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Commentary

Commentary on classic paper "Gimlich, R.L., Gerhart, J.C., 1984. Early cellular interactions promote embryonic axis formation in *Xenopus laevis*. Dev Biol 104, 117–130."

Some of the findings from experimental embryology have power through the clarity of an image. Such power is beautifully illustrated by the Spemann-Mangold organizer experiment, which used amphibian embryos to test the organizing properties of a piece of prospective dorsal tissue. Hilde Proescholdt (later Mangold) did preliminary experiments in which the graft and host were taken from two members of the same species, where host and graft could not be distinguished, and she showed that such a dorsal piece, grafted to the opposite side of a host embryo, resulted in a twinned tadpole. But what was the conclusion-was the secondary axis produced entirely by self-differentiation of the grafted piece, or might there have been an interaction between the graft and host tissues?

In the definitive experiments, Hilde Proescholt employed two newt species with differently pigmented eggs to examine the twinning phenomenon. Her drawings of sectioned embryos showed that a lightly pigmented graft taken from the dorsal region contributed to dorsal midline structures, including the notochord, while darkly pigmented host structures were diverted from their epidermal fate to make a second nervous system (Spemann, 1938; Harland, 2008). The clear and profound interpretation was that the graft must have induced host tissues to become the organized nervous system in the secondary axis, and that the second nervous system was not just self-differentiating from the graft.

In the featured paper, from Bob Gimlich and John Gerhart, the experiment addressed where dorsal identities lay in the earlier cleavage stages. Were they in the same cells that produced the organizer, or was there a difference between early and later organizing activities? There was already ample evidence from Peter Nieuwkoop and others that more vegetal cells had mesoderm inducing properties, but the special activity from dorsal-vegetal cells was made unusually striking in an image (Fig. 6 in Gimlich and Gerhart's 1984 paper) which showed that grafted dorsal-vegetal cells from a cleavage stage embryo induced a normally patterned axis, including a nervous system, but the grafted cells did not contribute to the axis. Therefore, the dorsal vegetal cells, themselves fated to become endoderm, could induce an axis at the early stage, without contributing directly to axial structures.

A similar conclusion was drawn by Dale and Slack (1987), though they used Nieuwkoop recombinates of vegetal cells with marked animal cap ectoderm. Again, they found that the dorsal most vegetal cells at the 32-cell stage were able to induce organizer structures. This property of vegetal cells that can induce the Spemann-Mangold organizer, was given the name "Nieuwkoop Center" by John Gerhart (Gerhart et al., 1989), as a region of the early cleaving embryo that has the ability to induce the Spemann-Mangold organizer. In contrast to Nieuwkoop, who argued that there was a graded signal from dorsal to ventral, the other groups found a sharp distinction between dorsal signalling restricted only to the dorsal-most vegetal cells, while the rest induced mesoderm of a ventral character. This finding of a binary signal was also important in supporting the view that signalling from the organizer at the later stage is essential for inducing the variety of fates found in the mesoderm, as part of the "three signal model" for patterning the early embryo (Dale and Slack, 1987).

The inducing activity of the dorsal vegetal-most cells in both these experiments has often led to the view that this is the main source of early dorsalizing signals, though a subsequent paper from Gimlich also showed that the tier of cells just above the vegetal tier also have dorsalizing activity (Gimlich, 1986). However, these more animal cells not only induce dorsal structures, but are also able to self-differentiate into organizer, so the clean experimental distinction of Nieuwkoop center from Spemann-Mangold organizer is not so striking as the experiment with the vegetal tier, where the grafted cells do not contribute at all to the induced axis.

These elegant manipulations using classic tools of experimental embryology were key to develop an understanding of the cascade of signalling events that begin with a simple egg, and progressively build the tissues of the complex tadpole. The embryological level of understanding demands a molecular explanation, but also defines the specific questions on timing, signalling specificity and differentiation that must be answered. We now understand the signals in terms of Wnt, Nodal, BMP and FGF, but the activity of these signalling factors has to be interpreted in light of the early activities of the vegetal dorsalizing center (Nieuwkoop Center), versus the later signals from the Spemann-Mangold organizer.

Acknowledgements

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Early Cellular Interactions Promote Embryonic Axis Formation in *Xenopus laevis*

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We have attempted to define the location and mode of action of axial determinants in the egg of *Xenopus laevis.* To this end, we transplanted small numbers of blastomeres from normal 64-cell stage embryos into synchronous recipient embryos which had been irradiated with ultraviolet light prior to first cleavage. Without transplantation, such embryos fail to develop dorsal structures of the embryonic body axis. We found that one to three blastomeres transplanted from the vegetal-most octet of cells can effect complete or partial rescue of axis development in a recipient, provided that the donor cells derive from the quadrant just under the prospective dorsal marginal region. These same cells, when transplanted into the ventral vegetal quadrant of a normal 64-cell embryo, cause the formation of a complete second body axis. In contrast, other cells from the vegetal octet of normal donors fail to cause axis formation. When the rescuing donor cells are labeled with a lineage-restricted fluorescent marker, we find that their progeny do not contribute to the axial structures of the recipient. Progeny of the transplanted cells are found below the level of the blastopore in the early gastrula and eventually give rise to portions of the gut, as is their fate in normal development. These results, in agreement with those of Nieuwkoop (P. D. Nieuwkoop, 1977, *Curr. Top. Dev. Biol.* **11**, 115–132), imply that the dorsal-most vegetal cells of the 64-cell embryo receive from the egg cytoplasm a set of determinants enabling them to induce neighboring cells to undertake axis formation. We discuss the relationship between axis induction in rescued irradiated embryos and axis determining processes in normal embryogenesis.

INTRODUCTION

Spemann and Mangold (1924) first demonstrated that the dorsal marginal zone of the amphibian early gastrula has the unique capacity to organize an entire second embryonic body axis when grafted into the ventral side of a recipient gastrula. This organizer activity is a property of cells of the prospective dorsal mesoderm, particularly the somite, notochord, and head mesoderm. Since the time of Spemann and Mangold, many researchers have analyzed the inductive interactions of the mesoderm and overlying ectoderm, but there have been only a few studies of the origins of this unique organizer region at pregastrula stages. It was assumed by Spemann himself (1938) and by others (Pasteels, 1964) that the organizer region is unique because its cells receive in their lineages a set of axial determinants localized in the equatorial grey crescent region of the uncleaved egg, opposite the point of sperm entry. This proposal has been placed in question by several observations (reviewed by Gerhart, 1980; Kirschner *et al.*, 1980), prominent among which are the results of Nakamura and Nieuwkoop, outlined as follows.

Nakamura and Takasaki (1970) and Nakamura *et al.* (1970) explanted groups of cells from the prospective dorsal marginal zone at various early cleavage stages, and found that explants from the 32- through 128-cell stages—despite their mesodermal fate map position—were unable to differentiate axial mesoderm, and instead produced only ciliated epidermis on prolonged culture. However, explants from the 256-cell stage onward were able to differentiate abundant axial mesodermal tissue. Such results would not, of course, be expected if cytoplasmically transmitted axial determinants had initially segregated to the equatorial egg regions. For this reason, Nakamura (1978) proposed that the organizer capacity was not inherent in the equatorial blastomeres from the outset but was acquired at the early blastula stage.

Nieuwkoop and his colleagues defined experimentally an inductive interaction by which the equatorial blastomeres might gain organizer properties. These researchers excised the entire prospective mesodermal zone at the midblastula stage (2000–8000 cells) of *Ambystoma mexicanum* (Nieuwkoop, 1969a) and *Xenopus laevis* (Sudarwati and Nieuwkoop, 1971). They found that the remaining animal hemisphere cap and vegetal base, when recombined, could nonetheless produce axial mesodermal structures, whereas each separately could not. Cell marking experiments (Nieuwkoop and Ubbels, 1972) showed that all mesodermal cells in the recombinates derived from the animal hemisphere piece. Furthermore, the dorsal mesoderm always formed on the prospective dorsal side with reference to the vegetal base, regardless of the orientation of the animal hemisphere cap (Nieuwkoop, 1969b). Nieuwkoop (1973, 1977) therefore concluded that the vegetal cells are responsible

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in these recombinates, and plausibly in normal blastulae as well, for inducing animal hemisphere cells to form mesoderm, including the dorsal mesoderm possessing Spemann organizer properties. By this interaction, certain cells would become the Spemann organizer not because they received axial determinants from the grey crescent region, but because they were privileged to reside directly above special inductive vegetal cells.

