Developmental Biology **225,** 370–380 (2000)
doi:10.1006/dbio.2000.9834, available online at http://www.idealibrary.com on IDEAL®

Anterior Neural Induction by Nodes from Rabbits and Mice

Hendrik Knoetgen, Ulrike Teichmann, Lars Wittler, Christoph Viebahn,* and Michael Kessel[1]

*Abteilung Molekulare Zellbiologie, Max-Planck-Institut für Biophysikalische Chemie, D-37077 Göttingen, Germany; and *Anatomisches Institut, Universität Bonn, Bonn, Germany*

The organizer of vertebrate embryos represents the major regulatory center for the formation of the embryonic axis during gastrulation. The early blastopore lip of amphibia and Hensen's node of the chick at the full-length primitive streak stage possess both a head- and a trunk-inducing potential. In mice, a head-inducing activity was identified in the extraembryonic, anterior visceral endoderm (AVE) by tissue ablation and genetic experiments. Evidence for a similar activity in the AVE from the rabbit was obtained by transplanting below the avian epiblast. However, it was still unclear whether the AVE is the exclusive origin of anterior neural induction or if this activity is recapitulated by the node and/or its derivatives. We report here that nodes from both rabbit and mouse embryos can induce a complete neural axis including forebrain structures upon grafting to chick hosts. Thus, in rabbits and mice not only the AVE, but also the node, possesses a potential for the induction of anterior neural tissue.

Key Words: **organizer; head; chick; mammalian; visceral endoderm; streak.**

INTRODUCTION

A small group of cells in a vertebrate embryo can evoke the generation of an embryonic axis when transplanted below competent ectoderm. Such organizing centers, or organizers, have been identified in fish, amphibia, birds, and mammals (Beddington, 1994; Harland and Gerhart, 1997; Shih and Fraser, 1996; Spemann and Mangold, 1924; Waddington, 1932). The extent of axis formation induced by a grafted organizer is dependent on the precise origin, cellular composition, and age of the transplant; on the characteristics of the grafting site; and on the species-specific possibilities of the embryological manipulation. Thus, an organizer-induced axis may consist of only a head, only a trunk, or both; it may possess paraxial mesoderm stemming from reprogrammed, surrounding mesoderm; it may contain an elongated notochord or just a mass of chordoid, graft-derived cells. The major difference between the inductive potentials of a young and an old blastopore lip led H. Spemann to the identification of an amphibian head and a trunk organizer, respectively (Spemann, 1931). In amniota, the homologous structure of the blastopore lip is the tip of the primitive streak, where the three germ layers are tightly associated. It was first described as a "node" by V. Hensen in a study on rabbit embryos, and since then the term "Hensen's node," abbreviated to the "node," is widely used (Hensen, 1876). The avian node possesses initially, in mid- to full-streak stages, both a head- and a trunk-organizing potential, while older nodes are limited to trunk induction (Dias and Schoenwolf, 1990; Storey *et al.*, 1992). A first analysis of mammalian organizers was performed by C. H. Waddington, who transplanted tissue from somite stage rabbit embryos to cultured chick embryos (Waddington, 1934, 1936, 1937). He found inductions and self-differentiations similar to those observed by him with chick-to-chick grafts, including the formation of neural tissue and notochord. These experiments could, however, not identify the regional quality of the developing neural tissue. More recent evidence indicated that the inductive potential of the murine node might be restricted to that of a trunk organizer, since no anterior structures or markers were observed after grafting murine nodes into cultured murine or avian embryos (Beddington, 1994; Tam and Steiner, 1999; Tam *et al.*, 1997; Zhu *et al.*, 1999). On the other hand, evidence for the activity of a head organizer in the anterior visceral endoderm (AVE) of mammals was obtained by embryological manipulations. Thus, the re-

[1] To whom correspondence should be addressed at the Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-37077 Göttingen, Germany. Fax: +49 551 2011504. E-mail: mkessel1@gwdg.de.

0012-1606/00 $35.00

moval of the AVE from cultured murine embryos prohibited the expression of the *Hesx1* gene, a specifically anterior marker (Thomas and Beddington, 1996). Transplantation of AVE from prestreak rabbit embryos below competent epiblast of cultured chick embryos induced the avian *Hesx1* ortholog *GANF* and the neural marker *SOX3,* as well as a morphologically evident neural epithelium, a response that could not be obtained with primitive endoderm of avian origin (Knoetgen *et al.,* 1999b). The initial coincidence in time and space of the head and the trunk organizers appears to apply to amphibia and birds, whereas the early and independent function of the mammalian AVE seems to represent a unique situation, perhaps a novel aspect in mammalian evolution (for reviews see Beddington and Robertson, 1999; Bielinska *et al.,* 1999; Knoetgen *et al.,* 1999a).

Many RNAs encoding transcription or secreted factors have been identified in vertebrate organizers (for review see Harland and Gerhart, 1997). The secreted proteins chordin and noggin induce neural ectoderm by directly binding and thus inactivating the epidermalizing protein BMP4 (Piccolo *et al.,* 1996; Zimmermann *et al.,* 1996). In order to initiate the development of secondary head structures in *Xenopus laevis,* in addition to BMP antagonism, other factors, such as Wnt or nodal proteins, must be inhibited by secreted factors such as cerberus, frzb, or dkk-1 (Niehrs, 1999; Piccolo *et al.,* 1999). In mice, mRNAs from "organizer genes" were identified not only in the node, but also in a temporally and spatially independent domain, the AVE (*Dkk-1, Cer-l, HNF3β, Gsc, FGF8, Lim1, OTX2, Nodal;* Ang *et al.,* 1994; Belo *et al.,* 1997; Filosa *et al.,* 1997; Glinka *et al.,* 1998; Shawlot and Behringer, 1995; Tsang *et al.,* 1999; Varlet *et al.,* 1997). The analysis of mutants ($Lim1^{-/-}$, $OTX2^{-/-}$, $Nodal^{-/-}$, $Hesx1^{-/-}$, $Gsc^{-/-}HNF3\beta^{+/-}$, $Lim1^{-/-}$, $HNF3\beta^{-/-}$) indicated the synergistic, partially redundant importance of these genes for anterior patterning in the mouse (Acampora *et al.,* 1995; Ang *et al.,* 1996; Ang and Rossant, 1994; Dattani *et al.,* 1998; Filosa *et al.,* 1997; Matsuo *et al.,* 1995; Shawlot and Behringer, 1995; Varlet, 1997; Weinstein *et al.,* 1994). However, since these genes are expressed in both the AVE and the node and its derivatives, the final cause for the head dysmorphologies remained unclear. Analysis of chimeras with mutant cells only in extraembryonic, and not in embryonic, tissues, proved the function of the AVE as an anterior organizing center (Rhinn *et al.,* 1998; Shawlot *et al.,* 1999; Varlet *et al.,* 1997). In the Cripto mutant the AVE develops in the absence of a node and turned out to be sufficient to initiate the formation of anterior neural ectoderm (Ding *et al.,* 1998). On the other hand, the targeted inactivation of *Wnt3* indicated that the AVE alone may not be enough for the development of anterior structures (Liu *et al.,* 1999). The murine genes for noggin and chordin are specifically expressed in the node and its derivatives and not in the AVE. Their concomitant inactivation results in severe prosencephalic dysmorphologies, indicating important requirements of non-AVE, but streak-related, structures for head development (Bachiller *et al.,* 2000). In conclusion, it still remains unclear whether the node of the mammals is involved either in the induction or in maintenance of the anterior neural structures or both.

