Developmental Biology **216,** 276–281 (1999)
Article ID dbio.1999.9508, available online at http://www.idealibrary.com on IDEAL®

Goosecoid Regulates the Neural Inducing Strength of the Mouse Node

L. Zhu,* J. A. Belo,†,[1] E. M. De Robertis,† and C. D. Stern*,[2]

**Department of Genetics and Development, Columbia University, 701 West 168th Street, New York, New York 10032; and †Department of Biological Chemistry and Howard Hughes Medical Institute, University of California, Los Angeles, California 90095-1662*

The homeobox gene *goosecoid* was the first specific genetic marker of Spemann's organizer in vertebrate embryos to be discovered. In the frog, misexpression of this gene by RNA injection produces duplication of the posterior axis. For these reasons, the recent finding that mice lacking *goosecoid* function have no early axial defects was rather surprising. Here we assay the neural inducing strength of wild-type and *goosecoid*-mutant mouse nodes by transplantation into primitive streak stage chick embryos. Wild-type mouse nodes strongly induce the neural-specific transcription factors *Sox2* and *Sox3* in the chick host. Homozygous *goosecoid*$^{-/-}$ nodes are severely impaired in their ability to induce both genes. Heterozygous *goosecoid*$^{+/-}$ nodes induce *Sox3* as well as wild-type nodes, but resemble −/− nodes in their limited ability to induce *Sox2*. We propose that *goosecoid* does play a role in regulating the neural inducing strength of the node and that regulative mechanisms exist which mask the early phenotypic consequences of *goosecoid* mutations in the intact mouse embryo.

INTRODUCTION

The organizer is a special region of gastrulating vertebrate embryos, defined by its ability to induce neural tissue when transplanted to an ectopic site. In amphibians, the organizer property is located at the dorsal lip of the blastopore and in amniotes, the organizer is Hensen's node (usually just called "the node" in mouse). The homeobox gene *goosecoid* was the first organizer-specific gene to be discovered (Cho *et al.*, 1991; Blum *et al.*, 1992; Izpisúa-Belmonte *et al.*, 1993): when ectopically expressed by injection into the ventral side of an early *Xenopus* embryo, a supernumerary organizer is generated (Cho *et al.*, 1991; Blum *et al.*, 1992) and cell migration during gastrulation is affected (Niehrs *et al.*, 1993); subsequently an almost complete secondary axis develops, suggesting that this gene is involved in defining the organizer region. Because of this information, two recently reported findings are surprising: first, the phenotype of mice lacking a functional *goosecoid* gene does not include obvious defects in gastrulation, neural induction, or any of the known early functions of the organizer (Yamada *et al.*, 1995; Rivera-Pérez *et al.*, 1995). Second, chick embryos from which all cells destined to form the organizer have been surgically removed regenerate a new organizer and subsequently develop normally, although the regenerated organizer does not express *goosecoid* (Psychoyos and Stern, 1996).

The chick embryo lends itself very well to assays of neural induction because a peripheral region, the area opaca, which does not normally contribute cells to any embryonic structure, can nevertheless respond to an organizer graft by generating neural tissue that is normally patterned and which expresses panneural markers (Storey *et al.*, 1992; Streit *et al.*, 1998). Here, we have made mouse–chick chimeras to assay the neural-inducing strength of nodes obtained from wild-type and *goosecoid*-mutant mouse embryos. We used chick-specific riboprobes to detect expression of the HMG-containing transcription factors *Sox3* and *Sox2*, which are early, specific, and reliable markers expressed throughout the neural plate and neural tube of the chick embryo (Streit *et al.*, 1998). We find that in the absence of *goosecoid* the neural-inducing ability of the mouse node is severely impaired in this assay. Even het-

[1] Present address: Mouse Development Laboratory, Instituto Gulbenkian de Ciência, Rua da Quinta Grande 6, Apartado 14, 2780-156 Oeiras, Portugal.

[2] To whom correspondence should be addressed. Fax: (212) 923 2090. E-mail: cds20@columbia.edu.

