DEVELOPMENTAL DYNAMICS 196:79–90 (1993)

Evidence That the Border of the Neural Plate May Be Positioned by the Interaction Between Signals That Induce Ventral and Dorsal Mesoderm

JING ZHANG AND ANTONE G. JACOBSON
Department of Zoology, Center for Developmental Biology, University of Texas, Austin, Texas 78712

ABSTRACT **In the early *Xenopus* embryo, a quadrant of endodermal cells that have descended from the vegetal dorsal localization in the zygote produces signals that pass into the animal hemisphere and induce dorsal mesoderm from the marginal zone. From the remaining three quadrants of the bordering endoderm, signals pass into the animal hemisphere and induce ventral mesoderm in the marginal region. There is evidence that suggests that these same mesoderm-inducing signals continue through the plane of the tissue of the animal hemisphere where they may at least begin the processes of neural and epidermal induction by changing the competence of the prospective ectodermal cells, and possibly influencing the early regional biasing of later expression of at least some gene products, such as Epi-1 whose expression in the future epidermal domain seems specified before gastrulation. We hypothesized that the interaction of the ventral and dorsal signals within the plane of the tissue of the animal hemisphere may position the border of the neural plate. If this is so, then transplantation into the animal pole of cells that signal induction of ventral mesoderm should drive the neural plate boundary back toward the blastopore and shorten the anterior-posterior axis. Removal of cells that induce ventral mesoderm should result in an axis that is longer than normal. Results of our experiments support these predictions. Also, by late pregastrula stage 9, increasing the ventral signals has no effect. Thus the evidence suggests that the position of the anterior neural plate boundary is established before gastrulation begins by the interaction of the signals that induce the ventral and dorsal mesoderm.**

Key words: Induction of dorsal mesoderm, Induction of ventral mesoderm, *Xenopus laevis*

INTRODUCTION

When the neural plate first appears, there is an abrupt morphological border or line between neural tissue and future epidermal tissue. How does this line get drawn? The answer appears to be in the induction history of the neural plate. The position of the neural plate border seems to be set, at least tentatively, before gastrulation begins as a result of the planar signaling that induces the dorsal and ventral mesoderm, and which also begins the progressive determination of the neural plate and epidermal regions.

The induction and organization of the dorsal axial structures of the vertebrate embryo have been a central theme of experimental embryology, especially after the Nobel Prize-winning work of Spemann and Mangold (1924). Spemann proposed the following for neural plate induction: "It would be entirely conceivable that [its] induction which occurs after exposure to the subjacent mesoderm is merely the continuation of another induction which was initiated when the [prospective mesodermal and ectodermal] materials were still lying side by side on the surface" (Spemann, 1927, page 950; quoted and translated to English by Hamburger, 1988, page 68).

The issue of planar signaling has been receiving new attention (see review by Jacobson and Sater, 1988). (In planar induction, signals move in the plane of contiguous tissues, while in vertical induction the signals move from an inductor tissue to a non-contiguous responding tissue that is apposed above or below it.) Induction of neural plate first by planar signaling from the chordamesoderm in the blastopore lip and then by vertical signaling from the chordamesoderm after it has involuted and become apposed beneath the prospective neural plate has been the object of several recent studies. For example, Dixon and Kintner (1989) used two independent molecular indicators of neural differentiation to assay the results of planar and vertical signaling in explants. They used a quantitative RNase protection assay developed by Kintner and Melton (1987) to assay for the expression of N-CAM transcripts which, in *Xenopus,* are expressed in early neural plates and are restricted to the neural tissue, and they also used NF-3 transcripts that are expressed in postmitotic neurons after neural tube closure. They found that neural tissue, as defined by these molecular

Received June 1, 1992.
Address reprint requests to Antone G. Jacobson, Department of Zoology, University of Texas, Austin, TX 78712.

markers, forms well as a result of planar signaling only, but forms extremely poorly when exposed only to vertical signals. A synergistic effect was observed when both signal methods were employed.

Keller et al. (1992b) used "Keller" explants of *Xenopus* dorsal lip and adjacent ectoderm in which only planar induction can occur to show that planar induction from the dorsal lip up to stage 11 is sufficient to induce the convergence-extension movements of the neural plate. Doniach et al. (1992) used similar explants and found that several position-specific neural marker genes were expressed in the ectoderm, suggesting that planar signals are sufficient to induce a patterned neural response. These examples confirm Spemann's idea that both methods of signaling do occur and are effective, and indeed suggest that planar signaling may be the more important.

In the newer territory of mesodermal induction that has exploded in the past two decades (see reviews by Nieuwkoop et al., 1985; Smith, 1989; Slack, 1990; Slack and Tannahill, 1992), Nieuwkoop and coworkers found that the mesoderm is induced from animal hemisphere tissue by signals from the endoderm. They state "We think that the most fundamental aspect of mesoderm induction is its spreading from cell to cell over a considerable distance and during a relatively long period of time; a spreading accompanied by a spatial decrease in intensity" (Nieuwkoop et al., 1985, page 107).

Jacobson and Sater (1988) speculated that the planar signaling that produces the induction of the mesoderm from the marginal zone of the animal cap of the amphibian embryo may continue past the border of the mesoderm into future ectoderm where it could induce other structures, such as the notoplate, or affect the competence of regions of the ectoderm to form dorsal or ventral structures. Part of the basis for these ideas came from the studies of the monoclonal antibody Epi 1 which is expressed only in the epidermal domain at neurula stage 14. London et al. (1988) found that the later expression of Epi 1 is already limited to the future epidermal area prior to gastrulation. Savage and Phillips (1989) found that the pattern and boundary characteristics of Epi 1 expression were defined by signals from the central blastopore lip region passing through the plane of the ectodermal sheet.

We hypothesize that, in *Xenopus laevis,* signals from the quadrant of endoderm that is known to induce the dorsal mesoderm (Gimlich and Gerhart, 1984), and signals from the three remaining quadrants of endoderm that induce ventral mesoderm, continue to spread through the entire animal cap, including through the prospective ectoderm beyond the induced mesoderm. These dorsal and ventral signals interact with one another to position the future border between the epidermis and the neural plate (Fig. 1). Stewart and Gerhart (1990) found that the width of the region in the late blastula that induces dorsal structures does not exceed 60°. We noted above that Keller et al. (1992b) and Doniach et al. (1992) found that planar signaling from the dorsal lip induces convergence extension and patterning in the neural plate. Our hypothesis is that even earlier planar signaling, from the vegetal cells at pregastrula stages, has a role in setting the boundaries of the neural plate.

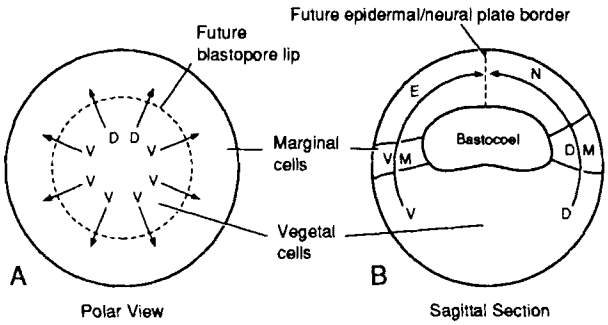

Fig. 1. **A:** View of the vegetal pole of a *Xenopus* blastula. Dorsal vegetal cells (D) are inducing (arrows) dorsal mesoderm in the marginal region, and larger numbers of ventral vegetal cells (V) are inducing ventral mesoderm. **B:** This drawing of a sagittal section of a *Xenopus* blastula illustrates our hypothesis that not only do the ventral vegetal cells (V) and the dorsal vegetal cells (D) induce from the tissue of the animal hemisphere the ventral mesoderm (VM) and the dorsal mesoderm (DM), but these two induction signals continue through the plane of the tissue, and their interaction near the animal pole establishes the position of the future anterior border between the neural plate (N) and the epidermis (E).

