Cytoplasmic localization and chordamesoderm induction in the frog embryo

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SUMMARY

The experiments described here were designed to reveal the distribution in the frog early embryo of components which are sufficient for specification of the dorsal structures of the embryonic body axis. The approach was to allow cleavage planes to divide the embryo into various well-defined regions and to transplant cells from each region into recipient embryos which would otherwise fail to form axial structures. Partial or complete body axis development could then be scored by the use of external criteria or histological methods. Recipients were embryos which had been irradiated before first cleavage with ultraviolet light on the vegetal surface. Irradiated embryos display a well-characterized set of deficiencies in the dorsal structures of the body axis, but their development can be 'rescued' toward normalcy in several ways. In particular, transplantation of certain small groups of blastomeres from the normal 32- to 64-cell embryo into irradiated recipients was sufficient to cause partial or complete axis development. Cell groups which could cause rescue were located in the vegetal and equatorial levels of one quadrant of the normal embryo – the quadrant centered on the future dorsal midline. Clonal marking analysis showed that the vegetal-most cells of this quadrant contribute primarily to endodermal structures in normal development. In rescued recipient embryos, these cells also contributed only to the endoderm; the dorsal mesoderm and central nervous system were formed exclusively by host cells which originated near the transplant. Rescue could also result from transplantation of equatorial cells from the dorsal quadrant of the normal embryo. As in normal development, these cells formed primarily the chordamesoderm of the rescued embryo. Host cells were induced to contribute the somitic mesoderm, central nervous system, and other structures which would have been missing but for the presence of the transplanted cells. The frequency and degree of rescue caused by equatorial and vegetal transplants is variable. This was explained by the discovery that the location of components needed for rescue varies among individual embryos without regard to the positions of cleavage planes. This was true even when donor embryos were selected on the basis of a precisely regular pattern of cleavage. In such selected embryos, particular blastomeres make a predictable contribution of progeny to the body axis. Thus it may be that the positions of components which can cause axis formation vary without exact regard to the fate map of prospective areas. The implications of this for the study of cytoplasmic localization in the early embryo are discussed. In any case, it is likely that regional interactions and a degree of developmental autonomy in the area of the prospective chordamesoderm are both involved in formation of the dorsal structures of the embryonic body axis.

INTRODUCTION

Fertilization or activation of the amphibian egg trigger a reorganization of the cytoplasm and cortex during the first cell cycle (Elinson, 1980; Klag & Ubbels,

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1975; Paleček, Ubbels & Rzehak, 1978; Vincent & Gerhart, 1985). It is my purpose to examine how this early event is related to the later formation of the dorsal structures of the embryonic body axis, namely, the notochord, prechordal, and somitic mesoderm, and the central nervous system.

In some species the precleavage rearrangement has as its morphological correlate the 'grey crescent,' which reflects an oriented displacement of the endoplasm relative to the egg surface (Ancel & Vintemberger, 1948; Elinson & Manes, 1978; Vincent & Gerhart, 1985). The material of the grey crescent is found in the region of the prospective chordamesoderm at the early gastrula stage (Banki, 1927; Vogt, 1929; Pasteels, 1937). With earlier studies of invertebrate embryogenesis as precedents (reviewed in Wilson, 1925), it was easy to imagine that the grey crescent was a locus of morphogenetic determinants which endowed a certain region of the embryo with special properties. Of course, the special properties of the prospective chordamesoderm included the ability to induce neural plate formation by the overlying ectoderm and to pattern the surrounding mesodermal layer (Spemann, 1938). The grey crescent could therefore be viewed as the organization centre for establishing the typical vertebrate body plan in the amphibian embryo (Dalcq & Pasteels, 1937; Brachet, 1977). This view received experimental support when Curtis (1960, 1962) succeeded in causing formation of second body axes by grafting pieces of grey crescent cortex to the ventral surface of *Xenopus* eggs.

Later studies of pattern regulation after excision of the entire prospective mesoderm told a different story. Upon deletion of the equatorial region the remaining parts of the embryo – the animal 'cap' of prospective ectoderm and the endodermal vegetal 'core' – were either recombined or cultured in isolation (Ogi, 1967, 1969; Nieuwkoop, 1969a; Nakamura, Takasaki & Mizohata, 1970a; Sudarwati & Nieuwkoop, 1971). Recombinates often formed mesodermal derivatives such as notochords, muscle, pronephric tubules, and red blood cells, but the animal and vegetal isolates did not.

Nieuwkoop (1969a,b) argued that an inductive interaction was responsible for mesoderm formation in his recombinates. The position of the dorsal mesoderm was strictly dependent upon the prospective dorsal–ventral polarity of the vegetal piece. As Spemann (1918) had earlier found, the prospective ectoderm was uniformly competent to form mesoderm and seemed to have no strong dorsal–ventral polarity of its own. Finally, Nieuwkoop & Ubbels (1972) showed by cell marking techniques that all of the mesoderm in their recombinates was formed from the ectodermal portion. Nieuwkoop (1973, 1977; Weyer, Nieuwkoop & Lindmayer, 1978) ultimately proposed that regionally specific inductive influences from the vegetal region were responsible for the mediolateral pattern of mesodermal differentiations in the normal embryo.

Other experimental support for this view came from studies of the state of determination of the prospective chordamesoderm at early cleavage stages. Nakamura and his colleagues (Nakamura & Takasaki, 1970; Nakamura, Takasaki & Ishihara, 1970b) found that pieces explanted from the dorsal equatorial region at the middle

blastula stage could autonomously form notochord and muscle in culture. Explants from the 32- to 64-cell stages, however, usually developed into ciliated epidermal balls. Thus the prospective chordamesoderm seemed not to be determined at the outset by virtue of having inherited the grey crescent cortex. Rather, this important region seemed to acquire its commitment gradually, perhaps due to the inductive influence of the vegetal core.

A more refined analysis of the processes involved in dorsal structure specification is now possible. This is due in part to the discovery of simple treatments which prevent normal body axis development by interfering with the precleavage reorganization process. These include ultraviolet irradiation of the vegetal egg surface (Grant & Wacaster, 1972; Malacinski, Brothers & Chung, 1977; Scharf & Gerhart, 1980), and brief cold or hydrostatic pressure shocks (Scharf & Gerhart, 1983). U.v., cold, and pressure are effective in perturbing axis formation only when applied during a limited period of sensitivity in the first cell cycle. All of the treatments produce a single syndrome of axis deficiency which can be described as a dose-dependent anterior-to-posterior truncation of the body axis (Scharf & Gerhart, 1983). At high doses, most embryos fail to form any of the dorsal axial structures, but develop as radially symmetrical 'belly pieces' (Fankhauser, 1930) with a ciliated epidermis, red blood cells, mesenchyme, and a rudimentary gut. These embryos survive until their nutritive yolk supply is depleted.

It is remarkably easy to 'rescue' embryos from the effects of axis-reducing treatments, and it is hoped that the means of rescue reveal something about the normal steps in axis formation. Taking their cues from earlier studies of the developmental effects of gravity on the amphibian egg (Ancel & Vintemberger, 1948; Pasteels, 1937), Scharf & Gerhart (1980) and Chung & Malacinski (1980) made an important discovery. A simple rotation of the u.v.-irradiated egg 90° away from its normal orientation in the gravitational field was sufficient to cause complete body axis development. It had just been observed that u.v. irradiation of the egg prevents the reorganization which causes grey crescent formation (Manes & Elinson, 1980). The rotation operation displaces the dense vegetal yolk mass, resulting in a cytoplasmic rearrangement similar to that which the egg normally makes (Ancel & Vintemberger, 1948; Pasteels, 1937; Black & Gerhart, 1985). This gravity-driven movement is clearly sufficient to mimic the developmental effects of the normal process completely. Rotation is even effective when applied prior to u.v., cold, or pressure shocks (Scharf & Gerhart, 1980). Therefore, axis-reducing treatments do not destroy preformed or prelocalized axial structure determinants, but instead interfere with a process involving cytoplasmic displacement by which determinants are effectively localized or locally activated.

