Restoration of the organizer after radical ablation of Hensen's node and the anterior primitive streak in the chick embryo

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SUMMARY

The region of the amniote embryo corresponding to Spemann's organizer in amphibians is Hensen's node, which lies at the tip of the primitive streak during gastrulation. It is a special site in the embryo that can be defined by the presence of progenitors of several axial tissues (notochord, prechordal mesoderm, somites, gut endoderm), by characteristic cell movements, by specific patterns of gene expression (e.g. *goosecoid*, *HNF-3β*, *Sonic hedgehog*) and, most importantly, by its ability to induce a complete axis, including host-derived neural tissue, when transplanted to an ectopic site.

Here, we show that complete removal not only of the node but also of the anterior 40% of the primitive streak leads to the development of normal embryos containing cells with all the fates normally produced by the node. Cell movement pathways through the regenerated node are identical to those seen in the normal embryo. The patterns of expression of *HNF-3β* and *Sonic hedgehog* are also restored, as is their left/right asymmetry, but *goosecoid* expression is not. When the regenerated node is transplanted to an ectopic site, it induces a complete embryonic axis that includes a fully patterned, host-derived central nervous system.

Analysis of the properties of cells surrounding the site of ablation shows that they acquire these properties gradually. We suggest that the organizer is a region of the embryo that is defined by cell interactions and that the node normally inhibits the organizer state in neighbouring cells.

Key words: Hensen's node, regeneration, primitive streak, gastrulation, neural induction, notochord, chick embryo, cell migration, cell fate

INTRODUCTION

Hensen's node, the amniote equivalent of the amphibian 'Spemann's organizer', is generally considered to be the most important region of the very early, gastrulating embryo. Not only does it generate the midline organs of the body (notochord, prechordal region, somites, gut), but is also responsible for inducing and patterning the whole of the central nervous system.

Like its amphibian counterpart (the dorsal lip of the blastopore), Hensen's node can be characterized by a well-defined set of cellular and molecular properties. In amniote embryos, the node is a bulb-like thickening lying at the cranial tip of the primitive streak during gastrulation. In the chick, where most studies have been conducted because of its ease of manipulation, the node is some 100 μm in diameter and contains about 2,000 cells (for reviews see Gallera, 1971; Nicolet, 1971; Leikola, 1976; Hara, 1978; Stern, 1994; Streit et al., 1994). Among the salient features of the chick node are:

(a) *the fate of its cells*: the node gives rise to the notochord/head process, the prechordal mesendoderm, the definitive (gut) endoderm, the medial halves of the somites and contributes to the midline (floor plate, or notoplate) of the future spinal cord (see Spratt, 1955; Rosenquist, 1966; 1983; Nicolet, 1970; Hara, 1978; Selleck and Stern, 1991; 1992; Schoenwolf, 1992);

(b) *its expression of a number genes in a stage- and region-specific manner:* these include the homeobox genes *goosecoid* (Izpisúa-Belmonte et al., 1993) and *cNot* (Stein and Kessel, 1995; Knezevic et al., 1995), the secreted factors HGF/SF (Streit et al., 1995), *Sonic hedgehog (Shh*; Riddle et al., 1993; Roelink et al., 1994) and *c-NR1* (Levin et al., 1995), the activin receptors cActR-IIA and cActRIIB (Stern et al., 1995), and the transcription factor *HNF-3β* (Ruiz i Altaba et al., 1995);

(c) *its role in the establishment of left/right asymmetry:* four of the above genes, *Shh*, *cActRIIA, cNR-1* and *HNF-3β* are expressed in or near the node in an asymmetric fashion and their misexpression alters the left-right polarity of heart looping (Levin et al., 1995);

(d) *its ability to induce an ectopic nervous system:* when grafted into an ectopic site (including regions fated to contribute only to extraembryonic membranes) at an appropriate stage of development (up to about stage 5), the node is able to change the fates of neighbouring epiblast cells by inducing them to form a complete nervous system (Waddington, 1932; 1933; Gallera, 1971; Hara, 1978; Dias and Schoenwolf, 1990; Storey et al., 1992);

(e) *its ability to pattern the neural plate of a host embryo:*

when grafted to appropriate position adjacent to the neural plate of a host embryo, even older nodes are able to *organize* a second axis from the neuralized cells of the host (Gallera, 1971; Storey et al., 1992; Izpisúa-Belmonte et al., 1993). Perhaps surprisingly, this ability operates across species and even across vertebrate classes (see Kintner and Dodd, 1991; Streit et al., 1994);

(f) *its ability to induce extra digits in the limb bud of a host embryo:* when grafted to the anterior margin of the limb bud of a host embryo, the node can induce digit duplications, mimicking the activity of the polarizing region of the limb (Hornbruch and Wolpert, 1986). However, this activity is different from neural inducing ability: the node starts to lose neural inducing activity from stage 4^{+}-5 (Storey et al., 1992), but it continues to induce extra digits until the 7 somite stage (stage 9; Hornbruch and Wolpert, 1986).

Hence, the node is a structure that can be defined morphologically, by the fate of its cells, by its unique inducing and patterning properties, and by the genes that it expresses at particular times in development. It is therefore perhaps surprising that several studies have reported an apparent ability of chick embryos to regulate after excision, reversal or replacement of the node (Abercrombie and Waddington, 1937; Abercrombie, 1950; Grabowski, 1953; 1956; Abercrombie and Bellairs, 1954; Gallera, 1965; Gallera and Dicenta, 1966; Butros, 1967; Gallera and Nicolet, 1974; Schoenwolf and Yuan, 1995). Since most of these studies were conducted without objective markers to identify the tissues formed, or without investigating whether regeneration involves changes in cell fates or reacquisition of inducing properties by cells that normally lack them, we have undertaken a reinvestigation of this issue. We find that embryos deprived not only of the node itself but also of the entire anterior 40% of the primitive streak can indeed regulate. They generate all the axial structures including a head, notochord, somites and gut endoderm, via cell movements that appear indistinguishable from those of unoperated embryos. The regenerated node expresses *HNF-3β* and *Sonic hedgehog* but not *goosecoid*, and also acquires the ability both to induce and to pattern a complete nervous system when transplanted to a host embryo. The experimental paradigm derived from these studies provides a novel means of investigating the molecular mechanisms that establish and maintain the organizer state in higher vertebrate embryos.