We present some simple experiments that directly test this hypothesis. We focus on the position of the anterior medial border of the neural plate since this is the part of the neural plate-epidermal border that is least distorted by convergence-extension movements within the neural plate, and the easiest part of the prospective neural plate border to locate on the pregastrula embryo. Some experiments strengthen the ventral signals near the prospective anterior border of the neural plate, and other experiments weaken the ventral signals at the prospective anterior border of the plate. We predict that the anterior neural plate border will be moved back into prospective neural plate in the first experiments, and out into prospective epidermis in the second experiments. These predictions are confirmed by marking experiments.

RESULTS

Control Experiments: Sham Transplantations and Identification of the Fate of the Animal Pole Cells

This first experiment is a control experiment. Its purpose is to establish the normal fate of cells at the animal pole, and to serve as a sham transplant experiment which removes, marks, and replaces the same cells into the animal pole. We removed a plug of cells from the animal pole and marked it with TRITC (tetramethyl rhodamine isothiocyanate), then implanted it into the animal pole of a host embryo from which a similar animal pole fragment had been removed (Fig. 2). After these host embryos developed to late neurula stage 20, the marked animal pole cells were found,

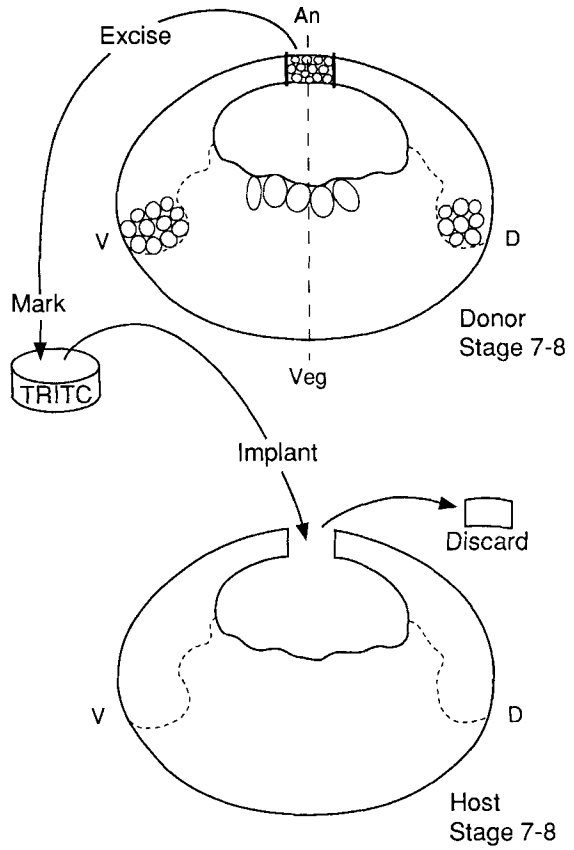

Fig. 2. Operations to create sham control embryos are diagrammed. To prepare a host embryo, a piece of tissue is excised from the animal pole and discarded. A similar fragment of tissue from the animal pole of a donor embryo is removed and placed briefly in TRITC to mark it, then the marked fragment is implanted in the prepared site at the animal pole of the host embryo. These drawings are from sagittal sections of *Xenopus laevis* embryos derived from photos in Danilchik and Denegre (1991, their Fig. 7). In the top section, cell sizes are shown in representative areas. The dashed lines indicate the boundary between larger and smaller cells. V, ventral; D, dorsal; An, animal pole; Veg, vegetal pole.

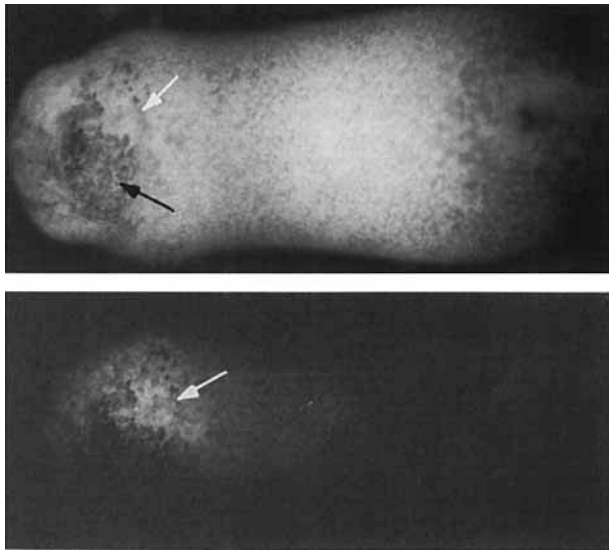

Fig. 3. Ventral views of a sham operated control embryo, reared to stage 20, that had received a marked animal-pole implant into the animal pole at stage 7+, photographed with visible light (**above**) and with ultraviolet light (**below**). The fluorescent implant (white arrow) is found at the site of the cement gland (black arrow). ×35.

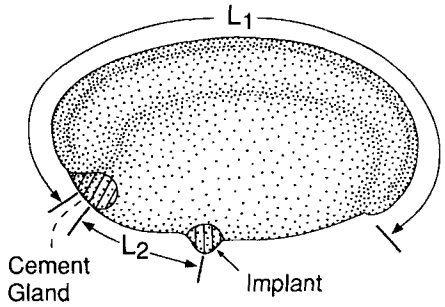

Fig. 4. The drawing is of a *Xenopus* embryo at stage 20 (late neurula), side view, anterior to the left. The length of the axis (length of the neural tube) (L_1), and of the distance from the cement gland to the ventral implant (L_2) were measured as indicated.

with epifluorescent microscopy of the whole embryo, to coincide with, or lie very near to, the site of the cement gland adjacent to the anterior end of the neural tube (Fig. 3). We measured the lengths of the axes of these embryos and also the distance between the cement gland and the implant (Fig. 4) (Table 1, Sham Control). The lengths of the axes of unoperated control embryos were also measured for comparison (Table 1, Normal).

Transplantation of Ventral Submarginal Tissue Into the Animal Pole of the Blastula at Stage 7+

The second set of experiments was a test of the hypothesis that ventral and dorsal signals interact to position the border of the neural plate. We removed and discarded plugs of cells from the animal poles of host embryos that were at stage 7 to 7+. We then excised ventral vegetal cells from the ventral submarginal zone of donor embryos that were at stage 10+ (26 cases), or at stage 7 to 7+ (11 cases), marked the excised cells with TRITC, and implanted them into the vacated animal pole sites of the hosts (Fig. 5).

After operated embryos reached late neurula stage 20, the implanted cells were located with epifluorescent microscopy. The implanted fragments were on the ventral side of the embryo some distance posterior to the site of the cement gland, and thus posterior to the anterior end of the nervous system (Fig. 6). The lengths of the axes of these embryos were measured, as were the distances between the site of the cement gland and the site of the implant (Fig. 4) (Table 1, Ventral implant). These embryos with shortened axes also usually had reduced heads.

Submarginal cells at stage 10+ are smaller than similar cells at stage 7+, making the operation easier, and this is the main reason we used stage 10+ donor

TABLE 1. Summary of Measurements of Axis Lengths (Mean mm ± s.d.)[a]

Operation	L_1	L_2	N
Normal controls	4.03 ± 0.24		44
Sham controls	4.02 ± 0.30**	0.12 ± 0.13	19
Ventral implants in stage 7+ hosts	2.79 ± 0.38*	0.97 ± 0.19	37
Ventral implants in stage 9 hosts	3.93 ± 0.22**		11
Ventral vegetal cells removed	4.49 ± 0.16*		17

[a]L_1 is the length of the axis. L_2 is the length from the cement gland to the implant. N is the No. of cases. Measurements were made at stage 20.
*Highly significant difference from normal controls ($P < .001$).
**Difference from normal controls is not significant (Student t-test).