In the experiments described below the susceptibility of u.v.-irradiated embryos to rescue was used to study the location and mode of action of the axial structure determinants in the early-cleavage-stage embryo of *Xenopus laevis*. The results support the contention that regional inductive interactions during blastula stages promote body axis formation in normal development. But they also indicate that

cells of the prospective chordamesoderm, which are presumably among the responding cells in such inductive interactions, are sometimes quite autonomous in developing as chordamesoderm even when separated from the known inductively active region at a very early stage. Body axis formation may thus involve both induction of mesoderm and a degree of autonomy on the part of the prospective chordamesodermal cell population.

MATERIALS AND METHODS

Xenopus laevis were obtained from Nasco Biological Science (Atkinson, WI.) 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, CA.), followed 36 h 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 mM-KCl, 2 mM-CaCl$_2 \cdot 2$H$_2$O, 1 mM-MgCl$_2 \cdot 6$H$_2$O, $50\,\mu$g gentamycin ml^{-1}, $25\,\mu$g mycostatin ml^{-1} buffered to pH 7.4 with 5 mM-sodium bicarbonate). The time of mixing of gametes was taken as the time of fertilization. Eggs were allowed to fertilize for about 10 mins at room temperature (18–22 °C), then dejellied with 2.5 % cysteine/HCl, 80 mM-NaCl (adjusted to pH 7.8–7.9 with 10 N-NaOH), washed thoroughly and cultured in 33 % MR. 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 were assigned developmental stages according to Nieuwkoop & Faber (1975).

Recipient embryos used in the transplantations were u.v. irradiated on the vegetal hemisphere by time 0.4 on the normalized time scale of the first cell cycle, as described previously (Scharf & Gerhart, 1980; Gimlich & Gerhart, 1984). Each irradiated embryo was assigned a score of 0–5 based on a standard set of body axis structures developed by the time non-irradiated control embryos had reached stage 41. The external criteria and scoring system used are those of Scharf & 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 non-irradiated embryo IAD was less than 0.4 in all donor batches. Eggs to be used as transplant recipients received a dose of 2.1–2.3×10^4 erg mm^{-2}, delivered in a single 60-second pulse. This treatment was empirically found to give an average IAD of 4.0–4.9.

The prospective dorsal side of normal non-irradiated donor embryos was located by marking the meridian containing the point of sperm entry (SEP) with a small spot of the vital dye Nile blue sulfate (MCB, Norwood, Ohio) while eggs were immersed in a 6 % (w/v) solution of Ficoll (Type 400, Sigma) in 33 % MR. Ficoll dehydrates the perivitelline space, preventing eggs from shifting within their vitelline envelopes (Kirschner, Gerhart, Hara & Ubbels, 1980). The average angle between

this meridian and the midline of the neural folds was measured at stage 13–14, 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 150° and 170° in the donor embryo batches used in these experiments. Some donor eggs were tipped 90° off the natural gravitational axis, with the SEP downwards, for 15 mins prior to normalized time 0·7 of the first cell cycle. Rotated eggs were then returned to their normal orientation, with the animal pole up. During rotation the uppermost meridian was marked with a spot of Nile blue. This mark was always found adjacent to the dorsal blastopore lip at the early gastrula stage, and within 15° of the neural plate at stage 13–14.

Blastomere transplantations

Cell transplantations were done on a bed of 2 % agarose (Type V, Sigma) in 100 % MR (with 50 μg gentamycin ml^{-1} and 25 μg mycostatin ml^{-1} buffered at pH 6·8 with 10 mM-sodium phosphate). Donor cells were isolated by teasing away surrounding cells with sharpened watchmaker's forceps and hair loops. To make vegetal and equatorial transplants from the same donor, the entire dorsal quadrant of the embryo was isolated, together with neighbouring vegetal and animal cells. The cell pairs to be transplanted were teased apart by tugging on the neighbouring cells, and then these were teased away from the prospective transplants. A cavity was made in the recipient embryo by plucking out an appropriate number of vegetal or equatorial blastomeres with forceps. The transplant cells were lifted into place with fine glass tools coated with poly-HEMA (dried onto the tool from a 0·5 % solution in 100 % ethanol; Hydron Polymer Type NCC, Hydron Laboratories, New Brunswick, NJ). Recipients were returned to their natural orientation, vegetal pole down, in round-bottomed wells in agarose. The host–graft boundary was usually healed within one hour. After a 2 h healing period, the medium was changed to 33 % MR (buffered at pH 7·4 with 5 mM-sodium bicarbonate) for culture.

To make transplants at later blastula stages, groups of cells were dissected from donor embryos with fine tungsten needles and washed free of broken cells by gentle pipetting. A cavity was made in the recipient by teasing cells away with forceps and hairloops, and transplants were set in place using poly-HEMA coated glass tools.

Injection and visualization of cell lineage tracers

Fluoresceinated dextran-amine (FDA) was prepared as described by Gimlich & Braun (1985; this is the same tracer as the 'FLDx' of Gimlich & Cooke, 1983, and Gimlich & Gerhart, 1984). FDA was injected into fertilized eggs or into individual blastomeres at a concentration of 50 mg ml^{-1} in distilled water. Blastomeres at the 32-cell stage were injected with 5 ng (0·1 nl) of FDA. Wholly labelled embryos were prepared by injecting fertilized eggs with 150 ng (3 nl) of lineage tracer. Needles for microinjection of lineage tracer were prepared as described by Gimlich & Gerhart, (1984).

Labelled embryos were fixed at stage 10–10½ and stage 30–32 in 4 % freshly

prepared paraformaldehyde, buffered with 0·1M-sodium cacodylate, pH 7·4, for 12–16 h at 8 °C and washed for at least 4 h 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, or 48 77 16).

RESULTS AND DISCUSSION

Rescue of u.v.-irradiated embryos by vegetal blastomere transplantation

The aim of the first set of experiments was to determine the regional distribution of the mesoderm-inducing activity in early *Xenopus* embryos, as Boterenbrood & Nieuwkoop (1973) had done in *Ambystoma mexicanum*. The strategy was to transplant cells from the vegetal-most level of 64-cell embryos into the same level of synchronous recipient embryos which had been u.v. irradiated over the vegetal hemisphere before first cleavage (Fig. 1A). It was expected on the basis of existing fate maps (Nakamura & Kishiyama, 1971; Hirose & Jacobson, 1979) that cells of this particular vegetal region would contribute mostly to the endoderm in normal development. Therefore, dorsal structure formation in the irradiated recipient embryo would not simply reflect the autonomous differentiation of transplanted dorsal mesoderm precursors. U.v.-irradiated embryos are ideal recipients for such transplants because they provide a supportive embryonic environment which is otherwise devoid of dorsal axial structures. Early-cleavage-stage vegetal blastomeres are large, fragile, and more difficult to transplant than the corresponding parts of a midblastula-stage embryo. However, the early transplants carry no broken cell debris, and they heal into place rapidly. This experimental design provides for the longest possible period of host–graft contact.

The results have been presented elsewhere (Gimlich & Gerhart, 1984), and will be summarized here. When several vegetal cells were transplanted from a quadrant centred about the future dorsal midline, the recipient embryos usually developed a partial or complete set of body axis structures. Control unoperated embryos, or irradiated recipients of transplants from irradiated donors, usually developed as radially symmetrical axis-deficient forms similar to the 'belly pieces' obtained by Fankhauser (1930) from ventral half egg fragments. Also, transplants from the lateral and ventral quadrants of the vegetal tier were unable to promote axial structure formation in the irradiated recipients. Therefore, the capacity of cells in the vegetal level of the 64-cell embryo to cause axis formation by irradiated recipient embryos is restricted to a few cells in the future dorsal quadrant. As illustrated in Fig. 1B, the same cells could cause a second body axis to develop when transplanted into the ventral region of a normal host embryo at the 64-cell stage (Gimlich & Gerhart, 1984).