We have investigated whether the potential for anterior neural induction resides exclusively in the AVE or in both AVE and node. We show by grafting rabbit or mouse tissue below avian ectoderm that anterior neuroectoderm is induced by both tissues. The mammalian node contains sufficient signals to elicit a complete neural axis with extensive anterior–posterior as well as dorsal–ventral patterning.

MATERIALS AND METHODS

Animals

Fertilized White Leghorn chick eggs were obtained from Lohmann Tierzucht (Cuxhaven, Germany). Fertilized New Zealand white rabbits were from Lammers (Euskirchen, Germany); NMRI mice were from Harlan Winkelmann (Paderborn, Germany).

Transplantation of Rabbit and Mouse Tissue into Chick Hosts

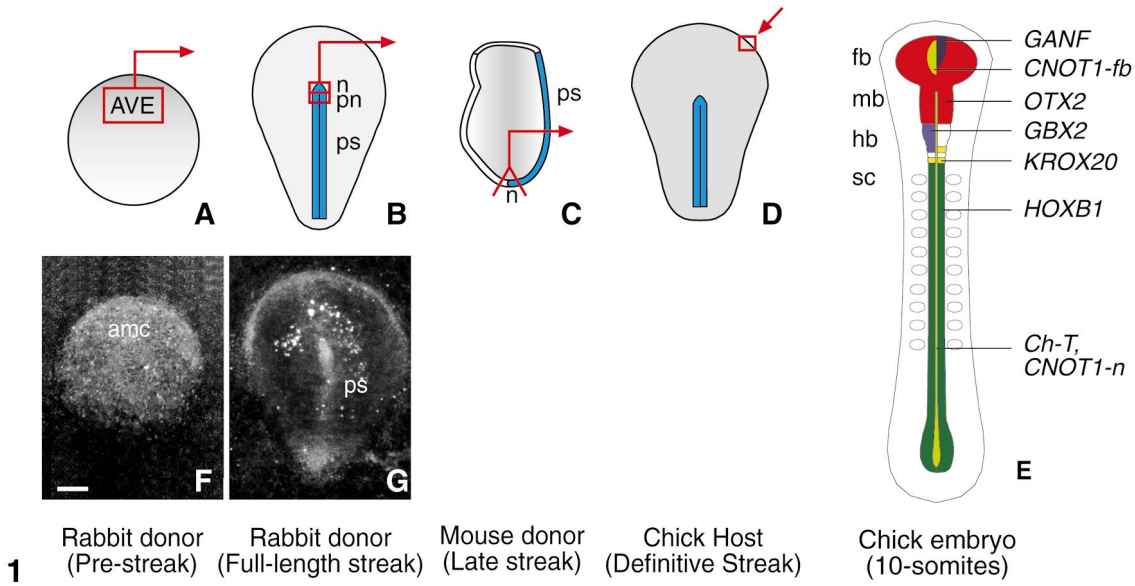
Chick embryos of stage HH3+/HH4 (Hamburger and Hamilton, 1951) were set up in NEW cultures (Stern, 1993), and grafts were transplanted to the anterior margin between the area pellucida and the area opaca (Fig. 1D). Grafts in this position induce structures in the area opaca or in the periphery of the area pellucida, which often is extended toward the blastoderm margin. We have no evidence for patterning influences or recruitment of cells from the primary embryo.

Fifteen- to 18-week-old rabbits were euthanized with 450 mg pentobarbital (Nembutal; Bayer, Germany). Embryos were recovered on day 6 postconception for AVE grafts or on day 7 for node grafts by flushing the uterus with prewarmed Dulbecco's modified Eagle medium (DMEM; GIBCO BRL, Life Technologies). We refer to the lower layer of prestreak rabbit embryos as "visceral" endoderm, in order to point out the topological and functional similarities to the extraembryonic, visceral endoderm of mice, in contrast to the yolk-rich hypoblast layer of chick embryos. AVE grafts (Fig. 1A) were prepared and transplanted as described previously (Knoetgen *et al.,* 1999b). Nodes or postnodal streaks (Fig. 1B) were excised from full-length streak rabbit embryos in DMEM containing 10% fetal calf serum (Biochrom) and marked with adhering particles of the water-insoluble dye Carmine (Sigma C-1022). The cultures containing grafted node or streak tissues were incubated overnight for an additional 20 h, whereas the cultures with the AVE grafts were incubated for only up to 10 h.

Mouse embryos were dissected from the uterus on day 7 postconception and staged (Downs and Davies, 1993; Hogan *et al.,* 1986). Late streak embryos were fixed by a holding pipette and manipulated in phosphate-buffered saline. The distal tip of the egg cylinder (Fig. 1C), containing the node, was excised with tungsten needles, marked with carmine, and transplanted as described above.

Whole-Mount in Situ Hybridization

Single whole-mount *in situ* hybridization was performed essentially as described by Wilkinson, except that the hybridization and



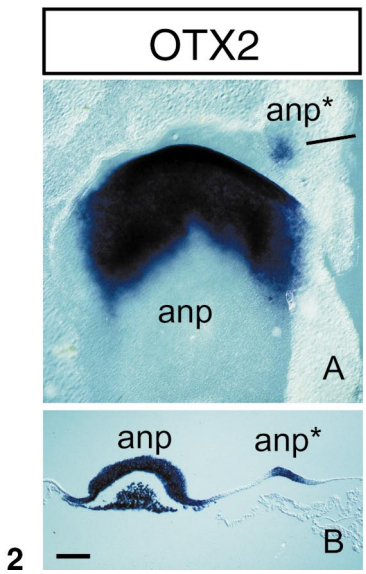


FIG. 1. Morphological and molecular landmarks of donor and host embryos. (A–D) Schematic representations of donor and host embryos. The primitive streak (ps) and the grafts are indicated, in particular the AVE, the node (n), and the postnodal streak fragment (pn). (E) The expression of marker genes at the 10-somite stage. For details of the expression domains see text. fb, forebrain; mb, midbrain; hb, hindbrain; sc, spinal cord. (F) Dark-field image of a prestreak rabbit embryo corresponding to A. Note that the anterior marginal crescent (amc) is thickened and morphologically conspicuous. (G) Dark-field image of a primitive streak stage rabbit embryo corresponding to B. The scale bar represents 175 μm in F and G.