Analysis of this interaction and its role in normal development has been hindered by the fact that recombined embryos often develop poorly, due to the large amount of tissue excised, and must be examined histologically in order to identify their tissue types. We have devised an alternate procedure in which surgical intervention is not so severe and development is more normal. We use *X. laevis* eggs irradiated with ultraviolet light prior to first cleavage, to block the subsequent development of all axial mesodermal and neural structures (Grant and Wacaster, 1971; Malacinski *et al.*, 1977; Scharf and Gerhart, 1980). When the eggs reach the 64-day stage, we replace two or more of the eight vegetal-most cells with their counterparts from nonirradiated donors of the same stage. We find that as few as two vegetal cells, if taken from the quadrant beneath the prospective dorsal marginal zone, can rescue the development of axial structures in recipients. This capacity is restricted to the "dorsal-most" quadrant in the vegetal hemisphere: transplantations of other cells from the vegetal-most level do not result in the rescue of axial development. Using an injectable cell lineage tracer (Weisblat *et al.*, 1980) we find that the progeny of vegetal donor cells remain in a subblastoporal location in rescued early gastrulae and themselves differentiate to ventral gut structures in the tadpole. Structures of the body axis in rescued recipients are composed entirely of host cells.

These results confirm and extend the work of Nieuwkoop (1973, 1977) and indicate that at very early cleavage stages the most vegetal cells possess regional differences which can later generate by induction the dorsal–ventral and anterior–posterior aspects of the embryonic body axis. Furthermore, the results suggest that these differences derive from cytoplasmic localizations which are established in the vegetal regions of the uncleaved egg by UV-, cold-, and pressure-sensitive mechanisms.

MATERIALS AND METHODS

Collection of Eggs and Fertilization

Xenopus laevis were obtained from Nasco Biological Science (Atkinson, Wisc.) and fed chicken liver three times per week. Ovulation was induced by injection of 50 units of gonadotropin from pregnant mare serum (Calbiochem-Behring Corp., La Jolla, Calif.), followed 36 hr later by 500 to 700 units of human chorionic gonadotropin (Sigma, St. Louis, Mo.). Eggs were stripped into a dry dish and mixed with a minced testis fragment in a small volume of 33% modified amphibian Ringer solution (MR; 100% MR: 0.1 *M* NaCl, 2 m*M* KCl, 2 m*M* $CaCl_2 \cdot 2H_2O$, 1 m*M* $MgCl_2 \cdot 6H_2O$, 50 μg/ml gentamycin, 25 U/ml mycostatin, buffered to pH 7.4 with $NaHCO_3$). The time of mixing of gametes was taken as the time of fertilization. Eggs were allowed to fertilize for about 10 min at room temperature (18–22°C), then dejellied with 2.5% cysteine · HCl (adjusted to pH 7.8–7.9 with 10 *N* NaOH), washed thoroughly, and cultured in 33% MR.

Staging of Eggs and Embryos

Eggs were staged using a normalized time scale in which time of fertilization equals 0.0 and the time of appearance of a cleavage furrow in 50% of control eggs equals time 1.0.

Embryos and larvae were assigned developmental stages according to Nieuwkoop and Faber (1975).

Ultraviolet Irradiation of Recipient Embryos and Assignment of Index of Axis Deficiency (IAD)

Recipient embryos used in the transplantations were UV-irradiated on the vegetal hemisphere by time 0.4 on the normalized time scale of the first cell cycle. Irradiation was carried out with 254-nm light as described by Scharf and Gerhart (1980). Later, each embryo was assigned a score of 0–5 based on a standard set of body axis structures developed by the time nonirradiated control embryos had reached stage 41. The external criteria and scoring system used are those of Scharf and Gerhart (1980, 1983). For each group of experimental embryos an average "index of axis deficiency" (IAD) was calculated by summing individual embryo scores and dividing by the number of individuals. Control nonirradiated embryo IAD was less than 0.4 in all donor batches. Eggs to be used as transplant recipients received a dose of $2.1–2.3 \times 10^4$ erg/mm^2, delivered in a single 55-sec pulse. This treatment was empirically found to give an average IAD of 4.0–4.9, without causing vegetal hemisphere cleavage abnormalities (Beal and Dixon, 1975; Malacinski *et al.*, 1977) or exogastrulation.

Nonirradiated Donor Embryos

The prospective dorsal side of normal nonirradiated donor embryos was located as follows: fertilized eggs were transferred to a 5% solution of Ficoll (Type 400, Sigma) in 33% MR in plastic tissue culture dishes. Ficoll dehydrates the perivitelline space, preventing eggs from shifting within their vitelline envelopes (Kirschner *et al.*, 1980). The envelope adheres slightly to the tissue culture plastic, so that embryos remain fixed in position. Eggs were oriented uniformly with respect to the position of the sperm entry point (SEP), a reliable indicator of the future ventral midline in most egg batches (Gerhart *et al.*, 1981). A small wound or dye spot was then made at the SEP at the 16- to 32-cell stage, to give a mark that was visible through neurulation. In this manner the average angle between the dorsal midline and a meridian containing the SEP could be measured at stage 14 when the outline of the neural plate is visible. This angle was measured through the shortest arc, whether in the clockwise or counterclockwise direction. The average angle varied between 98° and 169° in the donor embryo batches used in our experiments. An angle of 180° would represent the "ideal" topographic relationship—dorsal midline opposite the SEP.

Blastomere Transplantations

Cell transplantations were carried out on a bed of 2% agarose (Type V, Sigma) in 100% MR (with 50 μg/ml gentamycin and 25 U/ml mycostatin, buffered to pH 6.8 with 10 m*M* sodium phosphate). Donor cells were isolated by teasing away surrounding cells with sharpened watchmaker's forceps and hair loops. A cavity was made in the recipient embryo by plucking out one or two vegetal blastomeres with forceps. The transplant cells were lifted into place with fine glass tools coated with poly-HEMA (Hydron Polymer Type NCC, Hydron Laboratories, New Brunswick, N. J.). Recipients were returned to their natural orientation, vegetal pole down, in round-bottomed wells in agarose. The host-graft boundary was usually healed within 1 hr. After a 2- to 3-hr healing period, the medium was changed to 33% MR (buffered to pH 7.4 with sodium bicarbonate) for culture.

Preparation of Lineage Tracers

Fluorescein isothiocyanate-linked dextran (FDx) was kindly provided by Jochen Braun (University of California, Berkeley). The FDx preparation had an average molecular weight of 10.5×10^3 Da. It is similar to commercially available material (Sigma), but is more highly fluorescent. In order to make FDx cofixable with cell materials in buffered formaldehyde solutions, free amino groups were covalently added as follows. FDx in a 0.4% aqueous solution was activated by mixing with cyanogen bromide (CNBr, Sigma) at a 2:1 weight ratio of FDx to CNBr (a 1:1.25 molar ratio of CNBr to glucose). The pH was continuously monitored with a pH electrode and maintained at 10.7–10.9 by addition of 1 *N* NaOH. The reaction was carried out with constant stirring at room temperature, and was considered complete when the pH stopped falling (Mosbach *et al.*, 1976). The pH of the solution was then adjusted to 8.3 by addition of solid $NaHCO_3$ to 0.1 *M*, and solid *D,L*-lysine monohydrochloride was added to a give a molar ratio of about 10:1 with glucose residues. Coupling proceeded overnight at 8°C with gentle stirring, and then the reaction mixture was extensively dialyzed against distilled water. The molar amount of lysine incorporated was estimated by the trinitrobenzene sulfonate assay for free amino groups (Habeeb, 1966), using ϵ-aminocaproic acid (Sigma) as a standard and FDx as a blank. An average of two to four lysine residues were covalently attached per FDx chain. This preparation is designated "FLDx" for fluoresceinated, lysinated dextran. Samples were lyophilized and dissolved at 100 mg/ml in distilled water for injection. All solutions for injection were filtered through Millipore filters of 0.45-μm pore size. FLDx proved to be greater than 80% cofixable with cell materials treated with buffered formaldehyde, as described below. More information on preparation and characterization of FLDx will be provided elsewhere (Gimlich and Braun, in preparation).

