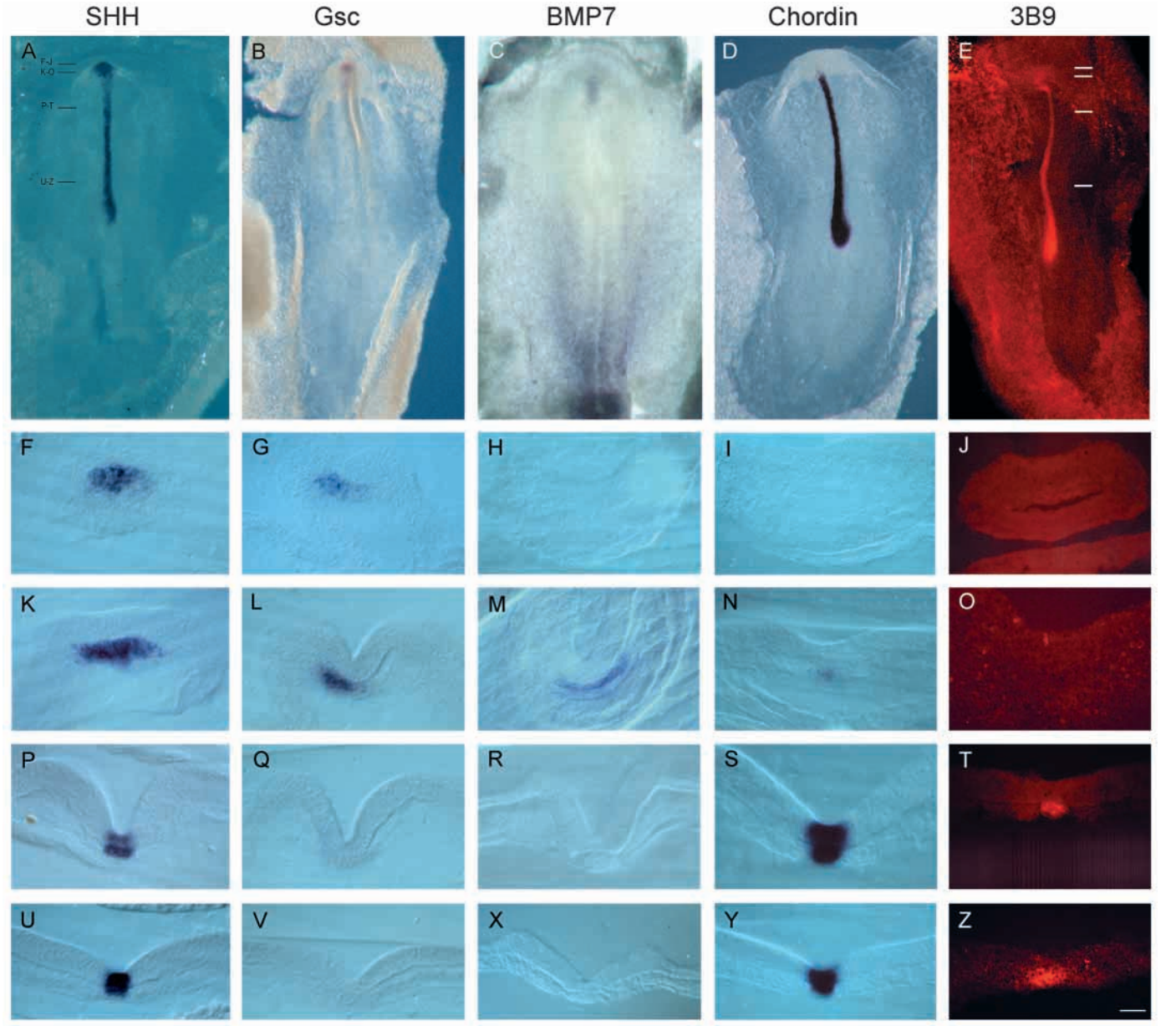

Fig. 1. Molecular characterisation of prechordal mesoderm and notochord. (A-E) Whole-mount analyses of stage 6+ to 7 embryos, after in situ hybridisation (A-D) or immunohistochemical labelling (E). (F-Z) Transverse sections of stage 6+ embryos (*Shh*, *chordin* and 3B9) or stage 7 embryos (*gsc*, *BMP7*), at the levels shown in A and E: prechordal plate (F-J), prechordal mesoderm (K-O), anterior notochord (P-T) and posterior notochord (U-Z). *Shh* is detected throughout axial mesoderm (A,F,K,P,U), expression of *gsc* is restricted to prechordal plate and prechordal mesoderm (B,G,L,Q,V), expression of *BMP7* is confined to prechordal mesoderm (C,H,M,R,X), *chordin* is expressed very weakly in prechordal mesoderm and strongly in notochord (N,S,Y), and 3B9 is expressed only in the notochord (E,J,O,T,Z). Scale bar: A-E, 200 μm; F-Z, 40 μm.

mesoderm thus initially co-expresses markers of notochord and prechordal mesoderm throughout its length (Table 1). This is likely to be due in part to a mixing of the two populations, as documented elsewhere (Foley et al., 1997). However, the high and uniform levels of expression of *netrin-1* and *chordin* on prechordal mesoderm cells argues additionally that, as it extends forwards, prechordal mesoderm expresses 'notochord-like' properties. The exclusive profile of prechordal mesoderm, including the downregulation of these markers and the selective expression of *BMP7*, occurs only once the prechordal mesoderm has migrated to its anteriormost position.

Cells in early extending axial mesoderm are not fully specified to a prechordal mesoderm identity

We next determined whether the marker profiles defining notochord and prechordal mesoderm in vivo (Fig. 1; Table 1) serve as definitive markers of in vitro cultured axial mesoderm explants. Explants of prechordal mesoderm and notochord from stage 5+ to 7 embryos were cultured and examined for expression of *gsc*, *BMP7*, *chordin* and 3B9. Prechordal mesoderm explants aged to the equivalent of stage 8 expressed *gsc* and *BMP7* but did not express *chordin* or 3B9 (100%, *n*=8; Fig. 4B-E). Notochord explants aged to the equivalent of stage