FIG. 2. The induction of anterior neural ectoderm by a graft of prestreak rabbit AVE. (A) Anterior neural plate (anp) of a 1-somite host embryo with strong *OTX2* expression in the prospective fore- and midbrain territory. A small anterior neural plate (anp*) was induced by the AVE graft on the margin between the area pellucida and opaca. The level of the cross section in B is indicated by a black line. (B) Section across the primary and secondary neural plates. Note that only the endogenous ectodermal expression domain of *OTX2* is underlain by the *OTX2*-expressing mesendodermal cells of the chick host, whereas the induced anterior neural plate is not. The scale bar represents 80 μm in A and 160 μm in B.

the first two washing steps were performed at 70°C in the presence of 0.1% Chaps detergent (Sigma), omitting a RNase treatment (Wilkinson, 1992). The *OTX2* (Bally-Cuif *et al.*, 1995), *GANF* (Knoetgen *et al.*, 1999b), *KROX20* (Sham *et al.*, 1993), *GBX2* (Niss and Leutz, 1998), *CNOT1* (Stein and Kessel, 1995), *Ch-T* (Kispert *et al.*, 1995), *HOXB1* (Guthrie *et al.*, 1992), and *SOX2* (Rex *et al.*,

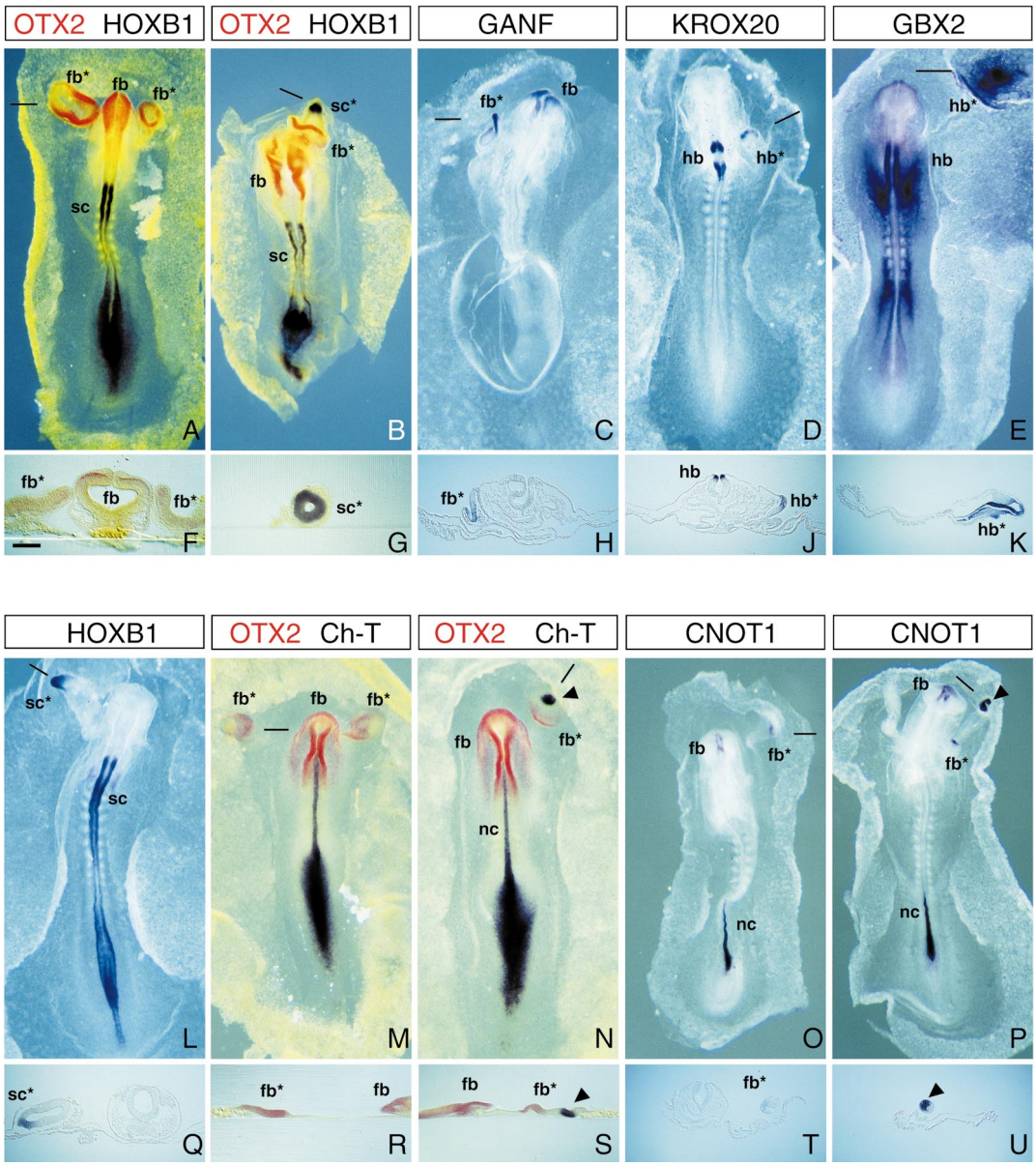


FIG. 3. The induction of complete neural axes by rabbit nodes. For a detailed discussion of the probes and the inductions see text. (A–E and L–P) Whole-mounted chick embryos analyzed by single or double *in situ* hybridization. (F–K and Q–U) The corresponding sections, performed at the levels indicated by black lines. The rabbit node grafts are schematically depicted in Fig. 1B and the transplantation sites in the avian hosts in Fig. 1D. Note that the ectopic structures contain neuroectoderm of fore-, mid-, and hindbrain and spinal cord character. Note that two types of inductions were observed with the mesodermal markers *Ch-T* and *CNOT1-n*. Either the secondary axes express both mesodermal and ectodermal markers (N, P) or they express exclusively the ectodermal markers (M, O). Note that the mesodermal markers are confined to the epiblast layer and no signs of ingression were observed (S, U). Indicated are forebrain (fb), spinal cord (sc), and notochord (nc) of the primary embryos. Induced structures identified by a molecular marker are marked with an asterisk (*), the arrowhead points to the expression domains of *Ch-T* or *CNOT1-n* in the epiblast. The scale bar in F represents 550 μm in A–E and L–P and 190 μm in F–K and Q–U.

1997) riboprobes were described previously. For paraffin sections (8 μm) stained embryos were dehydrated and embedded in Paraplast Plus (Sherwood Medicals). Double whole-mount *in situ* hybridization was performed using simultaneously a fluorescein- and a digoxigenin-labeled RNA probe, which were detected consecutively by the alkaline phosphate substrates fast red (Sigma) and NBT-BCIP (Roche; Dietrich *et al.*, 1997). Since fast red staining is lost during paraffin histology, double-stained embryos were embedded in a gelatin albumen mixture and cut into sections (30 μm) with a Vibratome (Pelco 101).

TABLE 1
Summary of Transplantation Experiments

	Chick node (n = 24)		Rabbit node (n = 122)		Rabbit postnodal streak (n = 40)		Mouse node (n = 57)	
Ectopic structure	19/24	79%	66/122	54%	28/40	70%	26/57	46%
OTX2	15/16	94%	18/23	78%	0/8	0%	8/18	44%
GANF	4/6	67%	6/15	40%	0/4	0%	2/8	25%
CNOT1-fb	5/5	100%	6/6	100%	0/6	0%	—	—
CNOT1-n	5/5	100%	4/6	67%	4/6	67%	—	—
GBX2	—		7/7	100%	2/2	100%	—	—
KROX20	—		2/6	33%	1/2	50%	—	—
HOXB1	5/6	83%	20/28	71%	6/6	100%	7/11	63%
SOX3	—		—		—		—	—
SOX2	—		—		—		—	—
Ch-T	5/5	100%	4/12	33%	4/4	100%	3/7	42%

Note. CNOT1 is expressed in two different domains: fb, dorsal forebrain, and n, posterior domain around the node.