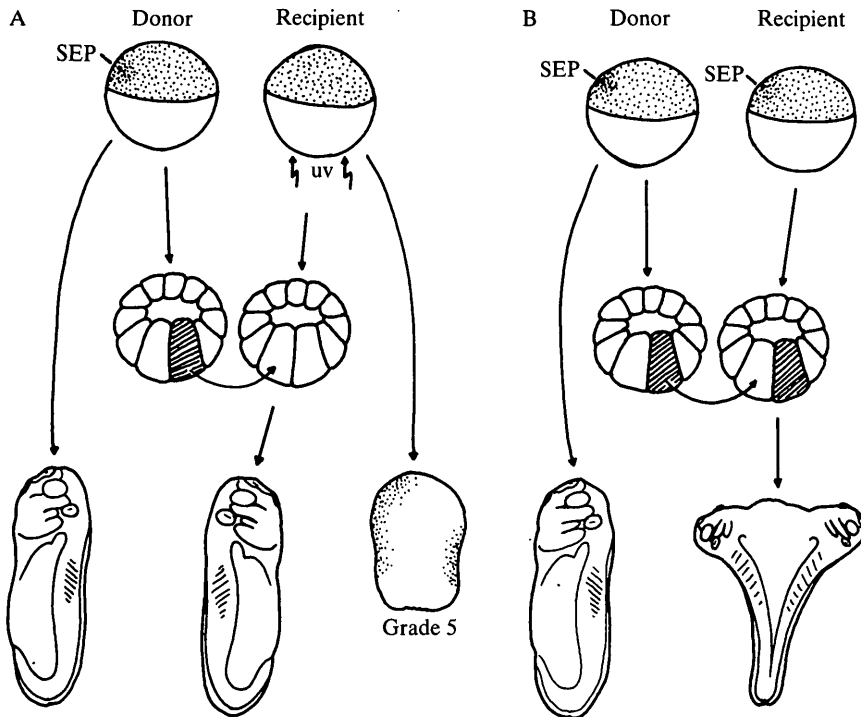A remarkable aspect of these results is that vegetal blastomere recipients show

Fig. 1. Schematic diagrams of vegetal blastomere transplantations. (A) Rescue of axis formation. Top: Donor eggs were marked along the meridian containing the sperm entry point (SEP). Sibling eggs were u.v. irradiated as described. Middle: At the 64-cell stage (shown in mid-sagittal section) the vegetal blastomeres of the dorsal-most quadrant of donor embryos were transplanted into a cavity made in an irradiated recipient by removing two cells at the same vegetal level. Bottom: Recipients of dorsal-most vegetal blastomeres show a rescue of axis development, whereas u.v.-irradiated controls usually develop into radially symmetrical 'grade 5' embryos with no body axis. (B) Second axis formation. Both donor and recipient eggs were marked at the SEP. At the 64-cell stage, dorsal-most vegetal blastomeres were transplanted into the ventral-most vegetal quadrant of a sibling normal embryo, after removing a pair of vegetal cells from the recipient. These recipients often develop partial or complete second body axes in the ventral midline (Gimlich & Gerhart, 1984).

the same series of axis deficiencies as embryos treated in the precleavage period with moderate u.v., cold, or hydrostatic pressure doses (Scharf & Gerhart, 1983). The effects of vegetal cell transplantation can therefore be expressed semiquantitatively according to the 'index of axis deficiency' (IAD) of Scharf & Gerhart (1980). The IAD assigns to each embryo a grade of 0 to 5, based on the completeness of body axis development. A grade of 0 denotes normal development, while grades 1–5 denote axis deficiencies ranging from microcephaly through acephaly to complete absence of dorsal axial structures. This scale can be used to relate monotonically the dose of u.v., cold, or pressure and the extent to which definitive axial structures are missing in the embryo. The IAD scale also describes the dose-dependent 'rescue' of axis formation by experimental gravity-induced cytoplasmic

rearrangements in u.v.-, cold-, or pressure-treated eggs (Scharf & Gerhart, 1980, 1983). Thus the effects of dorsal vegetal cell transplantation can be termed a rescue of axis formation in the same sense.

To understand how this rescue of axis formation occurs, it was necessary to determine the fates of the transplanted vegetal cells in normal development and in the recipient embryos. This was accomplished by labelling the cells with the microinjectable cell-lineage tracer fluorescein-dextran-amine (FDA; Gimlich & Braun, 1985). The progeny of the inductively active vegetal cells contribute ex-clusively to endodermal structures in normal development. At the early gastrula stage they are distributed as a coherent group from the sub-blastoporal surface to the floor of the blastocoel. In the tailbud-stage embryo, they are found in the yolky cell mass of the gut, and in the branchial and pharyngeal endoderm (Fig. 7B). Labelled transplanted cells make an almost identical endodermal contribution in well-rescued recipient embryos. An example of the distribution of transplant cell progeny at the trunk level of one rescued embryo is shown in Fig. 2. Here, all of the labelled cells are located in the gut, and all of the dorsal mesoderm and central nervous system are formed from unlabelled host cells (Gimlich & Gerhart, 1984).

These experiments, which extend those of Nieuwkoop & Ubbels (1971), show that mesodermal structures can form from competent animal or equatorial cells as the result of an inductive influence from the vegetal core of the embryo. In the best cases, the special properties of these few cells from the prospective endoderm can initiate the formation of a full set of body axis structures with an entirely normal organization. Though the result is quite clear, there are several indications that the normal process of axis formation is more complex. In the first place, the degree of rescue of axis formation is quite variable within a batch of vegetal cell recipients, and some embryos are not rescued at all (Gimlich & Gerhart, 1984). This variability cannot be ascribed to misidentification of the future dorsal midline. In some experi-ments donor embryos were tipped 90° off their normal gravitational orientation for a period prior to first cleavage in order to experimentally reposition the future dorsal midline by causing an artificial cytoplasmic rearrangement. In these em-bryos, the meridian which is uppermost during the tipping always becomes the dorsal midline (Gerhart et al. 1981). Nevertheless, the vegetal cell transplants from the true dorsal quadrant of these donors showed a high degree of variability within a batch in their capacity for rescue (Gimlich, 1985).