MATERIALS AND METHODS

Embryos and culture techniques

Fertile hens' eggs (White Leghorn, obtained from Spafas) were incubated at 38°C for about 12 hours to give embryos at stages 3^{+}-5 (Hamburger and Hamilton, 1951). Embryos were explanted in Pannett-Compton saline (Pannett and Compton, 1924) ventral side uppermost in modified New (1955) culture (Stern and Ireland 1981; Stern, 1993). This assembly was then transferred to a pool of albumen in a 35 mm plastic dish, where the operations were carried out.

Surgical manipulations

Ablations were done using fine glass needles made from 50 μl capillaries (Sigma) in a vertical electrode puller, while the embryo was submerged in Pannett-Compton saline. After the operation, the saline was removed from inside the glass ring and any remaining fluid within the site of ablation aspirated carefully with a very fine capillary, to

which suction was applied. This turned out to be very important to ensure healing of the opening. Reducing the tension of the vitelline membrane around the ring and the tension exerted by the blastoderm as it spread were also important to improve healing; this was achieved by nicking the outermost edge of the *area opaca* and by minimising the amount of albumen under the membrane.

In each case, the cuts were guided by recent fate maps of the primitive streak (Psychoyos and Stern, 1996); an eyepiece graticule was used to measure the length of the primitive streak and the position of the site of ablation with reference to the node as described previously (Psychoyos and Stern, 1996).

Carbocyanine dye labelling

To follow cell fates, small groups of cells were labelled with the carbocyanine dyes DiI (1,1′-dioctadecyl 3,3,3′,3′-tetramethyl indocarbocyanine perchlorate; Molecular Probes) and DiO (3,3′-dioctadecyl oxacarbocyanine perchlorate; Molecular Probes), applied as a 0.025% solution of the dye in 0.3 M sucrose/10% ethanol as described previously (Selleck and Stern 1991; Ruiz i Altaba et al., 1993; Psychoyos and Stern 1996). This was injected by air pressure through a micropipette made by pulling a 50 μl glass capillary in a vertical electrode puller. To follow the movements of labelled cells, embryos were photographed immediately after labelling and at intervals of one to a few hours thereafter as described previously (Psychoyos and Stern 1996).

After the operation and carbocyanine dye labelling in a lateral, anterior or posterior site relative to the ablation (described in the text and figure legends for each experiment), embryos were allowed to rest at 28-30°C for 3-4 hours and then cultured at 38°C in a humid chamber for up to 30 hours, when they had reached stages 9-12. The final position of the labelled cells was assessed both in whole mounts under fluorescence illumination and using wax histology following photooxidation of the dye as described previously (Izpisúa-Belmonte et al., 1993).

Assay of inducing ability of the regenerated node

To test whether the regenerated node has inducing properties, the assay described by Storey et al. (1992) was used. Briefly, the new node was excised from the blastoderm as soon as the healing was complete, before emergence of a head process, and transplanted to the inner margin of the area opaca of a host chick embryo at stages 3-3^{+}, placing it between the germ wall margin and the epiblast at the same level as the host node. To distinguish the grafted cells from those of the host, the transplanted node was labeled with DiI prior to grafting. Transplanted embryos were incubated until the host had reached stages 9-12, fixed and photooxidized as described above. In a few experiments, the ablation operation was carried out in a quail embryo and pieces of this subsequently grafted to a chick host. Quail cells were then revealed by immunoperoxidase with QCPN antibody, developed by Dr B. M. Carlson and obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and the Department of Biological Sciences, University of Iowa, Iowa City 52242, under contract N01-HD-2-3144 from the NICHD.

Whole-mount immunohistochemistry

To assess the phenotype of cells in the regenerated notochord, we used monoclonal antibody Not-1 (Yamada et al., 1991; kind gift of Drs J. Dodd and T. M. Jessell). After fixation of the cultured embryos in 4% formaldehyde in PBS for 1 hour, endogenous peroxidase was quenched by incubation in 0.3% H_2O_2 in the fixative. After rinsing in PBS and permeabilization in 0.5% or 1% Triton-X100 in PBS (PBT), they were blocked in 1% BSA and 1% heat-inactivated goat serum in PBT and incubated for 2 days in 1:1 dilution of supernatant in blocking buffer. After further washes in PBT, they were incubated overnight in 1:2,500 dilution of goat anti-mouse IgG-peroxidase

(Jackson Immunoresearch) in blocking buffer. Following further washes in PBT, the peroxidase activity was revealed with 500 µg/ml diaminobenzidine and 0.003% H_2O_2 in 0.1 M Tris (pH 7.4). QCPN staining was performed using an identical technique.

Whole-mount in situ hybridization

The procedure for whole-mount in situ hybridization using digoxigenin-labeled riboprobes was based on an unpublished protocol kindly provided by Drs D. Ish-Horowicz and P. Ingham, with some modifications. Briefly, probes were transcribed using T3, T7 or SP6 polymerase as appropriate for the plasmid vector: except for the *HNF-3β* probe (plasmid CT7 of Ruiz i Altaba et al., 1995), all other probes had been cloned into pBlueScript SK or KS (Stratagene). Embryos were fixed in 4% formaldehyde in PBS containing 2 mM EGTA overnight, and then stored in methanol at −20°C. Proteinase-K (Sigma) (10 µg/ml) treatment was at room temperature for 15 minutes, and this was stopped by fixation for 20-30 minutes in 4% formaldehyde containing 0.25% glutaraldehyde. Hybridization was carried out in 1.3× SSC (pH 5.3), 50% formamide containing 0.5% CHAPS and 0.2% Tween-20 at 70°C for most probes except *Krox-20*, where the temperature was 64°C. Post-hybridization washes were conducted at the hybridization temperature in the same buffer, followed by several washes in Tris-saline (pH 7.4) containing 1% Tween-20. Digoxigenin was detected by incubation in anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer-Mannheim) diluted 1:5,000 and, after further washes, the enzyme activity was visualised by the NBT/BCIP reaction in Tris-saline (pH 9.5).

In cases where it was necessary to combine in situ hybridization with antibody staining, immunocytochemistry was performed after the in situ procedure. Where in situ hybridization was combined with photooxidation of DiI, the in situ detection was done at the end (see Izpisúa-Belmonte et al., 1993).