Injection and Visualization of Lineage Tracers

Needles for the microinjection of lineage tracers were prepared from borosilicate capillaries (Frederick Haer and Co., Brunswick, Me.) pulled on a Brown-Flaming electrode puller (Sutter Instruments, San Francisco, Calif.). Outer tip diameter was 1–5 μm. Samples were expelled from the needles using an air-pressure system. The rate of flow of solution was calibrated before every few injections by expelling a droplet into a dish of light silicone oil (SF96 oil, Harwich Standard Chemical Co., San Francisco, Calif.) and measuring its volume with an ocular micrometer.

Individual vegetal blastomeres at the 64-cell stage were injected with 10 ng of FLDx (0.1 nl of solution). To prepare wholly labeled transplants, the two blastomeres on the prospective dorsal side (the side opposite the SEP) at the 4-cell stage were each injected with 150 ng of FLDx (1.5 nl of solution). Labeled vegetal cells were transplanted into irradiated recipients at the 64-cell stage as described above. When labeled transplanted blastomeres showed leakage of cytoplasm as a result of damage, the recipient was discarded.

Labeled embryos were fixed at stage 10 1/4 and stage 25–26 in Formalin (10% v/v of a 38% solution of formaldehyde) buffered with 0.1 *M* sodium cacodylate, pH 7.4, for 12–16 hr at 8°C and washed for at least 4 hr at room temperature in 0.1 *M* NaCl, 0.01 *M* sodium phosphate buffer, pH 7.4. Fixed embryos were dehydrated through an alcohol series to 100% ethanol, and embedded in JB4 glycolmethacrylate resin (Polysciences, Warrington, Pa.) for sectioning at 5 μm. Mounted sections were observed under epifluorescence optics optimized for fluorescein (Zeiss filter set 48 77 09).

RESULTS

Rescue of UV-Irradiated Embryos by Blastomere Transplantation

Previous results (Grant and Wacaster, 1971; Malacinski *et al.*, 1977; Scharf and Gerhart, 1980) have shown that amphibian eggs treated prior to first cleavage with ultraviolet light on the vegetal surface, or with cold shock or high hydrostatic pressure (Scharf and Gerhart, 1983), often fail to form dorsal axial structures. These embryos cleave normally and complete gastrulation, developing a simple three-layered anatomy with a radial symmetry derived from the animal–vegetal axis of the oocyte. They fail to neurulate but form ciliated epidermis and a rudimentary gut. Their only mesodermal tissues are mesenchyme and red blood cells ("grade 5" embryos).

There is evidence that UV, cold, and pressure act to prevent the egg from localizing or locally activating determinants needed for axis formation rather than to destroy preformed or prelocalized determinants (Scharf and Gerhart, 1980, 1983). In order to identify the location and mode of action of the postulated axial determinants in normal embryos we have taken the following approach. We remove a few blastomeres of an irradiated embryo at an early cleavage stage and replace them with their counterparts from an untreated embryo of the same stage, as diagrammed in Fig. 1. In this way, we test whether certain donor blastomeres are able to promote normal axis formation in the axis-deficient background of the irradiated recipient. We report here that axis formation can be rescued by transplanting certain of the vegetal-most blastomeres at the 64-cell stage. We were prompted to begin our study with these cells by the proposals of Nieuwkoop (1973, 1977) regarding the role of the vegetal regions of the midblastula in mesoderm induction.

The vegetal-most tier at the 64-cell stage usually contains six to eight blastomeres. We have transplanted cells from each of the four quadrants at this level, which we will distinguish as follows. The "dorsal-most" quadrant is centered on a meridian (the prospective dorsal midline) opposite that containing the SEP. The "lateral-most" and "ventral-most" quadrants are centered 90° and 180°, respectively, away from the prospective dorsal midline. In each instance all of the cells from a given quadrant were transplanted. Due to normal variations in the cleavage pattern, the actual number of blastomeres transplanted varied from one to three.

Figure 2 illustrates in more detail the experimental design and main results. As shown in Fig. 2a, one to three donor blastomeres were transferred into a cavity

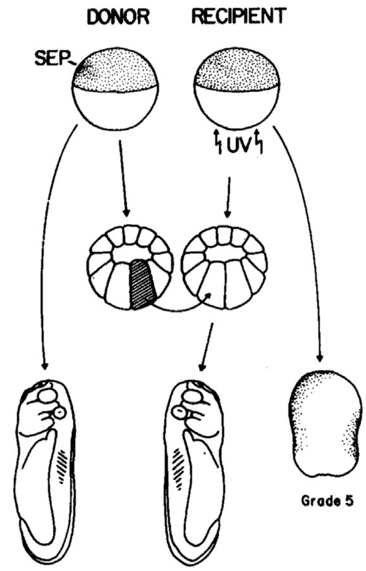

FIG. 1. Schematic diagram of the rescue of irradiated embryos by blastomere transplantation. Top: Eggs (external view) were fertilized and the SEP marked in potential donors. Sibling embryos were UV-irradiated as described under Materials and Methods. Middle: At the 64-cell stage (shown in sagittal section) the blastomeres of the dorsal-most quadrant (crosshatched) of the vegetal-most tier of cells were transplanted into a cavity made in an irradiated recipient by plucking out a like number of cells at the same vegetal level. Bottom: Recipients (external view) of dorsal-most vegetal blastomeres show rescue of axis development, whereas UV-irradiated controls, lacking grafted blastomeres, develop into radially symmetric "ventral pieces" with no body axis. Results were scored at stage 41 of unirradiated controls.

made in the recipient by removing a like number of cells from the vegetal-most region (as described under Materials and Methods). Transplants healed into place within 1 hr and recipients were allowed to develop until control nonirradiated siblings had reached stage 41 (Figs. 2c and d), at which time they were scored for the completeness of the body axis.

We found that unrescued or incompletely rescued recipients displayed the same types of axial deficiencies as embryos treated in the precleavage period with intermediate doses of UV, cold, or pressure, or rescued from the effects of these treatments by off-axis orientation (Scharf and Gerhart, 1980, 1983). These deficiencies are characterized by an anterior-to-posterior truncation series. For example, pigmented retinae were never present in the absence of otocysts and somites, otocysts never appeared without somites, and somites were situated farther posteriorly as they decreased in number (Figs. 2c and d). We could therefore quantify the results in terms of an individual or average "index of axis de-