There is other evidence that the vegetal core of the early embryo is not the only locus of components sufficient for body axis formation. Nakamura & Takasaki (1971) reported that deletion of all of the vegetal-most cells of the 32-cell (stage-6) embryo usually did not prevent the formation of notochord tissue and muscle by the remaining embryonic fragment. I repeated this operation to assess its effect on the organization of the body axis. The eight vegetal-most cells were removed from 32-cell embryos which displayed a regular cleavage pattern, with the vegetal cells separated from the equatorial neighbours by horizontal cleavage furrows. As will be described below, in such a cleavage pattern the progeny of the vegetal cells are
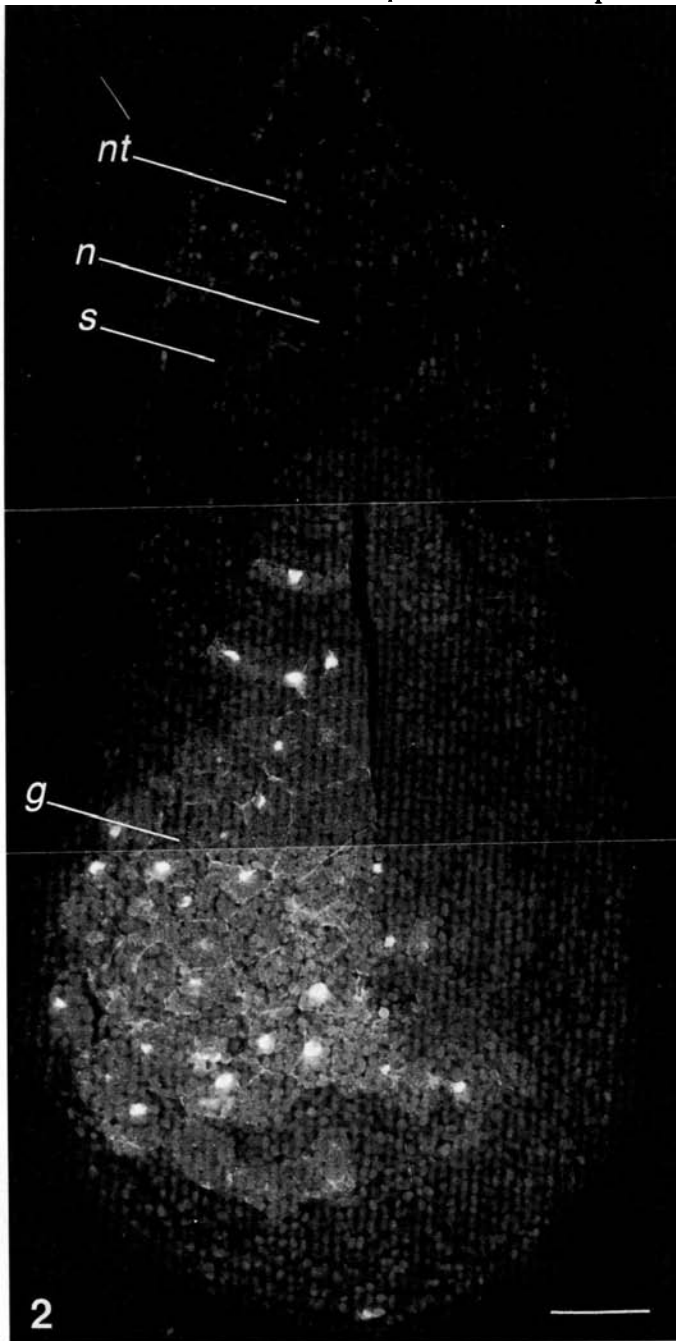
Fig. 2. Positions of labelled dorsal-most vegetal cell progeny in a rescued recipient embryo at stage 25. This epifluorescence image of a 5 μm transverse section at trunk level shows transplant cell progeny in the ventrolateral gut lining and yolky cell mass (*g*). The neural tube (*nt*), somites (*s*), and notochord (*n*) are all composed of unlabelled host cells. The sectional profiles of these structures are visible due to autofluorescence of yolk platelets in the cells. (Bar, 0·1 mm).

sub-blastoporal in position at the early gastrula stage, and contribute primarily to endodermal structures in the tailbud embryo. Fig. 3A shows two of the experimental embryos at the early gastrula stage. In these embryos the bottle cells of the early blastopore lip are all concentrated near the new vegetal pole, and there is little or no superficial sub-blastoporal material. Nevertheless, these embryos proceed to gastrulate, and some of them develop a well-organized and complete set of axial structures (Fig. 3B). In some cases the only externally obvious defect is the small size of the gut compared to that of control embryos. Several of these embryos have been raised to feeding larval stages, at which time they appear entirely normal. In other cases, severe deficiencies in axial structure formation result from vegetal cell deletion. Two such cases are shown in Fig. 3B. Defects include cyclopy, acephaly, and in 3 cases out of a total of 24, complete axis deficiency.
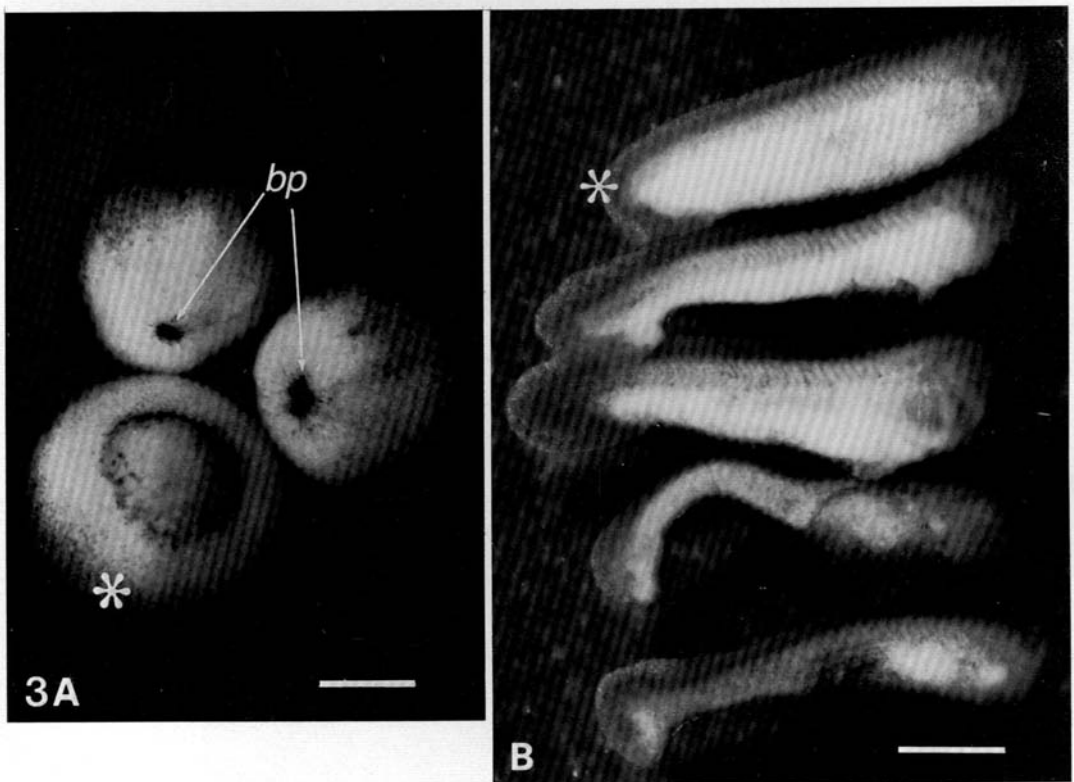

Fig. 3. The results of vegetal tier deletion. The eight cells of the most vegetal tier at the 32-cell stage (tier 4) were removed from normal embryos. The gap in these embryos healed within one hour. (A) Two such embryos are shown at the early gastrula stage, with a synchronous unoperated control embryo (asterisk). The bottle cells of the blastopore lip have all formed at the new vegetal pole. (Bar, 0·5 mm). (B) Four operated embryos at control stage 29, with an unoperated control embryo (asterisk). The bottom two embryos are acephalic, with otocysts, somites, and, as histological examination shows, an anteriorly truncated or completely absent notochord. (Bar, 1 mm).

Rescue of u.v.-irradiated embryos by transplantation of equatorial cells

The results of deleting the vegetal cells of the 32-cell embryo show that their influence is often not essential after this time for at least partial axis formation. It is possible that the inductive cells have already begun to exert their effects at this early stage, or that a part of the inductive activity is contained in the remaining equatorial cells, which then divide to form a deep inducing subpopulation and a more superficial responding population of prospective mesodermal cells. To obtain more precise information about steps in the specification of dorsal equatorial cells to form the chordamesoderm, these cells were transplanted into the equatorial level of synchronous u.v.-irradiated recipient embryos, as depicted in Fig. 4A. Transplantation was begun at the 32-cell stage, since this is the earliest time at which
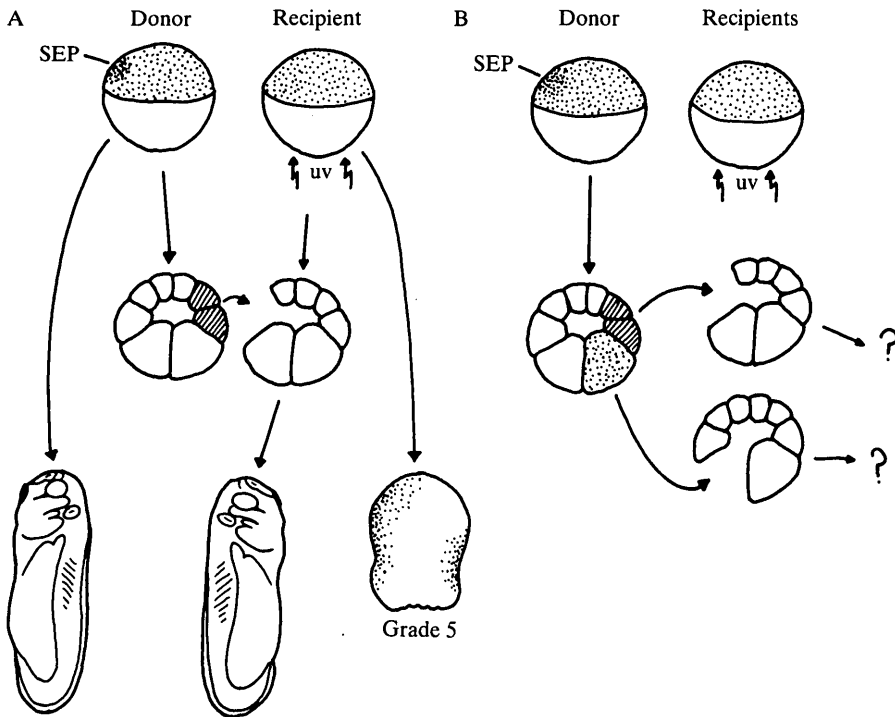