The cDNA probes used were as follows: *goosecoid* (Izpisúa-Belmonte et al., 1993); *HNF-3β* (Ruiz i Altaba et al., 1995; kind gift of Dr A. Ruiz i Altaba, New York University); *Sonic hedgehog* (Riddle et al., 1994; kind gift of Dr C. Tabin, Harvard University); *Krox-20* (180 base pair probe for chick *Krox-20*, kind gift of Dr D.

Wilkinson, NIMR, London); *Sox-2* (Uwanogho et al., 1995; Collignon et al., 1996; kind gift of Dr R. Lovell-Badge, NIMR, London) and *Tailless* (Yu et al., 1994; kind gift of Dr R. Yu, Nara Institute of Science and Technology, Japan).

Histological processing

Embryos that had been subjected to in situ hybridization were fixed in formaldehyde, dehydrated in absolute methanol for 5 minutes, in propan-2-ol for 10 minutes and cleared in tetrahydronaphthalene for 30 minutes. They were then embedded in Paraffin wax and sectioned at 10 µm. Other embryos were processed by standard histological techniques.

RESULTS

Embryos develop normally after extirpation of the node

We first wished to confirm the findings reported by Grabowski (1956) that embryos can develop normally after ablation of Hensen's node. We extirpated the node in 40 embryos from stages 3^+-6, and incubated them for up 30 hours. As described by Grabowski, we find that, when the node is ablated up to stage 4^-, the embryos develop with normal morphology, including a notochord and somites as well as head structures (Fig. 1). In contrast, embryos from which the node is extirpated at stages 4^+-6 form a head process (cranial notochord) and other head structures, but this ends abruptly; below this site, somites are present as a single, median row underlying the neural tube unless the site of the operation fails to heal, in which case somites are present on each side of the persisting gap (Fig. 1).

Notochord and somites develop after removal of the whole anterior 40% of the streak

Fate maps of normal embryos (Rosenquist, 1966; García-

Fig. 1. Formation of the embryonic axis in embryos from which the node or anterior primitive streak had been removed. The upper row shows the typical result obtained when the operation is done before stage 4, either by ablating just the node (left diagram), or the anterior 40% of the primitive streak (right diagram). In both cases (photograph), a normal axis including a head and notochord (here stained by immunoperoxidase with antibody Not-1) develops. The lower row shows the typical result obtained when the operation is done after stage 4 (diagram): the notochord ends abruptly with a caudal thickening but somites continue to form. In this case (photograph) the hole failed to heal, but the same result is obtained when healing is complete.

st. 3^+ - 4^-

st. 3^+ - 4^-

st. 4^+ - 9
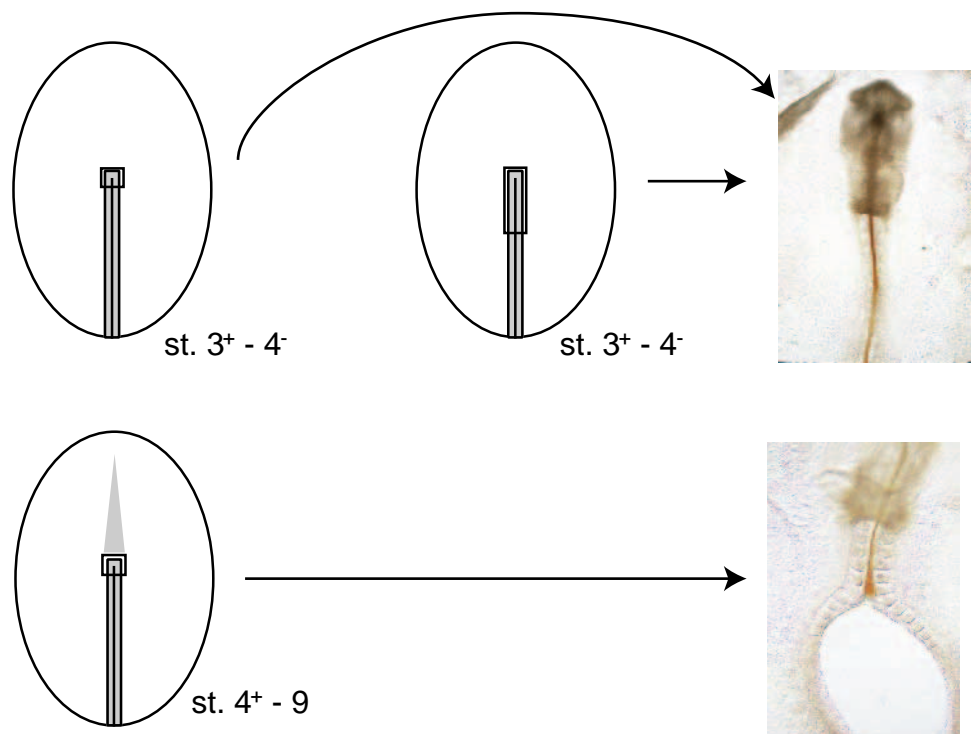
Martínez et al., 1993; Psychoyos and Stern, 1996) indicate that the region of primitive streak containing notochord and/or somite precursors extends far caudal to the node. It is therefore possible that the experiments of Grabowski (1956) and those described above lead to normal development simply because a sufficient number of these precursors escape excision. To eliminate this possibility, we ablated the entire prospective notochord region of the primitive streak, by cutting off the rostral 40% of the streak. Surprisingly, these embryos develop as normally as those from which only the node region is ablated, and contain a full-length notochord as well as somites (Fig. 1).

These results show that despite the absence of notochord and somite precursors in the primitive streak, embryos are able to generate a normal body pattern, containing both notochord and somites throughout the entire length of the body axis. Where do these cells come from?

Origin and movements of the cells contributing to the regenerated notochord and somites

To determine the origin of the cells that contribute to the notochord and other structures that form after ablation, we applied carbocyanine dyes (DiI or DiO) to label small groups of cells in different positions surrounding the site of ablation ($n=73$ embryos). The results were compared to the fates of cells in the same positions of unoperated embryos ($n=64$ embryos) (Table 1, Fig. 2). Only a few differences were found. The only statistically significant one ($P<0.01$) was an increase in the contribution of the stump of the primitive streak to notochord at the expense of lateral plate mesoderm.