RESULTS

We used molecular markers in order to analyze structures induced by various organizer grafts in the chick epiblast (Fig. 1E). Specifically, we applied the homeobox genes *OTX2* as a marker for forebrain and midbrain tissue (Bally-Cuif *et al.*, 1995), *GANF* for anterior forebrain (Knoetgen *et al.*, 1999b), *GBX2* for anterior hindbrain (rhombomere 1 to 3; Niss and Leutz, 1998), *KROX20* for posterior hindbrain (rhombomeres 3 and 5; Sham *et al.*, 1993), and *HOXB1* for posterior hindbrain and spinal cord (posterior to rhombomere 3; Guthrie *et al.*, 1992). *CNOT1* was applied as a marker for two domains, namely for a dorsal forebrain ("*CNOT1-fb*") and for a posterior domain of node, notochord, and postnodal neural plate ("*CNOT1-n*"; Stein and Kessel, 1995). We included *Ch-T*, the chicken *Brachyury* gene, as a marker for the prospective mesodermal cells in the primitive streak and for the notochord (Kispert *et al.*, 1995). The gene *SOX2* was used as a panneural marker (Rex *et al.*, 1997). Most of the marker genes are expressed from very early to late stages. However, *KROX20* is activated only at HH10 and *GANF* is transiently expressed only from HH4+ to HH10 in the forebrain. We performed transplantations of chick nodes as controls and confirmed the frequent induction of complete neural axes expressing *OTX2*, *GANF*, *CNOT1-fb*, and *HOXB1* (Table 1 and data not shown; e.g., Lemaire *et al.*, 1997; Storey *et al.*, 1992).

Rabbit AVE Induces Anterior Neural Ectoderm in Chick Embryos

In a previous study we had chosen the prestreak rabbit embryo as a source for mammalian, anterior visceral endoderm (Knoetgen *et al.*, 1999b). In rabbits, but not in mice, the prospective anterior–posterior axis can be recognized before the onset of gastrulation due to a morphologically evident anterior thickening, the anterior marginal crescent (Fig. 1F; Viebahn *et al.*, 1995). While young murine embryos form an egg cylinder, the rabbit embryos develop as a flat disc and exhibit a striking, superficial similarity to early chick embryos. AVE grafts elicit the formation of a small plate of a pseudostratified, columnar epithelium, which was identified by a general neural marker, *SOX3*, or by the early anterior neural markers *GANF* (Knoetgen *et al.*, 1999b) or *OTX2* (60%, n = 5, Fig. 2). We terminated the incubation of AVE transplanted cultures after 10 h, in order to avoid the eventual epidermalization of the small island of neural cells, as it would occur after the grafting of a plug of neural plate cells into the prospective epidermis (Garcia-Martinez *et al.*, 1997). AVE-induced epithelia did not express *SOX2*, a neural marker which becomes positive after more than 9 h of induction by node grafts (0%, n = 10; not shown; Rex *et al.*, 1997; Streit and Stern, 1999).

In summary, we found that rabbit AVE grafts induced the formation of anterior neural plates in chick hosts. These failed to form elevated neural folds or ectopic axes.

The Induction of a Complete Neural Axis by a Rabbit Node

We now extended our rabbit-to-chick transplantation study, in order to investigate the inductive potential of a mammalian node with focus on its anterior neural-inducing activities. As responsive tissue we used again chick ectoderm at the margin of the area pellucida and area opaca, which is fated to contribute to the epidermis or extraembryonic ectoderm, respectively (Garcia-Martinez *et al.*, 1993; Rosenquist, 1966; Schoenwolf and Sheard, 1990; Spratt, 1952). Thus, except for the different incubation periods, the results described below were obtained in the same setup as those of our previous study on the AVE and can be readily compared (Knoetgen *et al.*, 1999b). As expected, the grafted rabbit cells were not detectable after the overnight incubation period of approximately 20 h, due to culture conditions such as pH of 9.2, optimized for chick embryos. Therefore, they could not contribute to the in-

duced axes themselves, and the prominent self-differentiation of the grafted node into notochord-like tissues observed for chick nodes did not occur.

In our cross-species transplantation assay the rabbit nodes induced morphological alterations in 54% of the manipulations ($n = 122$, see Table 1 for details). We obtained evidence for inductions of a forebrain identity at a high frequency with *OTX2* (78%, $n = 23$, Figs. 3A, 3B, 3F, 3M, 3N, 3R, and 3S) and *CNOT1-fb* (100%, $n = 6$; Figs. 3O, 3P, and 3T) as marker genes and at a slightly reduced frequency for *GANF* (40%, $n = 15$, Figs. 3C and 3H). The ectopic neural axes always developed typically elevated neural folds, regionally fused to form a neural tube. This, and the specific dorsal restriction of markers like *CNOT1-fb*, indicated that also an extensive dorsoventral patterning had occurred, most probably due to factors like sonic hedgehog, known to be secreted from node cells (Roelink *et al.*, 1995). Also the anterior hindbrain marker *GBX2* (100%, $n = 7$, Figs. 3E and 3K) and the spinal cord marker *HOXB1* (71%, $n = 28$, Figs. 3A, 3B, 3G, 3L, and 3Q) were induced with high frequencies. The expression of the posterior hindbrain marker *KROX20* was less frequently induced (33%, $n = 6$, Figs. 3D and 3J), possibly reflecting that *KROX20* is expressed rather late in contrast to the other marker genes and that additional signals may be required for its induction.

It is conceivable that the neural induction by rabbit nodes might occur secondarily to a previously induced chicken organizer, which then in turn could induce a forebrain according to its normal and already described properties. Therefore, we analyzed the mesodermal markers *CNOT1-n* and *Ch-T*. Both are powerful markers for prospective or early mesodermal cells including those in Hensen's node. We found *Ch-T* activation with a frequency of 33% ($n = 12$, Figs. 3N and 3S), whereas *OTX2* expression was induced with a frequency of 78% ($n = 23$). These results demonstrate that the forebrain markers are induced in a significant proportion of our specimens without the simultaneous induction of *CNOT1-n* or *Ch-T*. This conclusion was confirmed by using double whole-mount *in situ* hybridization, in which we found the induction of forebrain neuroectoderm (*OTX2*) in the complete absence of the mesodermal marker *Ch-T* ($n = 4$; Figs. 3M and 3R). Furthermore, the forebrain expression domain of the *CNOT1* gene (*CNOT1-fb*) was detected in all cases (100%, $n = 6$, Figs. 3O, 3P, and 3T), whereas the posterior domain (*CNOT1-n*) occurred less frequently (67%, $n = 6$, Figs. 3P and 3U).

The results obtained with rabbit nodes differed in one respect significantly from comparable experiments with avian nodes. If a rabbit node was grafted some of the embryos expressed *Ch-T* and *CNOT1-n* in a posterior domain of the secondary axis, which was strictly confined to the ectoderm (Figs. 3S and 3U). In contrast, a comparable transplantation of chick nodes always results in posterior *Ch-T* or *CNOT1-n* domains, which are restricted to the self-differentiating graft. Chick and rabbit nodes have in common that they do not induce deepithelialization from the overlying cell layer and the basal membrane appears to remain intact.

In summary, we found that rabbit nodes induced secondary axes in chick hosts which invariably exhibited forebrain character. This induction did not necessarily involve the activation of avian mesodermal or organizer markers.

The Induction of a Complete Neural Axis by a Mouse Node

To test the inducing capacity of the murine node we grafted the distal tip of the late streak egg cylinder to cultured chick embryos under conditions identical to those used for the rabbit nodes. As for the rabbit node cells, the mouse node cells were not viable in the chick embryo culture assay. The nodes induced morphological alterations in 46% of the experiments ($n = 57$, see Table 1 for details). The ectopic structures developed elevated neural folds and in some cases neural tubes. To investigate the anterior–posterior patterning of these ectopic neural structures we analyzed the embryos with the markers *OTX2, GANF, HOXB1,* and *Ch-T*. The mouse grafts induced *OTX2* expression in 44% ($n = 18$, Figs. 4A–4C and 4E; for details see Table 1), *GANF* in 25% ($n = 8$, Figs. 4F and 4G), *HOXB1* in 63% ($n = 11$, Figs. 4B–4D), and *Ch-T* in 42% ($n = 7$, Fig. 4A) of the cases. Expression of *Ch-T* was again strictly confined to the ectoderm. Sixty-two percent of the specimens analyzed for *OTX2* plus *Ch-T* or *HOXB1* expression by double whole-mount *in situ* analysis displayed only *OTX2*-positive cells.