Fig. 4. (A) Rescue of axis formation by equatorial cell transplantation. Donor eggs were marked near the SEP, and sibling eggs were u.v.-irradiated on the vegetal surface. At the 32-cell stage (shown in mid-sagittal section, middle) four cells of tiers 2 and 3 from the dorsal quadrant were transplanted into a cavity made in a synchronous recipient embryo by removing four equatorial cells. Recipient embryos often showed rescue of axis formation, compared to control irradiated siblings (bottom). (B) Equatorial and vegetal cell transplantation from single donors. Donor and irradiated recipient embryos were prepared as usual. At the 32-cell stage, the four cells of tiers 2 and 3 in the dorsal quadrant were transplanted into one recipient, and the vegetal cell pair (tier 4) from the dorsal quadrant was implanted into another. Recipients were cultured as matched pairs, and the completeness of axis formation was scored at stage 39–41 of control embryos.

horizontal cleavage planes have divided an equatorial area which contains much of the prospective mesoderm from a vegetal level which contains mostly prospective endoderm (Nakamura & Kishiyama, 1971; Gimlich, 1985). Transplants consisted of four cells from the two middle tiers of the 32-cell embryo – these are designated tiers 2 and 3 (Fig. 4A).

When these equatorial cell transplants originated in the quadrant centred about the future dorsal midline, the irradiated recipients often developed partial or complete body axes (Fig. 5). Again, the set of morphologies which results from such transplantations closely resembles those described by the IAD scale (Scharf & Gerhart, 1980). Therefore, the result of dorsal equatorial blastomere transplantation will also be termed a rescue of axis formation.

Similar transplants from the two equatorial tiers in the quadrant centred on the future ventral midline (the SEP-containing meridian), or from the adjacent lateral quadrants did not cause a significant degree of rescue in irradiated recipients (Gimlich, 1985). Equatorial cells in both the lateral and ventral quadrants contribute to the somitic mesoderm in normal development (Nakamura & Kishiyama, 1971; A. Smallcombe and J. Cooke, personal communication), and consistent autonomous development of these cells should have been detectable as a high frequency of partial axis formation by irradiated recipients. Thus at the 32-cell stage, only the dorsal-most among the equatorial cells have the capacity to initiate body axis formation in the irradiated recipient embryo.

The locus of the rescue activity in the equatorial region is even further restricted. In 32-cell donor embryos with a strictly horizontal plane of third cleavage, only the
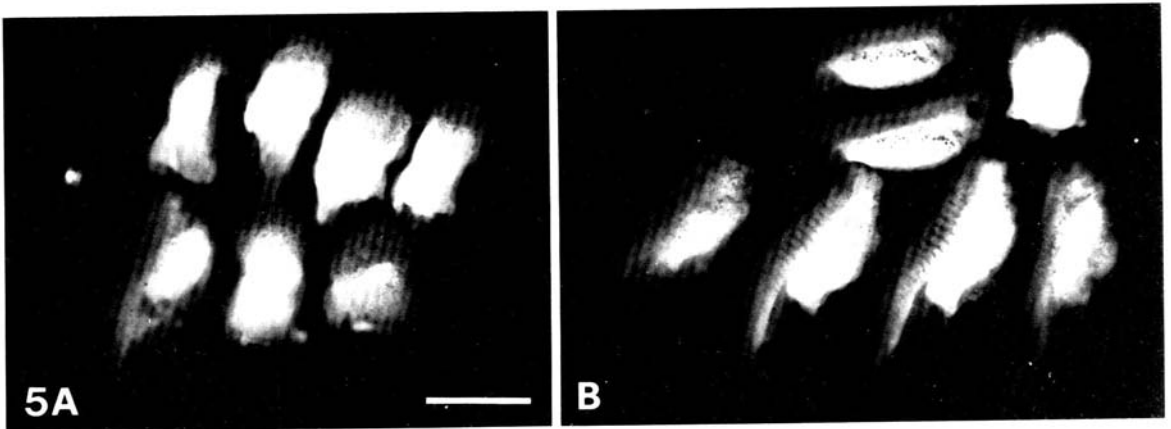

Fig. 5. Rescue of axis formation by dorsal equatorial cell transplants. Dorsal equatorial cells of tiers 2 and 3 were transplanted into recipient irradiated embryos as illustrated in Fig. 4A. Control recipients received similar equatorial cell transplants from irradiated sibling embryos. (A) Control recipients show severe axis deficiency. Five grade-5 and two grade-4 embryos are shown, for an average IAD of 4·7. (B) Dorsal equatorial cell recipients show a variable but striking degree of rescue in axis formation. Two grade-1, three grade-3, one grade-4, and one grade-5 embryo are shown, for an average IAD of 2·9. (Bar, 1 mm).

pair of cells in tier 3 of the dorsal quadrant are able to rescue body axis formation when transplanted into an irradiated recipient. Transplants of tier-2 cells from this quadrant cause partial rescue only when the third cleavage plane is oblique, so that they incorporate some of the unpigmented equatorial egg surface. This observation confirms those of Ruud (1925), Vintemberger (1935), Grunz (1977), Kageura & Yamana (1983, 1984), and Gurdon, Mohun, Fairman & Brennan (1985, and this volume), all of whom assessed the autonomous developmental capacities of isolated animal cell quartets from the 8-cell embryo in terms of dorsal structure formation or tissue-specific gene expression.

Rescue of axis formation by the dorsal equatorial blastomeres results from their autonomous ability to develop as axial mesoderm and to organize surrounding host cells into the axial structures. This was shown by clonal marking in normal embryos and in transplant recipients. The two tier-3 dorsal cells in normal embryos were microinjected with FDA cell lineage tracer. At the early gastrula stage their super-ficial cell progeny are found at and above the level of the dorsal blastoporal lip (Fig. 6A). In their deep extent, such clones reach the blastocoel floor and populate much of the area of the prospective prechordal mesoderm and notochord, according to the fate map of Keller (1975, 1976) for the *Xenopus* early gastrula. In normal tailbud-stage embryos the tier-3 equatorial cells in embryos with a regular cleavage pattern at the 32-cell stage make a very regular contribution to the body axis. Their
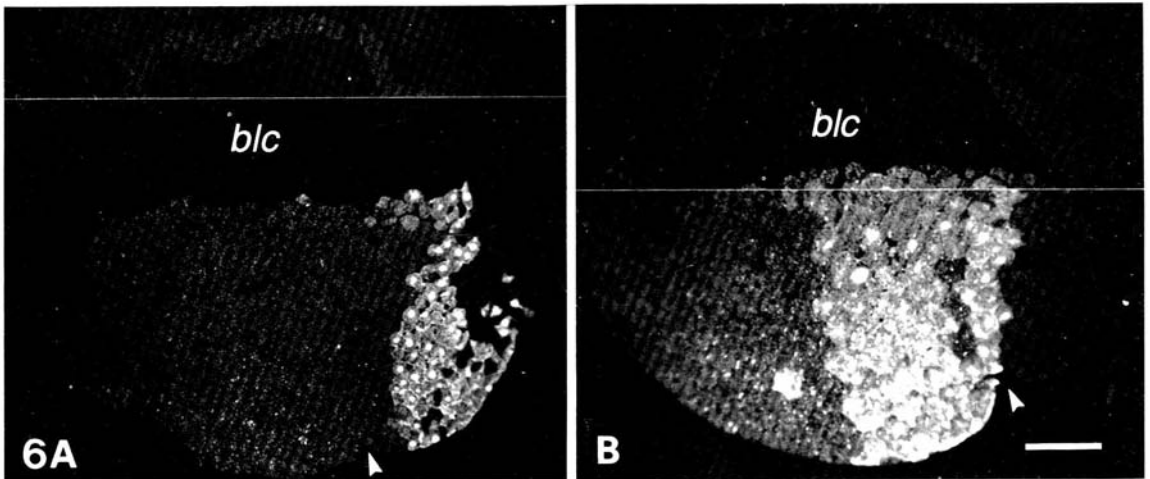