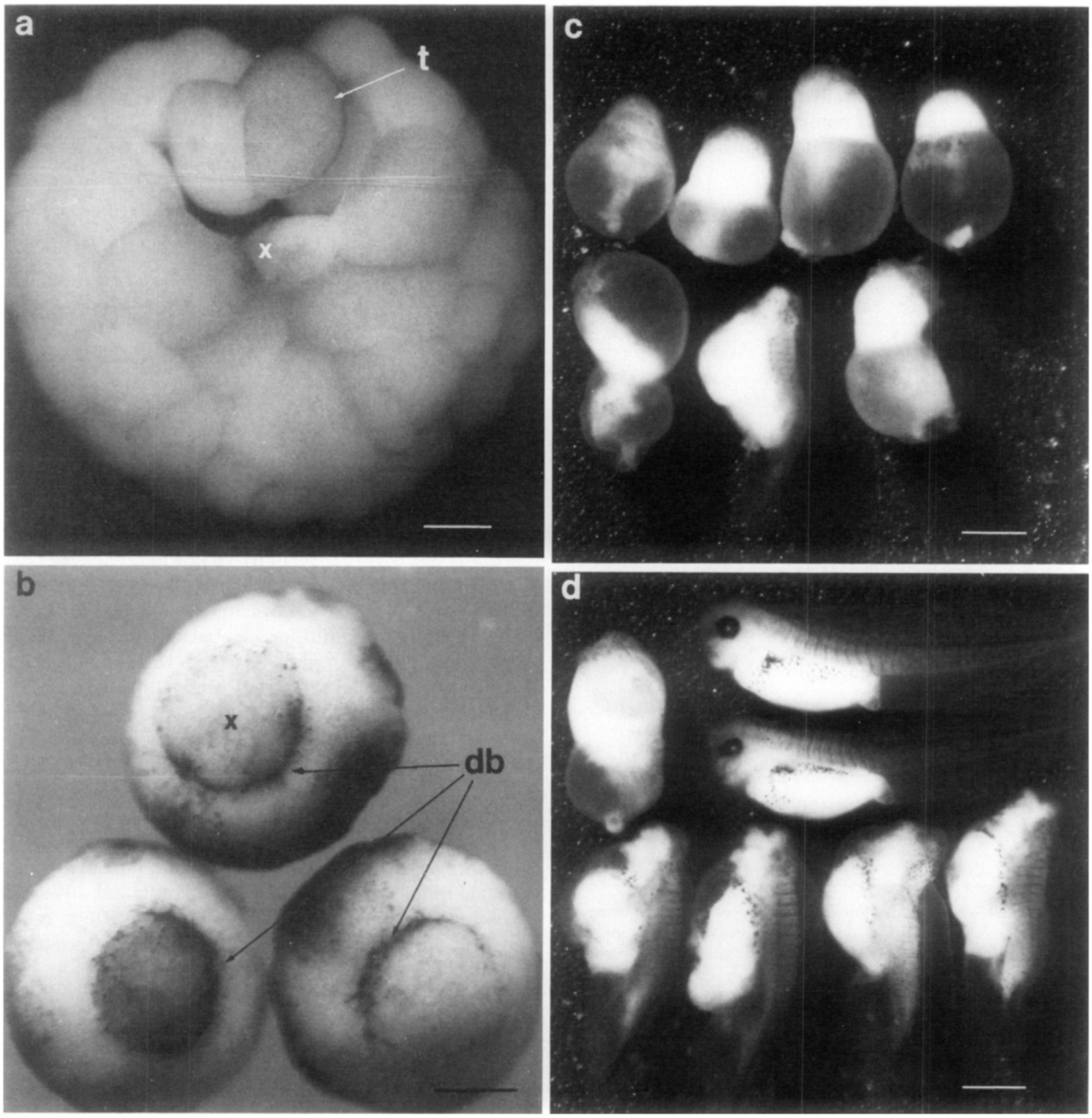

FIG. 2. Blastomere transplantation and results. (a) Vegetal pole view of an irradiated recipient embryo with a cavity in the vegetal hemisphere into which two dorsal-most vegetal blastomeres (t) from an unirradiated donor are being implanted at the 64-cell stage (x, vegetal pole). Bar, 0.2 mm. (b) Vegetal pole view of irradiated dorsal-most blastomere recipients at the early gastrula stage (stage 10 1/2). Like normal unirradiated embryos, these recipients show a typical dorsal blastopore lip (db) where invagination occurs first. The host-graft boundary is usually not visible at this stage. Bar, 0.5 mm. (c) Control recipients of vegetal blastomeres from synchronous irradiated siblings, shown at an age equivalent to stage 41 of control unirradiated embryos. Note the absence of rescue in this control group. Six grade 5 embryos and one grade 4 embryo are shown, for an average IAD of 4.9. Bar, 1 mm. (d) Batch of recipients of dorsal-most vegetal blastomeres, shown at an age equivalent to stage 41 of unirradiated controls. Note the extensive rescue compared to the control embryos of panel c. One grade 1, one grade 2, four grade 3, and one grade 5 embryo shown, for an average IAD of 2.9. Bar, 1 mm.

ficiency" (IAD), which denotes the level of axial development acheived.

The results reported in Table 1 and Fig. 3 show that when donor vegetal blastomeres were taken from the dorsal-most quadrant of nonirradiated embryos, recipients showed substantial rescue, quantified as a decrease in average IAD (from 4.7 to 2.9). In 85% of these operated embryos there were externally visible axial mesodermal structures, compared to only 20% of unoperated controls. Histological examination of embryos rescued to grades 0–2 showed that many differentiated a notochord, though some contained somites fused across the dorsal midline in place of a notochord (Youn and Malacinski, 1981). Also, 73% produced otocysts, compared to 9% of controls, and 37% produced pigmented retinae, which were present in only 2% of controls. Approximately 15% of the transplant recipients formed a complete axis (grade 0 or 1), whereas no irradiated controls did so.

TABLE 1
RESCUE BY TRANSPLANTATION OF VEGETAL BLASTOMERES

Experiment	Donor (n)[a]	IAD[b]	P_w[c]	UV batch IAD	SEP–Neural folds[d]
A	UV (5)	4.4	0.01	—	—
	Dorsal (3)	1.7			
B	UV (7)	4.0	0.0056	4.0	161°
	Dorsal (9)	2.3			
C	UV (5)	5.0	0.024	4.8	136°
	Dorsal (7)	3.4			
D	UV (6)	4.8	0.0051	4.7	98°
	Dorsal (8)	3.2			
E	Ventral (5)	5.0	0.0008	5.0	—
	Dorsal (5)	3.6			
F	UV (8)	4.6	0.0023	4.6	155°
	Ventral (8)	4.7			
	Dorsal (8)	2.9			
G	UV (9)	4.4	0.0056	4.5	169°
	Ventral (8)	4.5	(0.027)		
	Lateral (7)	4.4			
	Dorsal (8)	2.5			
H	UV (7)	5.0	0.0131	4.9	150°
	Ventral (9)	5.0	(0.0172)		
	Lateral (8)	4.9			
	Dorsal (7)	3.1			

[a] Blastomeres were transplanted from the vegetal-most level of donors in all cases. Donor blastomeres were identified as originating from the dorsal-most (dorsal), the lateral-most (lateral), or the ventral-most (ventral) quadrant of unirradiated embryos, or from a random position in the vegetal-most tier of blastomeres in irradiated embryos (UV) at the 64-cell stage. n, the number of embryos in each operated batch.

[b] The average IAD of each operated batch, calculated by summing individual embryo scores and dividing by the number of individuals.

[c] P_w denotes the probability that embryos with the individual IAD scores of the dorsal-most vegetal blastomere recipients could have been drawn from the same population as the UV vegetal cell recipients by sampling error. P_w values in parentheses indicate the probability that embryos with the IAD scores shown by lateral-most and dorsal-most blastomere recipients could have derived from the same population by sampling error. P_w was obtained using the Wilcoxon rank sum test for comparing small samples (Lehmann, 1975).

[d] The average angle from the SEP to the midline of the stage 14 neural plate in the batch of unirradiated embryos from which the 64-cell stage donors of dorsal-most, lateral-most, and ventral-most blastomeres were drawn. This figure gives a measure of the probability that the dorsal-most quadrant is located at a position opposite the SEP in donor embryos (see Materials and Methods).

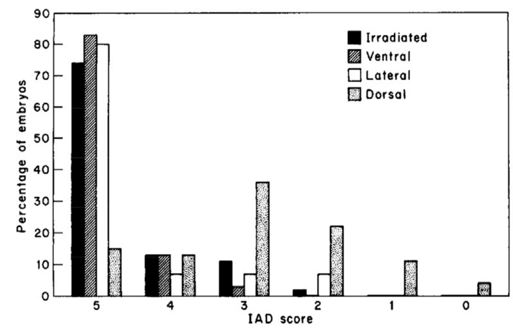

FIG. 3. Histogram of individual IAD scores in batches of irradiated recipients of vegetal blastomeres derived from dorsal-most (n = 55), lateral-most (n = 15), and ventral-most (n = 30) quadrants of normal embryos, or from random quadrants of the vegetal tier of irradiated embryos (n = 47). These data are derived from the experiments summarized in Table 1.

Not only was axis formation much more complete in dorsal-most vegetal cell recipients than in controls, but rescued embryos always formed a single axis. We had expected twinning in approximately 10% of our recipients, calculated as follows: 20% of the unoperated irradiated eggs could form a partial axis of their own (grades 3 and 4). If a host's center for axis formation is at least 90° of arc away from the graft, the two axes might be expected to develop separately (Cooke, 1972). Since the transplants were made into a random position around the recipient vegetal tier, approximately half of those recipients capable of forming axes should have formed twins. The absence of twins suggests that the transplant dominates the process of axis formation in irradiated recipients. As will be shown later, the body axis in rescued recipients always formed from cells directly above the transplant.