To examine the pathways taken by cells from these regions to their new destinations, we followed the position of the descendants of the labelled cells at intervals after the operation. Representative stills from this analysis are shown in Fig. 2. The gap created by the excision heals over a period of 6-12 hours, by which time the embryos appear normal at the full primitive streak stage (stage 4), the rostral tip of the primitive streak displaying a structure that resembles the normal node. Cells from the entire region surrounding the ablation site contribute to this new node. Interestingly, cell movements through this new node are similar to those in the normal node (see Psychoyos and

Stern, 1996) (Fig. 2): notochord precursors move anteriorly and emerge from the node at its midline, somite progenitors leave the streak by a route that avoids or barely passes through the lateral part of the node and gut endoderm cells appear to leave by insertion into the lower layer immediately underlying them.

In conclusion, cells from the entire region surrounding the site of ablation contribute to the regenerated node, which has normal morphology and normal organization into regions with different prospective fates. The routes taken by cells emerging from each of these regions of the regenerated node also resemble those of the normal node.

Expression of organizer markers by the regenerated node

The above results show that embryos from which the anterior 40% of the primitive streak has been ablated can reconstitute a node-like structure, from which cells with fates characteristic of the node arise. Does this regenerated node express node-specific markers? We used whole-mount in situ hybridization with digoxigenin-labelled riboprobes to examine the time course of expression of three such markers: *goosecoid*, *HNF-3β* and *Sonic hedgehog*. In normal embryos at stages 2-3[+], *goosecoid* is expressed in a broad domain surrounding the node but becomes confined to this structure by stage 4; thereafter it leaves this region with the prechordal plate (Izpisúa-Belmonte et al., 1993). *HNF-3β* is expressed at the tip of the primitive streak from stage 2 and thereafter remains expressed in the node as well as in the emerging chordamesoderm (Ruiz i Altaba et al., 1995). *Sonic hedgehog* is expressed in the node from stage 4[+] and extends into the head process from stage 5 (Riddle et al., 1993; Roelink et al., 1994; Levin et al., 1995).

Goosecoid expression was examined immediately after the operation and after about 6-12 hours, when the embryos had a morphology consistent with stage 4⁻-4 (full primitive streak). In 10/15 (67%) embryos fixed immediately after removal of the node and anterior streak, no expression of *goosecoid* could be observed. The remaining 5 (33%) embryos contained a small group of cells expressing *goosecoid* lying at the edge of the ablation site, either lateral or anterior to it. When operated

Table 1. Summary of the results obtained from an analysis of the fates of cells lying anterior, lateral or posterior to the site of ablation in terms of their contribution to different structures

	anterior region		lateral region		more lateral region		posterior region	
	control	ablated	control	ablated	control	ablated	control	ablated
amnion	13 (93%)	18 (82%)	4 (24%)	3 (25%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
gut endoderm	10 (71%)	12 (55%)	12 (71%)	5 (42%)	1 (13%)	1 (11%)	8 (32%)	15 (50%)
notochord	8 (57%)	10 (45%)	4 (24%)	2 (17%)	0 (0%)	0 (0%)	0 (0%)	4 (13%)
somites	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (11%)	0 (0%)	0 (0%)
lateral plate	0 (0%)	0 (0%)	5 (29%)	1 (8%)	7 (87%)	8 (88%)	20 (80%)	14 (47%)
no. of embryos analysed	14	22	17	12	8	9	25	30

The region marked as 'more lateral' lies some 80-120 μm lateral to the node.

Table 2. Summary of the results obtained from the experiment shown in Figs 5-7, illustrating the time course of acquisition of neural inducing ability by individual anterior, lateral and posterior explants

	Region grafted								
	Anterior Time after ablation (hours)			Lateral Time after ablation (hours)			Posterior Time after ablation (hours)		
	0	3	6-9	0	3	6-9	0	3	6-9
graft derived, non neural	10 (90%)	4 (67%)	3 (60%)	22 (67%)	14 (61%)	5 (29%)	33 (100%)	12 (100%)	7 (78%)
graft derived, self differentiated into neural	1 (10%)	0 (%)	0 (0%)	5 (15%)	0 (0%)	3 (17%)	0 (0%)	0 (0%)	0 (0%)
induced (host derived, neural)	0 (0%)	2 (33%)	2 (40%)	6 (18%)	9 (39%)	9 (53%)	0 (0%)	0 (0%)	2 (22%)
total analysed	11	6	5	33	23	17	33	12	9

'Time after ablation' corresponds to the age of the donor embryo, taken from the time of primitive streak excision, at which explants were obtained for grafting.

embryos were allowed to develop to stage 4 and then processed for in situ hybridization for *goosecoid*, 11/17 (65%) embryos had no detectable expression of this gene. The remaining embryos contained *goosecoid* expression in the node (Fig. 3). This suggests that the operation is successful in removing all *goosecoid*-expressing cells in about two thirds of the cases while, in the remaining instances, it spares some cells expressing the gene. These results suggest that embryos from which *goosecoid*-expressing cells have been removed successfully do not reacquire expression of this gene.

The results obtained with riboprobes to *HNF-3β* and *Sonic hedgehog* are different. Both genes are expressed as in normal embryos, both in the node and in emerging chordamesodermal cells in all cases (14/14 for *HNF-3β* and 15/15 for *Sonic hedgehog*). Interestingly, both genes display the normal left/right asymmetry of expression in the regenerated node (Fig. 3B,D). To test whether the operation succeeds in the complete removal of *HNF-3β*-expressing cells, we conducted in situ hybridization for this gene in 14 embryos that had been fixed immediately after the operation. None of these had any remaining detectable expression of *HNF-3β* close to the ablation site.

In conclusion, regenerated nodes express the node markers *HNF-3β* and *Sonic hedgehog* with the normal pattern, but *goosecoid* expression is not upregulated if all of the earlier expressing cells are removed.

The regenerated node can induce a complete nervous system

The above experiments indicate that, in the absence of Hensen's node, other cells in the embryo take on the fates and patterns of gene expression characteristic of cells that are normally situated in this region. Can they also acquire other node properties, such as the ability to induce a complete nervous system in a host embryo? To test this, we ablated the whole of the anterior 40% of the streak of embryos at stage 3+ in 23 embryos, incubated them until the healing was complete (in all cases, this was before any indication of a head process appeared), excised the new tip of the primitive streak and transplanted this into the lateral extraembryonic region (area opaca) of a stage 3-3+ host. To distinguish the grafted cells from those induced in the host, the transplanted tissue was either labelled with DiI or taken from a quail donor embryo. After incubation of the host embryo to stages 9-12, the embryo was processed to reveal the expression of markers in different regions of the nervous system and either photooxidised (DiI-labelled

embryos) or stained with the anti-quail antibody QCPN. Strikingly, in 18/23 (78%) embryos, a small ectopic axis had developed in the region adjacent to the graft, often containing somites and notochord in addition to a nervous system and a head. Mesodermal structures (somites, notochord) were always derived from the graft. The neural tube and head structures, as found with transplants of a normal node, were composed mostly of host cells and expressed the region-specific markers *Tailless* (Yu et al., 1994) and *Krox-20* (Chavrier et al., 1988; Wilkinson et al., 1989) in their correct spatial order (Fig. 4).