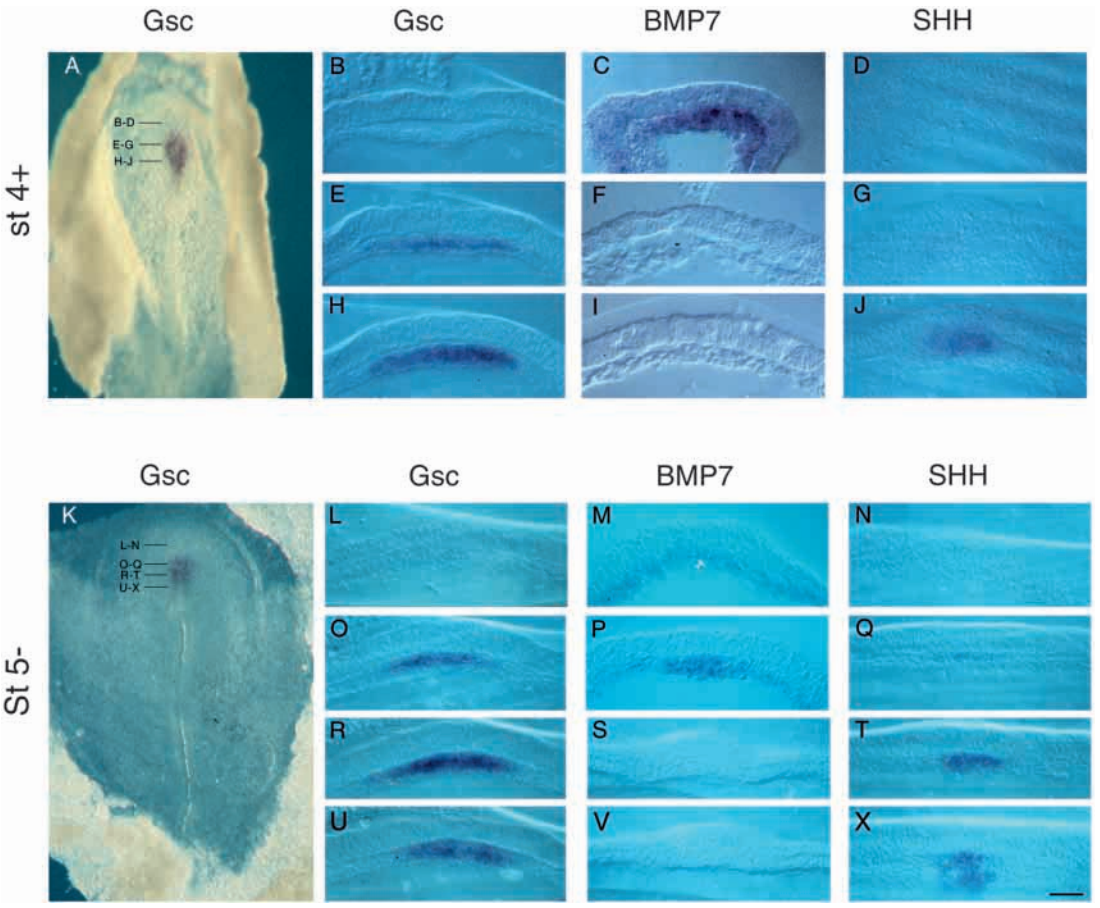

Fig. 2. Development of axial mesoderm. (A,K) Expression of *gsc* after in situ hybridisation in whole-mount embryos at stage 4+ (A) and 5– (K). The lines indicate the sections analysed in B-J and L-X. (B-J) Transverse sections of stage 4+ embryos after in situ hybridisation at the level of hypoblast (B-D), prechordal plate endoderm (E-G) and prechordal mesoderm (H-J). Hypoblast cells express *BMP7* (C), prechordal plate cells express *gsc* (E) and prechordal mesoderm cells express *gsc* and *Shh* but not *BMP7* (H-J). Note that we do not detect *gsc* in the hypoblast in this strain of chick (Bachvarova et al., 1998). (L-X) Transverse sections of stage 5– embryos after in situ hybridisation at the level of hypoblast/ prechordal plate (L-N), prechordal plate (O-Q), prechordal mesoderm (R-T) and notochord (U-X). Expression of *BMP7* is being downregulated in anteriormost endoderm cells (M). Prechordal plate cells express *gsc* and *BMP7* but not *Shh* (O-Q). Prechordal mesoderm and notochord both express *gsc* and *Shh* but not *BMP7* (R-X). At this level of resolution, we cannot establish whether *gsc* is expressed on all cells of the forming notochord, or whether *gsc*-positive and *gsc*-negative cells are intermingled. Scale bar: A,K, 110 μm; B-J, L-X, 35 μm.

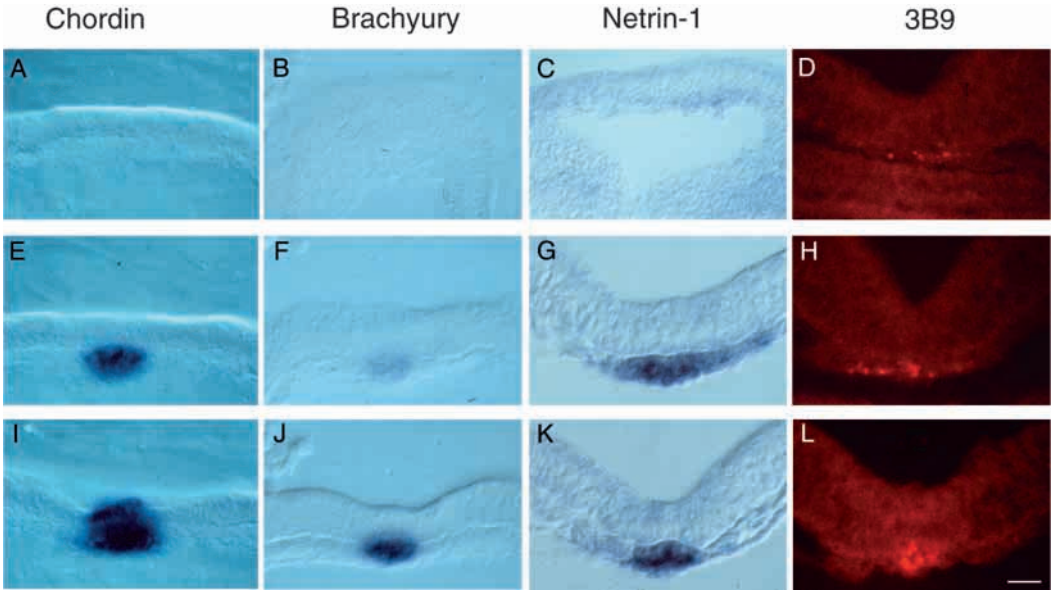

Fig. 3. Expression of notochord markers in early prechordal mesoderm. Transverse sections of chick embryos at the level of prechordal plate (A-D), prechordal mesoderm (E-H) and notochord (I-L) after whole-mount in situ hybridisation and sectioning or immunohistochemical analysis. (A,B,E,F,I,J) Stage 5+ embryos analysed for expression of *chordin* or *brachyury*. Neither is detected in prechordal plate cells (A,B). Both are expressed in medial cells of the prechordal mesoderm and in notochord cells (E,F,I,J). (C,D,G,H,K,L) Stage 6– embryos analysed for expression of *netrin* or 3B9. Both are detected weakly in prechordal plate cells (C,D) and more strongly in prechordal mesoderm and notochord (G,H,K,L). Scale bar: (A-L) 25 μm.

8 expressed *chordin* and 3B9 but did not express *gsc* or *BMP7* (100%, *n*=10; Fig. 6A-D). In conclusion, axial mesoderm that had extended anteriorly maintained regional characteristics after isolation and in vitro culture.

We next asked whether cells within Hensen's node are already specified to a notochord or prechordal mesoderm identity, since the late resolution of markers on these cells in vivo may simply reflect a late, but intrinsic, programme of differentiation. Hensen's node explants isolated and fixed immediately expressed *gsc* and *chordin* (100%, *n*=6, not shown) but did not express *BMP7* or 3B9 (0%, *n*=6, not shown). Hensen's node explants cultured to the equivalent of stage 8 continued to express *gsc* within the centre of the explant, but additionally expressed *chordin* and 3B9 on cells extending outwards at the periphery of the explant (100%, *n*=4; Fig. 4G,I,J). However, expression of *BMP7* was not detected (0%, *n*=7; Fig. 4H). In this experiment, we cannot distinguish whether *gsc*-positive cells within the explant mark early organiser cells, or prechordal-mesoderm cells. However, the absence of *BMP7* expression indicates that none of the cells are fully specified to a *BMP7*-expressing prechordal mesoderm identity.

The failure of Hensen's node to differentiate into *BMP7*-expressing prechordal mesoderm suggests that a later signalling event(s) from tissues other than the node may specify this characteristic. We next examined whether such signalling occurs as prechordal mesoderm cells first migrate from the node. Anteriormost regions of axial mesoderm from stage 5– embryos (containing prechordal mesoderm and notochord precursors: see Foley et al., 1997) were explanted and cultured in vitro. At the time of dissection, these explants expressed *gsc* and *chordin* but not *BMP7* (Fig. 2R,S; Dale et al., 1999). After culture to the equivalent of stage 8, the explants continued to express *chordin*, but downregulated *gsc*, and did not acquire *BMP7* expression (Fig. 4L-N), suggesting that normally, a signalling event that occurs after stage 5– results in the maintenance of *gsc*, the expression of *BMP7* and the downregulation of *chordin* to specify the characteristics of prechordal mesoderm.

Characterisation of stage 3+ to 4+ endoderm

Previous studies have shown that successive waves of endodermal populations migrate anteriorly in the chick embryo. Primary hypoblast cells are pushed anteriorly over the period stage 3-4, being replaced in more posterior regions by sickle hypoblast (endoblast). Prechordal plate (definitive, or embryonic) endoderm further displaces the primary hypoblast over the period stage 4-5 (Bachvarova et al., 1998; Bellairs, 1986). Two likely sources of a factor(s) that could specify prechordal mesoderm characteristics in anteriormost regions of extending axial mesoderm are primary hypoblast and/or prechordal plate endoderm. In the mouse, the anterior visceral endoderm (AVE), the mouse equivalent of the primary hypoblast, is required for formation of the forebrain (Beddington and Robertson, 1998). Although, in chick, primary hypoblast is not thought to function analogously to the AVE, directly regulating forebrain formation (Knoetgen et al., 1999), it remains possible that it plays a role in the specification of axial mesoderm.