0012-1606/99 $30.00

TABLE 1
Numbers of Donor Embryos Recovered and Used for *Sox2/3* Induction Experiments

gsc genotype	No. dissected (exp.)	Dead	Lost	Too old	Other	No. used
+/+	55 (41)	2	2	7	0	44
+/−	65 (82)	6	3	14	2	40
−/−	44 (41)	0	0	9	1	32
Total	164	8	5	30	3	116

Note. All mice are *β-gal*$^{+/-}$; the genotype for *goosecoid* is shown. The first column shows the number of embryos harvested, classified according to their genotype. The expected (exp.) number of embryos based on the Mendelian ratio (1:2:1) is shown in parentheses. The next three columns indicate the numbers of embryos that were not used because the donor tissue had died, the graft was lost, the donor was older than 0B stage, or the graft was accidentally placed in the wrong position in the host ("other"). The final column shows the numbers of embryos used for scoring.

erozygous mutant nodes have an impaired ability to induce *Sox2* expression in the chick, suggesting that *goosecoid* does play a role in the organizer. We suggest that the lack of an early phenotype in *goosecoid* mutant mice indicates the existence of regulatory mechanisms that can compensate for the loss of function of this gene.

MATERIALS AND METHODS

Generation of β-Galactosidase (β-Gal)-Expressing Goosecoid Mutant Mouse Embryos

Goosecoid heterozygotes (Yamada *et al.*, 1995) in a B6S JL/F1 background were crossed with the Rosa-26 mouse line (B6, 129-TgR) which expresses β-gal in all cells (Jackson Laboratories) to obtain *goosecoid*$^{+/-}$/*β-gal*$^{+/-}$ mice. Mice of this genotype were back-crossed with Rosa-26 to obtain *goosecoid*$^{+/-}$/*β-gal*$^{+/+}$ male mice. These were back-crossed with *goosecoid*$^{+/-}$/*β-gal*$^{-/-}$ females to generate mouse embryos of different *goosecoid* genotypes for subsequent experiments.

Preparation of Donor Mouse Node

Embryos on the seventh day of gestation were recovered from the matings described above, staged as described by Downs and Davis (1993), and collected in M2 medium (Sigma) containing 10% fetal calf serum. The distal tip of the embryos was excised by mechanical dissection with siliconized glass needles. In some experiments, a piece of similar size was dissected from either the anterior or the posterior part of the same embryo and used as a control graft. The extraembryonic tissues were used to genotype each individual embryo by PCR, as previously described (Belo *et al.*, 1998). The distribution of embryos according to genotype and the proportion of these that were used for induction experiments are shown in Table 1.

Transplantation of Mouse Node into Chick Host

Chick host embryos were staged according to Hamburger and Hamilton (1951). The dissected mouse node region was grafted into the area opaca of stage 3+/4 chick embryos, at the level of the host node. The chimeric embryos were set up in modified New culture as previously described (Stern, 1993), except that mouse explant medium M2 plus 10% fetal calf serum was used instead of chick albumen in the culture. The grafted embryos were then cultured for 20–24 h at 38°C before analysis.

Visualization of β-Gal Activity and Whole Mount in Situ Hybridization

Chimeric embryos were fixed at room temperature in 4% paraformaldehyde (PFA) for 1 h. After fixation, they were rinsed in PBS several times before incubation at 30–32°C for 1–3 h in β-gal staining solution [0.4 mg/ml 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (Molecular Probes), 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% Nonidet P-40 in PBS], which yields a magenta color. After staining, embryos were postfixed in 4% PFA overnight at 4°C.

Embryos were then assessed for the expression of the early panneural markers *Sox3* and *Sox2* (kind gifts from Drs. R. Lovell-Badge and P. Scotting) by whole mount *in situ* hybridization as previously described (Psychoyos and Stern, 1996; Streit *et al.*, 1998). Digoxigenin-labeled riboprobes were transcribed from the 3′-untranslated region and were chick specific. The alkaline phosphatase reaction was visualized with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate to yield a purple/blue color. Following *in situ* hybridization, the embryos were postfixed and embedded in paraffin wax for histological sectioning.

