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Commitment of cell fate in embryo of the zebrafish, *Danio rerio*

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Introduction

During embryonic development of vertebrates, pluripotent stem cells are allocated to separate lineages and each cell lineage pursues a limited and stereotyped cell fate. Eventually, dividing cells can autonomously maintain commitments* (for definition, see footnote) to their lineages. The questions as to when and how embryonic cells receive positional cues and become committed to the region-specific fate are still one of the major concerns of developmental biologists. To address these questions, it would be ideal to have a single species with which cellular, molecular and genetic analyses could be carried out on individually identified cells. The zebrafish *Danio rerio* (Fig. 1) has the potential to be such a species. Recently, a great advance has been made in the zebrafish mutant search by the groups of C. Nüsslein-Volhard (Tübingen, Germany) and W. Driever (Boston, USA). They succeeded in a large-scale saturation mutagenesis which had produced about two thousand developmental mutants by April, 1994 (Mullins and Nüsslein-Volhard, 1993; Driever *et al.*, 1994). Together with the genetic linkage map being constructed by several groups (Postlethwait *et al.*, 1994), those isolated mutants will make a great contribution to the field of life science.

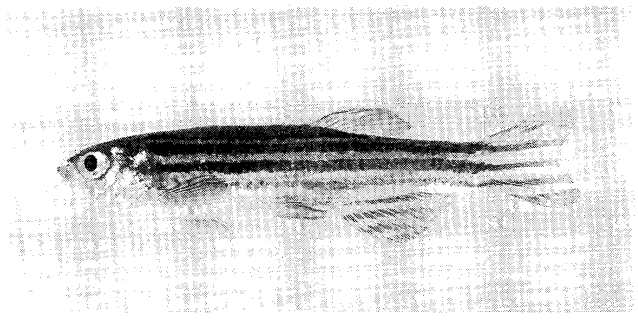

Fig. 1. Adult male zebrafish (*Danio rerio*). Zebrafish and small (3–4 cm long) tropical freshwater fish that originate from India. They reach sexual maturity at about three months. One female lays about two hundred eggs weekly, a feature that greatly facilitates genetic analysis.

In addition to the mutational analysis, the zebrafish embryo has long been regarded as an excellent material for cellular studies of early embryogenesis because it is easy to manipulate and optically clear (reviewed by Kimmel, 1989). These favorable properties are also shared with the embryos of related teleost species, such as the medaka, *Oryzias latipes*. Because of these properties, a number of experiments have been carried out so far to analyze the cell movement, cell lineage, fate map and cell commitment. In this article, we will survey normal development and the fate map of the zebrafish, and describe cellular commitment events and underlying molecular mechanisms examined in the zebrafish embryo with more recently published data and our unpublished results.

Normal development

The development of the zebrafish during the first embryonic day (at 28.5°C) is shown in Fig. 2. Embryonic development is very rapid; about 12 hours from the time of fertilization one can visualize the establishment of a body plan that is typically vertebrate. In zebrafish, a structure called a micropyle restricts sperm entry to the animal pole of the egg (Wolenski and Hart, 1987). After fertilization, yolk-free cytoplasm segregates towards the animal pole. The embryo undergoes the first cleavage about 40 min after fertilization. The first five cleavages are partial or 'meroblastic' and are vertical with respect to the animal-vegetal axis and alternate 90° from one another. The sixth cleavage is horizontal and yields an upper set of blastomeres completely separated from neighboring blastomeres while the lower set of blastomeres retains a connection with the yolk. Cleavages continue synchronously every 15 min, resulting in a densely packed cap of cells, blastoderm, at the animal pole of the uncleaved yolk.

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*Here, 'commitment' is defined as the irreversible, autonomous and heritable restriction of the potential of a cell such that it expresses only one fate (Stent, 1985).