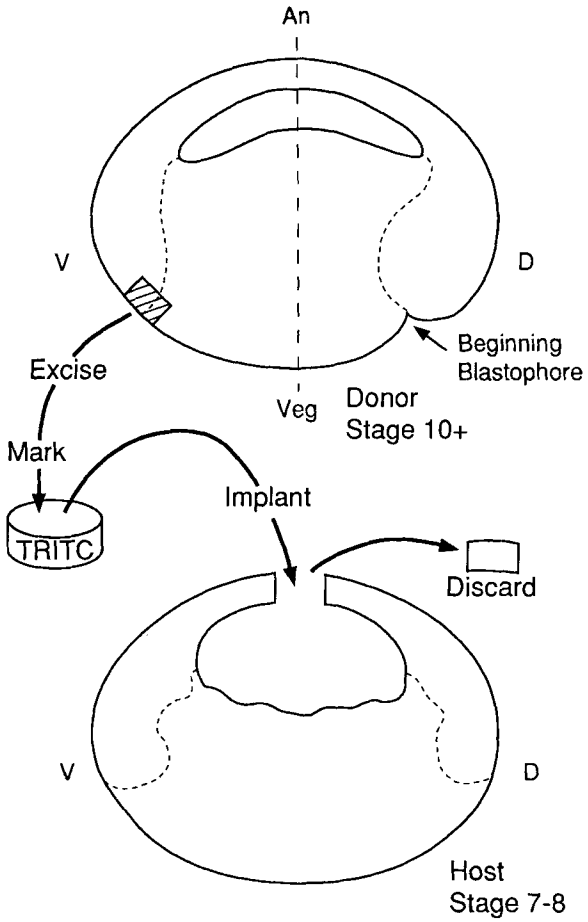

Fig. 5. Diagram of operations to implant a ventral fragment into the animal pole. A host embryo is prepared by removing and discarding some tissue from the animal pole. A ventral-signaling fragment of tissue is removed from the ventral submarginal region of a donor embryo and placed briefly in TRITC to mark it. The marked fragment is then implanted into the animal pole of the host embryo.

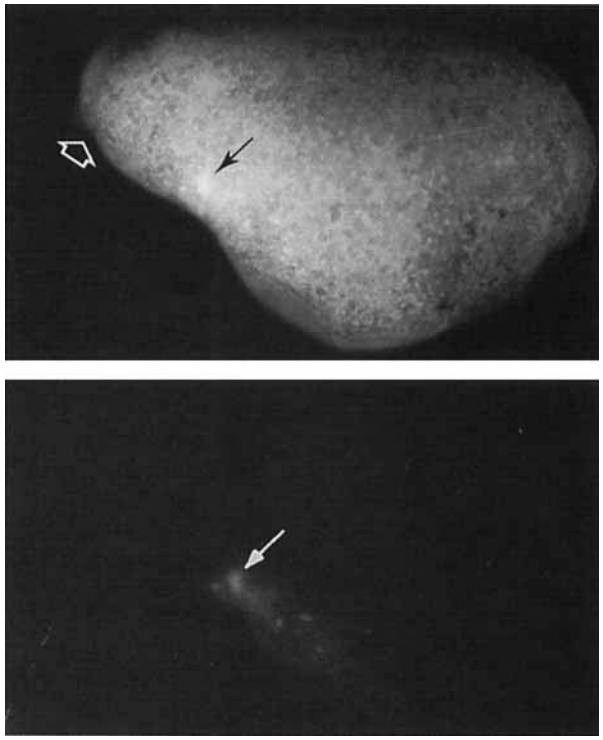

Fig. 6. Views of the left side of a host embryo that had received a ventral implant into the animal pole at stage 7+, was reared to stage 20, then photographed by reflected visible light (**top**) and ultraviolet light (**bottom**). The implant (white arrow), located by fluorescent microscopy, is beneath the ventral epidermis. The site of the implant (black arrow) is some distance posterior to the cement gland (open arrow). ×40.

tissue for some of the experiments. In the 26 cases with stage 10+ donor tissue, the mean axis length at stage 20 was 2.80 ± 0.43 mm, and in the 11 cases with stage 7 to 7+ donor tissue, the mean axis length was 2.78 ± 0.23 mm. There is no statistical difference in the results of these two sets of experiments, so heterochronic implants apparently do not have different effects.

Examination of sections through the implanted tissue with fluorescent microscopy reveals that the implant becomes incorporated into the local endoderm (Fig. 7), in a ventral position well posterior to the heart. The appearance of the heart in a normal position relative to the anterior neural boundary, but well anterior to the implant that was placed in the animal pole, suggests that the positions of the mesodermal and dorsal ectodermal structures match despite the axes being shorter.

Some experimental embryos that were raised to stage 39–40 are illustrated in Figure 8, and compared there with normal control embryos. The experimental

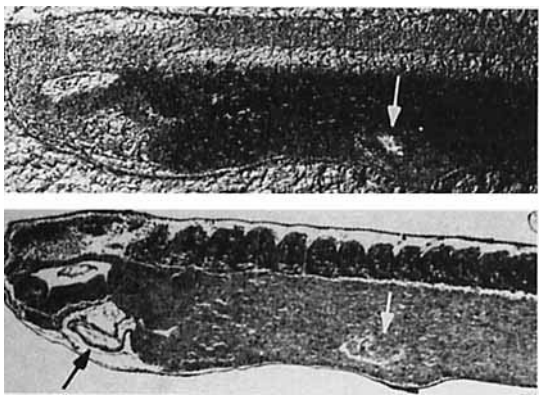

Fig. 7. A parasagittal section of an embryo at stage 36 that had received at stage 7+ an implant into the animal pole of marked ventral vegetal cells. The upper photo was taken with UV light to locate the fluorescent cells, and the lower photo was taken with white light to reveal the histology. The fluorescent implanted cells (white arrows) are incorporated into the local gut endoderm in a ventral position well posterior to the heart (dark arrow). ×29.

embryos have shorter axes, reduction or loss of some anterior head structures such as eyes, and tails have been induced by the implant in some of the embryos. Kao and Elinson (1988) present an index (DAI index) of morphologies of ventralized and dorsalized *Xenopus* embryos that ranges from 0 (totally ventralized) through 5 (normal) to 10 (totally dorsalized). Our experimental embryos most closely approximate a DAI index of 3.

Marking Embryos to Determine the Fates of Points Between the Animal Pole and the Blastopore in Control and Experimental Embryos

A third set of experiments consisted of marking the host embryo by placing it briefly in TRITC and punching holes with an eyebrow hair knife in the prospective dorsal midline at one-third and at two-thirds of the distance between the animal pole and the prospective dorsal lip (Fig. 9). The embryos then took up TRITC in the punctured areas, and these positions could be identified with fluorescent microscopy at later stages. The point of these experiments is to follow the fates of the cells in these positions in sham operated embryos and in embryos that have received a ventral implant into the animal pole. In sham operated embryos, the two points end up in the nervous system (Fig. 10, four cases). The more anterior mark remains fairly punctate, but the posterior mark becomes elongated along the midline of the neural plate, presumably by convergence-extension movements. In host embryos that received a ventral implant into the animal pole, in most cases the posterior mark is found in the anterior nervous system and the anterior mark is found in the anterior epidermis on the ventral side of the embryo between the implant and the anterior border of the nervous system. In the eight cases of this sort, three cases were as illustrated in Figure 11, two cases had both marks in the ventral epidermis, and the remaining three cases had both marks displaced laterally from the midline. These latter cases most likely result from