This experiment shows that the regenerated node possesses inducing ability that is as strong and as complete as that of the normal node. However, one possible explanation for this phenomenon is that cells surrounding the ablated region also possess inducing ability in the unoperated embryo. To test this, cells anterior, lateral and posterior to the site of usual operation were tested for inducing ability in the same assay (*n*=77 grafts; Figs 5-7, Table 2). We find that none of these regions has strong inducing activity. Anterior and posterior explants never induced ectopic nervous system in the host embryo (*n*=44), while lateral explants did so only occasionally (6/33 cases; 17%).

A further possibility is that cells lying anterior, lateral or posterior to the anterior primitive streak acquire inducing ability when they are juxtaposed. To test this, three explants (one from each of these regions) were grafted together into a host embryo (*n*=5). None of the grafts led to the development of an ectopic axis. Taken together, these results show conclusively that cells that do not normally have inducing ability can acquire this property when the node is ablated, but that mere juxtaposition of the cells surrounding the ablation site is not sufficient for them to acquire neural inducing ability.

Time course of acquisition of neural inducing properties

The above experiment suggests that cells surrounding the ablation site acquire neural inducing ability only if they have remained in their normal position in the embryo for some time after excision of the organizer. To determine the length of this period, we excised each of the three regions (anterior, lateral and posterior to the ablation site) at intervals following ablation of the anterior primitive streak, and assessed them for neural inducing ability in the assay described above (Fig. 5). The results are shown in Figs 6 and

7 and Table 2. They indicate that each of the three regions acquires neural inducing activity over time, but the region lying posterior to the site of ablation takes longest to do so (6-9 hours).

In another set of experiments, we investigated the time course of acquisition of neural inducing activity by a combination of anterior, lateral and posterior pieces grafted together into a host embryo (*n*=71 experiments). No induction was seen if the explants were taken from embryos 0 or 3 hours following excision (*n*=7). By contrast, 10/23 (43%) embryos grafted with a combination of pieces obtained from donors at 6-9 hours following ablation contained ectopic neural tissue derived from the host.

These results show that, in order for cells surrounding the anterior primitive streak to acquire the ability to induce a nervous system, they have to remain in their positions adjacent to the ablation site for 6-9 hours. We suggest that the acquisition of neural inducing ability by cells at this site results from the interplay of two factors: an inhibition emanating from the node, which prevents them from inducing in the normal embryo, and positional cues from the rest of the embryo.

DISCUSSION

Abercrombie and Waddington (1937) were the first to investigate the regulative capacity of gastrulating chick embryos by placing grafts of primitive streak from different regions and different stages underneath the primitive streak of host embryos. Although in the absence of markers they had difficulty in identifying graft and host tissues, they found that the graft appeared to reverse its orientation to suit that of the host and, in general, that it differentiated according to the position where it was grafted rather than its site of origin.

Some years later, Abercrombie (1950) carried out anteroposterior reversals of large portions of the primitive streak of the chick embryo at different stages of development, and concluded that "*The reversal of the primitive streak does not at any stage control the orientation of the embryo and is labile with respect to its developmental potentialities.*" ... "*The primitive streak is subject to control by*

the surrounding blastoderm". From these experiments, the streak was viewed as a mere passageway for cells from ectoderm to mesoderm, and the properties of the node itself nothing more special than being at the tip of this structure. Later experiments (Abercrombie and Bellairs, 1954) contributed to refine this view further. In these experiments, the node was replaced by a graft of posterior primitive streak. In more than half the embryos, partial duplication of the axis resulted, but no structures were missing. Thus, other cells appeared to acquire node-like fates. The duplications of the axis were explained by the presence of the graft, which did not contribute to the notochord (assessed by labeling with ^{32}P) and seemed to act as a barrier, splitting the notochord produced by the remaining anterior end of the primitive streak into two.

At about the same time, Grabowski (1953, 1956) was exploring more directly and specifically the effect of removal of Hensen's node. He conducted his experiments very carefully, measuring the size of the excised piece and varying both this and the stage of the embryo at the time of the operation in a systematic way. He also incubated the operated embryos for various periods from 5 minutes to 80
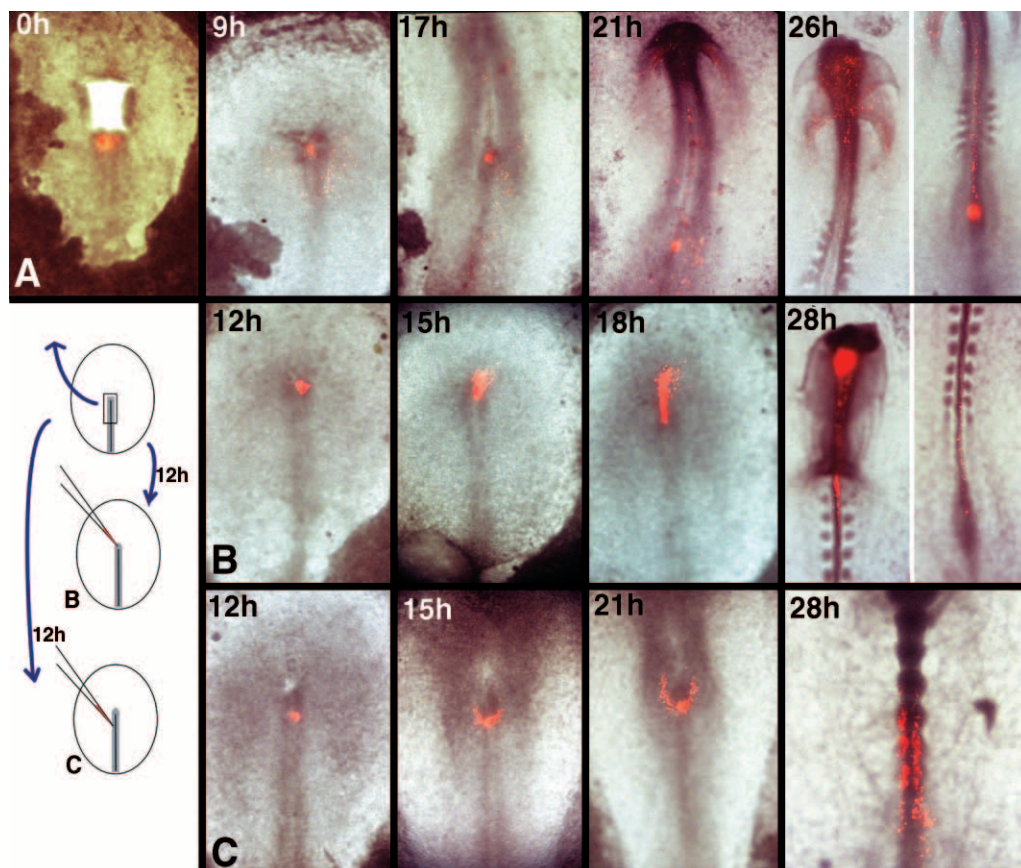