RESULTS

The Grafted Mouse Node Survives and Develops in the Chick Host

In initial experiments, we grafted mouse nodes into the area opaca of chick host embryos and examined the morphology of the grafted node and surrounding host regions as well as the expression of a marker for the notochord of the mouse [mouse *Sonic hedgehog* (*Shh*)] after 24 h of culture. When the chick embryo culture was set up conventionally, using egg albumen as substrate, the mouse node did not

change morphology during culture and did not express *Shh* after culture (0/5). We reasoned that this could be due to the high pH of albumen (about 9.2), which is likely to be deleterious to the mouse tissue. For this reason we replaced the egg albumen culture medium with mouse M2 medium containing 10% fetal calf serum. These culture conditions allowed the grafted mouse node to survive, to express β-gal strongly, and to differentiate, elongating during the culture period and frequently forming a notochord that expressed *Sonic hedgehog* (9/10) (Fig. 1A).

The Mouse Node Induces the Early Neural Markers Sox3 and Sox2 in the Chick Host

Next, we assayed the ability of grafted E7.0 (Rosa-26) mouse nodes to induce neural markers in the area opaca of the chick host. Following culture, we stained the chimeras with β-gal staining solution (to reveal the grafted mouse cells) and then for either *Sox2* or *Sox3* (chick specific) by *in situ* hybridization, followed by histological sectioning of the embryos. A high proportion of chimeras (28/44; 64%) showed expression of the chick neural markers (Figs. 1B, 1C, 1E, and 1F), and the chick epiblast immediately above the grafted mouse node had elevated and thickened, similar to what is seen after a graft of a chick node (Storey *et al.*, 1992; Streit *et al.*, 1998) (Fig. 1E, F; inp). *Sox3* was induced more frequently (86%) than *Sox2* (44%). As controls, we grafted other portions of the egg cylinder: neither the anterior (0/12) nor the posterior region (0/6) induced *Sox3* expression or columnar morphology in the chick host (Fig. 1D).

We also classified the results of this graft according to the stage of the donor mouse embryo. No statistically significant difference was found in the inducing ability of nodes obtained from mice at early streak (ES), mid-streak (MS), late streak (LS), or no allantoic bud (0B) stages, but nodes obtained from early bud (EB) stage embryos had reduced ability to induce *Sox3* (1/9; 11%).

Goosecoid Mutant Nodes Have Impaired Ability to Induce Chick Neural Markers

The same experiment was done with nodes obtained from embryos derived from Rosa-26/*goosecoid*$^{-}$ crosses, and the genotype of each donor mouse node was determined retrospectively by PCR (Belo *et al.*, 1998). The results are summarized in Table 2. Mouse nodes lacking both copies of *goosecoid* are greatly impaired in their ability to induce either marker. Nodes lacking one copy of *goosecoid* are able to induce *Sox3* as well as wild-type nodes, but induce *Sox2* much less efficiently (Table 2).

DISCUSSION

Our results show that the mouse node can induce the expression of the neural markers *Sox3* and *Sox2* in a host chick embryo. This was expected because other combinations of organizer and ectoderm across different vertebrate classes (including rabbit nodes placed into avian embryos and vice versa; Waddington, 1930, 1932, 1933, 1934; Waddington and Schmidt, 1933) can also induce neural tissue from the host (Waddington, 1930, 1932, 1933, 1934; Waddington and Schmidt, 1933; Blum *et al.*, 1992; Kintner and Dodd, 1991; Hatta and Takahashi, 1996). Nodes obtained from older (late streak to no allantoic bud stages) mouse donor embryos generate ectopic expression of *Sox2* at a slightly lower frequency than those from younger donors, but the difference is not apparent for *Sox3*, which appears to be an easier gene to induce overall (Table 2).

In addition, we show that lack of even a single copy of *goosecoid* reduces the ability of the mouse node to induce *Sox2* and that lack of both copies impairs its ability to induce both genes. It is interesting to compare this finding with a previous report of crosses between *goosecoid* mutant mice and those defective in another gene expressed in the organizer, *HNF3β*. Double heterozygous (*HNF3β*$^{+/-}$/*goosecoid*$^{+/-}$) embryos have a severe early phenotype even though neither heterozygote alone shows any early defects (Filosa *et al.*, 1997).