In summary, we could show that, like the rabbit node, the node of the late streak mouse embryo possesses the ability to induce a secondary neural axis in chick hosts, exhibiting forebrain and spinal cord character. This neural induction is independent of the activation of avian mesodermal or organizer markers.

Rabbit Postnodal Streak Grafts Induce Secondary Axes Lacking Anterior Neuroectoderm

The inductive potential of primitive streak grafts depends significantly on their anterior–posterior origin (see Discussion). We used the rabbit streak tissue adjacent to a node explant as a separate graft (Fig. 1B). Such postnodal streak transplants resulted in 70% of the cases in significant morphological alterations of mostly neural, certainly not streak-like, structures ($n = 40$, see Table 1 for details, Fig. 5). None of the ectopic neural structures expressed the anterior markers *OTX2* (0%, $n = 8$, Figs. 5A, 5F, and 5L), *GANF* (0%, $n = 4$, Figs. 5B and 5G), or *CNOT1-fb* (0%, $n = 6$, Figs. 5M and 5O). However, the frequencies of induction of the anterior hindbrain marker gene *GBX2* (100%, $n = 2$, Figs. 5D and 5J), posterior hindbrain marker *KROX20* (50%, $n = 2$, Figs. 5C and 5H), and posterior hindbrain/spinal cord marker *HOXB1* (100%, $n = 6$, Figs. 5E and 5K) were high and comparable to the results of the node grafts. The induction of the mesodermal marker genes *Ch-T* and *CNOT1-n* in the overlying epiblast occurred also at a high

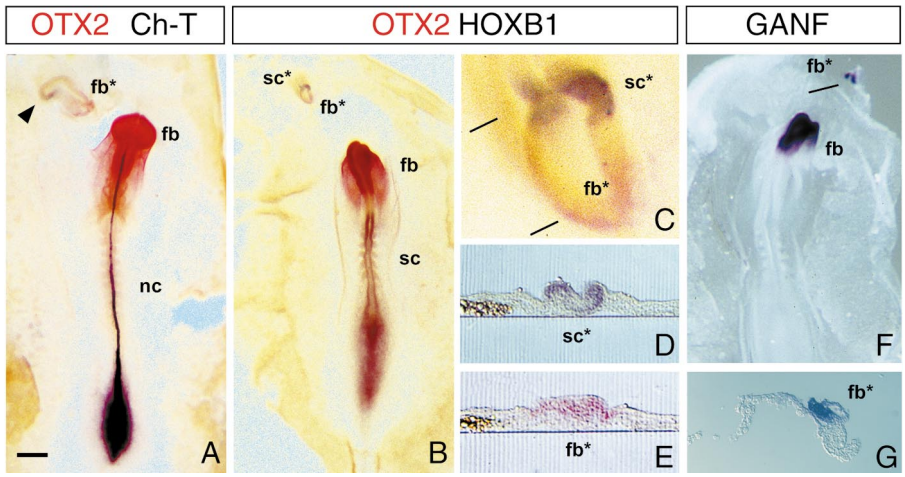


FIG. 4. The induction of anterior neural ectoderm by mouse nodes. For a detailed discussion of the probes and the inductions see text. The specimens in A and B are whole-mounted chick embryos analyzed by double *in situ* hybridization with the indicated probes. (A) The grafted murine node (see Fig. 1C) induced an ectopic axis including anterior neural ectoderm (fb*) identified by *OTX2* and an expression domain of *Ch-T* in the host epiblast (arrowhead). (B) The specimen displays a small ectopic neural axis positioned in the area opaca. (C) A higher magnification of the induced structure shown in B. *OTX2* is expressed at the pole pointing toward the host embryo (cross section in E) and *HOXB1* at the other pole (cross section in D). The levels of the sections are indicated by black lines. (F) Anterior neural ectoderm is induced and identified by *GANF* expression (cross section in G). Indicated are forebrain (fb), spinal cord (sc), and notochord (nc) of the primary embryos. Induced structures identified by a molecular marker are marked with an asterisk (*). The scale bar represents 550 μm in A, B, F; 50 μm in C; and 70 μm in D, E, G.

rate (100%, $n = 4$, Fig. 5N and 67%, $n = 6$, Fig. 5O). These data suggest that the induction of mesodermal markers by node grafts (see above) is dependent on the presence of postnodal streak tissue accidentally present in the grafts.

In summary, we found that postnodal streak grafts of the rabbit induced incomplete axes in chick hosts, lacking anterior neural tissue. This induction was in the majority of cases accompanied by the activation of mesodermal markers.

DISCUSSION

In this study we demonstrate for the first time that a mammalian node possesses a potential for the induction of a complete embryonic axis including anterior neural tissue. In the following paragraphs we will discuss the distribution of inductive potentials along the primitive streaks of the rabbit and the chick. We compare our data with other transplantation studies in the mouse and will finally summarize the implications for our understanding of head induction in mammals.

Inductive Potentials along the Primitive Streak

Different sections of the primitive streak have distinct inductive potentials, which appear to be conserved between the rabbit (this study) and the chick (Gallera and Nicolet, 1969; Joubin and Stern, 1999; Lemaire *et al.*, 1997; Storey *et al.*, 1992). These different cell populations are not readily separable with the dissection needle. However, we can deduce a convincing logic from the multitude of operations and molecular markers performed by us and others. The anterior tip of the streak induces anterior neural ectoderm, a varying amount of more posterior neural tissue, but no mesodermal markers. The postnodal streak induces neural tissue of hindbrain and more posterior neural identity, which is invariably accompanied by the activation of mesodermal marker genes. If an anterior streak graft includes tissue of the postnodal streak the ectopic secondary axis displays anterior as well as posterior neural identities as well as mesodermal marker genes, i.e., a fully patterned axis. The middle of the chick streak induces a secondary primitive streak lacking neural-specific and anterior streak markers (Lemaire *et al.*, 1997). The latter are induced only if more anterior cells are present in the graft.

In conclusion, pure neural induction is restricted to the tip and pure mesoderm induction to the middle of the streak. In-between, there is an anterior–posterior decrease of a neural-inducing, and an increase of a mesoderm-inducing, potential. In addition, more anterior grafts induce more anterior markers in the ectoderm.