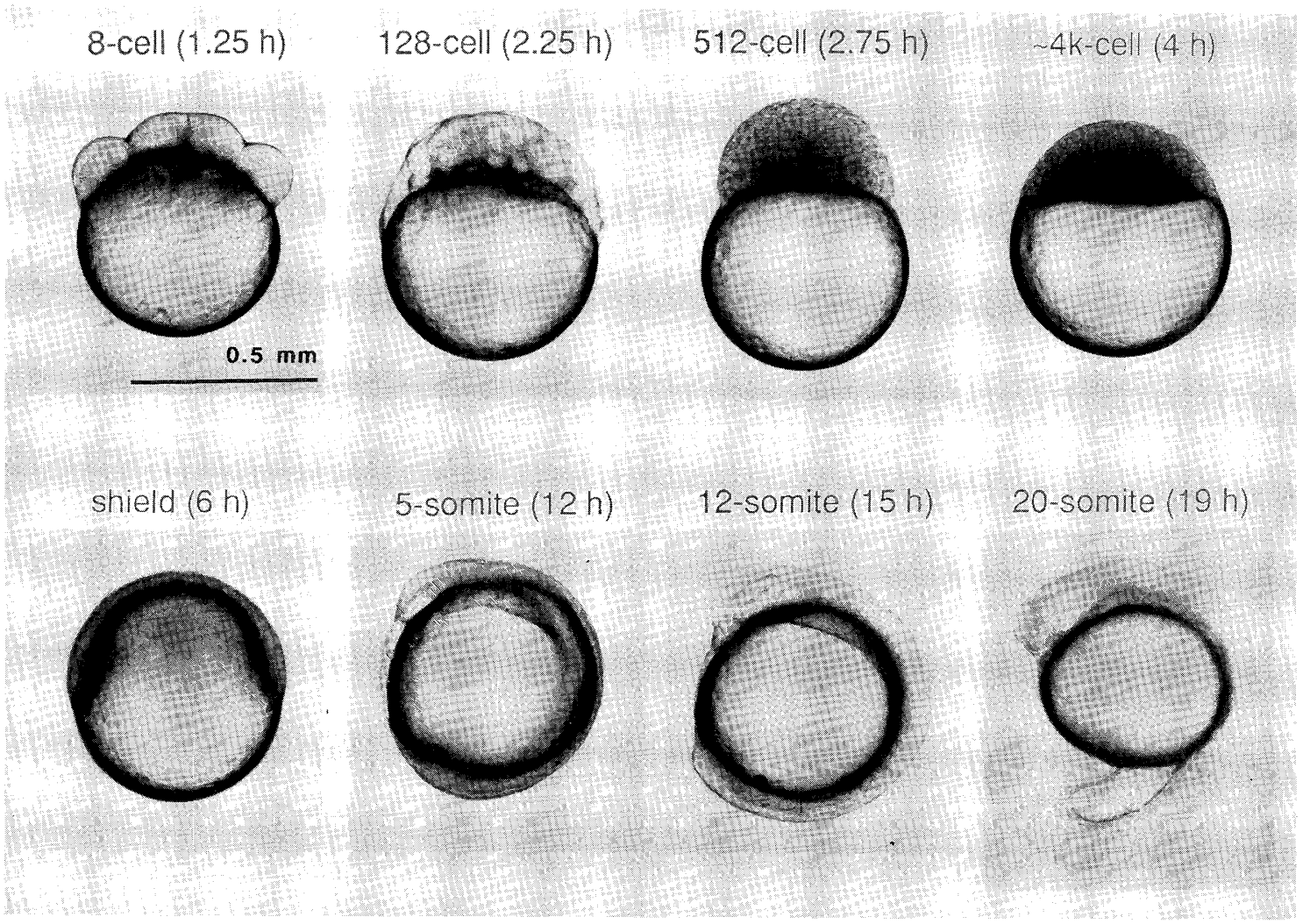

Fig. 2. Development of the zebrafish during the first day of embryogenesis (h = hours of development at 28.5°C). Lateral views of dechorionated embryos are shown and the animal pole is at the top in every embryo. Eggs are fertilized externally and develop rather synchronously. As the bulk of the oocyte is taken over by the yolk, cell division is limited to a small disk of yolk-free cytoplasm atop the yolk. At the shield stage (50%-epiboly), cells around the margin have first involuted around the margin to form the germ ring. In this and following figures, the dorsal side of the embryo is to the right. The body plan of the fish is almost complete and the major organs are clearly visible by 20–24 h. Hatching usually occurs at 36–48 h.