When assaying the neural inducing ability of the mouse node, consideration should be given to a contribution from anterior visceral endoderm (AVE), a tissue that been shown to be important for the induction and/or patterning of anterior regions of the nervous system (Thomas and Beddington, 1996; Varlet *et al.*, 1997; Belo *et al.*, 1997). *Goosecoid* is expressed in the AVE in addition to the node and may play a role in both tissues. Between the early and late streak stages, the AVE appears to move anteriorly such that at the early streak stage, the *goosecoid* expression domain overlaps the distal tip of the egg cylinder, while at late streak/no bud stage it has separated from the node. We might therefore expect to find differences in inducing ability of nodes (distal tips) dissected from early (ES–MS) compared to late (LS–0B) embryos. However, this is not the case (Table 2); in our assay, the distal tips of embryos from ES to 0B stages are able to induce both *Sox3* and *Sox2*.

More surprisingly, our assay reveals a requirement for *goosecoid* in the mouse node that is not apparent in the intact mouse embryo, where lack of even both copies of the gene does not affect early development (Yamada *et al.*, 1995; Rivera-Pérez *et al.*, 1995). It has been suggested that the lack of an early phenotype in the whole mutant animal could be due to functional redundancy with other *goosecoid*-related genes (Belo *et al.*, 1998; Lemaire *et al.*, 1997; Funke *et al.*, 1997). In chick embryos, the *goosecoid*-related gene *GSX* (Lemaire *et al.*, 1997) is expressed in the primitive streak but not in the organizer, suggesting that *goosecoid* expression represses *GSX* expression in the same cells (Lemaire *et al.*, 1997). If the mouse has a gene homologous to *GSX*, one possibility is that the absence of *goosecoid* expression in the node allows *GSX* expressing cells to