Fig. 2. Cell movements after ablation of the anterior primitive streak and node. (A) In this embryo, the anterior 40% of the primitive streak was ablated and a small mark of DiI placed into the stump of the primitive streak. 9 hours later the hole has healed and labelled cells can already be seen scattered around the labelling site, in the endoderm. Subsequent development shows more extensive contribution to the endoderm, particularly at the dorsal midline. (B,C) Two embryos studied as shown on the diagram on the left: after ablation of the anterior 40% of the primitive streak, the embryo was allowed to develop for 12 hours until healing had occurred. Then a DiI mark was placed into the tip of the new node (B) or just behind it (C). As in normal embryos labelled at these sites, the injection in B contributed cells to midline structures like notochord and prechordal mesendoderm, and that in C to somites.
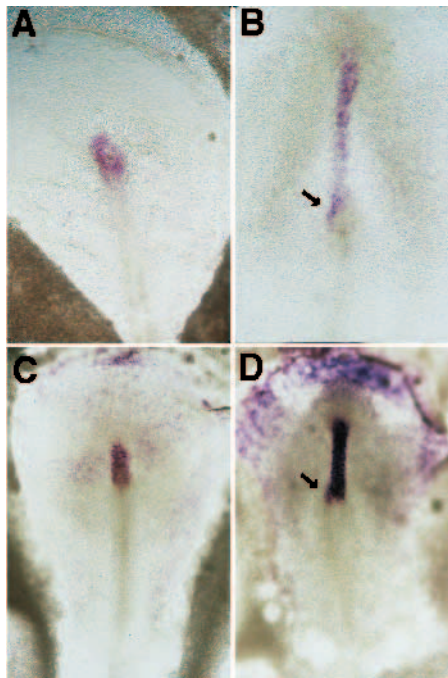
Fig. 3. Expression of node markers in embryos developing after ablation of the node and anterior 40% of the primitive streak. (A) *goosecoid*. An example of an embryo in which *goosecoid* expression was detected after healing had occurred. This result was obtained in about 35% of cases; in the remaining ones, no expression of this gene was seen. (B) *Sonic hedgehog*. An embryo that had developed for some 16 hours after removal of the anterior primitive streak and node; normal expression of *Sonic hedgehog* is seen on the left side of the node (arrow) and in the emerging head process. (C,D) *HNF-3β*. Embryos seen at about 13 hours (C) and 16 hours (D) following the operation; the pattern of expression of *HNF-3β* is indistinguishable from that in normal embryos, including the slight asymmetry of transcript levels towards the left side of the node (arrow in D).

hours. From these experiments, he concluded that embryos can regulate perfectly for excision of the node region, and, provided they heal well, develop with no structures missing and no obvious anomalies. He determined the maximum size of the excised piece allowing regulation as being 0.5×0.5 mm at the definitive primitive streak stage (stage 4), 0.3×0.3 mm at the head-process stage (stages 5-6), and 0.1×0.2 mm between stages 6 and 8.

However, these studies were all conducted before any tissue-specific markers were available, and the inducing properties of the supposedly regenerated node were never tested. Grabowski (1956) himself acknowledges this possibility: *"other possible functions of the node, such as neural induction, may be assumed by the anterior end of the streak after node removal ..."*, although he did not investigate it further. Another complication is that no detailed fate maps of the primitive streak were available when these studies were published, and it is possible that cells normally derived from the node are not as restricted to this structure as was generally assumed. This possibility is supported by the findings of Rosenquist (1966), Schoenwolf (1992), García-Martínez et al. (1993) and Psychoyos and Stern (1996), who found that cells posterior to the node contribute to somite and notochord at the definitive

primitive streak stage. More recently, Yuan et al. (1995a,b) reported that cells that do not normally give rise to the notochord can do so when large portions of the embryo (including the entire primitive streak) are ablated. They proposed that cells about 100 μm lateral to the node can be induced to give rise to notochord by a population of cells situated between this and the node itself.

We have therefore undertaken a detailed study of the capacity of the early chick embryo to regulate following ablation not only of the node itself, but also of the whole anterior 40% of the primitive streak, deleting the entire portion of the streak that contains precursors of the prechordal
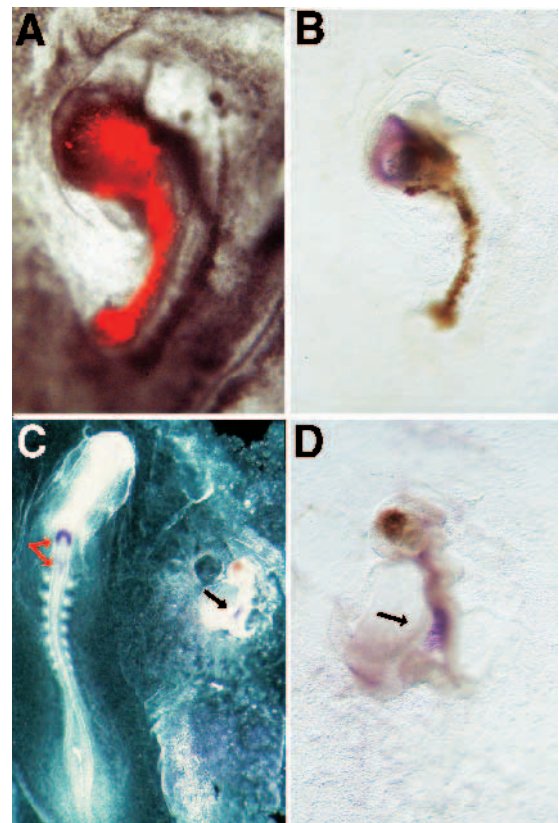