Fig. 6. Clonal contributions at the early gastrula stage of dorsal tier-3 and tier-4 blastomeres of the 32-cell embryo. (A) The two dorsal tier-3 cells were microinjected with FDA, and labelled embryos were fixed at stage 10–10$\frac{1}{2}$. The epifluorescence image of an approximately mid-sagittal section is shown. The dorsal blastopore lip is marked with an arrowhead, and the embryo profile is visible due to yolk platelet autofluorescence. Labelled cells occupy a surface domain which approaches the blastopore, and a deep region ascending to the blastocoel (*blc*) floor. (B) Two dorsal tier-4 cells were FDA labelled. Their progeny at stage 10–10$\frac{1}{2}$ occupy a sub-blastoporal region extending to the vegetal pole, and a coherent deep domain which comprises part of the blastocoel floor. (Bar, 0·2 mm).
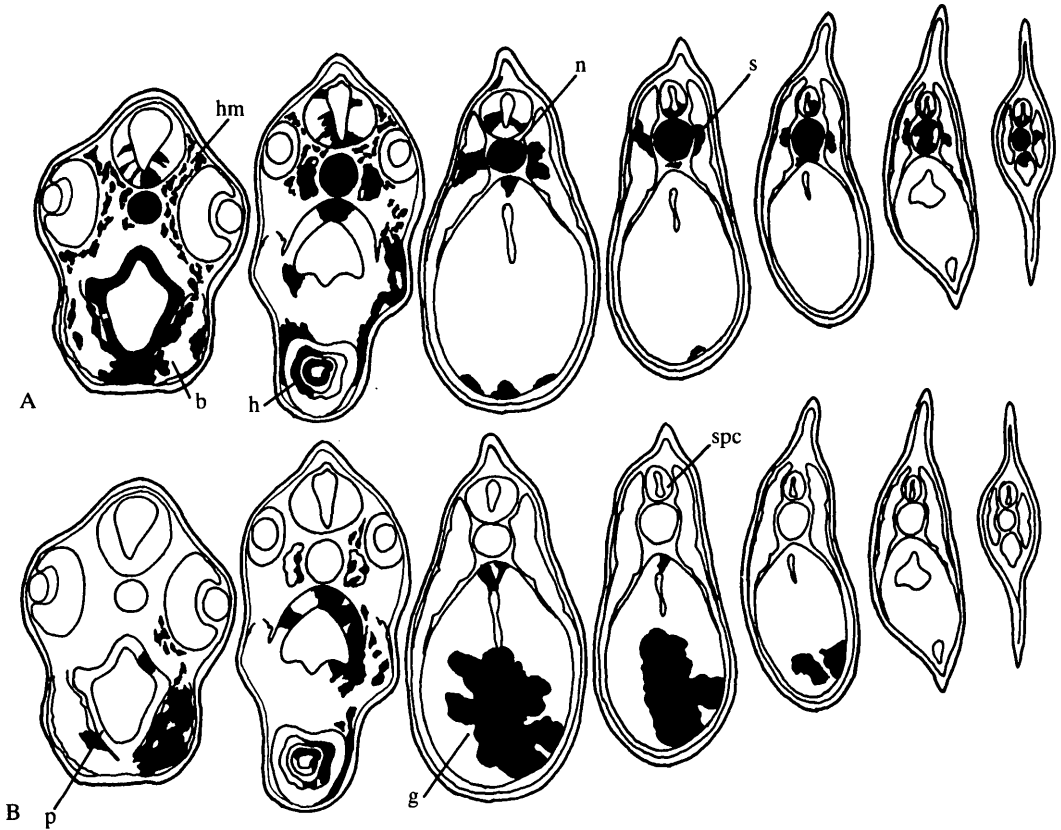
Fig. 7. Clonal contributions of dorsal tier-3 and tier-4 blastomeres in the normal tailbud-stage embryo (stage 30–32). The dorsal tier-3 or tier-4 cell pair was labelled by microinjection of FDA at stage 6, and embryos were fixed at stages 30–32, sectioned at 5 μm from plastic, and viewed in epifluorescence optics. For comparison, the labelling pattern in two individual embryos is represented in solid black on a standard set of sectional profiles. (A) Tier-3 cell progeny in this embryo populate the head mesenchyme (*hm*), pharyngeal (*p*) and branchial (*b*) endoderm, and heart (*h*). They also contribute much of the notochord (*n*) and some parachordal somite mesoderm (*s*), ventral cephalic neurectoderm and spinal cord (*spc*), and archenteron roof endoderm. (B) Tier-4 cell progeny populate the yolky endodermal cell mass (*g*), a patchy strip of the archenteron roof endoderm, the branchial endoderm, and the heart. They also make a minor contribution to the head mesenchyme (*hm*).

progeny form much of the notochord and also contribute to parachordal somitic mesoderm, head mesenchyme, spinal cord floorplate, archenteron roof, and anteroventral endoderm (Fig. 7A; Gimlich, 1985).

When transplants of these cells cause extensive rescue of axis formation, clonal marking shows that they make a contribution to the body axis which is strikingly similar to their contribution in normal development. Fig. 8A shows the positions of the transplanted cells' progeny in a case of rescue to IAD grade of 1. In this
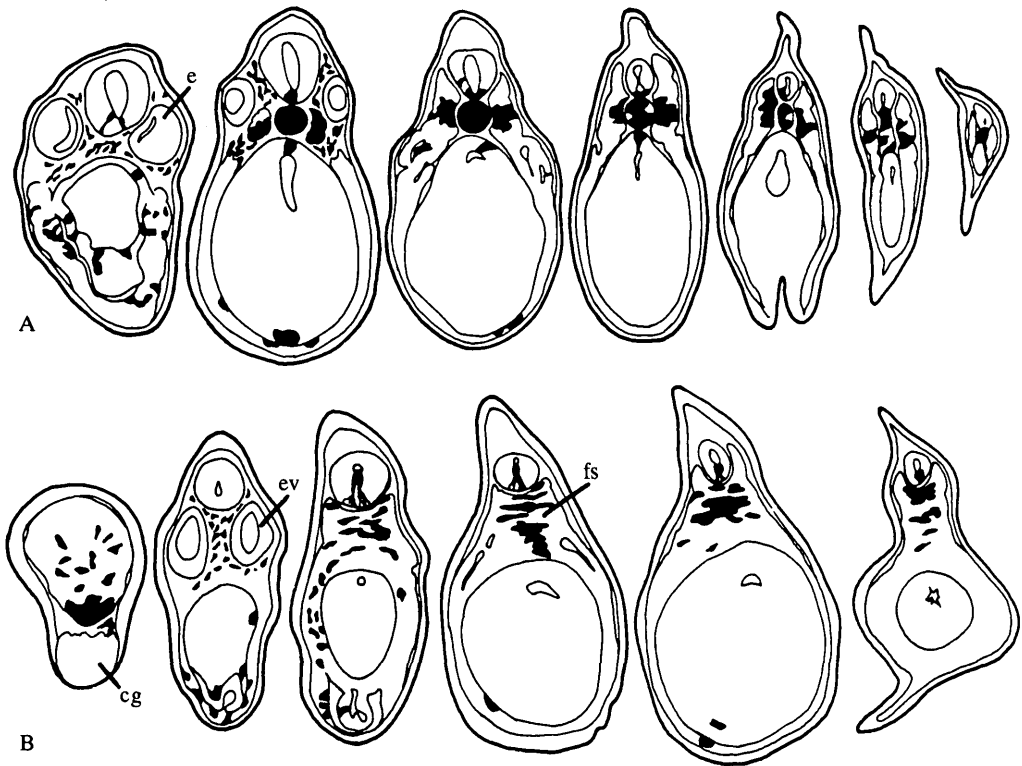
Fig. 8. Clonal contributions of the transplanted dorsal tier-3 blastomere pair in rescued recipients. Donor eggs were fully labelled by microinjection with FDA. Dorsal tier-3 cell pairs were transplanted, as in Fig. 4A, into unlabelled irradiated recipients. Embryos were fixed at a time equivalent to control stage 30, and sectioned transversely at 5 μm. The distribution of labelled cell progeny is represented in solid black on camera-lucida drawings of several sectional profiles. (A) Recipient rescued to IAD grade 1. Transplant contributed much of the chordamesoderm, along with branchial and pharyngeal endoderm, head mesenchyme, parachordal somitic mesoderm, archenteron roof, and ventral neurectoderm. (*e*, optic vesicle). (B) Transplant cell contribution in a recipient which developed a grade-3 level of axial organization. Here the transplanted cells formed anterior mesenchyme, muscle, and contributed to the lateral plate mesoderm, heart, and ventral neurectoderm. The notochord is absent, and somites join across the dorsal midline beneath the neural tube. (*ev*, optic vesicles; *cg*, cement gland; *fs*, fused somites).