Within three hours, the early blastula stage is reached. The zebrafish has a frog-like midblastula transition at the 10th cleavage, during which cleavage cell cycles become longer and asynchronous, with associated increases in RNA synthesis and cell motility (Kane and Kimmel, 1993). By this time, three types of blastoderm cells are present: a monolayer of the enveloping layer (EVL), a yolk syncytial layer (YSL), and a large mass of deep layer cells (DEL) which lie in between EVL and DEL (Fig. 3). The blastoderm starts to spread evenly over the yolk in the process of epiboly at the end of the blastula stage. As epiboly movement bring the margin of the blastoderm to the equator of the yolk (i.e., 50%-epiboly), the morphogenetic movements of gastrulation begin. Gastrulation occurs as DEL cells around the blastoderm margin involute and migrate towards the animal pole giving rise to the hypoblast. At the same time both involuted and non-involuted DEL cells converge to the dorsal side of the embryo to form the embryonic shield

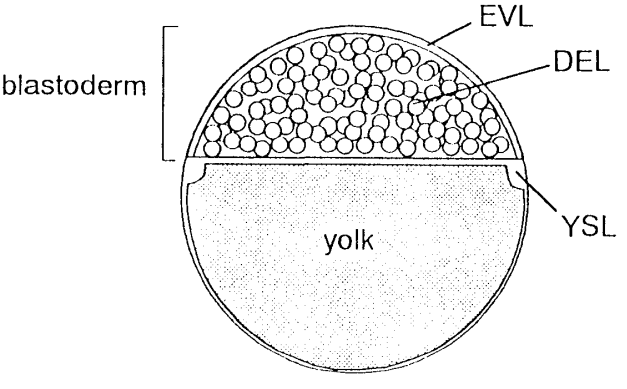

Fig. 3. Schematic cross section through a zebrafish at late-blastula stage, showing the various cell types. For abbreviations, see text.

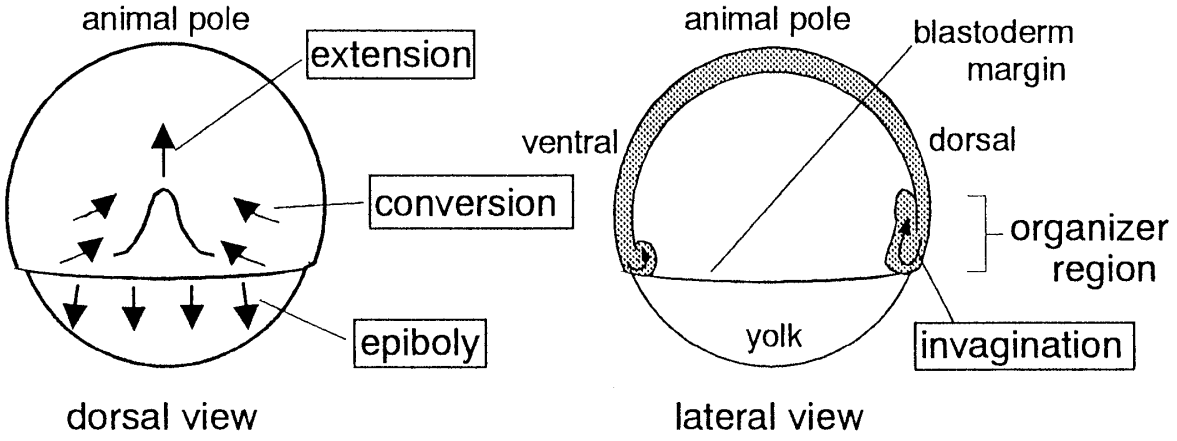

Fig. 4. Early morphogenesis of the zebrafish embryo. During gastrulation, the processes of epiboly, involution, convergence and extension occur simultaneously, producing the extended body axis visible at the dorsal side of the embryo.

(fish organizer). As cells enter the shield, they also intercalate between other cells leading to the lengthening or 'extension' of the embryonic axis in the anterior-posterior (A-P) direction. Ultimately, these movements result in a yolk completely covered by the blastoderm with the future body axis visible as distinct thickening on the dorsal side of the embryo (Fig. 4). After the completion of epiboly, somitogenesis and neurulation proceed (2–3 somites formed per hour). By 24 hpf (hours postfertilization), most of the organs are visible and early organogenesis is nearly complete.