The Inductive Potential of Mammalian Nodes

At first sight, our results obtained in rabbit-to-chick and mouse-to-chick transplantations are in contrast to previous findings, in which two stages of a murine gastrula organizer were studied in mouse-to-mouse grafting experiments. The epiblast anterior of the young streak was designated the

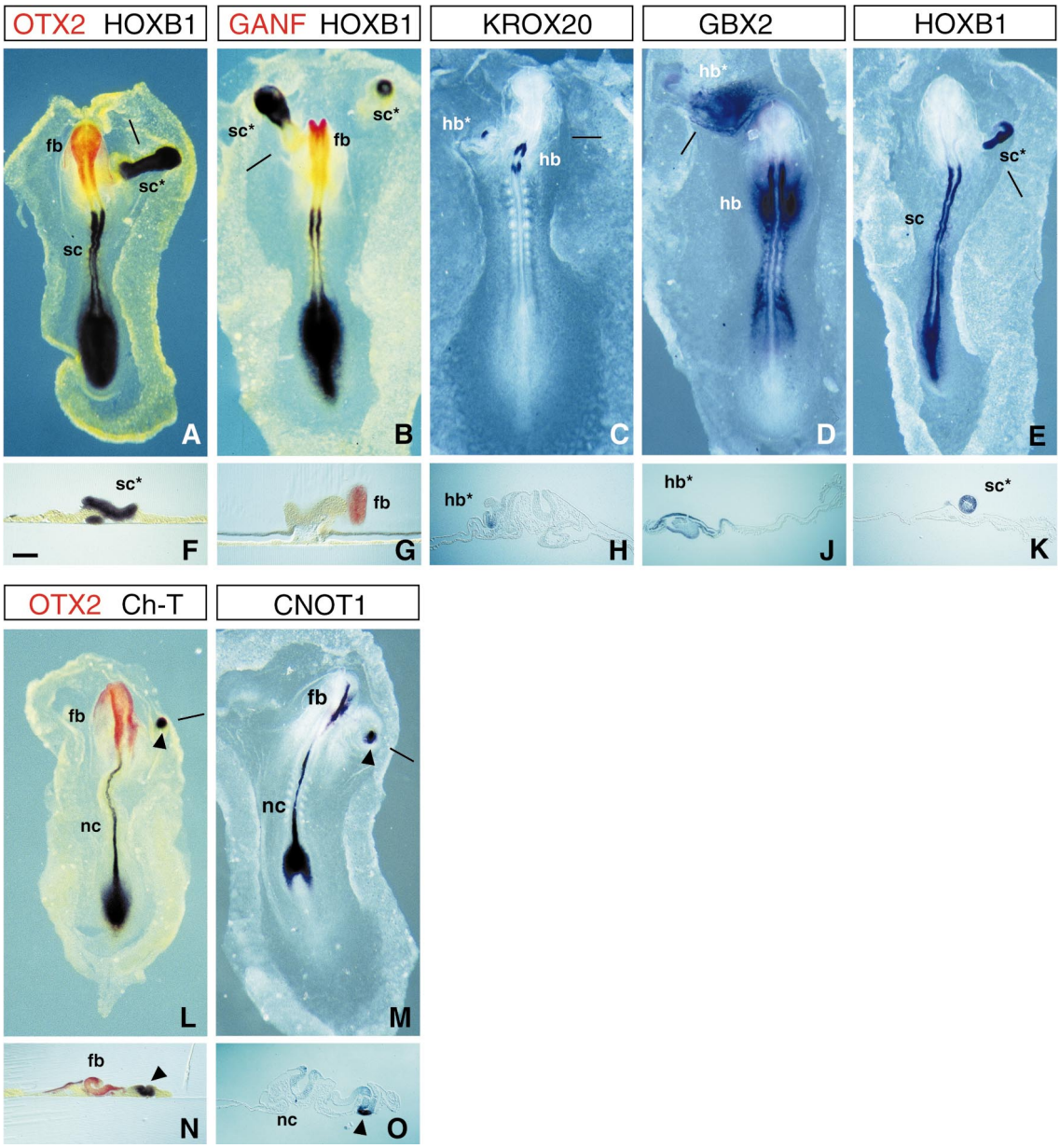


FIG. 5. Rabbit postnodal streak grafts induce an incomplete neural axis lacking fore- and midbrain. For a detailed discussion of the probes and the inductions see text. (A–E, L, M) Whole-mounted chick embryos analyzed by single or double *in situ* hybridization. (F–K, N, O) The corresponding sections, performed at the levels indicated by black lines. The rabbit postnodal streak grafts are schematically depicted in Fig. 1B and the transplantation sites in the avian hosts in Fig. 1D. Note that the ectopic structures contain neuroectoderm of hindbrain and spinal cord character, but lack fore- and midbrain character. Note that the grafts induced also mesodermal markers in the overlying epiblast, such as *Ch-T* (L, N) or *CNOT1-n* (M, O). Indicated are forebrain (fb), hindbrain (hb), spinal cord (sc), and notochord (nc) of the primary embryos. Induced structures identified by a molecular marker are marked with an asterisk (*). The scale bar represents 500 μm in A–E, L, M and 160 μm in F–K, N, O.

early gastrula organizer (EGO) and the node of the fully elongated streak the late gastrula organizer (LGO; Tam and Steiner, 1999; Tam *et al.*, 1997). Both were capable of inducing neural tissue and small embryonic axes after transplantation to cultured murine embryos. However, neither the EGO nor the LGO nor the early streak AVE induced anterior markers, and also various dual combinations were negative (Beddington, 1994; Tam and Steiner, 1999; Tam *et al.*, 1997). Only a triple graft, consisting of EGO, AVE, and anterior epiblast isolated from early streak

embryos activated *OTX2* expression in about 25% of the cases (Tam and Steiner, 1999). We think that the differing findings and conclusions can be explained by the specific experimental conditions. We studied prestreak AVE, whereas Tam and colleagues grafted early streak AVE. More importantly, also the fate and competence of the responding ectoderms were significantly different. Mouse-to-mouse transplants were inserted below an ectoderm which is already on its way to becoming posterior neural tissue, i.e., hindbrain and spinal cord, and is already reached by the mesodermal wings. In an equivalent grafting site in chick hosts the influence of posteriorizing substances like FGFs and retinoic acid, and an as yet unknown factor secreted from the paraxial mesoderm, was identified (Muhr *et al.*, 1999). However, avian or murine prechordal mesoderm (HH5) was attributed with counteracting and thereby rostralizing prospective rhombencephalic neuroectoderm (Foley *et al.*, 1997; Tam and Steiner, 1999). It is conceivable that the triple grafts provided enough of a "rostralizer" to counteract posteriorizing factors.

The grafting sites in our rabbit-to-chick and mouse-to-chick experiments differed significantly from that in the mouse-to-mouse experiments. The normal fate of the responding avian ectoderm is mostly extraembryonic, marginally epidermal, and not neural, and there is no underlying mesoderm present before the transplantation. In our rabbit-to-chick experiments, the grafted rabbit cells did not survive, and therefore their signals must have been emitted directly after transplantation. It is known that also in chick-to-chick transplantations the grafted tissues need to be in contact with the ectoderm only for a few hours (Streit *et al.*, 1998). We observed that the eventual absence of rabbit organizer cells allowed the activation of *Ch-T* and the *CNOT1-n* in the overlying epiblast, whereas in the chick persisting organizer cells repressed this epiblastic expression, most probably by their "antidorsalizing" activity (Joubin and Stern, 1999; Yuan *et al.*, 1995).

In conclusion, we suggest that differences in the competence of the responding epithelium, but not in the inducing node tissue, are responsible for the inductions we observed. We think that the capacity for anterior neural induction represents a general feature of mammalian nodes.

Anterior Neural Induction in Mammals

Previously, we had already demonstrated that the rabbit AVE has an anterior neuroectoderm-inducing capacity (Knoetgen *et al.*, 1999b). The responses elicited in the chick ectoderm by the AVE represent only a subset of the node-mediated activities described in this communication. The AVE leads to the formation of an ectopic anterior neural plate which expressed only anterior and early neural marker genes, but did not subsequently develop into elevated neural folds or neural tube-like structures. On the other hand, the transplantation of rabbit and mouse nodes caused the formation of a fully patterned ectopic neural axis. Based on these data and evidence from genetic studies (see Introduction), we see the following scenario for mammalian head induction.