To identify whether the primary hypoblast and prechordal

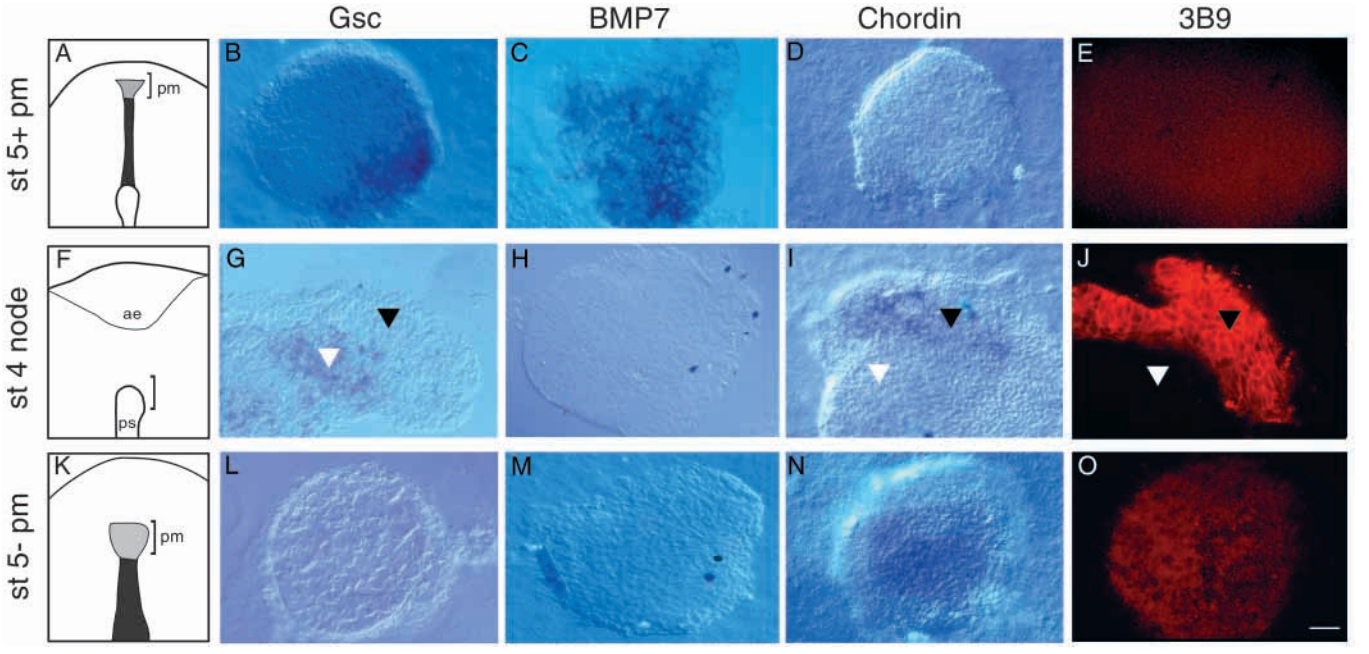

Fig. 4. Specification of *BMP7* in prechordal mesoderm occurs at stage 5+. Expression of axial mesodermal markers in cultured explants after in situ hybridisation (B-D,G-I,L-N) or immunohistochemical labelling (E,J,O). (A,F,K) Diagrams, showing regions isolated and cultured (brackets). (B-E) After 18 hours in culture, stage 6 prechordal mesoderm expresses *gsc* in a subset of the cells (B), expresses *BMP7* uniformly (C) but does not express *chordin* (D) or 3B9 (E). (G-J) After 24 hours in culture, a stage 4 node explant expresses *gsc* in cells that remain in the centre of the explant (white arrowhead, G), and expresses *chordin* (I) and 3B9 (J) in cells that appear to extend at the perimeter of the explant (black arrowhead), but does not express *BMP7* (H). (L-O) After 22 hours in culture, stage 5– anteriormost axial mesoderm expresses *chordin* (N) but does not express *gsc* (L), *BMP7* (M) or 3B9 (O). Scale bar: (B-E,G-J,L-O) 30 μm.

plate are capable of patterning extending axial mesoderm, we first established that we could accurately recognise each. At stage 3+ to 4–, anterior endoderm, which is composed primarily of hypoblast cells, co-expresses *BMP2*, *Otx-2* and high levels of *Hex* (Andree et al., 1998; Bally Cuif et al., 1995; Yatskievych et al., 1999). In the Hysex hybrid chicks analysed here, we detect a similar profile (not shown), but additionally detect both *BMP4* (not shown) and *BMP7* (Fig. 5A) in the hypoblast. Similarly, at stage 4+ anteriormost endoderm (composed of the remnants of anterior hypoblast together with prechordal plate) expresses *BMP2*, *BMP4*, *BMP7* (Fig. 5B and not shown) and *Hex* (Fig. 5C). In addition, *gsc* but not *Shh* is now detected in the anterior endoderm (Fig. 2E,G).

Posteriorly located embryonic endoderm did not share the same profile of markers as anterior endoderm. Weak expression of *Hex* and *BMP4* was occasionally detected in endoderm immediately adjacent to Hensen's node at stage 3+ (not shown), but was never detected after stage 4 (Fig. 5B). In addition, although *BMP7* was weakly detected in lateral hypoblast and sickle endoblast, no expression was detected in posterior-medial embryonic endoderm over the period stage 3+ to 4+ (Fig. 5A).

The markers defining distinct regions of endoderm were then used to establish that we could accurately dissect subdomains of endoderm over the period stage 3+ to 4+. Endodermal explants were dissected as shown (boxed regions in Fig. 5A,C), cultured and examined with combinatorial markers. Explants from each region faithfully expressed appropriate markers after culture (Table 2; Fig. 5D-F), indicative of their accurate dissection.

Table 2. Marker profile in cultured endoderm explants

	Stage 3+			Stage 4–			Stage 4+	
	ae	ee (m)	ee (l)	ae	ee (m)	ee (l)	ae	ee
BMP7 (<20 hours)	+	–	–	+	–	–	+	–
BMP7 (>20 hours)	–	–	–	–	–	–	–	–
hex	+	–	–	+	–	–	+	–
Otx2	+	–	–		ND		ND	
Shh	–	–	–	–	–	–	–	–
gsc	–	–	–	–	–	–	+	–

n=4 to 6 explants each.

Abbreviations: ae, anterior endoderm; ee (m) medial posterior embryonic endoderm; ee (l), mediolateral posterior embryonic endoderm. See Bachvarova et al., 1998.

Specification of *BMP7* in prechordal mesoderm by anterior endoderm

To determine whether anterior endoderm can affect the character of migrating axial mesoderm, recombinates of stage