The zebrafish fate map

Before the onset of gastrulation, the movements of individual cells are somewhat random and unpredictable. Kimmel and Warga (1986) have demonstrated that an early zebrafish blastomere contributes progeny to many diverse tissues of the embryo. Therefore, the early lineages of the zebrafish are indeterminate with respect to the future fates of cells. Because of this, it is not possible to construct an accurate fate map until the onset of gastrulation. However, during gastrulation, the morphogenetic movements of the blastoderm cells become more restricted and predictable, although the processes of epiboly, involution, convergence

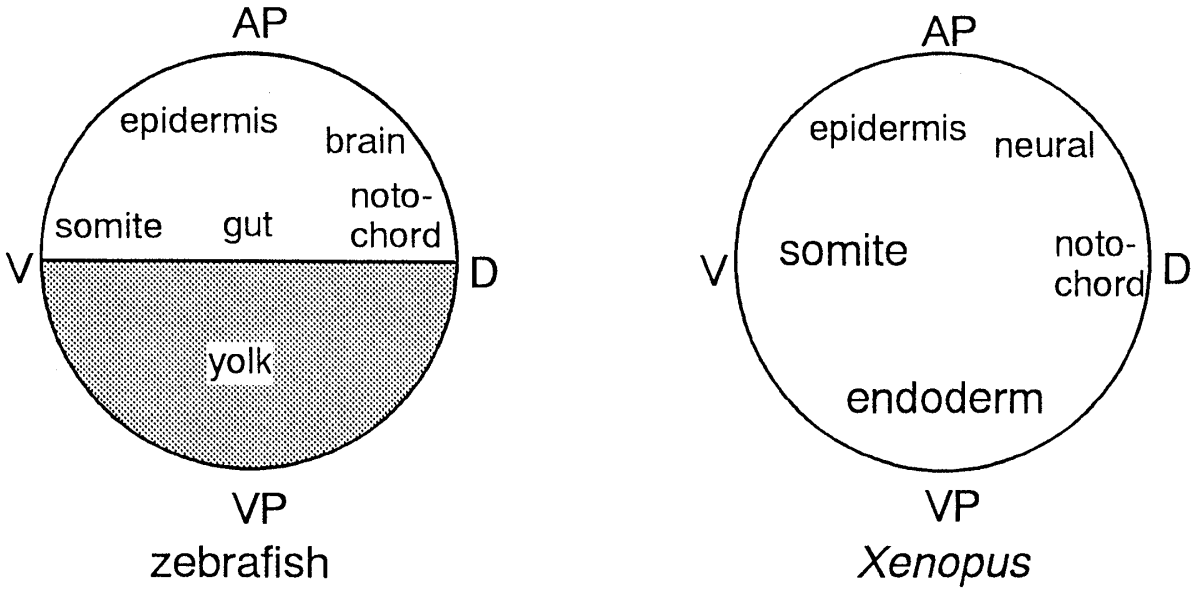

Fig. 5. Fate maps for the zebrafish at the beginning of gastrulation (Kimmel *et al.*, 1990) and *Xenopus* at the 32-cell stage (Dale and Slack, 1987). The zebrafish map resembles that of *Xenopus*. The zebrafish embryo is organized similarly to the frog, but with its vegetal pole opened up and the yolk cell inserted at this location. Abbreviations: AP; animal pole, VP; vegetal pole, V; ventral, D; dorsal.

and extension are occurring simultaneously (Fig. 4). By cataloguing the positions of fluorescently labeled cells at the gastrula stage and identifying the types of progeny cells that they eventually produced, Kimmel *et al.* (1990) were able to construct the fate map for the zebrafish gastrula (approximately 8000 cells) (Fig. 5). This is the first stage in zebrafish development in which the fates of individual cells become tissue-restricted. As compared with the *Xenopus* embryo in which lineage restrictions can be described as early as the 32-cell stage (Dale and Slack, 1987), lineage restrictions in the zebrafish embryo occur later, as in mammals. More importantly form a comparative point of view, the overall organization of the zebrafish fate map very closely resembles similar maps devised for other chordate embryos such as ascidians (Nishida, 1987) and amphibians (Fig. 5).