Fig. 4. Neural inducing ability of the regenerated node. After ablation of the node and anterior 40% of the primitive streak, the embryos were allowed to develop until the hole had healed and a structure resembling a new node had formed (about 9-12 hours). This structure was then labelled with DiI, excised and grafted into the margin of the area opaca of a host embryo at stage 3⁺. (A,B) A miniature axis has formed, seen in A by fluorescence microscopy to reveal the DiI-labelled cells in the living embryo, and in B after fixation, photooxidation of the DiI (giving a brown precipitate marking the donor-derived cells in the notochord) and in situ hybridization with a probe for *Tailless*, expressed in a head-like structure which is entirely derived from host cells. (C,D) A miniature axis has formed, seen at low magnification in C and higher magnification in D. Photooxidized, donor-derived cells (brown) are seen in what appears to be prechordal mesoderm in a head-like structure and along the midline of the ectopic axis (out of focus in the photograph); expression of *Krox-20* is seen as one band in the ectopic axis (black arrow in C and D), which according to its intensity and shape resembles that of rhombomere 3 of the host (compare with red arrows in C).
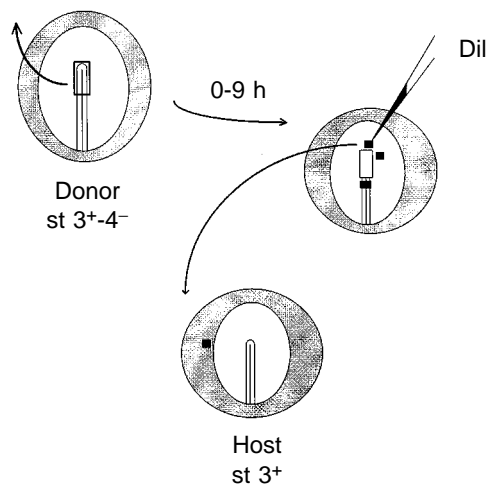
Fig. 5. Diagram illustrating the operation performed to test the neural inducing ability of anterior, lateral and posterior explants from the region surrounding the ablated streak. Embryos were operated by removing the anterior 40% of the streak at stages 3^+-4^-, incubated for various periods from 0 to 9 hours, and small regions anterior, lateral or posterior to the ablation site labelled with DiI. The pieces were then excised and transplanted, either singly or in combination, to the margin of the area opaca of a host embryo at stage 3^+. The results were analyzed after photooxidation of the DiI and in situ hybridization for neural markers as shown in Figs 4, 6 and 7.

Fig. 6. Examples of the results obtained after the experiment shown in Fig. 5. (A) Miniature axis formed after grafting a lateral explant obtained from a donor 6 hours after streak ablation. Here the donor embryo was a quail and the donor cells identified by immunostaining with QCPN antibody. Host cells express the general neural marker *Sox-2*, and donor quail cells are seen in mesodermal structures along the axis and next to it. (B,C) Neural structures formed after grafting a DiI-labelled lateral piece obtained from a donor 9 hours after streak ablation, following photooxidation and in situ hybridization for the forebrain marker *Tailless*. C is a transverse section through these structures at the level indicated in B. Donor-derived (brown) cells are confined to mesendodermal structures. The neural tube, which expresses *Tailless*, is derived from the host.

mesoderm, notochord, somites and most of the definitive endoderm. We explore whether such embryos can produce these structures, the cell movements by which they are generated, the expression of node-specific genes in regenerating embryos and the time course of reacquisition of neural inducing activity by the remaining cells. We find that embryos deprived of this large region regulate perfectly: apart from a delay of about 6-9 hours as compared to controls, they produce all the axial organs including the head and chordamesoderm and display normal patterns of cell movements; the expression of two organizer-specific genes (*HNF-3β* and *Sonic hedgehog*) is normal (albeit a third gene, *goosecoid* does not appear to be subject to regulation) and they gradually reacquire the ability to induce a complete nervous system upon transplantation to a host embryo. These results strongly suggest that the organizer property is actively maintained in the embryo even at late stages of gastrulation, by cell interactions.

Regulation of the organizer in other vertebrates

Is the ability to regulate following ablation of the organizer confined to avian embryos? Data from different vertebrate classes have provided conflicting conclusions. In teleosts, Lewis (1912) and Brummett (1968, 1969), after ablation of either the anterior or the posterior part of the shield, concluded that there is only limited regeneration, and that this is generally confined to the posterior shield. Nicholas and Oppenheimer (1942) and more recently Shih and Fraser (1996) report a much more regulative behaviour: ablation of a large region centered on the shield is followed by relatively normal development, and axial structures and a head appear to form normally. Since Shih and Fraser used zebrafish while the other authors used *Fundulus*, the differences between their results are more likely to be due to the surgi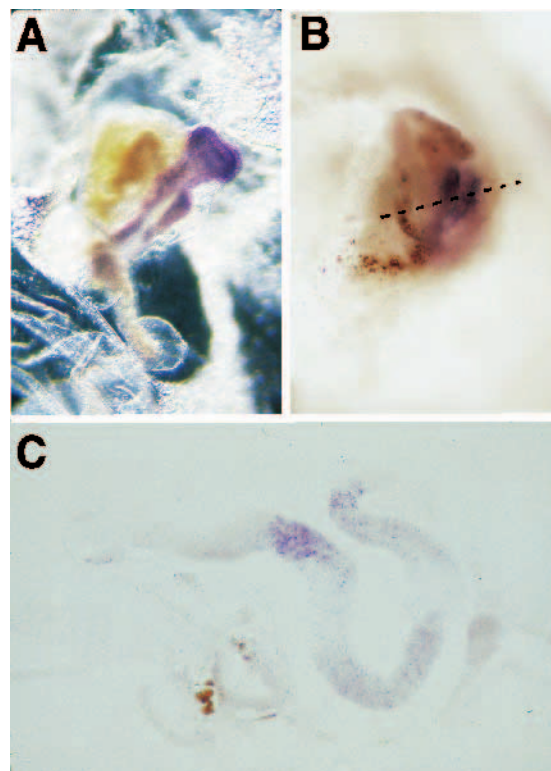cal techniques used rather than to species differences. However, none of these studies used tissue-specific markers, or investigated whether any cells change fate following ablation or whether the regenerated shield can induce a second axis from a host embryo.