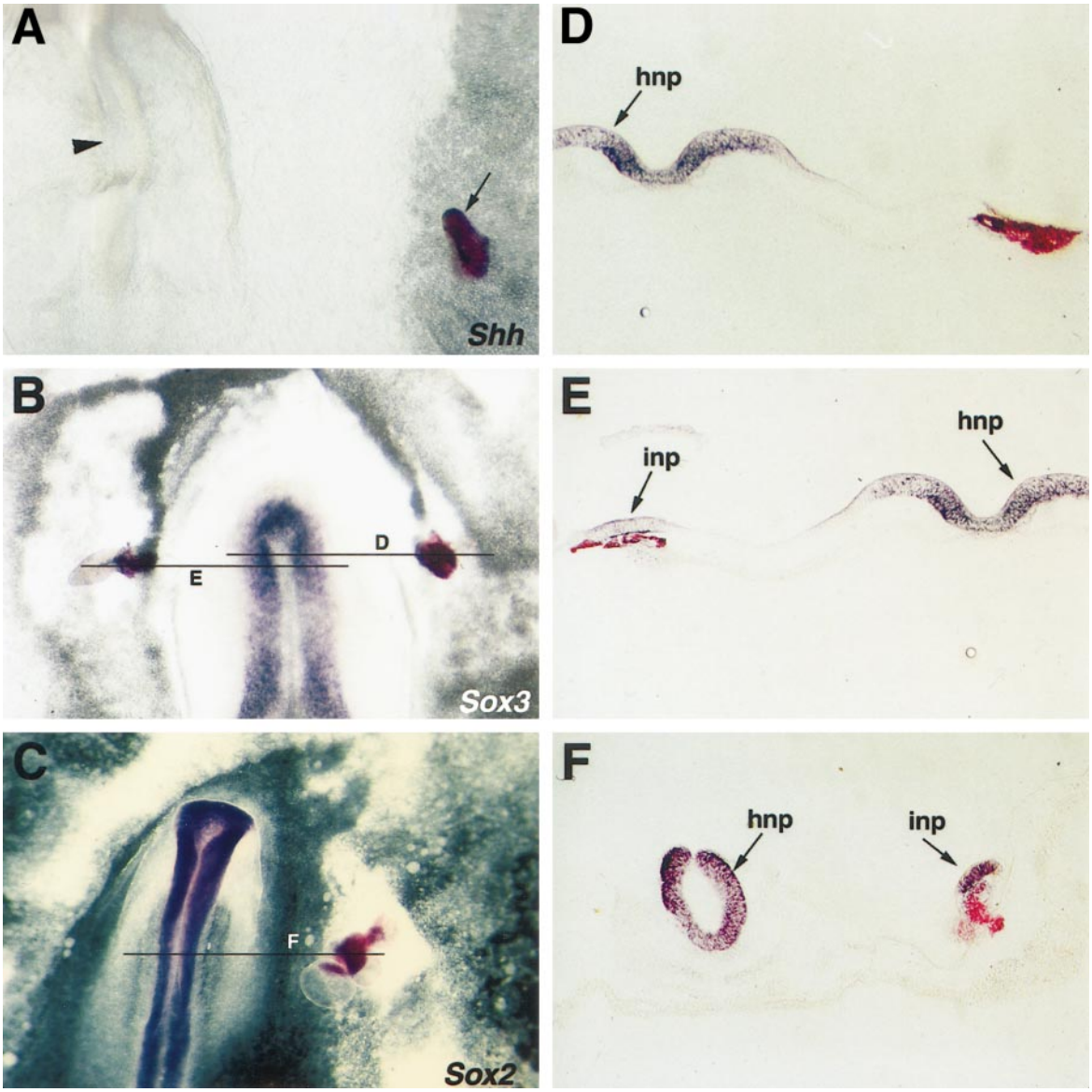

FIG. 1. (A) A mouse node grafted into the area opaca of a host chick embryo survives, elongates, and expresses the node/notochord marker *Sonic hedgehog* (*Shh*). *In situ* hybridization using a mouse-specific probe for *Shh* detects expression by the mouse node derivatives (arrow) but not in the notochord of the chick host (arrowhead). (B) Induction of chick *Sox3* by the grafted mouse node. This chick embryo received a graft of a mouse node on the left and a similar-sized fragment of mouse posterior primitive streak on the right. The node graft has elongated while the streak graft has not. *In situ* hybridization with chick-specific *Sox3* probe (purple) and in toto staining with salmon-gal (red) to reveal Rosa-26 donor cells. In section, the posterior primitive streak graft (D) expresses β-gal but no *Sox3* expression or any effect on cell morphology is seen in the overlying chick epiblast. The node graft, on the other hand (E), has induced a thickening of the epiblast as well as expression of chick *Sox3* (inp). hnp, host neural plate. (C) Induction of chick *Sox2* by the mouse node. In (F), the grafted node cells (expressing β-gal, red) lie adjacent to a region of chick epiblast that has adopted columnar morphology and expresses chick *Sox2* (purple). hnp, host neural plate. inp, induced neural plate.

take on node functions and compensate for the lack of *goosecoid*. In grafts of the node to a region remote from the endogenous domain of *GSX* expression, as done here, no such regulation would occur because of the absence of a population of *GSX*-expressing cells close to the graft.

A second, and perhaps more likely, explanation is that

TABLE 2

Induction of Chick *Sox3* and *Sox2* by Mouse Nodes of Different Genotype

gsc genotype	Mouse node stage: ES–MS	Mouse node stage: LS–0B	Total
	Sox3		
+/+	8/9	10/12	18/21 (86%)
+/−	0/1	16/19	16/20 (80%)
+/+	1/4	8/19	9/23 (39%)*
	Sox2		
+/+	5/10	5/13	10/23 (43%)
+/−	1/5	3/15	4/20 (20%)***
−/−	0/3	2/6	2/9 (22%)**

Note. All mice are β-*gal*$^{+/-}$; the genotype for *goosecoid* is shown. Stages (Downs and Davies, 1993) of donor mouse embryos: ES, early streak; MS, mid-streak; LS, late streak; 0B, no allantoic bud. All chick hosts were at stage 3+/4 (Hamburger and Hamilton, 1951). Statistical analysis (χ^2, 2 × 2 contingency test, compared to wild type): *$P < 0.002$; **$P < 0.02$; ***$P < 0.05$.

the chick area opaca assay requires stronger inducing activity than does the prospective neural plate of the normal embryo and is therefore able to uncover more subtle consequences of the mutation. Whichever of these interpretations turns out to be correct, our results do reveal that *goosecoid* activity is required in the mouse node for its full inducing strength and that the phenotypic consequences of the lack of just one copy can be detected with this assay.