The first identified step of mammalian head induction is a signal from the AVE to the ectoderm, which is enough to trigger neural development before the onset of gastrulation. From the outset of gastrulation, the developing neural plate has an anterior identity, as identified by the *Hesx1* marker (Hermesz *et al.*, 1996; Thomas and Beddington, 1996). The early neural plate has to compete with epidermalizing activities. It becomes stabilized by a second neural induction signal coming from the gastrula organizer, the node, and its direct derivative, the prechordal mesendoderm. Thus, head induction appears to be a redundant, double insurance system, possibly involving the same genes at different developmental stages.

In evolutionary terms the existence of a pregastrula organizer in the extraembryonic endoderm in addition to a gastrula organizer in the node appears to be a novel characteristic of mammals. The dual anlage of the mammalian head organizer contrasts with the single anlage described for amphibia and birds, in which it resides exclusively in the original organizing center, i.e., the blastopore lip and Hensen's node, respectively, and in their direct derivative, the prechordal plate. The common genetic repertoire of the pregastrula and the gastrula organizers in mammals would suggest that also inductive capacities were conserved. Our analysis of the rabbit and mouse node confirms this expectation.

ACKNOWLEDGMENTS

We thank W. Behrens for excellent technical assistance and W. Vukovich for discussions. Molecular probes were kindly provided by E. Boncinelli, G. Eichele, B. Herrmann, K. Niss, P. Sharpe, and D. Wilkinson. The authors were supported by the Max-Planck-Gesellschaft, the Boehringer Ingelheim Fonds, and DFG Grants SFB271/A3 and Vi151/3-1.

REFERENCES

Acampora, D., Mazan, S., Lallemand, Y., Avantaggiato, V., Maury, M., Simeone, A., and Brulet, P. (1995). Forebrain and midbrain regions are deleted in *otx2*(−/−) mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121,** 3279–3290.

Ang, S.-L., Conlon, R. A., Jin, O., and Rossant, J. (1994). Positive and negative signals from mesoderm regulate the expression of mouse *Otx2* in ectoderm explants. *Development* **120,** 2979–2989.

Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L., and Rossant, J. (1996). A targeted mouse Otx2 mutation leads to severe defects in gastrulation and formation of axial mesoderm and to deletion of rostral brain. *Development* **122,** 243–252.

Ang, S. L., and Rossant, J. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78,** 561–574.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J. A., Anderson, R. M., May, S. R., McMahon, J. A., McMahon, A. P., Harland,

R. M., Rossant, J., and De Robertis, E. M. (2000). The organizer factors Chordin and Noggin are required for mouse forebrain development. *Nature* **403,** 658–661.

Bally-Cuif, L., Gulisano, M., Broccoli, V., and Boncinelli, E. (1995). *c-otx2* is expressed in two different phases of gastrulation and is sensitive to retinoic acid treatment in chick embryos. *Mech. Dev.* **49,** 49–63.

Beddington, R. S. (1994). Induction of a second neural axis by the mouse node. *Development* **120,** 613–620.

Beddington, R. S. P., and Robertson, E. J. (1999). Axis development and early asymmetry in mammals. *Cell* **96,** 195–209.

Belo, J. A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M., and De Robertis, E. M. (1997). Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68,** 45–57.

Bielinska, M., Narita, N., and Wilson, D. B. (1999). Distinct roles for visceral endoderm during embryonic mouse development. *Int. J. Dev. Biol.* **43,** 183–205.

Dattani, M. T., Martinezbarbera, J. P., Thomas, P. Q., Brickman, J. M., Gupta, R., Martensson, I. L., Toresson, H., Fox, M., Wales, J. K. H., Hindmarsh, P. C., Krauss, S., Beddington, R. S. P., and Robinson, I. (1998). Mutations in the homeobox gene Hesx1/Hesx1 associated with septo-optic dysplasia in human and mouse. *Nat. Genet.* **19,** 125–133.

Dias, M. S., and Schoenwolf, G. C. (1990). Formation of ectopic neurepithelium in chick blastoderms: Age-related capacities for induction and self-differentiation following transplantation of quail Hensen's nodes. *Anat. Rec.* **228,** 437–448.

Dietrich, S., Schubert, F. R., and Lumsden, A. (1997). Control of dorsoventral pattern in the chick paraxial mesoderm. *Development* **124,** 3895–3908.

Ding, J. X., Yang, L., Yan, Y. T., Chen, A., Desai, N., Wynshawboris, A., and Shen, M. M. (1998). Cripto is required for correct orientation of the anterior–posterior axis in the mouse embryo. *Nature* **395,** 702–707.

Downs, K. M., and Davies, T. (1993). Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118,** 1255–1266.

Filosa, S., Riveraperez, J. A., Gomez, A. P., Gansmuller, A., Sasaki, H., Behringer, R. R., and Ang, S. L. (1997). Goosecoid and Hnf-3-beta genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124,** 2843–2854.

Foley, A. C., Storey, K. G., and Stern, C. D. (1997). The prechordal region lacks neural inducing ability, but can confer anterior character to more posterior neuroepithelium. *Development* **124,** 2983–2996.

Gallera, J., and Nicolet, G. (1969). Le pouvoir inducteur de l'endoblaste presomptif contenu dans la ligne primitive jeune de poulet. *J. Embryol. Exp. Morphol.* **21,** 105–118.

Garcia-Martinez, V., Alvarez, I. S., and Schoenwolf, G. C. (1993). Locations of the ectodermal and nonectodermal subdivisions of the epiblast at stages 3 and 4 of avian gastrulation and neurulation. *J. Exp. Zool.* **267,** 431–446.

Garcia-Martinez, V., Darnell, D. K., Lopez-Sanchez, C., Sosic, D., Olson, E. N., and Schoenwolf, G. C. (1997). State of commitment of prospective neural plate and prospective mesoderm in late gastrula/early neurula stages of avian embryos. *Dev. Biol.* **181,** 102–15.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391,** 357–362.

Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R., and Lumsden, A. (1992). Neuroectodermal autonomy of Hox-2.9 expression revealed by rhombomere transpositions. *Nature* **356,** 157–159.

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Harland, R., and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell. Dev. Biol.* **13,** 611–617.

Hensen, V. (1876). Beobachtungen über die Befruchtung und Entwicklung des Kaninchens und Meerschweinchens. *Z. Anat. Entw. Gesch.* **1,** 353–423.

Hermesz, E., Mackem, S., and Mahon, K. A. (1996). *Rpx*—A novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. *Development* **122,** 41–52.

Hogan, B. L. M., Constantini, F., and Lacy, E. (1986). "Manipulating the Mouse Embryo: A Laboratory Manual." Cold Spring Harbor Laboratory Press, New York.

Joubin, K., and Stern, C. D. (1999). Molecular interactions continuously define the organizer during the cell movements of gastrulation. *Cell* **98,** 559–571.

Kispert, A., Ortner, H., Cooke, J., and Herrmann, B. G. (1995). The chick Brachyury gene: Developmental expression pattern and response to axial induction by localized activin. *Dev. Biol.* **168,** 406–415.

Knoetgen, H., Teichmann, U., and Kessel, M. (1999a). Head-organizing activities of endodermal tissues in vertebrates. *Cell. Mol. Biol.* **45,** 481–492.