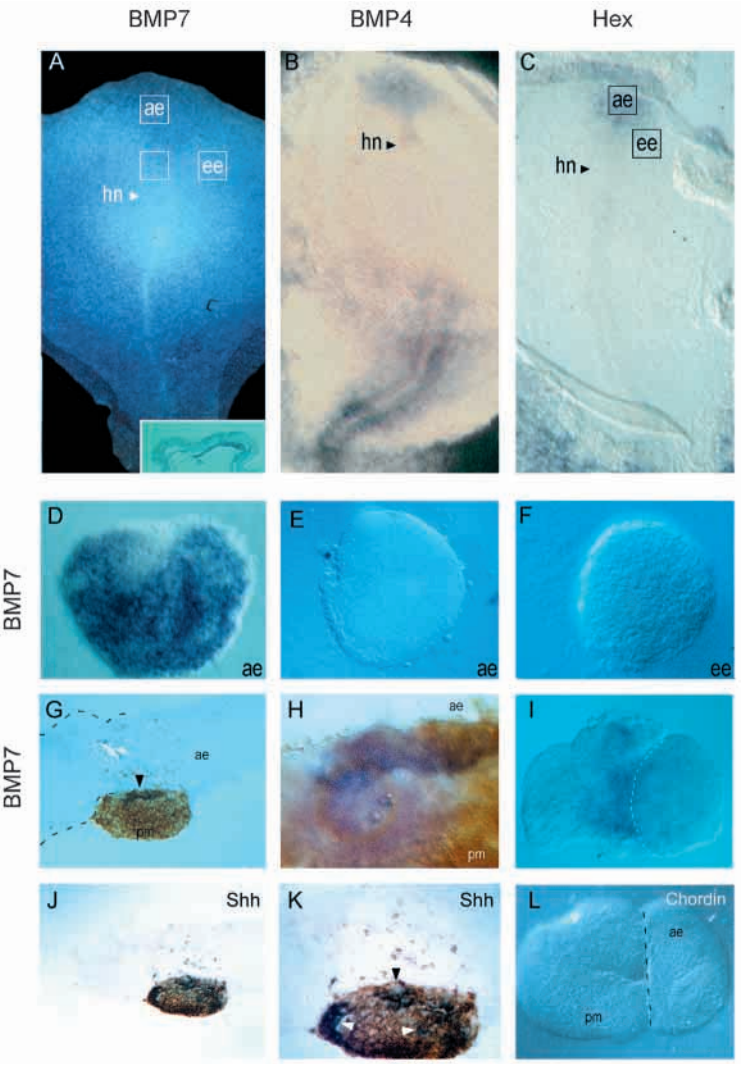

Fig. 5. Specification of prechordal mesoderm by anterior endoderm. (A-C) Stage 4– and 4+ embryos, after whole-mount in situ hybridisation with *BMP7* (A; stage 4–), *BMP4* (B; stage 4+) and *hex* (C; stage 4+). Expression of all three genes is detected in anteriorly situated endoderm comprising hypoblast and prechordal plate. Boxes depict the regions assayed for ability to specify prechordal mesoderm character. Inset in A shows section at level of hypoblast. (D-F) Endodermal explants, cultured and analysed for expression of *BMP7*. (D) Stage 3+ anterior endoderm cultured for 18 hours expresses *BMP7*. (E) No expression of *BMP7* is detected in a stage 3+ anterior endoderm explant cultured for 24 hours. (F) No expression of *BMP7* is detected in stage 3+ posterior embryonic endoderm cultured for 18 hours. (G,H) Section through a recombinate of stage 3+ chick hypoblast and stage 5– quail anterior axial mesoderm, double-labelled to detect QCPN (brown) and *BMP7* (blue). *BMP7* is detected within the quail tissue in a confined region closest to the hypoblast. Migrating quail cells can be detected within the chick tissue. H shows a high-power magnification of part of G. (I,L) Serial adjacent sections through recombinates of stage 3+ chick hypoblast and stage 5– chick anterior axial mesoderm analysed for expression of *BMP7* (I) or *chordin* (L). Expression of BMP7 is detected in axial mesoderm that abuts the hypoblast. No expression of Chordin is detected in the prechordal mesoderm. (J,K) Serial adjacent section to that shown in G,H, double-labelled to detect QCPN (brown) and *Shh* (blue). *Shh* is detected both in *BMP7*-positive cells (black arrowheads in G,K) and in *BMP7*-negative cells throughout the mesodermal explant (white arrowheads). Abbreviations: ae, anterior endoderm; hn, Hensen's node; ee, embryonic endoderm; pm, prechordal mesoderm. Dotted lines depict the interface between prechordal mesoderm and hypoblast (anterior endoderm).

3+ anterior endoderm and anteriormost regions of stage 5– quail axial mesoderm were cultured. Induction of *BMP7* was observed in cells that co-expressed the quail-specific marker QCPN (100%, *n*=5; Fig. 5G,H) and was most apparent in cells closest to the anterior endoderm. Concommitantly, *chordin* expression was downregulated in the quail tissue (0%, *n*=3; Fig. 5L) and *gsc* expression was maintained (100%, *n*=4, not shown). The specificity of this effect was tested by monitoring the ability of other regions of endoderm to induce *BMP7* within similar explants. HH stage 4– to 4+ anterior endoderm could mimic the action of the stage 3+ anterior endoderm (100%, *n*=5 each). In contrast, posterior embryonic endoderm from stage 3+ to 4+ embryos did not induce expression of *BMP7* within anteriormost regions of axial mesoderm (0%, *n*=6 each).

These experiments support the idea that signals that derive from anterior endoderm govern the character of extending axial mesoderm, causing it to downregulate *chordin*, maintain expression of *gsc* and express *BMP7* once it migrates anteriorly. However, they do not distinguish whether the anterior endoderm causes extending axial mesoderm to adopt a definitive prechordal mesoderm fate, or to assume the fate of prechordal plate endoderm (see Table 2). To examine this, recombinates were examined for expression of *Shh*. *Shh* was detected throughout the mesodermal explant(100%, *n*=4; Fig. 5J,K), overlapping with *BMP7* in regions directly abutting the endoderm (Fig. 5G,K arrowheads). In the embryo, prechordal mesoderm co-expresses *Shh* and *BMP7* (Fig. 1K,M), whereas the two genes appear mutually exclusive in prechordal plate (Figs 1F,H, 2P,Q). This suggests that axial tissue remains mesodermal in character after culture with endoderm.

Anterior endoderm can induce prechordal mesoderm from notochord in vitro

Fate-mapping experiments have shown that early extending axial mesoderm contains a mixture of prechordal mesoderm and notochord cells that ultimately sort along the anterior-posterior axis (Foley et al., 1997). We wished to examine whether, in addition to such sorting, a cell-fate switch may occur, resulting in anteriormost axial mesoderm cells displaying uniform prechordal mesoderm character. The finding that anterior endoderm can repress *chordin* and induce *BMP7* in anteriormost regions of extending axial mesoderm does not address whether it affects only the character of partially specified prechordal mesoderm cells or whether it can additionally affect intermingled notochord cells. We therefore wished to establish whether signalling by the anterior endoderm can affect the character of notochord cells. To do so, we asked whether anterior endoderm can alter the fate of notochord cells to a prechordal mesoderm identity, both suppressing notochord characteristics and inducing prechordal mesoderm characteristics. Endodermal explants were cultured as conjugates with stage 5+ to 7 notochord, and the recombinates examined for expression of *gsc*, *BMP7*, *chordin* and 3B9. Posteriorly situated embryonic endoderm did not affect the character of cultured notochord (Table 3). In contrast, anterior endoderm from stage 3+ to 4+ embryos induced prechordal mesoderm characteristics and suppressed notochord characteristics in the notochord (Fig. 6; Table 3). Expression of *gsc* and *BMP7* was induced within notochord that bordered the anterior endoderm (compare Fig. 6A with E, and B with F). Concommitantly, expression of the notochord markers *chordin* and 3B9 was either completely lost or dramatically reduced (compare Fig. 6C with G, and D with H) within the notochord explant. Expression of *Shh* was detected throughout the notochord explant (not shown). Anterior endoderm can therefore provide signal(s) that act directly on axial mesoderm, changing the fate of notochord to prechordal mesoderm.

A comparison of serial adjacent sections from recombinates of anterior endoderm and notochord suggested that the induction of *gsc* and *BMP7* occurred in the mesoderm immediately adjacent to the anterior endoderm, whereas expression of *chordin* and 3B9 was suppressed over a greater distance. To determine whether this reflects that anterior endoderm exerts both short-range and long-range actions to pattern axial mesoderm, anterior endoderm explants were cultured at a distance from notochord explants. In these instances, 3B9 was again lost from the notochord explants (Fig. 6L). In contrast, notochord explants continued to express *chordin* and did not express *gsc* or *BMP7* (Fig. 6I-K; Table 3).

Finally, we used the in vitro assay to determine the period over which the notochord remains competent to repond to the anterior endoderm. Newly formed notochord that emerges from Hensen's node over the period stage 5+ to 11 (the latest stage examined) displayed an equal ability to respond to anterior endoderm by adopting prechordal mesoderm characteristics (Table 3). However, the competence of notochord to respond to this signal rapidly declines: notochord explants aged in vitro for 8-12 hours no longer lose expression of 3B9, or express *BMP7* in response to anterior endoderm signals (Table 3).

Anterior endoderm can induce prechordal mesoderm from notochord in vivo

To ascertain whether anterior endoderm can alter the character

Table 3. Induction of prechordal mesoderm characteristics by endoderm

	Stage 3^+ endoderm-stage 5^+/6 NC			Stage 4^+ endoderm-stage 5^+/6 NC		Stage $3^+/4^+$ ae-stage 5^+/6 NC (distant)	Stage 3^+ ae-stage 5^+-10 nascent NC	Stage $3^+/4^+$ ae-stage 5^+-10 aged NC
	ae-NC	ee (m)-NC	ee (l)-NC	ae-NC	ee-NC			
gsc	+	–	–	+	–	–	+	ND
*BMP7**	+	–	–	+	–	–	+	–
chordin	–	+	+	–	+	+	ND	ND
3B9	–	+	+	–	+	–	–	+

The chart shows the marker profile of notochord (NC) explants cultured with endoderm from stage 3^+ or 4^+ embryos.
Abbreviations: as in table 2.
*All analyses with *BMP7* were performed at times >20 hours in culture, when *BMP7* expression is downregulated in the anterior endoderm explants.
n=3 to 8 each.