Commitment of cell fate

In many organisms, the earliest precursor cells are thought to be pluripotent, that is, developmentally undefined and capable of expressing a large number of possible phenotypes. Eventually, individual cells become committed to expressing a particular fate. The characterization of the cellular changes that accompany and produce a developmentally committed state has been attracting many developmental biologists. However, there is still much that remains to be studied or reexamined using modern techniques.

As already described above, lineage tracing experiments have revealed that the future, tissue-specific identities of embryonic cells can be predicted initially at the onset of gastrulation. This suggests that the embryonic cells in zebrafish possess pluripotency until the blastula stage and that the commitment of cell fate occurs during gastrulation. However, the cellular commitment to a particular fate can not be inferred through observations of normal development or from the fate map; it can only be determined through experimental manipulation of embryos. The transplantation of cells from one area of the embryos into another is one of the best methods with which to study commitment events. If the transplanted cell expresses a fate appropriate to its new position, then it was still pluripotent and uncommitted; if it retains the fate of its old position, it was committed to expressing its original fate at the time of transplantation.

To analyze commitment events in early zebrafish embryos, we performed cell-transplantation experiments at various stages of development between various regions. In these experiments, a mixture of rhodamine- and/or biotin-dextran was injected into the yolk of donor embryos at the 2–8-cell stage. The injected dye spread through intercellular cytoplasmic connections to all cells of the blastoderm. Donor cells taken from an appropriate region at a certain stage were transplanted to a new position in an unlabeled host embryo, and fates of transplanted donor cells in the host were examined.

We first examined pluripotency of the early embryonic cells. Cells (about 50 to 100 cells) at the blastula stage (1000–4000 cells, 3–4 hpf) were transplanted to various positions in host blastulae, and the host embryos were allowed to develop up to 20–24 hpf (Fig. 6A-C). Since the zebrafish embryo is radially symmetric around the animal-vegetal axis just before gastrulation (Fig. 2), the relationship between their original and new positions could not be determined precisely in the experiments. However, the results obtained did not vary much among the experiments; the donor cells were widely distributed in the host embryo, and they contributed to many organs which were derived from all germ layers (endoderm, mesoderm and ectoderm) (Fig. 6D-F). The donor cells expressed fates appropriate for their new position (Fig. 6G), showing that they were pluripotent at the time of transplantation, the blastula stage. The results are consistent with those obtained by lineage tracing analysis (Kimmel and Warga, 1986) showing that the cells at the blastula stage are undetermined in their cell fate. Those pluripotent cells, when transplanted into host embryos, can contribute to host organs until adulthood. Lin *et al.* (1992) produced germ-line chimeras of zebrafish by introducing a small number of cells from genetically pigmented into albino embryos at midblastula stage. Furthermore, work towards establishing a zebrafish embryonal stem (ES) cell line from pluripotent blastomeres is underway in several laboratories. In fact, murine ES cells, which are now powerful tools for developmental genetics, are derived from mouse pluripotent cell lineage, i.e., the inner cell mass.

When and how uncommitted cells lose their pluripotency is the next question. From the results of fate mapping, the stage of gastrulation was thought to be an important period for cellular