In control operations, irradiated embryos receiving grafts of vegetal blastomeres from their irradiated siblings had an average IAD quite similar to that of the UV batch (4.6). In addition, irradiated embryos cultured after removal of the fertilization envelope showed average IAD values indistinguishable from that of the batch (data not shown). Statistical comparison of individual embryo scores for dorsal-most vegetal cell recipients and UV-vegetal cell recipients shows that the differences in average IAD are very unlikely to result from sampling error (see legend to Table 1).

It should be noted that grade 5 embryos are generally delayed in the onset of gastrulation. Bottle cells of the blastopore lip appear around the entire circumference of the lower marginal zone at about the time when the blastopore lip would have spread to the ventral extreme in normal embryos (Scharf and Gerhart, 1980, 1983). When irradiated recipients of dorsal-most vegetal cells are examined at the early gastrula stage, many show a normal morphology, with a distinct dorsal blastopore lip (Fig. 2b). It is these embryos which go on to develop structures of the body axis, while recipients with a more radially symmetric early blastopore lip can usually be predicted to form grade 4 or 5 embryos (data not shown). Thus the introduction of normal dorsal-most vegetal cells into the vegetal hemisphere of irradiated embryos often causes them to develop normally with respect to both the externally visible cell movements of gastrulation and the formation of mesodermal and neural structures of the dorsal body axis.

To assess the regional distribution of this rescue activity among vegetal blastomeres, we made similar-sized grafts of lateral-most and ventral-most vegetal cells. The data in Table 1 and Fig. 3 show that neither of these transplantations was able to effect rescue of axial development by irradiated recipients. These recipients showed about the same average level of UV syndrome as the recipients of vegetal cells from other irradiated embryos or as the unoperated irradiated batch itself. Therefore, the activity in the 64-cell vegetal region which rescues axial development in irradiated recipients is rather sharply localized to the two or three blastomeres of the dorsal-most quadrant.

Fate of Vegetal Cells in Donors and Recipients

On the basis of existing fate maps (Nakamura and Kishiyama, 1971; Keller, 1975, 1976; Jacobson and Hirose, 1981) it was expected that the vegetal-most cells of the 64-cell stage would provide progeny exclusively to the ventral gut region of the larva, and not to dorsal axial structures. In order to confirm these lineage relations, we injected vegetal-most cells of normal 64-cell embryos with the fluorescent linage tracer FLDx (described under Materials and Methods). Figure 4a shows that these blastomeres extend from the vegetal surface to the floor of the blastocoel. When we label a cell of the dorsal-most quadrant at this level, we observe that its superficial progeny at the early gastrula stage lie just below the dorsal lip of the blastopore. The inner progeny of the same marked blastomere extend to the blastocoel floor (Fig. 4b) and lie inwards to the presumptive pre-

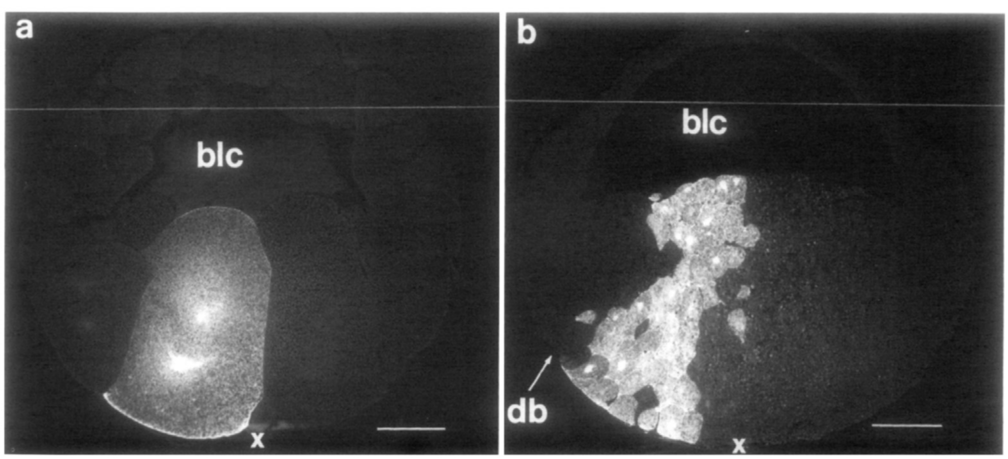

FIG. 4. Position and fate of dorsal-most vegetal blastomeres in normal embryos. A single dorsal-most vegetal cell was injected with FLDx at the 64-cell stage as described under Materials and Methods. Plastic (5 μm) sections were taken near the mid-sagittal plane, and viewed under fluorescein-optimized epifluorescence optics. (a) Injected embryo fixed during the seventh division (giving a 128-cell blastula). The FLDx-containing cell extends from the egg surface near the vegetal pole (x), up to floor of the blastocoel (blc). Bar, 0.2 mm. (b) Labeled progeny of such an injected cell, at the early gastrula stage. Labeled clone extends from the vegetal subblastoporal surface to the blastocoel floor (db, dorsal blastopore lip; x, vegetal pole). Bar, 0.2 mm.

chordal mesoderm, as defined by Keller's (1976) fate map of deep regions of the early gastrula.

At stage 25 (tailbud embryo) the progeny of the dorsal-most vegetal blastomeres are found in the ventral gut lining and the yolky endodermal cell mass, but not in the dorsal structures of the body axis, such as the notochord, somites, and central nervous system (data not shown; Jacobson and Hirose, 1981). These results illustrate that the dorsal-most vegetal cells, which can cause rescue of dorsal axial development when transplanted into irradiated recipients, normally contribute progeny only to the subblastoporal presumptive endoderm.

It was necessary to repeat these lineage studies with vegetal blastomeres transplanted into irradiated recipients. Although we did not expect it to be the case, axial structures arising in rescued recipients could conceivably be made by progeny of the transplanted cells. In this event, rescue would only amount to self-differentiation of the graft. To determine the fates of transplanted cells in rescued recipients, we labeled the prospective dorsal halves of donor embryos, as described under Materials and Methods. Then, at the 64-cell stage, one to three dorsal-most vegetal blastomeres were transplanted from these marked donors into unlabeled irradiated recipients. The distribution of labeled progeny of the transplanted cells was determined in a population of embryos at the early gastrula stage, and in rescued embryos at stage 25–26.

Recipients which showed a clear dorsal blastopore lip at the early gastrula stage (the earliest indication of extensive rescue) were fixed and examined in whole mount or in section with epifluorescence optics. The superficial progeny of the transplanted blastomeres form a patch of cells located just beneath the dorsal blastopore lip (Fig. 5a). Figure 5b shows a 5-μm section in the midsagittal plane of an irradiated recipient early gastrula. Labeled cells extend from the vegetal subblastoporal surface to the blastocoel floor, as a coherent "plug" of cells deep in the core of the vegetal hemisphere, just as they would in normal embryos.

At an early tailbud stage the progeny of the transplanted cells are contained within the endoderm. Figure 6 shows a transverse section at the level of the spinal cord of a rescued recipient at stage 26. Labeled cells in this region are located exclusively in the yolky endodermal mass and in the ventral gut lining. The histological reconstruction in Fig. 7 summarizes the distribution of labeled progeny in a sample (n = 6) of rescued recipients with individual IADs of 0–2.

In all cases, labeled cells comprise a fairly coherent group extending from the anterior ventral and ventrolateral archenteron lining, through the yolky endoderm, and ending anterior to the proctodaeum, or former blastopore. Dorsal axial structures of the head and trunk, including the central nervous system, notochord, somites, pronephros, and prechordal mesoderm, are entirely devoid of labeled graft cell progeny. The dorsal axial structures must therefore have developed from cells of the irradiated recipient and not from the transplanted donor cells.