ACKNOWLEDGMENTS

This work was supported by NIH Grant GM53456 to C.D.S. and NIH Postdoctoral Fellowship CA74432 to L.Z. E.M.D.R. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

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 neuralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech. Dev.* **68,** 45–57.

Belo, J. A., Leyns, L., Yamada, G., and De Robertis, E. M. (1998). The prechordal midline of the chondrocranium is defective in Goosecoid-1 mouse mutants. *Mech. Dev.* **72,** 15–25.

Blum, M., Gaunt, S. J., Cho, K. W., Steinbeisser, H., Blumberg, B., Bittner, D., and De Robertis, E. M. (1992). Gastrulation in the mouse: The role of the homeobox gene goosecoid. *Cell* **69,** 1097–1106.

Cho, K. W., Blumberg, B., Steinbeisser, H., and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene goosecoid. *Cell* **67,** 1111–1120.

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., Rivera-Pérez, J. A., Gómez, A. P., Gansmüller, A., Sasaki, H., Behringer, R. R., and Ang, S. L. (1997). Goosecoid and HNF-3β genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124,** 2843–2854.

Funke, B., Saint-Jore, B., Puech, A., Sirotkin, H., Edelmann, L., Carlson, C., Raft, S., Pandita, R. K., Kucherlapati, R., Skoultchi, A., and Morrow, B. E. (1997). Characterization and mutation analysis of goosecoid-like (GSCL), a homeodomain-containing gene that maps to the critical region for VCFS/DGS on 22q11. *Genomics* **46,** 364–372.

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

Hatta, K., and Takahashi, Y. (1996). Secondary axis induction by heterospecific organizers in zebrafish. *Dev. Dynam.* **205,** 183–195.

Izpisúa-Belmonte, J. C., De Robertis, E. M., Storey, K. G., and Stern, C. D. (1993). The homeobox gene goosecoid and the origin of organizer cells in the early chick blastoderm. *Cell* **74,** 645–659.

Kintner, C. R., and Dodd, J. (1991). Hensen's node induces neural tissue in *Xenopus* ectoderm: Implications for the action of the organizer in neural induction. *Development* **113,** 1495–1505.

Knoetgen, H., Viebahn, C., and Kessel, M. (1999). Head induction in the chick by primitive endoderm of mammalian, but not avian origin. *Development* **126,** 815–825.

Lemaire, L., Roeser, T., Izpisúa-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.

Niehrs, C., Keller, R., Cho, K. W., and De Robertis, E. M. (1993). The homeobox gene goosecoid controls cell migration in *Xenopus* embryos. *Cell* **72,** 491–503.

Psychoyos, D., and Stern, C. D. (1996). Restoration of the organizer after radical ablation of Hensen's node and the anterior primitive streak in the chick embryo. *Development* **122,** 3263–3273.

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.

Rivera-Pérez, J. A., Mallo, M., Gendron-Maguire, M., Gridley, T., and Behringer, R. R. (1995). Goosecoid is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. *Development* **121,** 3005–3012.

Stern, C. D. (1993). Avian embryos. *In* "Essential Developmental Biology: A Practical Approach" (C. D. Stern and P. W. 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.

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.

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.

Waddington, C. H. (1930). Developmental mechanics of chick and duck embryos. *Nature* **125,** 924–925.

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

Waddington, C. H. (1933). Induction by the primitive streak and its derivatives in the chick. *J. Exp. Biol.* **10,** 38–46.

Waddington, C. H. (1934). Experiments on embryonic induction. III. A note on inductions by the chick primitive streak transplanted to the rabbit embryo. *J. Exp. Biol.* **11,** 223–228.

Waddington, C. H., and Schmidt, G. A. (1933). Inductions by heteroplastic grafts of the primitive streak in birds. *Roux Arch. EntwMech. Org.* **128,** 522–563.

Yamada, G., Mansouri, A., Torres, M., Stuart, E. T., Blum, M., Schultz, M., De Robertis, E. M., and Gruss, P. (1995). Targeted mutation of the murine goosecoid gene results in craniofacial defects and neonatal death. *Development* **121,** 2917–2922.

Received for publication July 22, 1999
Revised September 20, 1999
Accepted September 20, 1999