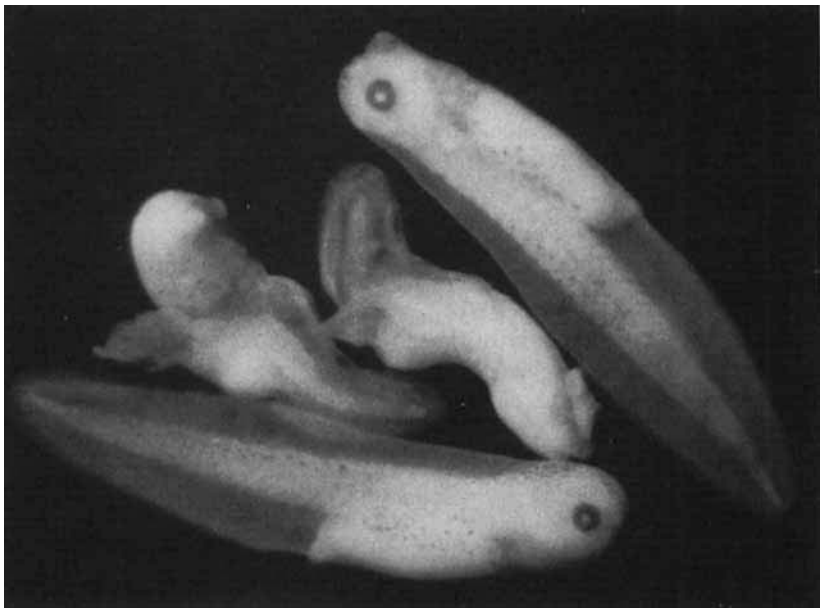

Fig. 8. A comparative view of normal and operated embryos at stage 39–40. The two middle larvae received implants of ventral vegetal cells into their animal poles at stage 7+. These larvae have shorter axes, extra tails, and loss of some dorso-anterior structures. The outer larvae are normal controls from the same spawning. ×12.3.

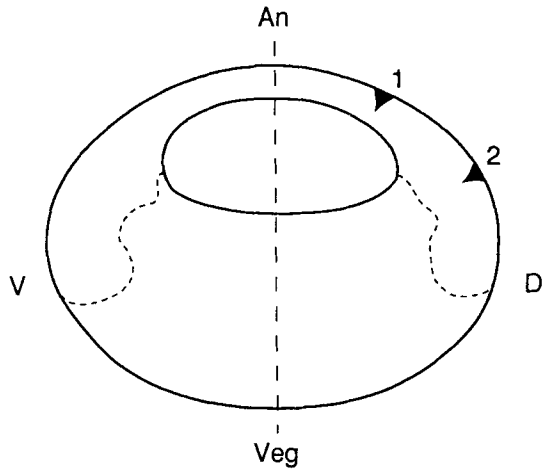

Fig. 9. A host embryo that has received an implant into the animal pole, either a ventral fragment or a sham control, is placed briefly into TRITC and punctured at the two sites marked 1 and 2, which are one-third and two-thirds the distance from the animal pole to the future blastopore lip. The fluorescent marker is taken up at the puncture sites and followed to later stages.

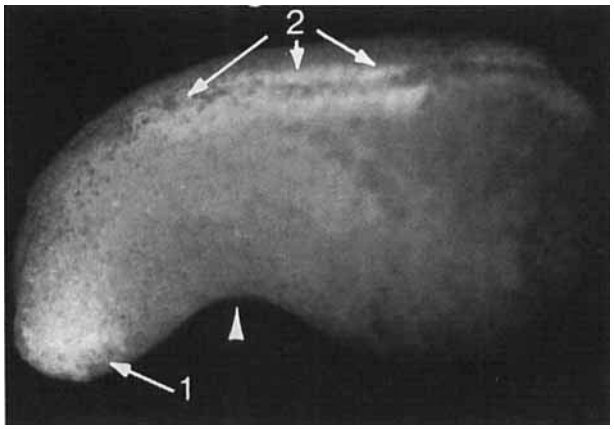

Fig. 11. A host embryo that has received an unmarked ventral fragment into the animal pole, then was marked as in Figure 8, was reared to stage 20 then photographed by double exposure with both reflected visible light and ultraviolet light. The photograph is a lateral view, anterior to the left, dorsal at the top. The fluorescent marked regions are indicated with arrows. Mark 2, elongated by convergence-extension movements of the neural plate, is found in the anterior spinal cord region of the nervous system, and mark 1 in the anterior epidermis as indicated. The ventral implant is approximately at the position indicated by an arrowhead. ×40.

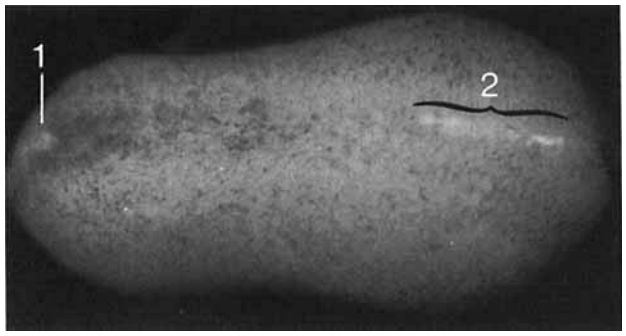

Fig. 10. Sham-operated embryos, marked as in Figure 8, were reared to stage 20 then photographed by double exposure with visible light and with ultraviolet light. The two marks (1,2) are found within the neural tube. The more posterior mark has been strung out along the midline by convergence-extension movements. ×40.

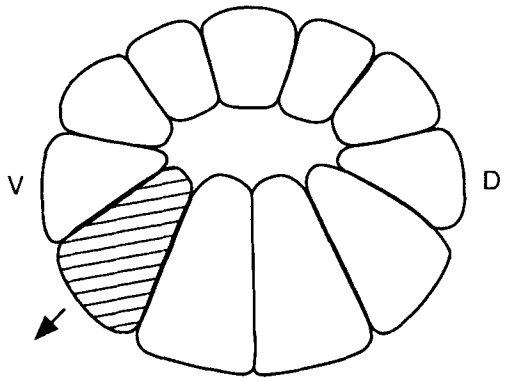

Fig. 12. Drawing of a sagittal section of a 64-cell stage embryo. Two to three cells at the level of the ventral vegetal cells (hatched) were removed to reduce the level of ventral signal at the animal pole.

misjudging the future midline when the marks are made at stage 7+. These experiments demonstrate that in embryos with a ventral implant, the fate of the region between the implant and the anterior border of the nervous system is changed from neural plate to ventral epidermis.

In seven additional marking experiments on stage 7+ embryos with ventral implants into the animal pole, four marks rather than two were made at equal distances between the animal pole and the position of the future blastopore. This marking procedure was of less use because the four marks tended to fuse along the midline as a result of convergence-extension movements. In five cases, some of the marks were in the anterior epidermis and the rest were in the midline of the nervous system. These cases confirm that prospective anterior neural plate is converted into ventral anterior epidermis when ventral vegetal cells are transplanted into the animal pole. In two more cases, the marks were displaced laterally from the midline, probably as a result of misjudging the position of the midline at the time of marking.

In all of the marking and transplant experiments, the position of the blastopore was observed as soon as it formed. In every case the blastopore formed at or near the blue mark made on the tipped zygote to define the dorsal side, and the experimental embryos did not differ in the site of blastopore formation from normal embryos. Thus modifications in the length of the neural axis in embryos with ventral implants into the animal pole resulted from the repositioning of the anterior bor-

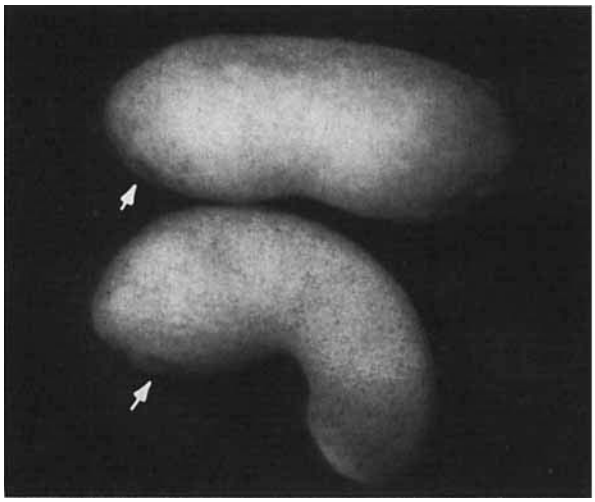

Fig. 13. Lateral views of embryos at stage 20, normal embryo at the top and an embryo from which ventral vegetal cells were removed at the 64-cell stage at the bottom. Arrows indicate the cement glands near the anterior borders of the nervous systems. ×21.