These results clearly show that the introduction of normal dorsal-most vegetal cells into an embryo blocked in its own early dorsalization processes is sufficient to

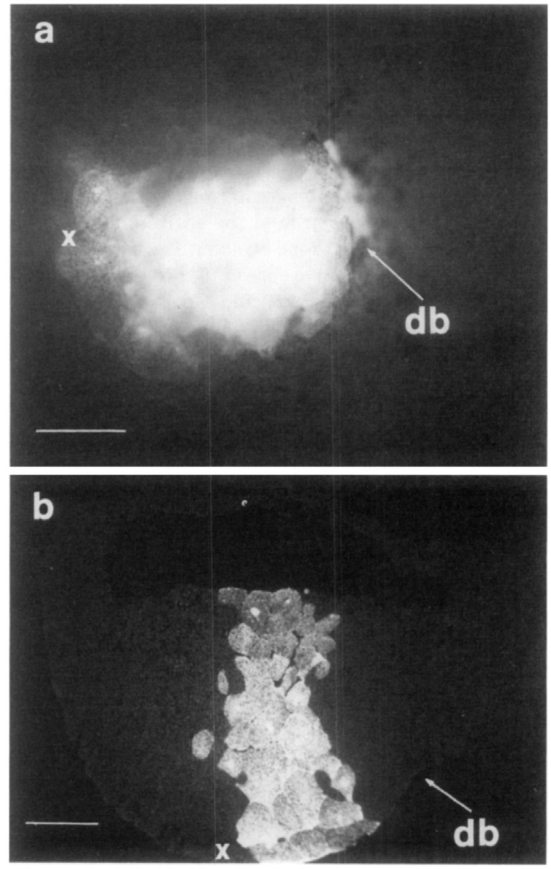

FIG. 5. Distribution of progeny of labeled blastomeres transplanted at the 64-cell stage, in a recipient developed to the early gastrula stage. FLDx-labeled dorsal-most vegetal blastomeres were transplanted into an unlabeled irradiated host as described under Materials and Methods. (a) Vegetal external view of whole fixed recipient at the early gastrula stage, using epifluorescence optics. The dorsal lip of the blastopore (db) had formed just above the superficial labeled progeny of the transplanted blastomeres (x, vegetal pole). Bar, 0.2 mm. (b) Plastic (5 μm) section near the midsagittal plane of a recipient at the early gastrula stage. Labeled progeny of the transplanted blastomeres occupy the subblastoporal and deep presumptive endoderm, just as in normal development. Bar, 0.2 mm.

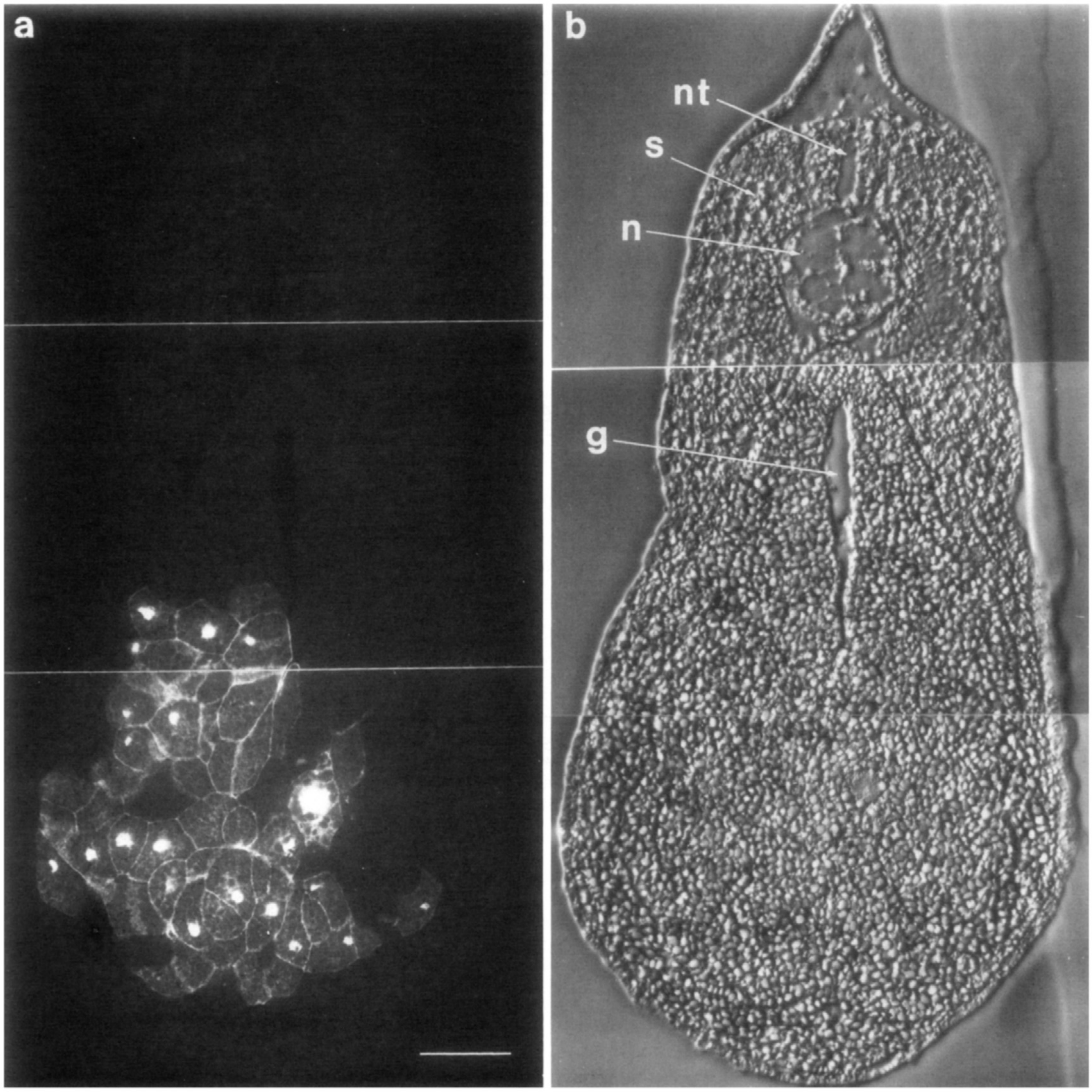

FIG. 6. Fates of labeled transplant cell progeny in rescued recipients at stage 25. The plastic section (5 μm) was cut transversely at the level of the anterior spinal cord. (a) Epifluorescence image shows positions of labeled transplant progeny in the ventral gut lining and yolky cell mass. No labeled cells are found in the dorsal structures of the body axis. Bar, 0.1 mm. (b) Differential interference contrast image of the same section, showing the neural tube (nt), somite mesoderm (s), notochord (n), and the gut lumen (g).

cause complete axis development. The transplanted cells develop according to their normal fate as subblastoporal endoderm, while some cells of the impaired host change fate to produce axial mesoderm and neural tissue. The presumptive axial mesoderm is none other than the classically defined "primary embryonic organizer" (Spemann and Mangold, 1924; Spemann, 1938). Thus, in these rescued recipients, the organizer is formed as the result of an inductive interaction with the normal dorsal-most vegetal cells.