embryo the transplant formed the entire chordamesoderm, together with parachordal somite mesoderm, neural tube floorplate, anterior mesenchyme, and branchial and pharyngeal endoderm. Cells of the irradiated host embryo contributed most of the somitic mesoderm, central nervous system, and other structures which would not have developed in the absence of the transplanted normal cells (Gimlich, 1985).

When rescue of axis formation is only partial, the clonal marking shows that the transplanted cells fail to develop chordamesoderm autonomously. Fig. 8B shows

the distribution of transplant cell progeny in a recipient which developed only a grade-3 level of axial organization. In this embryo the transplant cell progeny formed only somitic mesoderm, with the somites fused across the dorsal midline. The failure of most of the 32-cell-stage equatorial transplants to develop autonomously as chordamesoderm is not attributable to mechanical or physiological damage during transplantation. Dorsal equatorial cell transplantation from one normal embryo to another seldom causes axis-deficient development by the recipient.

The frequency of chordamesoderm and muscle development by recipients of dorsal equatorial cells reflects a much greater frequency of axial mesoderm differentiation by these cells than has been observed in simple explantation experiments. When Nakamura and his co-workers (Nakamura, Takasaki & Ishihara, 1970b) explanted dorsal cells of tiers 2 and 3 at the 32-cell stage into culture, only about 5 % of the explants formed notochord or muscle. In the present experiments, 91 % of the recipients formed at least somites. This difference certainly reflects the much more supportive conditions of a recipient embryo for expression of the autonomous developmental capacities of cells of the prospective dorsal mesoderm.

The results of equatorial cell transplantation are compatible with the recent biochemical observations of Gurdon and his colleagues. This group has reported on the accumulation of messenger RNA transcribed from α-actin genes in *Xenopus* embryos. They find that these transcripts accumulate specifically in the somitic mesoderm during neurulation, and so they consider α-actin mRNA accumulation as an early marker of cellular commitment to muscle differentiation (Mohun, *et al.* 1984). In a series of experiments involving egg constrictions, blastomere ablation, and *in vitro* culture of dissociated embryonic cells, this group has obtained evidence that all of the components necessary for muscle commitment are localized from the earliest stages in the equatorial region (Gurdon, Brennan, Fairman & Mohun, 1984, Gurdon *et al.* 1985, and this volume). They suggest that vegetal–equatorial cell interactions during pregastrula stages are not necessary for the proper initiation and accumulation of α-actin gene transcripts. In confirmation of this suggestion, 91 % of the dorsal equatorial cell recipients in the present experiments developed some morphologically detectable muscle. However, lateral equatorial cells of the prospective somitic mesoderm were not autonomous in forming muscle when transplanted at the 32-cell stage. Thus the early autonomy of the equatorial zone in initiating α-actin gene transcription may be due to muscle differentiation by the prospective chordamesodermal region, as seen in embryos rescued to a grade-3 level of axial organization by dorsal equatorial cell transplants (Fig. 8B). Alternatively, muscle differentiation in early-stage complete-equatorial-zone isolates would sometimes result from an interaction of the lateral and ventral somite precursors with an autonomous chordamesoderm rudiment. In either case, muscle differentiation by the normal precursors to the somites would result from inductive interactions during blastula and gastrula stages.

Equatorial cell rescue capacity increases during cleavage

If the rescue of body axis formation in irradiated recipient embryos is an adequate assay of the specification of cells to form the chordamesoderm, then the average degree of rescue should progressively increase as the transplants are made at successively later cleavage stages. Even in explantation experiments the dorsal equatorial region is autonomous in forming the notochord and acting as the organizer by the late blastula stage (Nakamura *et al.* 1970*b*). In fact, this is the case. Whereas recipients of stage-6 dorsal equatorial transplants had an average IAD of 3·0, stage-7 recipients scored an average IAD of 2·5, stage-8 recipients an average of 1·7, and stage-9 recipients scored an average IAD of 1·1. That is, by stage 9, the dorsal equatorial region in over 90 % of the donor embryos is capable of causing development of an entire set of dorsal axial structures in an irradiated recipient (Gimlich, 1985).

Complete distribution of rescue activity in individual donor embryos

The results described so far show that the dorsal equatorial region of the early embryo sometimes contains the components needed to initiate body axis development in the absence of the normally adjacent vegetal cells. The frequency with which equatorial region transplants express developmental autonomy increases gradually throughout the blastula period. At the late blastula stage, most dorsal equatorial transplants can rescue complete axis formation in an irradiated recipient embryo. It is reasonable to suppose that developmental autonomy in the region of prospective chordamesoderm is promoted by the inductive activity of vegetal prospective endodermal cells. However, vegetal cell transplants within each batch of stage-6 donors also show a quite variable capacity for rescue. One way to rationalize these results is to propose that the components needed to rescue axis formation are not predictably partitioned by the early cleavage planes. To test this proposal, I transplanted the vegetal cells and the equatorial cells of the dorsal quadrant into separate irradiated recipient embryos, as diagrammed in Fig. 4B.

These paired transplantations were made from 32-cell-stage donors into synchronous recipients. Dorsal quadrant cells of tiers 2 and 3 replaced four cells from the same tiers of one recipient. The pair of tier-4 blastomeres replaced two vegetal cells in another recipient. Donor embryos were selected on the basis of a very regular pattern of cleavage, with the four cell tiers separated by horizontal planes of division. Vegetal or equatorial cells of matched unoperated embryos in some experiments were injected with FDA to study their normal fates. The recipients were cultured as matched pairs and scored for the completeness of the body axis at a time equivalent to stage 40–41 of controls.