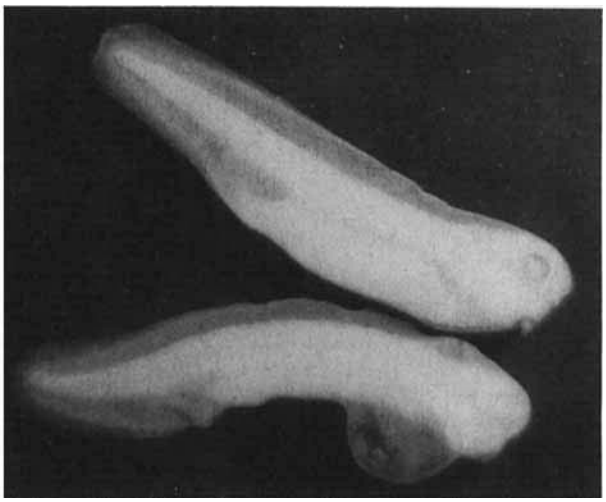

Fig. 14. Lateral view of a normal (**top**) and an experimental embryo (**bottom**) at stage 36–37. Three vegetal cells were removed from the ventral side of the experimental embryo at the 64-cell stage. The lengths of the nervous systems, measured along the dorsal side from the level of the cloaca to the cement gland, are 3.86 mm on the normal embryo and 4.70 mm on the operated embryo. ×12.3.

der of the nervous tissue, and not of the position of the blastopore lip.

Transplantation of Ventral Submarginal Tissue Into the Animal Pole of the Blastula at Stage 9

It is possible that the ventral tissue, when implanted into the animal pole, attempts to sort out or to behave differently from the behavior of the local cells, due to adhesive or other differences. This local disruptive behavior of the dislocated tissue might be the cause of the shortening of the axis. The sham experiment of removing, marking, and replacing the animal pole cells does not control for this possibility since the implanted cells are not foreign to the site of implant. If our hypothesis is correct, then all other cells of the embryo are involved either in producing and/or transmitting either (or both) dorsal and ventral signals, so a suitable control is somewhat elusive.

We reasoned that if our basic hypothesis is correct, then a time should be reached when the epidermal/neural plate boundary becomes fixed in position and ventral implants into the animal pole should have no effect on the position of the boundary. Thus, for this fourth set of experiments, we have transplanted exactly the same ventral vegetal cells from stage 10+ donors into the animal poles of hosts older than stage 7+. We have been surprised to find that this operation has no effect on the boundary when done on late pregastrula stage 9 hosts. This operation is identical with that shown in Figure 5, except the host embryos were at late stage 9 rather than at stage 7+.

Since the donor tissue is the same as in the second experiment, this experiment also serves well as a control for the possibilities mentioned above.

The mean length of the neural axes at stage 20 of the 11 cases of implants of ventral vegetal tissue into the animal pole of stage 9 hosts was 3.93 ± 0.22 mm (standard deviation). There is no statistical difference between the lengths of the axes of these experimental embryos at stage 20 and those of normal embryos at the same stage (whose mean lengths were 4.03 ± 0.24 mm), nor with the lengths of the axes of the sham controls.

Removal of Ventral-Signaling Cells at the 64-Cell Stage

In the second set of experiments, we augmented the amount of ventral signals that were received in the region of the animal pole and the axis was shortened. We thus reasoned that removal of some of the ventral vegetal cells from their normal ventral positions might accomplish the inverse and reduce the amount of ventral signals received by cells in the animal pole region. This should change the interaction between dorsal and ventral signals in that region such that the axis would become longer.

In this fifth set of experiments, we removed two to three ventral vegetal cells from embryos at the 64-cell stage (Fig. 12). We measured the axes of the operated embryos at stage 20 and found that the axes of these experimental embryos were highly significantly longer ($P < .001$) than the lengths of the axes of normal embryos. The 17 experimental embryos in this series had a mean axis length of 4.49 ± 0.16 mm compared to the mean axis length of normal embryos at the same stage of 4.03 ± 0.24 mm. A normal embryo and an embryo with ventral vegetal cells removed are compared at stage 20 in Figure 13, and at stage 36–37 in Figure 14.

To see if prospective ventral epidermis is converted

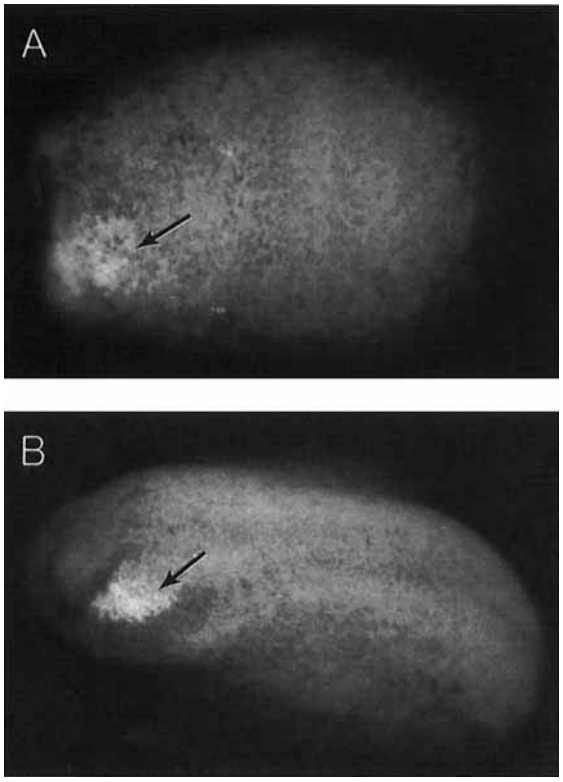

Fig. 15. These embryos are photographed with both UV and white light, anterior to the left. **A:** Ventral view of a normal embryo, at stage 15, that was marked with TRITC ventral to the animal pole at stage 7+. The fluorescent cells (arrow) are in the epidermis adjacent to and overlapping with the cement gland. **B:** Dorsolateral view of an experimental embryo, at stage 15, from which ventral vegetal cells were removed at the 64-cell stage and which was then marked at stage 7+ with TRITC as in the normal embryo. The marked cells are in the forebrain of the open neural plate (arrow). The length of the neural plate at stage 15 is 2.83 mm for the normal embryo, and 3.07 mm for the experimental embryo. ×32.7.

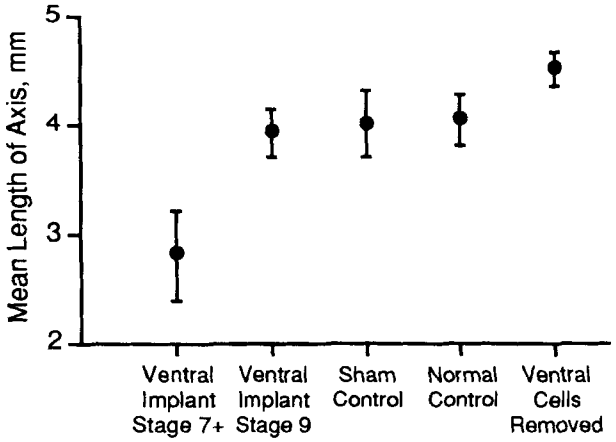

Fig. 16. The lengths of the axes at stage 20 of normal embryos and four experimental sets of embryos is graphed. The lines and bars indicate standard deviation. There is no significant difference between normal controls, sham controls, and embryos with ventral implants at stage 9. The embryos with ventral implants at stage 7+ and those with ventral vegetal cells removed are highly significantly shorter or longer, respectively, than the normal controls (Student t-test).

to neural plate in these experiments, both normal and experimental embryos were marked at stage 7+ with TRITC just ventral to the animal pole in nine cases. In normal embryos, the marks were found at stage 15 or stage 20 to be in the epidermis immediately ventral to the cement gland (Fig. 15A). In the experimental embryos that lack some ventral vegetal cells, the mark is found in the forebrain (Fig. 15B).