Factors Affecting the Frequency and Extent of Rescue

In the experiments summarized in Fig. 3, 14% of the recipients of dorsal-most vegetal cell transplants did

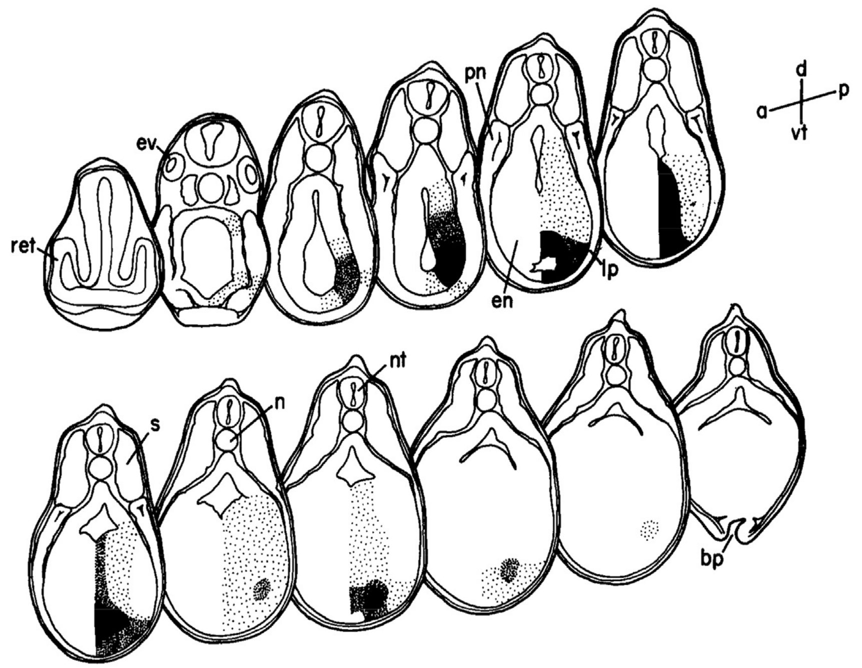

FIG. 7. Superimposed positions of labeled transplant cell progeny at stages 25–26, from six rescued recipients scoring IAD 0–2. Transverse plastic (5 μm) sections were taken at 12 standard levels of each recipient. The positions of labeled transplant cell progeny were scored on a 50 × 50-μm grid of squares overlaid on sections typifying the 12 standard body levels. Since the distribution of labeled cells was not strictly bilaterally symmetric ineach embryo, all labeled positions were projected onto one half-profile at each level. In this diagram, those positions labeled in one to two recipients are indicated by light stippling; those positions labeled in three to four recipients by heavy stippling; and those labeled in five to six recipients, by solid areas. Labeled cells were found in branchial endoderm and lateral plate mesoderm in one of the six cases. All other labeled cells were in the ventrolateral and ventral gut lining and the yolky endodermal cell mass. (ret, retina; ev, ear vesicle; pn, pronephros; en, endoderm; lp, lateral plate mesoderm; s, somite mesoderm; n, notochord; nt, neural tube; bp, blastopore; d, dorsal; vt, ventral; a, anterior; p, posterior).

not show any evidence of rescue; they developed into grade 5 embryos. Another 13% showed little if any rescue; they formed somites and possibly a small amount of notochord, but lacked otocysts and pigmented retinae. These grade 4 embryos occurred at about the same frequency as in batches which received vegetal transplants from irradiated siblings, and in unoperated batches. Even the 73% of embryos showing rescue (grades 0–3) do not usually form perfectly complete axes (grade 0), though many embryos scoring grade 1 are only very slightly microcephalic. There are several possible reasons for the incompleteness of rescue.

First, damage to cells of the graft or host during transplantation could interfere with interactions involved in rescue. We routinely excluded recipients which showed lysis or cleavage arrest by the transplanted cells. These embryos, when allowed to develop, usually either exogastrulated or formed grade 5 embryos. Less obvious kinds of cell damage would have escaped our notice, but might have reduced the success of rescue. All recipients in which the transplanted cells healed into place and continued to divide (greater than 70% of the total number of operations) are included in the data of Table 1 and Fig. 3.

Second, we cannot be certain that in every case the original orientation of the transplant was maintained in the recipient embryo. Cells were always placed with the pigmented apical surface outward, and this inside-outside orientation was easy to control. However, cells might occasionally have been implanted in an upside-down orientation (originally marginal surface toward the vegetal pole), and this would be difficult to recognize. Such abnormal arrangements might be ineffective if localized cell surface interactions are involved in rescue. This possibility remains to be tested experimentally.

Third, since only one quadrant of the vegetal-most tier is able to elicit rescue on transplantation (Fig. 3), we expected that the level of rescue might depend upon the fidelity with which the true dorsal midline could be located. This is probably an important factor, as there is a fair correlation ($r = 0.71$) between the extent of rescue of a recipient batch and the average angle from the SEP to the stage 14 neural folds in the corresponding donor batch (i.e., the degree of certainty of identifying the dorsal-most donor cells). If the dorsal midline of a donor is not opposite the SEP, as we assume, the transplant will often include only part of the dorsal-most quadrant or will miss this quadrant entirely. Preliminary results indicate that one transplanted dorsal-most blastomere is on the average less effective than two in causing rescue, so that accurate transplantation of the entire dorsal-most quadrant is crucial for full rescue.

Fourth, some recipients may be more responsive to the transplant than others. We find that the lower the average IAD of the irradiated embryos, the better they are as recipients. That is to say, the rescue operation is, on the average, more effective (the rescue batch IAD is lower) on recipients which preserve a slight ability to form their own axes (Table 1). Although such recipients never form twins, it is possible that they contribute their own residual axis-forming abilities to the graft-induced axis. In this way, transplantation might rescue a grade 5 embryo to a score of 3, but only promote a grade 3 embryo to grade 0 or 1. While participation of the recipient may contribute to the success of rescue, we have nonetheless observed individual rescue to a level of grade 0 or 1 even in experiments in which all controls scored grade 5. For this reason we consider it likely that dorsal-most vegetal blastomeres can promote complete rescue in at least a minority of cases. Since it is not possible to pick out potential grade 3 and 4 embryos from a mixed population at the 64-cell stage, the contribution of an irradiated recipient's residual axis-forming capacity would be difficult to assess on an individual basis.

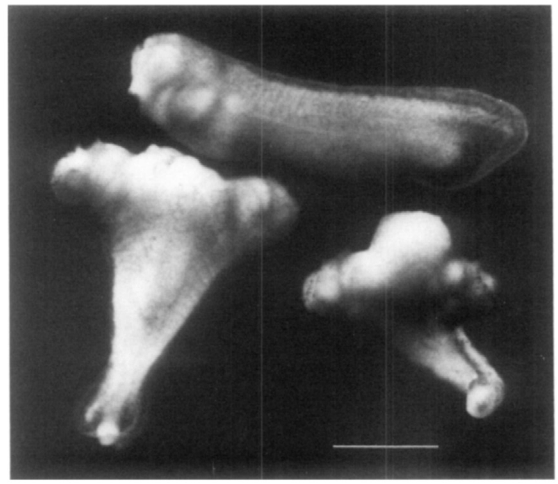

FIG. 9. Twin axes resulting from transplantation of dorsal-most vegetal blastomeres into the ventral-most quadrant. The top embryo resulted from a control transplantation of ventral-most blastomeres into the ventral-most quadrant. Each of the twin embryos (bottom) has two well-formed heads and trunks, though the twin on the right has poorly formed tail structures. Twins were obtained in four out of four cases in this experiment, while ventral recipient controls and unoperated embryos never formed twins. Bar, 1 mm.

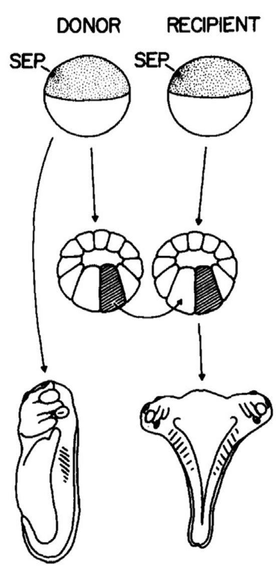

FIG. 8. Schematic diagram of second axis formation resulting from transplantation of dorsal-most vegetal cells (crosshatched) into the ventral-most vegetal quadrant of a normal embryo at the 64-cell stage. Control embryos have ventral-most blastomeres transplanted into the ventral-most quadrant (not shown).

It is possible that a combination of these several factors affects the extent and frequency of rescue of experimental individuals. The important fact is that in every experiment we observe (1) a significant shift in average IAD resulting from dorsal-most vegetal cell transplantation and (2) a significant number of embryos displaying an entire set of axial structures.

Second Axis Induction in Normal Embryos

In preliminary experiments we found that transplantation of one to three dorsal-most vegetal blastomeres into the ventral-most vegetal quadrant of a normal 64-cell embryo (Fig. 8) caused the formation of a second axis in the prospective ventral midline of the host. Figure 9 shows two larvae which received transplants of dorsal-most vegetal cells into the ventral-most vegetal quadrant at the 64-cell stage. In this experiment, four out of four recipients produced second body axes, while control recipients of ventral-most vegetal blastomeres never formed dual axes. Histological sectioning showed that both host and second axes contain a full set of neural and mesodermal structures. We do not yet know the exact fate of transplanted cells in these twins, but we expect that they contribute to the shared gut, just as in normal development and in rescued irradiated recipients. Thus, transplanted dorsal-most vegetal cells can direct axial development in normal as well as irradiated recipients.