In *Xenopus*, Cooke (1972a,b, 1973, 1975) (see also Gerhart, 1980) reports that removal of the organizer region results in a delay of some hours in the subsequent developmental events, but this is followed by fairly normal development including the formation of structures normally derived from the ablated tissue. Again, no tissue-specific markers were available and neither the inducing ability of the regenerated dorsal lip nor the possibility of cells changing their fates were investigated systemmatically.

In the mouse, ablation experiments such as these are very difficult to perform. However, mice engineered to carry a deletion in the gene encoding *HNF-3β* lack a visible node and notochord (Ang and Rossant; 1994; Weinstein et al., 1994). Nonetheless, these mice generate a nervous system with anterior-posterior pattern. The above sets of authors have inter-
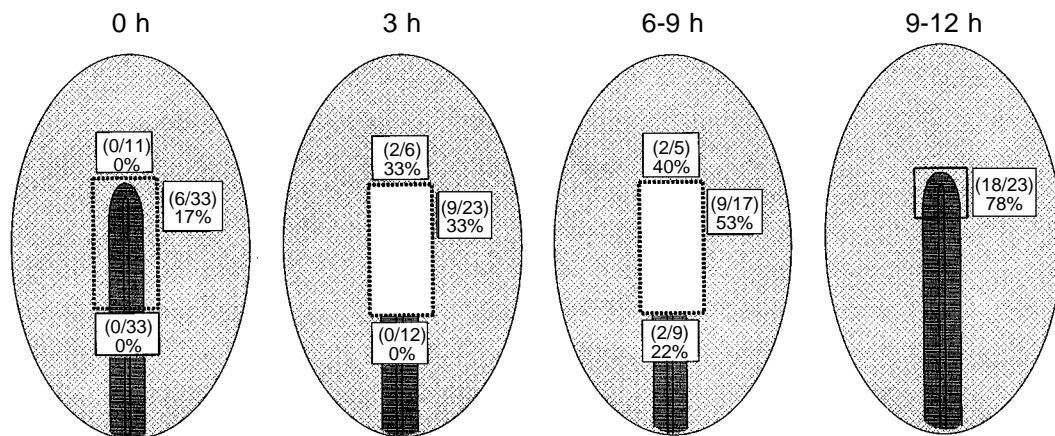
Fig. 7. Summary of the results of the experiment performed to determine the time course of acquisition of neural inducing activity by cells anterior, lateral and posterior to the ablation site (Figs 5 and 6). Lateral explants are the first to acquire inducing ability, followed by anterior pieces and finally those obtained from the stump of the primitive streak. The final diagram shows the result obtained when the whole of the regenerated node was transplanted (as in Fig. 4).

preted this result to indicate that mouse embryos can dispense with the node and notochord for generating and patterning the nervous system, but their experiments are unable to reveal whether the embryos have some remaining node cells which, although unable to generate a notochord, still possess organizer activity.

In conclusion, it appears as if embryos in all major vertebrate classes may be able to regulate following ablation of the organizer region, although considerably more work will be required to establish this as a general rule, particularly to determine whether cells that do not normally reside in the node can be made to change fates to axial mesendoderm or to acquire the ability to induce and pattern a nervous system.

goosecoid is not required to define the organizer

In our experiments, embryos deprived of the node and anterior primitive streak reacquire the expression of the node-specific markers *HNF-3β* and *Sonic hedgehog* in both the node and notochord. By contrast, two-thirds of the embryos do not show reexpression of *goosecoid*. Since this proportion is similar to the number of embryos in which the *goosecoid*-expressing region was successfully ablated, this result suggests that no regulation of this gene occurs if all *goosecoid*-expressing cells are removed. Given that a much higher proportion of the regenerated nodes (78%) have neural inducing ability than embryos in which some residual *goosecoid*-expressing cells remained after the operation (33%), this indicates that *goosecoid* expression is not required in the organizer during the period of neural induction. This does not rule out the possibility that this gene plays a role in defining populations of cells that can acquire the organizer status (see below). However, recent results with targeted disruption of the *goosecoid* gene (Rivera-Pérez et al., 1995; Yamada et al., 1995) show that this gene is indeed dispensable during the early stages of development because the only defects seen in such mice (affecting cranio-facial, rib and limb morphogenesis) concern later developmental mechanisms.

Evidence for an inhibitor produced by the organizer

The results presented here strongly argue that the node may

exert an inhibitory influence on neighbouring cells, such that their tendency to acquire organizer properties is suppressed during normal development but they are released from this inhibition when the node is ablated. One possible candidate molecule that fulfills the criteria required from such an inhibitor is Anti-dorsalizing Morphogenetic Protein (ADMP), a putative secreted factor belonging to the TGF-β family and with some homology to BMP-3 which is expressed by the organizer in *Xenopus* yet antagonizes the dorsalizing effects of the organizer (Moos et al., 1995). Experiments are in progress in our laboratory to identify a chick homolog and to test this hypothesis directly.

A second factor is required in addition to an inhibitor

Production of a molecule like ADMP by the node is not suffi-cient to explain the results, because when explants surrounding the site of ablation are implanted immediately into the area opaca of a host embryo, they lack inducing activity even if they are combined. They must remain in their position adjoining the site of ablation for 3-9 hours before they acquire this ability. This finding suggests that a second factor is required, along with the removal of an inhibitor produced by the node, for these cells to take on the properties of the organizer. One possibility is that the earlier domain of expression of *goosecoid* (at stage 3), which is larger than the anterior half of the primitive streak and also extends laterally at the level of the node (Izpisúa-Belmonte et al., 1993), defines the group of cells that is competent to become organizer when the node is removed. If this is the main function of *goosecoid* at these early stages of development, this could explain two findings: (a) the lack of an early phenotype in mice carrying a targeted disruption of the *goosecoid* gene (see above) and (b) that injection of *goosecoid* in a ventral blas-tomere generates a partial ectopic axis in *Xenopus* (Cho et al., 1991; Izpisúa-Belmonte et al., 1993).

It is also possible, however, that the second factor represents some sort of graded positional signal, independent of *goosecoid* expression, informing cells of their location with respect to the site of the organizer, so that only the nearest cells can respond when the inhibitory signals from the node are

removed. Further experiments are required to distinguish between these possibilities.

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