Knoetgen, H., Viebahn, C., and Kessel, M. (1999b). Head induction in the chick by primitive endoderm of mammalian, but not avian origin. *Development* **126,** 815–125.

Lemaire, L., Roeser, T., Izpisua-Belmonte, J.-C., and Kessel, M. (1997). Segregating expression domains of two goosecoid genes during the transition from gastrulation to neurulation in chick embryos. *Development* **124,** 1443–1452.

Liu, P. T., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R., and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat. Genet.* **22,** 361–365.

Matsuo, I., Kuratani, S., Kimura, C., Takeda, N., and Aizawa, S. (1995). Mouse *otx2* functions in the formation and patterning of rostral head. *Genes Dev.* **9,** 2646–2658.

Muhr, J., Graziano, E., Wilson, S., Jessell, T. M., and Edlund, T. (1999). Convergent inductive signals specify midbrain, hindbrain, and spinal cord identity in gastrula stage chick embryos. *Neuron* **23,** 689–702.

Niehrs, C. (1999). Head in the WNT: The molecular nature of Spemann's head organizer. *Trends Genetics* **15,** 314–319.

Niss, K., and Leutz, A. (1998). Expression of the homeobox gene Gbx2 during chicken development. *Mech. Dev.* **76,** 151–155.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397,** 707–710.

Piccolo, S., Sasai, Y., Lu, B., and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: Inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86,** 589–598.

Rex, M., Orme, A., Uwanogho, D., Tointon, K., Wigmore, P. M., Sharpe, P. T., and Scotting, P. J. (1997). Dynamic expression of chicken Sox2 and Sox3 genes in ectoderm induced to form neural tissue. *Dev. Dyn.* **209,** 323–332.

Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., Le Meur, M., and Ang, S. L. (1998). Sequential roles for Otx2 in visceral

endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125,** 845–856.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81,** 445–455.

Rosenquist, G. C. (1966). A radioautographic study of labeled grafts in the chick blastoderm. Development from primitive-streak stages to stage 12. *Carnegie Contrib. Embryol.* **38,** 31–110.

Schoenwolf, G. C., and Sheard, P. (1990). Fate mapping the avian epiblast with focal injections of a fluorescent-histochemical marker: Ectodermal derivatives. *J. Exp. Zool.* **255,** 323–339.

Sham, M. H., Vesque, C., Nonchev, S., Marshall, H., Frain, M., Gupta, R. D., Whiting, J., Wilkinson, D., Charnay, P., and Krumlauf, R. (1993). The zinc finger gene Krox20 regulates HoxB2 (Hox2.8) during hindbrain segmentation. *Cell* **72,** 183–196.

Shawlot, W., and Behringer, R. R. (1995). Requirement for *Lim1* in head-organizer function. *Nature* **374,** 425–430.

Shawlot, W., Wakamiya, M., Kwan, K. M., Kania, A., Jessell, T. M., and Behringer, R. R. (1999). Lim1 is required in both the primitive streak-derived tissues and visceral endoderm for head formation in the mouse. *Development* **126,** 4925–4932.

Shih, J., and Fraser, S. E. (1996). Characterizing the zebrafish organizer—Microsurgical analysis at the early-shield stage. *Development* **122,** 1313–1322.

Spemann, H. (1931). Über den Anteil von Implantat und Wirtskeim an der Orientierung und Beschaffenheit der induzierten Embryonalanlage. *Arch. Mikrosk. Anat. Entwicklungsmech.* **123,** 390–516.

Spemann, H., and Mangold, H. (1924). Über die Induktion von Embryoanlagen durch Implantation artfremder Organisatoren. *Roux Arch. Entwicklungsmech.* **100,** 599–638.

Spratt, N. T. (1952). Localization of the prospective neural plate in the early chick blastoderm. *J. Exp. Zool.* **120,** 109–130.

Stein, S., and Kessel, M. (1995). A homeobox gene involved in node, notochord and neural plate formation of chick embryos. *Mech. Dev.* **49,** 37–48.

Stern, C. D. (1993). Avian embryos. *In* "Essential Developmental Biology—A Practical Approach" (C. D. Stern and P. W. H. Holland, Eds.), pp. 45–54. IRL Press, Oxford.

Storey, K. G., Crossley, J. M., De Robertis, E. M., Norris, W. E., and Stern, C. D. (1992). Neural induction and regionalisation in the chick embryo. *Development* **114,** 729–741.

Streit, A., Lee, K. J., Woo, I., Roberts, C., Jessell, T. M., and Stern, C. D. (1998). Chordin regulates primitive streak development and the stability of induced neural cells, but is not sufficient for neural induction in the chick embryo. *Development* **125,** 507–519.

Streit, A., and Stern, C. D. (1999). Establishment and maintenance of the border of the neural plate in the chick: Involvement of FGF and BMP activity. *Mech. Dev.* **82,** 51–66.

Tam, P. P. L., and Steiner, K. A. (1999). Anterior patterning by synergistic activity of the early gastrula organizer and the anterior germ layer tissues of the mouse embryo. *Development* **126,** 5171–5179.

Tam, P. P. L., Steiner, K. A., Zhou, S. X., and Quinlan, G. A. (1997). Lineage and functional analyses of the mouse organizer. *Cold Spring Harbor Symp. Quant. Biol.* **62,** 135–144.

Thomas, P., and Beddington, R. (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Curr. Biol.* **6,** 1487–1496.

Tsang, T. E., Kinder, S. J., and Tam, P. P. L. (1999). Experimental analysis of the emergence of left–right asymmetry of the body axis in early postimplantation mouse embryos. *Cell. Mol. Biol.* **45,** 493–503.

Varlet, I., Collignon, J., and Robertson, E. J. (1997). nodal expression in the primitive endoderm is required for specification of the anterior axis during mouse gastrulation. *Development* **124,** 1033–1044.

Viebahn, C., Mayer, B., and Hrabe de Angelis, M. (1995). Signs of the principle body axes prior to primitive streak formation in the rabbit embryo. *Anat. Embryol.* **192,** 159–169.

Waddington, C. H. (1932). Experiments on the development of chick and duck embryos, cultivated in vitro. *Philos. Trans. R. Soc. London B* **221,** 179–230.

Waddington, C. H. (1934). Experiments on embryonic induction. *J. Exp. Biol.* **11,** 211–227.

Waddington, C. H. (1936). Organizers in mammalian development. *Nature* **138,** 125.

Waddington, C. H. (1937). Experiments on determination in the rabbit embryo. *Arch. Biol.* **48,** 273–290.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M., and Darnell, J. E., Jr. (1994). The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78,** 575–588.

Wilkinson, D. G. (1992). "In Situ Hybridisation: A Practical Approach." Oxford Univ. Press, London.

Yuan, S., Darnell, D. K., and Schoenwolf, G. C. (1995). Mesodermal patterning during avian gastrulation and neurulation: Experimental induction of notochord from non-notochordal precursor cells. *Dev. Genet.* **17,** 38–54.

Zhu, L., Belo, J. A., De Robertis, E. M., and Stern, C. D. (1999). Goosecoid regulates the neural inducing strength of the mouse node. *Dev. Biol.* **216,** 276–281.

Zimmermann, L. B., De Jesus-Escobar, J. M., and R. M., H. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86,** 599–606.

Received for publication March 24, 2000
Revised June 12, 2000
Accepted June 12, 2000