of nascent notochord in an in vivo context, we next exposed nascent notochord to anterior endoderm in vivo. Anterior endoderm explants were grafted into stage 10 chick embryos beneath newly formed notochord (Fig. 7G,N) that co-expressed Shh and 3B9 but expressed neither *BMP7* nor *gsc* (*n*=10; Fig. 7A-F). After incubation to stage 12 or stage 13, operated embryos were examined with the axial mesoderm markers Shh, 3B9, *BMP7* and *gsc* in two regions: (1) a region 300-400 μm posterior to the graft and (2) a region above the graft. In the non-operated region posterior to the graft, notochord cells maintained their normal identity, expressing Shh and 3B9 but neither *BMP7* nor *gsc* (Fig. 7H-J,L,M). In contrast, in regions adjacent to the graft, Shh expression was maintained in the axial mesoderm (Fig. 7P), but the notochord downregulated 3B9 and showed a weak upregulation of *BMP7* (Fig. 7Q,S). In addition, in chicks cultured to stage 12 (*n*=4), upregulation of the prechordal mesoderm marker, *gsc*, was observed in notochord cells (Fig. 7U). This upregulation was not detected in embryos cultured to stage 13 (*n*=4, Fig. 7T), paralleling the normal transient expression of *gsc* in prechordal mesoderm in situ (Table 1 and not shown). Downregulation of the notochord marker 3B9 was detected over a distance of approx. 300 μm, centred around the grafted endoderm. In contrast, upregulation of *BMP7* and *gsc* occurred in a restricted domain (approx. 70 μm) that correlated exactly with the length of the underlying grafted endoderm. The differences in the ability of anterior endoderm to downregulate 3B9 at a distance, but to induce *BMP7* and *gsc* at close range mimic the effects observed in vitro (Fig. 6).

The in vivo results provide further support for the idea that signalling from anterior endoderm can convert notochord into prechordal mesoderm. Nonetheless, both the in vitro and the

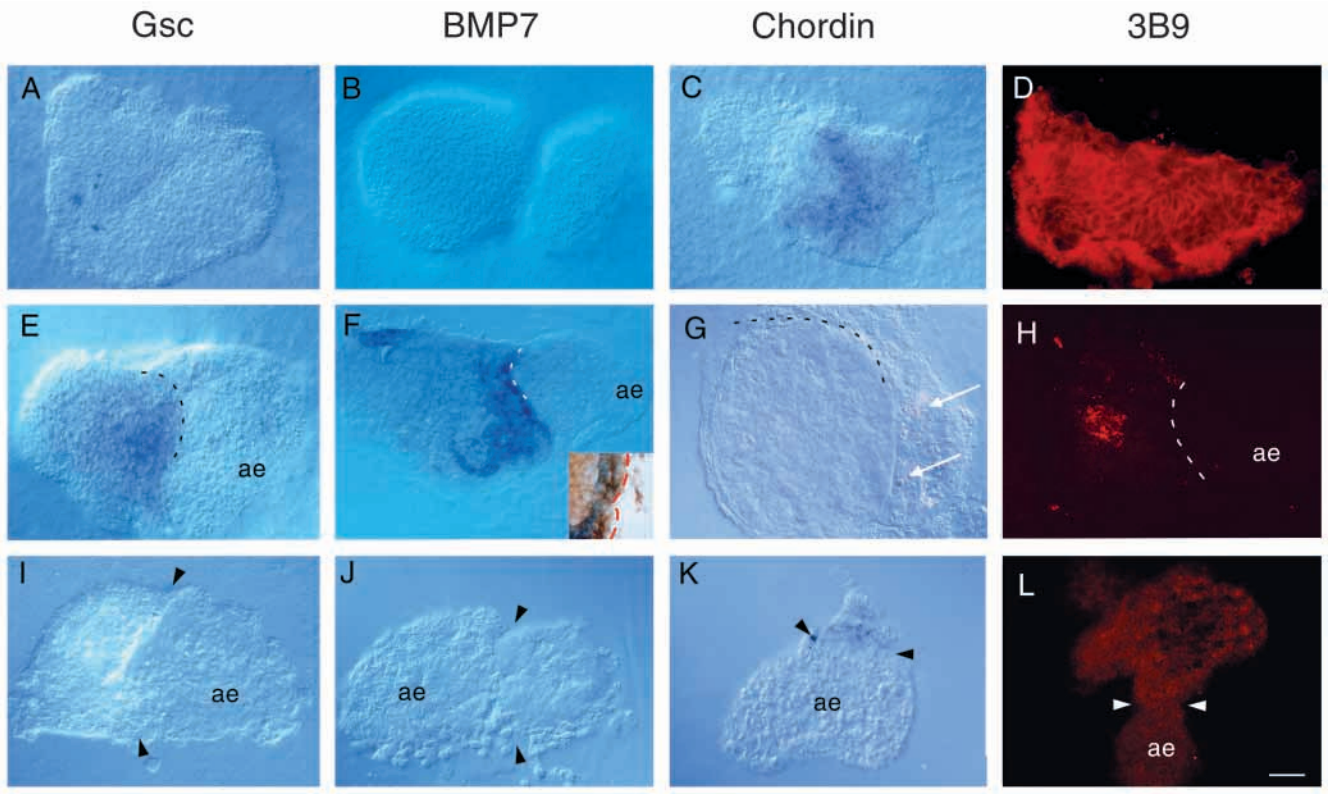

Fig. 6. Anterior endoderm changes the identity of notochord to prechordal mesoderm in vitro. Explants, cultured in vitro and assayed by in situ hybridisation and/or immunohistochemical labelling. (A-D) Notochord explants, cultured alone (A-C, whole-mount views; D, a section). (A) Cultured stage 7 notochord does not express *gsc*. (B-D) Stage 6 notochord explants cultured for 18 hours do not express *BMP7* (B), but express *chordin* (C) and 3B9 (D). (E-H) Conjugates of anterior endoderm and notochord (E, whole-mount view; F-H, sections). (E) Stage 7 chick notochord cultured with stage 3+ quail anterior endoderm. *gsc* expression is detected in the notochord, at the junction with the anterior endoderm. (F) Stage 6 chick notochord cultured with stage 4 chick anterior endoderm. *BMP7* expression is detected in the notochord, at the junction with the endoderm. Inset: stage 6 quail notochord cultured with stage 4 chick anterior endoderm, double-labelled to detect *BMP7* (blue) and the quail-specific marker, QCPN (brown). Expression of *BMP7* is detected in quail cells that are immediately adjacent to the anterior endoderm. (G) Stage 4 quail anterior endoderm cultured with stage 6 chick notochord, double-labelled to detect the quail marker QCPN (silvery-brown, marked with arrows) and *chordin* (blue). No expression of *chordin* is detected in the notochord explant. (H) Serial adjacent section to that shown in F. 3B9 is repressed throughout almost the entire notochord explant. (E-H) Dotted lines indicate half of the border of notochord and anterior endoderm. (I-L) Explants of anterior endoderm and notochord cultured at a distance (I,J, whole-mount views; K,L, sections). Note that the explants have just grown together (borders indicated by arrowheads). (I) Stage 7 chick notochord cultured with stage 4+ anterior endoderm in the absence of initial contact. No expression of *gsc* is observed in the notochord. (J) Stage 6 chick notochord cultured with stage 4+ anterior endoderm in the absence of initial contact. No expression of *BMP7* is detected in the notochord. (K) Stage 6 chick notochord cultured with stage 4 anterior endoderm in the absence of initial contact. *chordin* is expressed in the notochord. (L) Stage 6 chick notochord cultured with stage 4 anterior endoderm in the absence of initial contact. No expression of 3B9 is detected in the notochord. Scale bar: (A-L) 40 μm; inset in F, 10 μm.