One might argue that removing ventral vegetal cells produced longer axes simply because the lack of some large yolky vegetal cells would allow more convergence extension. We know this is not the case since we have removed equal numbers of dorsal cells at the 64-cell stage and all such experimental embryos (six cases) produced shorter than normal axes (data not shown).

DISCUSSION

We hypothesized that the position of the anterior border of the *Xenopus* neural plate is determined by interactions between the signals that induce ventral mesoderm and the signals that induce dorsal mesoderm. These signals are viewed as passing from the endoderm through the marginal region of the tissue of the animal hemisphere from which they induce mesoderm and continuing through the rest of the animal cap where they may initiate the determination processes that lead to formation of the neural and epidermal regions. One could call these early effects on the future ectodermal regions either an initial induction or a change in competence. This situation is similar to the finding that the "changing competence" of the prospective lens ectoderm is due to early induction events (Jacobson, 1966). In any event, the ventral and dorsal inductive signals could cause each of the two ectodermal regions to embark on a process of progressive determination, early stages of which may be labile or reversible. If the dorsal signal induces neural differentiation and suppresses epidermal differentiation, while the ventral signal suppresses neural differentiation and supports epidermal differentiation, then a boundary should form where the two signals have about equal effects.

Our experiments support this hypothesis. The sham control experiments (Figs. 2, 3) do two things. Since the marked animal pole cells placed into the animal pole end up in or very near to the cement gland which is adjacent to the anterior border of the neural plate, these experiments establish that the animal pole region of the stage 7+ embryo is normally just ventral to the future anterior end of the neural plate. Second, the replacement of animal pole cells with animal pole cells has no effect on the position of the border of the neural plate, so the transplantation operation by itself does not disturb normal development. Axes of these sham operated embryos were the same lengths as axes of unoperated normal embryos (Fig. 16; Table 1).

The replacement of animal pole cells with cells from the ventral submarginal region that signal induction of ventral mesoderm (Figs. 5–8) should change the level of interaction between dorsal and ventral signals near the anterior end of the embryo. If our hypothesis is correct, one could expect that the anterior border of the neural plate would be driven back toward the blastopore, shortening the axis. This is what we observed. The marked implants, placed into the animal pole, ended up on the ventral faces of the embryos nearly a millimeter posterior to the cement glands. The axes of these embryos (the lengths of their nervous systems at stage 20) were, on the average, about 1¼ mm shorter than those of sham operated or normal control embryos (Table 1; Fig. 16). The same results were obtained on stage 7+ hosts whether the donor tissues came from stage 10+ or stage 7+ embryos, so heterochronic implants do not have a different effect.

These results imply that tissue that normally would have become nervous system was changed in its fate by these experiments to become epidermis instead. This implication is confirmed by the marking experiments (Fig. 9). Marks placed between the animal pole and the future dorsal lip of the blastopore normally end up in the nervous system (Fig. 10), but in embryos with a ventral implant in the animal pole, some of the marks end up in ventral epidermis (Fig. 11). There is no indication that anterior ectoderm was induced by the implant to form ventral mesoderm since all marked cells end up in the ventral epidermis.

The experiments so far were with host embryos at blastula stage 7+. When hosts at stage 9 were used, the same ventral-signaling cells implanted into the animal pole had no effect on the position of the boundary of the neural plate. These results lead to the surprising implication that the neural plate boundary is at least tentatively set before gastrulation begins. These experiments also serve as control experiments that eliminate the idea that the different cellular behaviors of the dislocated cells somehow reduced axis length in a non-specific manner.

The position of the boundary of the anterior neural plate is not changed by implanting ventral vegetal cells at stage 9, but the boundary position might still be able to be modified by other signals such as those from the dorsal vegetal cells, or those from mesoderm that would underlie the neural plate if the positions of those tissues were to be modified experimentally.

The ventral implants at stage 7+ sometimes, but not always, induced tails, which then tended to migrate posteriorly through time (Fig. 8). Induction of local host tissues by the implant might interfere with axis formation in the area and thus be an alternative explanation for the shorter axes of the operated embryos. This is one reason that we proceeded to test our hypothesis by doing the inverse experiments, that is, to remove some of the cells that are the source of ventral signals before those signals have much chance to act. These experiments disturb only tissue of the vegetal hemisphere and can affect the animal pole region only by reducing the source of ventral signals.

The experiments that were designed to reduce the amount of ventral signaling at the animal pole by removing some of the ventral-signaling cells at the 64-cell stage were expected to produce embryos with longer axes, and they did. The argument that signals from the vegetal cells normally pass all the way to the animal pole is strongly supported by the results of these experiments. The results of both increasing and decreasing signal strength may be explained by our hypothesis. Alternative explanations are less parsimonious, requiring multiple hypotheses, and seem unlikely to us, especially when taking into account the results of the marking experiments that show that prospective neural plate becomes epidermis when the ventral signals are increased near the animal pole, while prospective anterior ventral epidermis becomes neural plate when the ventral signals are decreased.

In the DAI index (Kao and Elinson, 1988), all the embryos have shorter axes than 5, the normal embryo. Our embryos with extra-long axes, whose ventral vegetal cells were removed, do not fit into this series.

The experiments so far deal only with the augmentation or the reduction of the ventral signal. It is clear that ventral and dorsal signals, besides influencing the future dorsal or ventral nature of the mesoderm and the ectoderm, also have some effects on the anterior-posterior differences along the axis. Our experiments do not distinguish whether the ventral signal(s) are one or more molecular species. The expanding literature on molecular candidates suggests that there may be several ventral signals and several dorsal signals. In the context of our hypothesis, the head (anterior end of the neural plate) forms where planar signals for both ventral and dorsal mesoderm induction should be most attenuated. The boundary of the neural plate appears to be set in position before gastrulation begins in the *Xenopus* embryo, so it becomes problematical whether later normal vertical inductions, as the mesoderm comes to underlie the anterior ectoderm, would have much affect on deciding what will be epidermis and what will be neural plate.

Our evidence suggests, therefore, that the quadrant of endodermal cells that signals induction of dorsal mesoderm, and the other three quadrants of endodermal cells that signal induction of ventral mesoderm, send their signals through the plane of the animal hemisphere tissue during cleavage and blastula stages, and these signals, besides inducing the ventral and dorsal mesoderm, begin the progressive determinations that specify the regions in the future ectoderm that will become neural plate and epidermis. As the signals pass through the plane of the tissue, their strengths should be attenuated to some extent. The border between neural plate and epidermis is defined by interactions between the two different signals, and probably forms where the two signals are about equal (where induction equals suppression).

At the blastula stages when we did our experiments, our sham operations with marked cells indicate that the animal pole cells are fated to become cement gland and to lie adjacent to the anterior border of the nervous system. Implanting cells sending out strong ventral signals into the animal pole changed the fate of the dorsal ectoderm near the animal pole so the cement gland and the border of the nervous system formed in a more "dorsal"-posterior position. Sive et al. (1989, 1990) have evidence that prior to gastrulation, the position of the cement gland and of the anterior border of the nervous system may advance progressively toward the animal pole. If this is so, then our experiments with ventral implants into the animal pole may prevent the full poleward advance of the neural plate border.