DISCUSSION

These results show that the two or three dorsal-most vegetal cells of the 64-cell embryo of *X. laevis* have the ability to induce the formation of a complete set of dorsal body axis structures in a UV-irradiated recipient, which would itself fail to develop an axis. The inductive interaction of the transplanted vegetal cells with marginal cells of the host takes place across a boundary which will later become the blastopore. As expected from their subblastoporal position, the progeny of the transplanted rescuing blastomeres develop according to their normal fate as endoderm of the ventral gut, and do not contribute to the axis, which forms exclusively from host cells. Successful rescue can first be recognized at gastrulation, when a blastopore lip with dorsal characteristics appears directly over the graft. This dorsal lip forms at approximately the normal time, and then spreads laterally around to the prospective ventral side. By comparison, unrescued UV-irradiated embryos usually form a delayed, radial blastopore which invaginates shallowly, showing only ventral characteristics (Malacinski *et al.*, 1977; Chung and Malacinski, 1980; Gimlich, unpublished observations). Furthermore, the dorsal-most vegetal blastomeres can induce the formation of a complete second body axis when transplanted into the ventral-most vegetal quadrant of another normal 64-cell embryo. This small group of vegetal blastomeres clearly has the capacity to induce cells of the adjacent marginal zone to display dorsal characteristics—early bottle cell activity, dorsal convergence and extension in the mesodermal mantle, and Spemann organizer activity—which lead to normal or nearly normal development. These results confirm and extend the previous findings of Nieuwkoop (1973, 1977) and his colleagues on the ability of endodermal cells to induce mesoderm formation in the animal hemisphere of midblastula stage embryos from which the marginal zone had been excised.

Since induction by the dorsal-most vegetal blastomeres is both necessary and sufficient for dorsal patterning of the marginal zone, at least in the circumstances of our rescue experiment, it will be important to ask how these cells acquire their inductive ability. Here we assume that the vegetal cells receive specific morphogenetic determinants which make them inductively active. It is known from earlier experiments that the egg "differentiates" its contents prior to first cleavage, with topographic reference to the point of sperm entry (Gerhart *et al.*, 1981). One aspect of this differentiation is grey crescent formation in the marginal cortex opposite the SEP. The grey crescent has long been considered a locus of morphogenetic determinants which direct the development of axial structures (Dalcq and Pasteels, 1937; Curtis, 1960, 1962). Our results illustrate, however, that it is the deep vegetal region *beneath* the grey crescent which later shows axis-inducing activity. It is therefore possible that the same vegetal region of the egg, and not the grey crescent, contains the crucial determinants. Alternatively, it is possible that they originate in the grey crescent equatorial zone and then move to the deep vegetal region during early cleavages, before furrows block their path. In either case, the processes which confer axis-inducing capacity on the vegetal blastomers are inhibited by precleavage UV-irradiation, cold shock, and hydrostatic pressure.

Our results allow us to make two proposals with respect to the mechanism of axis induction. First, there is an apparent quantitative parameter to the induction. When rescue is incomplete, it is always the more anterior axial structures that are missing. We never find embryos with rescued anterior structures but missing posterior ones. We think that this anterior-to-posterior truncation series reflects directly the quantitative strength of induction exerted by the vegetal blastomeres on their marginal neighbors. When induction is weak, only trunk and posterior axial development occur, while a stronger inductive signal causes anterior development also. According to this interpretation, incomplete rescue is due to our failure to transplant all of the inductive blastomeres, since we sometimes cannot accurately predict their position relative to the SEP. If we happen to transplant only one of these blastomeres, along with one or two lateral cells, we expect only partial rescue. In preliminary studies with grafts of single blastomeres we find, in fact, that rescue is never as complete as it is when all the blstomeres of the dorsal-most quadrant are transplanted (Gimlich, unpublished observations).

Another important point related to the quantitative nature of the induction is that we were able to score the various extents of partial rescue according to the "index of axial deficiency" scale, which was devised for an entirely different purpose. This scale is adapted from the "UV syndrome" series of Malacinski *et al.* (1977), which related monotonically the precleavage UV dose and the extent to which definitive axial structures are absent in the embryo. This same scale can also be used to describe the dose-dependent defects in embryonic axis formation by eggs treated with cold or hydrostatic pressure, and, more surprisingly, the dose-dependent rescue of UV-, cold-, or pressure-treated eggs by precleavage oblique orientation (Scharf and Gerhart, 1983). This latter rescue treatment causes an artificial gravity-driven rearrangement of egg materials (Gerhart *et al.*, 1981). It is dose dependent in that a longer treatment results in a greater anterior-to-posterior completeness of axis development. Presumably, a greater degree of rearrangement causes formation of a greater quantity of axial determinants, whether by their localization to one region or by the local activation of their precursors. At least some of these determinants would then enter the lineages of the dorsal-most vegetal blastomeres, and would determine the intensity of their induction. We can rationalize the observation that all of the axis impairment and rescue treatments, including blastomere grafting, produce embryos related by the same quantitative scale, if it is, in fact, a scale reflecting the strength of induction. It remains to be seen how these early intensity effects could later be translated into the anterior-to-posterior completeness of body axis development.

A second prediction from our results concerns the role of gene expression in the inductive activity of the dorsal-most vegetal cells. The results of Nakamura and Takasaki (1970) and Nakamura *et al.* (1970), and the similar observations of Koebke (1977) suggest that marginal cells gain information needed for axial development during the 64- to 512-cell stages, a period of about 1.5 hr. We suggest that this information is provided by induction from the dorsal-most vegetal cells underlying the marginal zone. If the induction proves to take place at these early blastula stages, it would be quite possible that the vegetal cells do not need gene expression for their inductive activity, since detectable RNA synthesis begins only after the "midblastula transition" at the 4000-cell stage (Newport and Kirschner, 1982). In such a case, the determinants present in the cytoplasm of the dorsal-most vegetal cells would have to act on post-transcriptional aspects of metabolism to establish these cells' inductive capabilities. We are testing this possibility more rigorously by using lineage-restricted inhibitors of gene expression in transplanted blastomeres.

These points of discussion apply to cases of rescue and second axis formation by blastomere transplantation, but we cannot yet extrapolate them to normal embryogenesis. This reservation is necessary because we do not know the entire distribution of axis-controlling determinants in the 64-cell embryo. It is possible that cells of the marginal zone, some of which will form dorsal axial structures, also contain such determinants and therefore are not normally dependent on induction by vegetal cells. In blastomeres contributing materially to the axis, the axial determinants would meet the standard criteria for a morphogenetic determinant—they would be localized within the egg cortex or cytoplasm and segregated to certain cell lineages, within which they would direct developmental choices by affecting transcriptional or post-transcriptional activities. By contrast, those determinants in the vegetal-most cells of the presumptive subblastoporal endoderm, which can act on the marginal cells from outside their lineages, would be defined as "axis-inducing determinants." While the marginal zone explantation experiments which we have discussed (Nakamura, 1978; Koebke, 1977) favor the idea that normal marginal cells do not receive their own axial determinants, this must be tested in transplantation experiments, since explantation of early blastomeres may grossly affect their developmental abilities. The "ventralized" embryo from UV-, cold-, or pressure-treated eggs provides an ideal recipient for such transplantations, since it lacks the ability to form dorsal structures of its own.

In summary, normal embryos at the 64-cell stage contain a group of two or three vegetal blastomeres capable of inducing neighboring marginal cells to form dorsal axial structures in the embryo, even though these vegetal blastomeres will not themselves contribute progeny to the axis. These special vegetal cells receive "axis-inducing determinants" from the egg cytoplasm through a process which is sensitive to UV irradiation, cold shock, or hydrostatic pressure treatment during the first cell cycle. The role of vegetal axis-inducing activity in normal embryogenesis will be explored in future experiments.

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