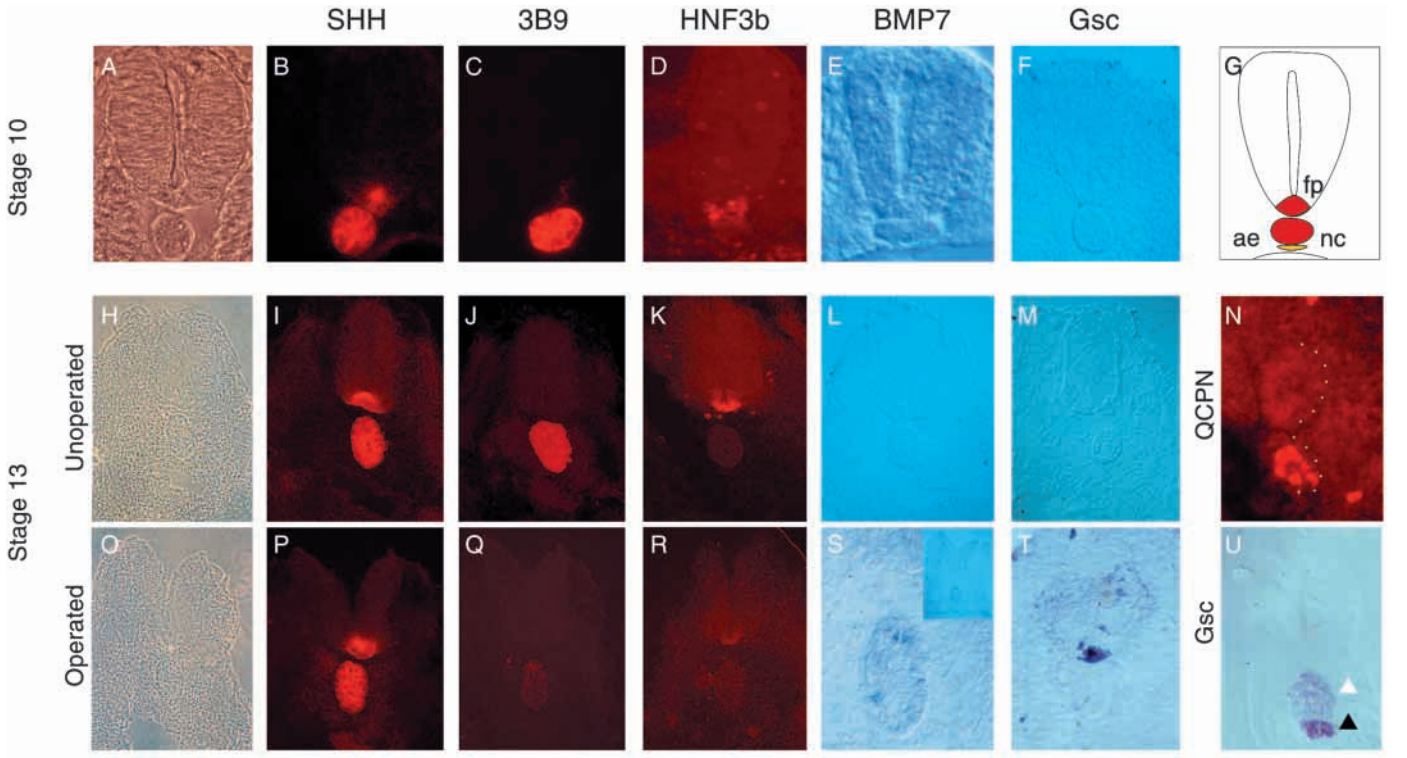

Fig. 7. Anterior endoderm changes the identity of notochord to prechordal mesoderm in vivo. (A-F,H-M) Expression profile of midline markers in controls. Serial adjacent transverse sections through (A-F) posterior spinal cord regions of control stage 10 embryos and (H-M) spinal cord regions posterior to operated regions of a stage 13 embryo (see diagram, G). (A,H) Phase images; (B,I) SHH expression in notochord and floor plate; (C,J) 3B9 expression in notochord; (D,K) HNF3β expression in floor plate; (E,L) no detectable expression of *BMP7* expression in notochord or floor plate; (F,M) no expression of *gsc* in notochord or floor plate. (G) Schematic diagram, illustrating the position of the anterior endoderm graft beneath the notochord. (N) Transverse section of stage 12 embryo after a graft of quail anterior endoderm cells beneath the notochord at stage 10. The grafted cells, immunolabelled to detect QCPN, are seen beneath the notochord (half outlined). In addition, a quail cell can be seen migrating away from the graft. (O-T) Serial adjacent transverse sections through posterior spinal cord regions of a stage 13 embryo that had received a stage 4– chick anterior endoderm graft at stage 10. (O) Phase-contrast image. (P) Notochord and overlying neural tube cells express SHH. (Q) No expression of 3B9 is detected in notochord. (R) HNF3β is downregulated in ventral midline cells. (S) High-power image showing that axial mesoderm cells adjacent to the graft weakly express *BMP7*. Inset shows the same section at low magnification. (T) Ventral midline cells express *gsc*. (U) Transverse section through a stage 12 embryo after an anterior endoderm (stage 4+) graft at stage 10, showing *gsc* expression. Strong expression is detected in the graft (black arrowhead). In addition, weaker expression is detected in the adjacent notochord (white arrowhead). In addition to the changes in axial mesoderm and ventral midline neurectoderm cells, the general morphology of the neural tube was affected; in particular, a failure of dorsal closure was observed. Note that the graft in U was from a stage 4+ embryo, whereas that in T was from a stage 4 embryo, accounting for the differential expression of *gsc* (Table 2). Note also that expression of *gsc* in the ventral neural tube in T, but not in U, reflects the older (stage 13) embryo in T. Scale bar: (A-F,H-R) 28 μm; (S) 15 μm; (T) 25 μm; (U) 20 μm.

in vivo experiments rely on the combinatorial expression of Shh, *BMP7* and *gsc* to define prechordal mesoderm, the analysis of which is performed on serial adjacent sections. To further strengthen the evidence that exposure to anterior endoderm can convert notochord to a prechordal mesoderm identity, we therefore examined whether exposure to anterior endoderm results in the acquisition of novel functional properties by the notochord. Previously we have shown that the restricted expression of *BMP7* in prechordal mesoderm confers it with functional properties distinct from those of the notochord. The co-expression of BMP7 and Shh in prechordal mesoderm appears to enable this tissue to induce ventral forebrain cells that downregulate *HNF3β*, and co-express *Shh*, *BMP7* and *gsc* (Dale et al., 1999 and not shown). Analysis of the ventral neural tube within operated regions of stage 13 embryos in the current experiments showed a downregulation of HNF3β (compare Fig. 7K,R), a maintenance of Shh (Fig. 7P) and an upregulation of *gsc* (compare Fig. 7M,T), while embryos cultured to stage 14 weakly expressed *BMP7* in the ventral midline (*n*=2, not shown). Together these experiments suggest that ventral midline cells of the neural tube within the operated territory have been specified to a rostral diencephalic midline fate and provide indirect evidence that proximity to anterior endoderm confers the axial mesoderm with the functional properties of prechordal mesoderm.

TGFβs mediate the action of anterior endoderm

We next asked whether known factors present in anterior endoderm can mimic its ability to pattern axial mesoderm, focusing on the TGFβ superfamily members BMP2, BMP7 and activin because of their restricted expression in chick anterior endoderm and their documented ability to affect the character of chick cardiac mesoderm (Figs 2C, 5A,B; Andree