The line between epidermis and neural plate is apparent morphologically when the neural plate first forms at stage 14. At this time, Epi 1 is expressed only in the epidermis and not in the neural plate, as discussed in the introduction. The limitation of Epi 1 expression to the future fate map position of the epidermis is decided in cleavage and blastula stages by planar signals that determine the dorsal side (London et al., 1988; Savage and Phillips, 1989). Some reports also suggest that expression of certain molecular markers for neural plate (e.g., XIHbox6) are more easily induced in dorsal than in ventral ectoderm from pregastrulae (Sharpe et al., 1987). It is likely that not all aspects of the differentiation of the epidermal and neural domains are determined at the same time. The process of epidermal and neural differentiation begins before gastrulation in *Xenopus*.

Spemann long held the idea that induction of the neural plate began with an inductive effect passing through the plane of the ectoderm from the forming dorsal lip before gastrulation, and was completed when the chordamesoderm involuted and underlay the prospective neural plate (as noted in the introduction). Holtfreter (1933), on the basis of his experiments with exogastrulae, declared that the early planar induction did not occur, and Hamburger (1988, page 115) says "No experimental evidence for Spemann's view has ever come forth; it has long been laid to rest, and Holtfreter has prevailed." This was written before the experiments of Dixon and Kintner (1989), described in the Introduction, that give evidence that not only does planar signaling from the dorsal lip occur, but it may be more important than the vertical signaling from the chordamesoderm when that tissue finally comes to underlie the prospective neural plate.

We are stressing that these signaling events may have effects in the ectoderm before the dorsal lip becomes a signaling area. That is, during cleavage stages and in the blastula the signals emanate from the endoderm and pass through the plane of the tissue of the entire animal hemisphere. By early gastrula stages, dorsal signaling emanates from the dorsal mesoderm (dorsal lip) and may cease in the endoderm (Gerhart et al., 1991). It is possible that a similar transfer of signaling capability occurs in the ventral tissues by gastrula stages. Progressive determination of both the mesoderm and the ectoderm may begin in cleavage stages and be completed at the end of gastrulation. Major boundaries in the embryo are defined as a part of the progressive determination of the parts.

This paper has stressed the role that the ventral vegetal cells have in establishing boundaries in the pregastrula embryo because the majority of previous studies mostly ignore any active or interactive role of the ventral vegetal cells and focus instead on the dorsal vegetal cells that induce the dorsal axial structures. The same sorts of experiments that we report above with ventral vegetal cells that induce ventral structures could be done with dorsal vegetal cells that induce dorsal structures.

Stewart and Gerhart (1990) report that reducing the amount of dorsal organizer at late blastula stages reduces the dorsal axis. As the axis is reduced, the more anterior dorsal structures are progressively lost. They conclude that the amount of organizer determines the anterior extent of dorsal development. Our data suggest that axis length is determined by the interaction of dorsal and ventral signals.

We have done preliminary studies to reduce the dorsal vegetal cells by removing two or three cells at the 64-cell stage from the dorsal side, rather than from the ventral side as shown in Figure 11, and we get embryos with shorter axes; some with reduced heads and some with normal heads (data not shown). The opposite experiment, to increase the amount of dorsal signals in the animal pole by transplanting dorsal submarginal cells into the animal pole, we have also done. The total length of dorsal axis indeed is increased by this experiment, but the "axis" consists of a normal axis with a secondary axis abutted head-to-head (data not shown). The dorsal inductor seems very much to be involved with setting both dorsal and anterior-posterior development. Because of the complexity of these problems (see review by Slack and Tannahill, 1992), we are leaving further discussion of the dorsal induction system for further studies.

We chose to examine how the medial-anterior border of the neural plate is set because we know from fate maps that this border stays at the midline. Keller et al. (1992a) started with the Eagleson and Harris (1990) map of the neural plate of *Xenopus* at stage 15 and followed the positions of parts and borders back to stage 10+ by running video recordings of the intervening stages backwards. A similar remapping had been done earlier on the newt *Taricha torosa* by Jacobson and Gordon (1976). The maps at gastrula stages of the prospective neural parts and borders in the two species are quite similar. In each case, the anterior midline border of the neural plate stays at the midline. The prospective lateral borders of the hind brain and spinal cord parts of the neural plate are initially largely out of sight around on the "ventral" side of the embryo and become displaced toward the dorsal midline and elon-

gated along the axis during gastrulation and early neurulation. Convergence and extension mostly are limited to the prospective hind brain and spinal cord regions.

If the future positions of the lateral borders of the neural plate are affected during pregastrula stages by interactions of dorsal and ventral signals from the vegetal cells (and we believe they are), then experimental analysis of the positions of these prospective borders will be greatly complicated by the distortion caused by the powerful convergence-extension movements of the notoplate and the notochord. The notoplate is the medial portion of the neural plate, and the notochord is the medial portion of the dorsal mesoderm. Both of these areas may be induced by signals from dorsal vegetal cells (Jacobson and Sater, 1988; Keller et al., 1992b), and these two regions are largely responsible for the extensive convergence-extension movements during gastrulation and neurulation. Computer simulations and analyses will probably be necessary to define the changing landscape of these lateral borders in relation to the temporal, spatial, and intensity differences of the two signals.

EXPERIMENTAL PROCEDURES

All experiments were done with embryos of *Xenopus laevis*, staged according to the staged series of Nieuwkoop and Faber (1967). Embryos were reared, and experiments performed, in a modified Ringer's salt solution (MMR) (Newport and Kirschner, 1982) which consists of 0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM sodium Hepes, 0.1 mM EDTA, adjusted to pH 7.35 with NaOH. Eggs were obtained from female frogs brought to ovulation by injections of human chorionic gonadotropin. Fertilization was by artificial insemination using sperm obtained from minced testes that had been removed surgically from male frogs. Embryos are dejellied in a solution containing 0.03 g cysteine HCl and 0.005 g NaCl per ml of water, brought to pH 7.8 to 8.1 with 5 N NaOH.

Marking the Position of the Future Dorsal Midline

At the blastula stages at which some of our experiments were done, it was necessary to know the dorsal-ventral orientation of the embryos. Stages 7 to 7+ are blastula stages just before pigment concentration begins to make the beginning blastopore visible. We used the technique described by Stewart and Gerhart (1990) to mark at the zygote stage with Nile blue the site of the future dorsal lip of the blastopore. The zygote is placed in a solution containing 7% (w/v) Ficoll in 1 part 33% (v/v) MMR and 1 part Sorenson's solution, which consists of 4 parts 0.25 M NaH_2PO_4. H_2O and 46 parts 0.25 M Na_2HPO_4, diluted 1 to 3.75 (pH 7.8). The zygote is then tipped 90° off the gravitational axis before 0.4 of the first cell cycle. Tipping and marking was completed within 30 minutes after fertilization at room temperature. A small crystal of Nile blue is used to stain a spot on the uppermost meridian of the tipped egg, and later this spot is within 15° of the middle of the dorsal lip in nearly all of the embryos. The position of the blue mark was used to locate the dorsal side of experimental embryos at the blastula stages used for some of our experiments.

Tissue Transplantations

Embryos were placed in 100% MMR solution in operating dishes coated with a mixture of paraffin and plasticine. Two pairs of sharpened watchmaker's forceps were used to remove the vitelline membranes. Host embryos were at stages 7 to 7+. Some donor embryos were at stage 10+, and some were at the same stage as the host. One experiment used host embryos at stage 9.