The degree of rescue achieved in these pairs of recipients is illustrated in Fig. 9. In general, when the stage-6 equatorial cell transplant showed extensive developmental autonomy by causing rescue to IAD grade 1, the associated vegetal inductive activity was weak. Conversely, when the vegetal transplant could cause nearly
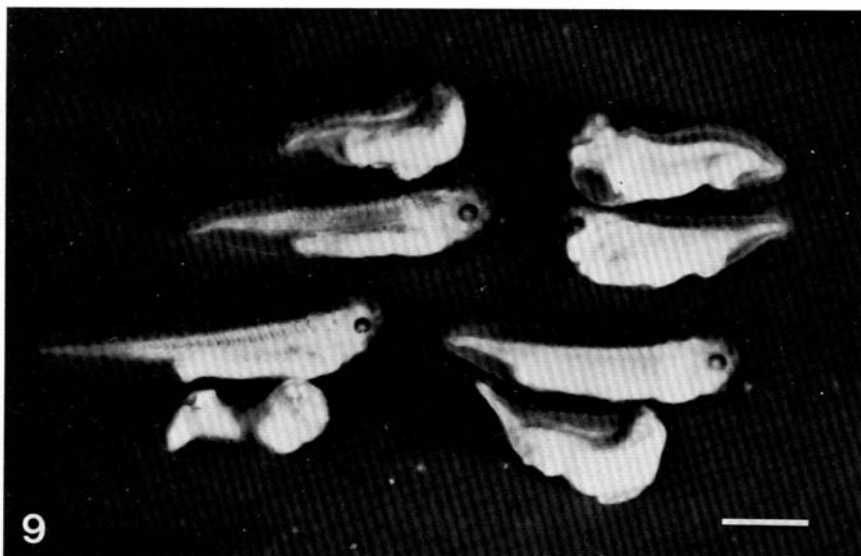
Fig. 9. Paired recipients of vegetal and equatorial cell transplants from the dorsal quadrant of the same donor. Four pairs of recipients are shown. In each pair, the top embryo was the recipient of a dorsal tier-3 blastomere transplant, and the bottom embryo received a transplant of the adjacent dorsal tier-4 cells. Recipients are shown at a time equivalent to stage 39 of controls. (Bar, 1 mm).

complete rescue, the dorsal equatorial cells showed limited autonomy, supporting development of only a grade-3 to-5 level of axial organization. In roughly half of the 32-cell-stage donors, both transplants caused an intermediate grade of rescue (grades 2–3). There were no donors in which the equatorial and vegetal cells could both cause a grade-1 or-2 level of axis development (Gimlich, 1985).

This experiment shows very clearly that the positioning of 'rescue activity' in normal embryos varies without exact regard to the positions of early embryonic cleavage planes. This is true even within a set of embryos selected on the basis of a particular regular cleavage pattern. Correlated clonal marking in such selected embryos shows that the progeny of dorsal equatorial and vegetal blastomeres make a predictable contribution to the various structures of the tailbud-stage embryo. The region of overlap between dorsal tier-3 and tier-4 clonal contributions is mostly in the area of the head mesenchyme, the heart, and the pharyngeal and branchial endoderm (Fig. 7A,B).

Transplants made at stage 9 gave a different result. At this stage rescue induced by grafts of the dorsal vegetal region was infrequent and not extensive. Dorsal equatorial transplants, however, uniformly gave extensive rescue, as mentioned previously. Because stage-9 vegetal transplants take more time to heal into place than transplants made at earlier stages, they may fail to induce dorsal structure development because they do not have time to influence the equatorial region before the onset of gastrulation. To test this possibility, dorsal vegetal transplants

from stage-9 donors were made into stage-7 irradiated recipients. These transplants also failed to cause extensive rescue of axis development, even though they had several hours to heal into place before the beginning of gastrulation (Gimlich, 1985). Thus, in agreement with Boterenbrood & Nieuwkoop (1973), I find that the axial mesoderm-inducing activity of the vegetal core has declined by the late blastula stage.

On the basis of the present results and those of others, a tentative sequence of events in embryonic axis formation can be proposed. A precleavage rearrangement involving a shift of endoplasm relative to the egg surface (Elison, 1980; Vincent & Gerhart, 1985) initially specializes a particular egg region. This specialization becomes incorporated into blastomeres of a unique subequatorial quadrant of the embryo during the early cleavage period. These blastomeres thus inherit the capacity to initiate axis formation, as shown in the transplantation experiments. Purely for convenience, the components which confer on cells the ability to initiate axis formation will be spoken of as 'axial determinants'. Cells which inherit axial determinants include endoderm precursors in the future dorsal quadrant of the stage-6 embryo, and also equatorial cells which will contribute both endoderm and dorsal axial mesoderm. The latter cells extend deeply within the blastula, and later divide paratangentially to produce a deep cell population and a more superficial layer which, as preliminary experiments show, consists almost entirely of prospective chordamesoderm and pharyngeal endoderm. Yet, as early as these cell layers can be separated for transplantation, the prospective chordamesoderm component shows some frequency of developmental autonomy (Gimlich, unpublished observations). Thus it may be difficult to decide whether axial determinants function strictly by establishing inductive activity in a particular set of cell lineages separate from those which produce axial mesoderm. It is clear that, at least in some embryos, the developmental autonomy of the chordamesoderm rudiment is acquired gradually through the blastula period, probably as a result of the inductive influence of nearby vegetal cells. At gastrulation, the prospective chordamesoderm undertakes a vigorous and autonomous repacking of its constituent cells, and this behaviour is thought to be crucial for involution of the mesodermal layer, blastopore closure, and anteroposterior elongation of the embryo (Keller, 1984; Keller, *et al.* this volume). As gastrulation proceeds, a dorsal strip of the mesodermal layer acts to induce neural plate formation by the overlying ectodermal sheet (Spemann, 1938).

An early specialization of one dorsal-most sector of the embryo is sufficient to account for the generation of mediolateral and anteroposterior pattern in the mesodermal layer. In the present experiments, rescue of axis formation by a dorsal equatorial blastomere transplant involved induction of host cells to form axial mesodermal structures, sometimes with entirely normal organization (Gimlich, 1985). The time course of this induction is not known. Other workers (Spemann & Mangold, 1924; Cooke, 1982; Smith & Slack, 1983) have shown clearly that patterning of the mesodermal mantle can be accomplished by inductions from the chordamesoderm rudiment which occur even after the onset of gastrulation. Since

transplants of equatorial or vegetal cells from outside the dorsal-most quadrant are ineffective in causing a rescue of axis formation (Gimlich & Gerhart, 1984; Gimlich, 1985), it seems likely that similar interactions are involved in normal embryogenesis. In contrast, Cooke (1985; and this volume) has recently suggested that components needed for specifying posterolateral mesoderm development are in place around the circumference of the embryo as early as the 4-cell stage. His suggestion was stimulated by a re-evaluation of classical experiments in which the early embryo was split along cleavage planes into 'dorsal' and 'ventral' halves (Ruud, 1925; Spemann, 1928). Ventral fragments of *Xenopus* eggs formed anaxial 'belly piece' embryos (Fankhauser, 1930) much less frequently than previously reported for other species. Instead, they often formed half-sized acephalic embryos with axial organization similar to that of the grade-3 and-4 embryos which develop after precleavage u.v. irradiation, cold shock, or pressure shock (Scharf & Gerhart, 1983). Egg constriction and blastomere separation experiments are subject to the complication that gravity-induced cytoplasmic shifts can 'dorsalize' the isolated fragments (reviewed in Gerhart, 1980). Resolution of the problem of mediolateral mesoderm specification in normal development awaits further experiments.

What have these experiments revealed about components which are involved in initiating axis formation? First, since their positions may vary without precise regard to the fate map boundaries, it is not likely that they act directly to specify the differentiation of any particular cell type. They can function by conferring mesoderm-inducing ability on cells which contain them. They might also function from within prospective mesodermal cell lineages, conferring not only the autonomous capacity to form axial mesoderm, but also the ability to 'dorsalize' (Slack & Forman, 1980) nearby parts of the mesodermal cell layer. Further clues as to the nature of the regional specialization which the egg makes and its effects on cellular activities may come from a study of the mechanism by which equatorial cell progeny can be induced to form the chordamesoderm. Analysis of the timing of this inductive interaction in experimental situations will be useful (Gurdon, *et al.* this volume). It should also be possible to investigate the mode of intercellular communication which is necessary for chordamesoderm induction. For instance, using probes which interrupt intercellular communication via the common low resistance electrical pathway, the gap junction (Warner, Guthrie & Gilula, 1984; A. Warner, this volume), the involvement of ion or small molecule exchange in the induction can be determined.

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