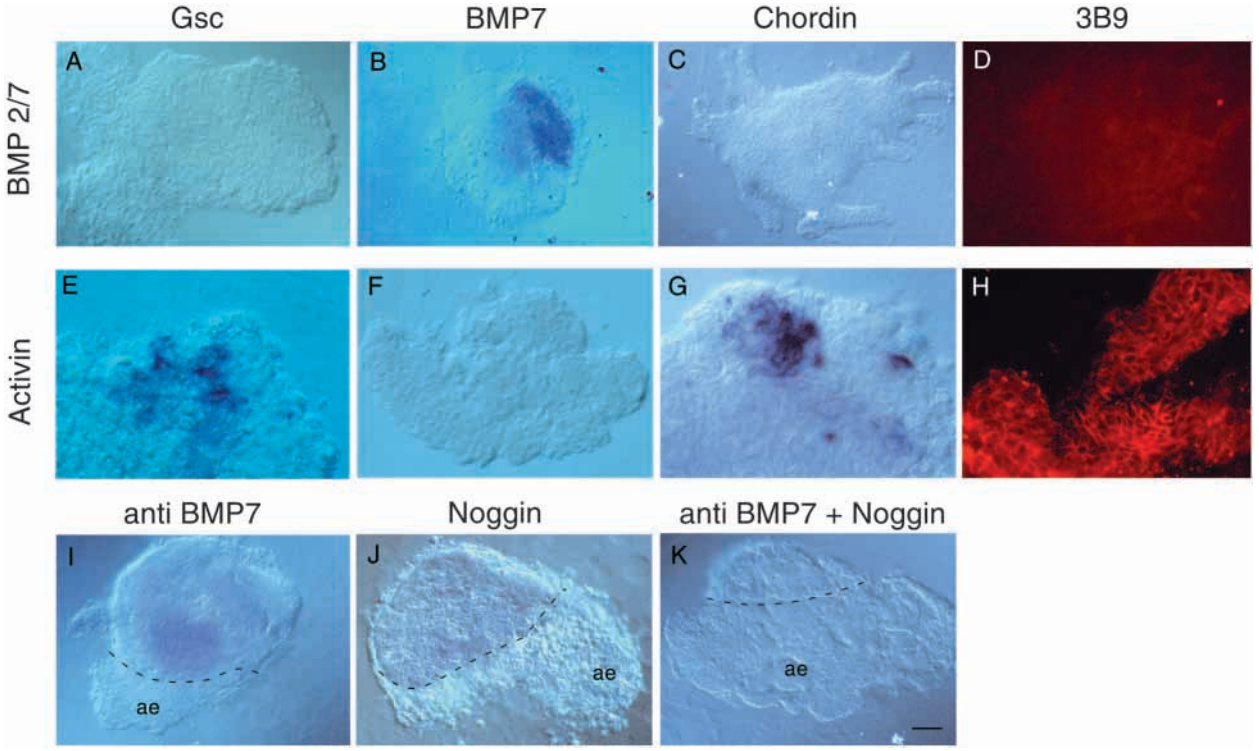

Fig. 8. TGFβs mediate the ability of anterior endoderm to induce prechordal mesoderm character. (A-D) Notochord explants cultured with a combination of 5 nM BMP2 and 5 nM BMP7. Explants do not express *gsc* (A), express *BMP7* in a portion of the explant (B) and lose expression of *chordin* and 3B9 throughout the explant (C, D). (A-C) Whole-mount views; (D) a section. (E-H) Notochord explants cultured with 20 units activin. Explants express *gsc*, *chordin* and 3B9 (E,G,H) but do not express *BMP7* (F). (I,J) Weak expression of *BMP7*, detected through whole-mount in situ hybridisation, in notochord-anterior endoderm recombinates, cultured with (I) anti-BMP7 antibody or (J) Noggin. (K) No expression of *BMP7* is detected in a notochord-anterior endoderm recombinate, cultured with a combination of anti-BMP7 antibody and Noggin. The dotted lines in I-K demarcate the boundary of notochord and anterior endoderm. Scale bar: (A-D,F-H,I-K) 45 μm; (E,G) 40 μm.

et al., 1998; Ladd et al., 1998; Lough et al., 1996; Schultheiss et al., 1997; Sugi and Lough, 1995; Yatskievych et al., 1997). Stage 5-7 notochord explants were cultured in the presence of BMP2, BMP7 or activin, then examined for expression of *gsc*, *BMP7*, *chordin* and 3B9. BMP2 and BMP7 exhibited similar activity, both repressing notochord markers, inducing the prechordal mesoderm marker, *BMP7* but not inducing expression of *gsc*. At low concentrations (1 nM), both BMPs began to suppress expression of 3B9 in the notochord explants (Table 4). At higher concentrations (5-10 nM), they each suppressed expression of both 3B9 and *chordin* and induced expression of *BMP7* within the notochord explants (Table 4; Fig. 8B-D). Activin did not suppress the expression of the notochord markers 3B9 or *chordin*, but did induce *gsc* expression in the notochord explants (Fig. 8E-H). This suggests that expression of BMP2, BMP7 and activin in anterior endoderm can mediate distinct aspects of its ability to specify prechordal mesoderm.

Table 4. TGFβ molecules differentially suppress notochord character and induce prechordal mesoderm character in notochord explants

	gsc	*BMP7*	*chordin*	3B9
Notochord	0% (6)	0% (15)	100% (4)	100% (20)
Notochord+BMP7 1 nM	0% (3)	0% (5)	100% (2)	50% (8)
Notochord+BMP2 1 nM	ND	0% (3)	100% (3)	50% (4)
Notochord+BMP7 10 nM	0% (3)	83% (6)	0% (3)	20%* (5)
Notochord+BMP2 10 nM	ND	100% (3)	33% (3)	40%* (5)
Notochord+BMP7 5 nM +BMP2 5 nM	0% (3)	100% (5)	0% (3)	0% (5)
Notochord+Activin	100% (4)	0% (4)	100% (3)	100% (4)

Number of explants analysed indicated in parentheses.
*In these cases, some suppression of 3B9 was observed, but not throughout the explant.

The ability of activin to induce expression of *gsc* in notochord explants, but not to inhibit the expression of notochord markers, is consistent with previous observations (Knezevic et al., 1995; Mitrani et al., 1990; Stern et al., 1995; Ziv et al., 1992). However, the finding that BMPs specify prechordal mesoderm character is more unusual, given their ability to ventralise/posteriorise mesoderm (Harland and Gerhart, 1997; Slack, 1994; Tonegawa et al., 1997). We therefore performed further experiments to determine whether BMPs are required for the ability of anterior endoderm to pattern axial mesoderm. To do so we preincubated anterior endoderm with function-blocking antibodies to BMP7 then recombined it with notochord, or cultured anterior endoderm-notochord recombinates in the presence of noggin. Exposure of the explants to either function-blocking BMP7 antibody, or to noggin alone did not prevent the induction of *BMP7* in the notochord explants, although the labelling was weak and diffuse (Fig. 8I,J; *n*=7 each). However, exposure to a

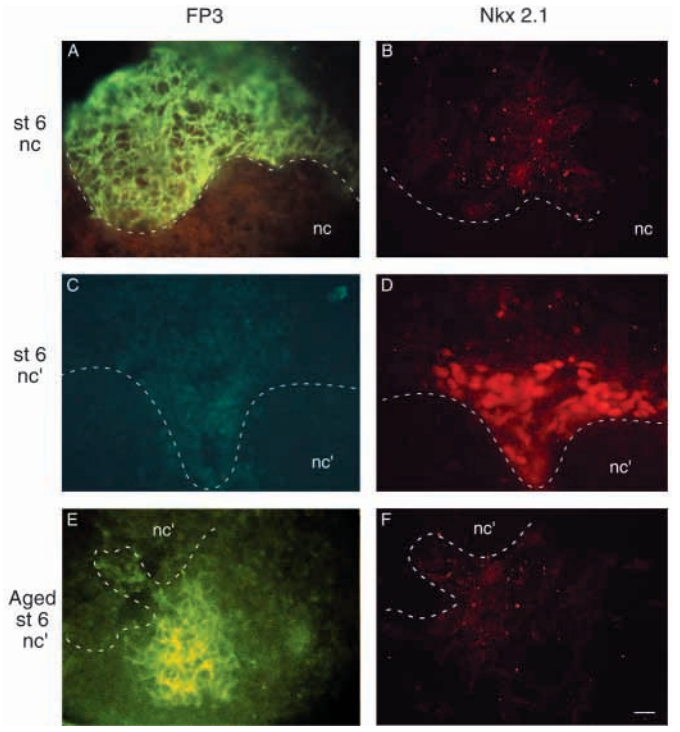

Fig. 9. Induction of prechordal mesoderm function by BMP7. (A,B) A stage 6 notochord explant (nc) recombined with rat lateral neural plate induces a floor plate marker, FP3 (A), and does not induce a forebrain ventral midline marker, Nkx2.1 (B). (C,D) A stage 6 notochord explant, pre-exposed to BMP7 (nc′) and then recombined with rat lateral neural plate does not induce FP3 (C) but instead induces Nkx2.1 (D). (E,F) A stage 6 notochord explant aged for 10 hours, then exposed to BMP7 (nc′), and recombined with rat lateral neural plate induces FP3 (E) and not Nkx2.1 (F). The dotted lines demarcate the boundary of notochord and neural tissue. Scale bar: (A-F) 17 μm.

combination of anti-BMP7 antibody and noggin prevented the ability of anterior endoderm to induce *BMP7* within notochord explants (Fig. 8K; *n*=6).

Finally, to provide evidence that BMPs can induce, in axial mesoderm, the functional properties of prechordal mesoderm, we performed a 2-step functional assay. Notochord explants were treated with BMP2 and BMP7 under conditions that would induce them to express *BMP7* itself (see Fig. 8B), and were then conjugated with responsive neural tissue. Control untreated notochord explants induced the differentiation of floor plate cells that expressed FP3 and did not express the forebrain ventral marker Nkx2.1 (Fig. 9A,B). Similarly, notochord explants aged in vitro to a time where they have lost competence for *BMP7* induction (Table 3), then treated with BMP2 and BMP7, induced floor plate cells and not forebrain ventral midline cells (Fig. 9E,F). This control suggests that the exogenous BMPs do not simply stick to the notochord explants, and then co-operate with the notochord to induce Nkx2.1. In contrast, explants of nascent notochord pre-exposed to BMP2 and BMP7 induced the differentiation of forebrain ventral cells that expressed Nkx2.1 and not FP3 (Fig. 9C,D). Exposure to BMP2/BMP7 therefore can cause axial notochord to acquire certain phenotypic and functional properties of prechordal mesoderm.