To obtain ventral-signaling tissue, an eyebrow hair knife was used to cut out a fragment of tissue from the submarginal area of the ventral side of a donor embryo. If the donor embryos were at stage 10+, then the ventral side could be readily identified as on the side opposite the forming blastopore lip. If the donor embryos were at stage 7 to 7+, then the ventral side was identified as opposite the marked future dorsal lip area, as explained above. The excised ventral fragment was transferred to a depression slide and immersed in a drop of TRITC (tetramethyl rhodamine isothiocyanate, Molecular Probes) at a concentration of 15 to 25 μg/ml for 1 minute. (A stock solution was made of TRITC dissolved in DMSO to a concentration of 2–3 mg per ml. This was diluted in saline to a final concentration of 15–25 μg per ml. Stock solution must be made fresh before each set of experiments.) The fragment was then washed three times in 100% MMR and implanted into the animal pole of a host embryo. This technique was modified from Heasman et al. (1984), who reported that, by three different criteria, the dye remained cell autonomous when dyed cells were re-implanted. The host embryos were prepared by excising a fragment of tissue from the animal pole, equal in size to the fragment to be implanted. The animal pole is easily identified as the point that naturally floats uppermost at these stages. Transfer of the fragments was done with hair loops. The fragments heal into place in about 15 minutes, at which time the host embryo is transferred into 20% MMR in a small plastic petri dish that had been coated with 1% agarose, and cultured until sibling normal control embryos reached late neurula stage 20.

Similar procedures were used to remove, mark, and implant a fragment from the animal pole of embryos at stage 7 to 7+ for sham control experiments.

Measurements of Axis Length and Distance from Cement Gland to Implant

When control embryos reached stage 20 (a stage at which the neural plate has just completed forming the neural tube), the lengths of the axes and the distances between the implant and the cement gland of experimental embryos were measured. These measurements

were computer assisted using Bioquant IV morphometries software. Measurements were made at stage 20, which is at the end of neurulation when the neural tube is just closing. At this stage, the anterior medial point of the nervous system is easily distinguished, and the posterior extent of the nervous system is at the level of the cloaca. The tail bud has not yet made its contribution (possibly variable) to the length of the axis.

Histology

Histology was done on embryos that had been reared to stage 36 after tissue transplantation. These embryos contained grafts of tissue labeled with TRITC. They were fixed in Kahle's solution (6 parts formaldehyde, 15 parts 95% ethanol, 1 part glacial acetic acid, 30 parts distilled water) for 48 hours at room temperature. Embryos were then dehydrated through an ethanol-butanol series, embedded in paraplast, and sectioned at 8 μm. Sections were collected on gelatin-coated slides. They were viewed and photographed using epifluorescence optics (Zeiss) before removal of the wax, since the process of wax removal causes quenching of the fluorescence. The sections were then dewaxed through a xylene-ethanol series and stained with 0.1% Janus green and 0.2% neutral red, then mounted in 80% glycerol containing 4% N-propyl gallate (Sigma). The sections were photographed in white light for comparison with the fluorescent image.

ACKNOWLEDGMENTS

We thank our colleagues Jon Nuelle and Ying Lu for their suggestions and help. This research is supported by NIH grant HD 25902.

REFERENCES

Danilchik, M.V., and Denegre, J.M. (1991) Deep cytoplasmic rearrangements during early development in *Xenopus laevis*. Development 111:845–856.

Dixon, J.E., and Kintner, C.R. (1989) Cellular contacts required for neural induction in *Xenopus* embryos: evidence for two signals. Development 106:749–757.

Doniach, T., Phillips, C.R., and Gerhart, J.C. (1992) Planar induction of anteroposterior pattern in the developing central nervous system of *Xenopus laevis*. Science 257:542–545.

Eagleson, G.W., and Harris, W.A. (1990) Mapping of the presumptive brain regions in the neural plate of *Xenopus laevis*. J. Neurobiol. 21:427–440.

Gerhart, J., Doniach, T., and Stewart, R. (1991) Organizing the *Xenopus* organizer. In: "Bodega Marine Laboratory Marine Science Series," Vol. 3, Keller, R., Clark, W., Jr., and Griffin, F. (eds). New York: Plenum Press, pp 55–77.

Gimlich, R.L., and Gerhart, J.C. (1984) Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. Dev. Biol. 104:117–130.

Hamburger, V. (1988) "The Heritage of Experimental Embryology: Hans Spemann and the Organizer." Oxford: Oxford University Press.

Heasman, J., Wylie, C.C., Hausen, P., and Smith, J.C. (1984) Fates and states of determination of single vegetal pole blastomeres of *Xenopus laevis*. Cell 37:185–194.

Holtfreter, J. (1933) Die totale Exogastrulation, eine Selbstablösung des Ektoderms vom Entomesoderm. Roux Arch. Entw. Mech. 129: 669–793.

Jacobson, A.G. (1966) Inductive processes in embryonic development. Science 152:25–34.

Jacobson, A.G., and Gordon, R. (1976) Changes in the shape of the developing vertebrate nervous system analysed experimentally, mathematically and by computer simulation. J. Exp. Zool. 197:191–246.

Jacobson, A.G., and Sater, A.K. (1988) Features of embryonic induction. Development 104:341–359.

Kao, K.R., and Elinson, R.P. (1988) The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos. Dev. Biol. 127:64–77.

Keller, R., Shih, J., and Sater, A. (1992a) The cellulart basis of the convergence and extension of the *Xenopus* neural plate. Dev. Dyn. 193:199–217.

Keller, R., Shih, J., Sater, A.K., and Moreno, C. (1992b) Planar induction of convergence and extension of the neural plate by the organizer of *Xenopus*. Dev. Dyn. 193:218–234.

Kintner, C.R., and Melton, D.M. (1987) Expression of *Xenopus* N-CAM RNA is an early response of ectoderm to induction. Development 99:311–325.

London, C., Akers, R., and Phillips, C.R. (1988) Expression of Epi 1, an epidermal specific marker, in *Xenopus laevis* embryos is specified prior to gastrulation. Dev. Biol. 129:380–389.

Newport, J., and Kirschner, M. (1982) A major developmental transition in early *Xenopus* embryos: 1. Characterization and timing of cellular changes at the midblastula stage. Cell 30:675–686.

Nieuwkoop, P.D., and Faber, J. (1967) "Normal Table of *Xenopus laevis* (Daudin)." 2nd ed. Amsterdam: North-Holland.

Nieuwkoop, P.D., Johnen, A.G., and Albers, B. (1985) "The Epigenetic Nature of Early Chordate Development. Inductive Interaction and Competence." Cambridge: Cambridge University Press.

Savage, R., and Phillips, C.R. (1989) Signals from the dorsal blastopore lip region during gastrulation bias the ectoderm toward a non-epidermal pathway of differentiation in *Xenopus laevis*. Dev. Biol. 133:157–168.

Sharpe, C.R., Fritz, A., DeRobertis, E.M., and Gurdon, J.B. (1987) A homeobox-containing marker of posterior neural differentiation shows the importance of predetermination in neural induction. Cell 49:749–758.

Sive, H.L., Hatori, K., and Weintraub, H. (1989) Progressive determination during formation of the anteroposterior axis in *Xenopus laevis*. Cell 58:171–180.

Sive, H.L., Draper, B.W., Harland, R.M., and Weintraub, H. (1990) Identification of a retinoic acid-sensitive period during primary axis formation in *Xenopus laevis*. Genes Dev. 4:932–942.

Slack, J.M.W. (1990) Growth factors as inducing agents in early *Xenopus* development. J. Cell. Sci. [Suppl.] 13:119–130.

Slack, J.M.W., and Tannahill, D. (1992) Mechanism of anteroposterior axis specification in vertebrates. Lessons from the amphibians. Development 114:285–302.

Smith, J.C. (1989) Mesoderm induction and mesoderm-inducing factors in early amphibian development. Development 105:665–677.

Spemann, H. (1927) Neue Arbeiten über Organisatoren in der tierischen Entwicklung. Naturwissenschaften 15:946–951.

Spemann, H., and Mangold, H. (1924) Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. Roux Arch. Entw. Mech. 100:599–638.

Stewart, R.M., and Gerhart, J.C. (1990) The anterior extent of dorsal development of the *Xenopus* embryonic axis depends on the quantity of organizer in the late blastula. Development 109:363–372.