DISCUSSION

The work described here provides evidence that prechordal mesoderm specification in the chick may be dependent upon signals deriving from anterior endoderm. Although formal proof for this requires the selective elimination of anterior endoderm, an experiment that we have not yet performed, our initial experiments suggest that the normal anterior-migration of axial mesoderm results in its exposure to signals that derive initially from the hypoblast and then from the prechordal plate, that may act to specify prechordal mesoderm identity. The signals that derive from anterior endoderm appear to both suppress notochord markers (see also Brickman et al., 2000) and elicit prechordal mesoderm markers, hence governing the late specification of prechordal mesoderm and, additionally, changing the fate of notochord cells to a prechordal mesoderm identity. Together, such signalling may act to produce uniform prechordal mesoderm character in a mixed cell population and govern the spatial extent of the prechordal mesoderm. Our studies suggest that TGFβs mimic different aspects of the ability of anterior endoderm to specify prechordal mesoderm identity in extending axial mesoderm. Potential candidates are BMP2, BMP4, BMP7 and activin. We show here that *BMP4* and *BMP7* are initially expressed in the anterior hypoblast during early gastrulation and subsequently expressed also on prechordal plate endoderm, while previous studies have shown that activin and *BMP2* are expressed in anterior endoderm (Andree et al., 1998; Schultheiss et al., 1997; Sugi and Lough, 1995; Yatskievych et al., 1997). BMP2 and BMP7 can mimic the action of anterior endoderm in suppressing notochord characteristics and inducing expression of *BMP7* in axial mesoderm, while activin can mimic the ability of the anterior endoderm in maintaining or inducing *gsc* expression in axial mesoderm. All three may therefore co-operate in the embryo to specify axial mesoderm to a prechordal mesoderm identity.

Distinct signalling centres act to specify prechordal mesoderm

A number of reports have suggested that, in a range of species, notochord and prechordal mesoderm precursors arise in the organiser in response to signalling by members of the TGFβ superfamily such as activin,Vg-1 and nodal (reviewed in Harland and Gerhart, 1997; Kodjabachian and Lemaire, 1998; Schier and Shen, 2000).

Our studies suggest that, in the chick, axial mesoderm cells may not be fully committed in the node, but instead can be subverted by anterior endoderm. Although *gsc* is restricted to cells in Hensen's node and is then expressed on migrating prechordal mesoderm cells, other aspects of prechordal mesoderm specification occur only later, in apparent response to signalling by anterior endoderm. Taken together with other studies, our experiments suggest a model in which the differentiation of chick axial mesoderm occurs in a stepwise fashion, in which TGFβ signalling operates sequentially. In the first step, notochord and prechordal mesoderm precursor cells form within an area of the embryo devoid of BMP signalling (Fig. 5A), in response to Vg1- and activin-like signals (Joubin and Stern, 1999; Stern et al., 1995). Our experiments show that as they migrate anteriorly, axial mesoderm cells continue to express markers indicative of their exposure to activin-like

signalling, including *gsc*, *chordin* and 3B9. We suggest that, in the second step, the convergent extension of axial mesoderm results in the exposure of anterior and posterior regions to distinct signals. We find that the continued absence of BMP signalling is a prerequisite for the maintenance of notochord character in posterior regions of the axial mesoderm, but that the anterior migration of axial mesoderm results in its re-exposure to TGFβ signals that are now confined to anterior endoderm. Our findings are consistent with the idea that the exposure of anteriormost regions of axial mesoderm to BMPs and activin-like signals are required for its specification to a prechordal mesoderm identity.

Such a 2-step model for prechordal mesoderm specification bears a remarkable similarity to that suggested for the specification of cardiac mesoderm. Cardiac myogenesis is believed to be initiated in posterior epiblast cells by high doses of activin that derive from posteriorly located hypoblast prior to stage 3 (Yatskievych et al., 1997). As these cardiac precursors migrate anteriorly, their exposure to BMP signals that derive from anterolateral endoderm results in the specification of heart (Andree et al., 1998; Ladd et al., 1998; Lough et al., 1996; Schultheiss et al., 1997). Our experiments suggest that, like cardiac mesoderm, prechordal mesoderm precursors are generated in a posterior domain (Hensen's node) by mesoderm inducers such as activin and Vg-1, but that subsequent exposure to BMP signalling from anterior endoderm is required for their specification. The similarities in patterning of both prechordal mesoderm and cardiac mesoderm raise the possibility that the anterior endoderm may operate generally to govern the character of anteriorly migrating mesoderm.

Short-range signalling by anterior endoderm suggests a cascade of inductive interactions

Our experiments suggest that TGFβs deriving from anterior endoderm can exert three effects in extending axial mesoderm, namely the downregulation of notochord markers, the transient maintenance of *gsc* and the induction of *BMP7*. With the exception of 3B9 down-regulation, all aspects appear to be mediated by short-range interactions. Both in vitro and in vivo, the endodermal-mediated induction of *gsc* and *BMP7* occurs over a short distance (approximately 70-100 μm), a distance that correlates well with the length of the prechordal mesoderm in vivo (Dale et al., 1999). Furthermore, induction of *gsc* and *BMP7* and the downregulation of *chordin* are not observed when anterior endoderm is cultured at a distance from notochord explants in vitro. Lastly, the effective concentration of BMPs required to induce *BMP7* and downregulate *chordin* is higher than that required to downregulate 3B9.

Our experiments show that the hypoblast can cause the differentiation of prechordal mesoderm from notochord. However, two lines of evidence suggest that in vivo, the prechordal plate, rather than the hypoblast, is likely to directly mediate this phase of prechordal mesoderm specification. First, *BMP7* and *gsc* can be induced in the notochord only at the interface with anterior endoderm. Although we cannot exclude the possibility that migrating axial mesoderm encounters some remnants of hypoblast cells, it is more likely to encounter prechordal plate that migrates out of Hensen's node ahead of axial mesoderm (Bellairs, 1986; Rosenquist, 1966; Schoenwolf et al., 1992; Schoenwolf and Sheard, 1990; Selleck and Stern, 1991; Spratt, 1955). Second, our analyses reveal that, over time, expression of *BMP7* appears to progress in an anterior-posterior wave – waxing, then rapidly waning, first in hypoblast, then in prechordal plate, then in prechordal mesoderm cells (Figs 5A, 2C,M,P). Together, these results suggest a model in which a cascade of homeogenetic induction events involving BMPs specify anterior midline regions of the chick. We suggest that BMPs expressed in the hypoblast first induce expression of *BMPs* in prechordal plate. These BMPs in turn induce *BMP7* expression in prechordal mesoderm, hence enabling the prechordal mesoderm to induce *BMP7* in RDVM cells. Such homeogenetic induction of BMPs is not without precedent: in posterior regions of the neuraxis, BMPs that derive from the surface ectoderm appear to induce their own expression within dorsal spinal cord cells (Lee and Jessell, 1999). At present, it is unclear whether signalling by anterior endoderm-derived BMPs results in the induction of markers other than BMPs themselves. Our experiments show that not all markers of anterior endoderm are homeogenetically induced in prechordal mesoderm: *hex* remains confined to anterior hypoblast and anterior endoderm and is never detected in prechordal mesoderm (not shown). It is possible, therefore, that the primary action of BMP signalling is to suppress notochord characteristics from prechordal mesoderm and to induce expression of BMPs.

In summary, our observations suggest that an anteriorising signalling centre exists in chicks, and suggests that signals from this centre may operate to pattern extending axial mesoderm (see also Knoetgen et al., 1999). The ability of anterior endoderm to pattern axial mesodermal cells provides a means of promoting anterior identity in cells whose differentiation programme is initially established in the posterior organizer, Hensen's node, achieving an 'intermediate'-like cell type. Our data, together with recent experiments showing that chick hypoblast cannot directly pattern forebrain (Knoetgen et al., 1999), suggest that the key role of hypoblast in chick may be to pattern, directly or indirectly through prechordal plate, axial mesoderm, which acts in turn to pattern overlying neurectoderm (Dale et al., 1997; Foley et al., 1997; Pera and Kessel, 1997). We have shown previously that BMP7 co-operates with Shh in prechordal mesoderm to pattern the overlying forebrain ventral midline (Dale et al., 1997). The work described here suggests that the localisation of *BMP7* in prechordal mesoderm is dependent on its induction by BMPs that derive from anterior endoderm. Thus, in the chick, BMPs may play a role in patterning both the mesodermal and the neural midline to an anterior-like (intermediate) fate, inducing prechordal mesoderm and thence forebrain ventral midline, hence achieving a co-ordinated identity in midline cells of different germ layers within anterior-ventral regions of the embryo.

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