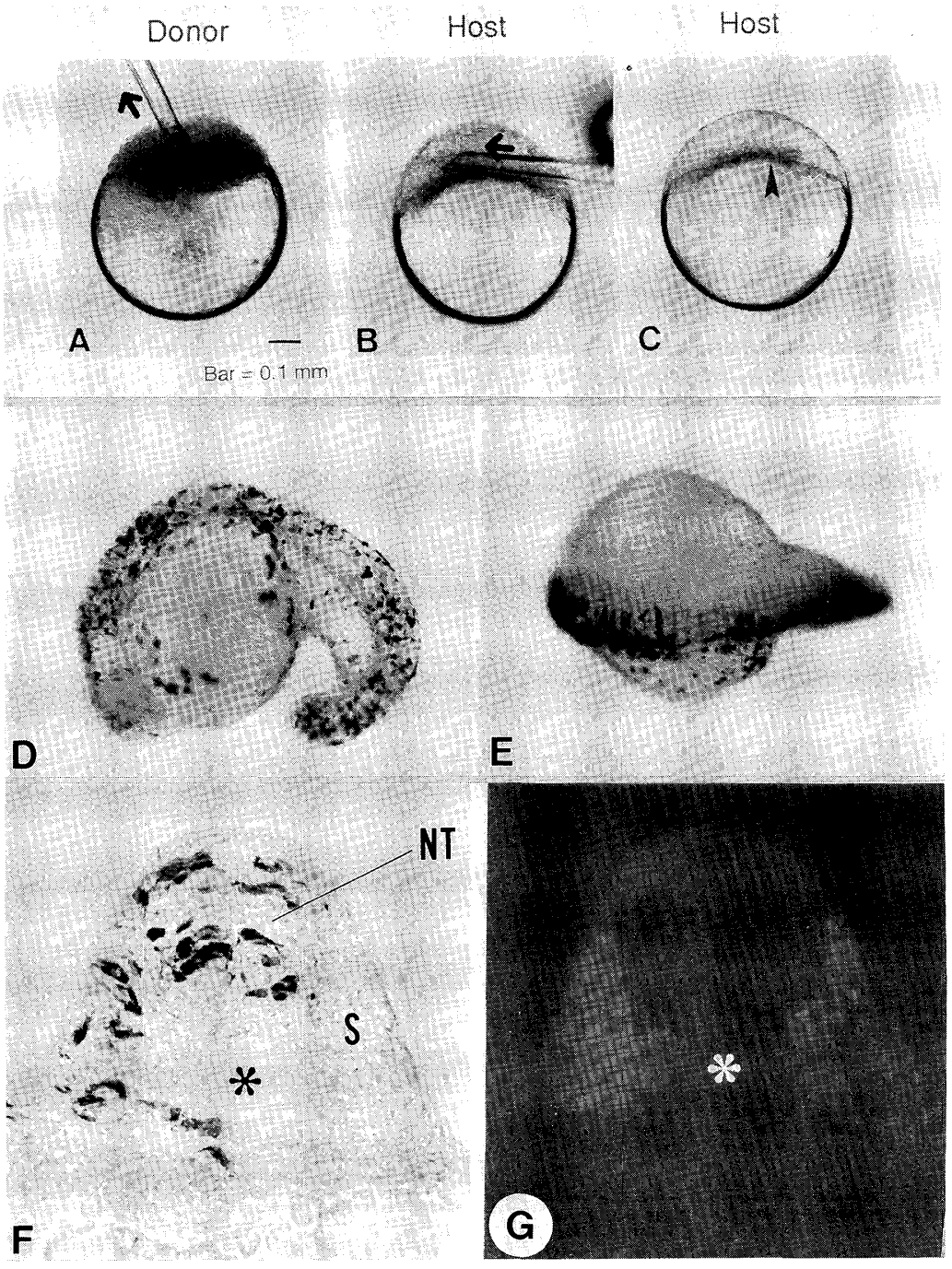

Fig. 6. Cell-transplantation experiments at the blastula stage and the fates of transplanted donor cells. (A)–(C) The process of cell transplantation. All cells of a donor embryo are labeled with rhodamine-dextran and biotin-dextran. Donor cells are drawn directly from labeled donor embryos into the capillary through back pressure. The capillary is then inserted into an unlabeled host embryo, and the labeled donor cells are gently expelled. In this series of figures, cells from the animal pole region are transplanted into the marginal region of the host. The transplanted cells can be detected under a fluorescent microscope (arrowhead in C). (D) and (E) The host embryo was allowed to develop up to 24 hpf, fixed and processed for avidin-peroxidase staining to visualize the biotinylated donor cells in the host (the donor cells are stained brown but appear black in these B/W photographs). The cells in the animal-pole region are normally distributed in the anterior region of a 24-h embryo; however, those cells transplanted to the marginal region are distributed widely along the body axis. A lateral view (D) and a dorsal view (E) of such a host embryo are shown. The embryos are oriented with the anterior end to the left. (F) Frozen section through the trunk region of the stained host embryo in D. The stained (donor) cells contributed to the somite (S) and the neural tube (NT). The asterisk indicates the notochord. (G) The same section of E stained with anti-myosin antibody (immunofluorescent analysis) indicates that the donor cells distributed in the somite do express a molecular marker for muscle differentiation.

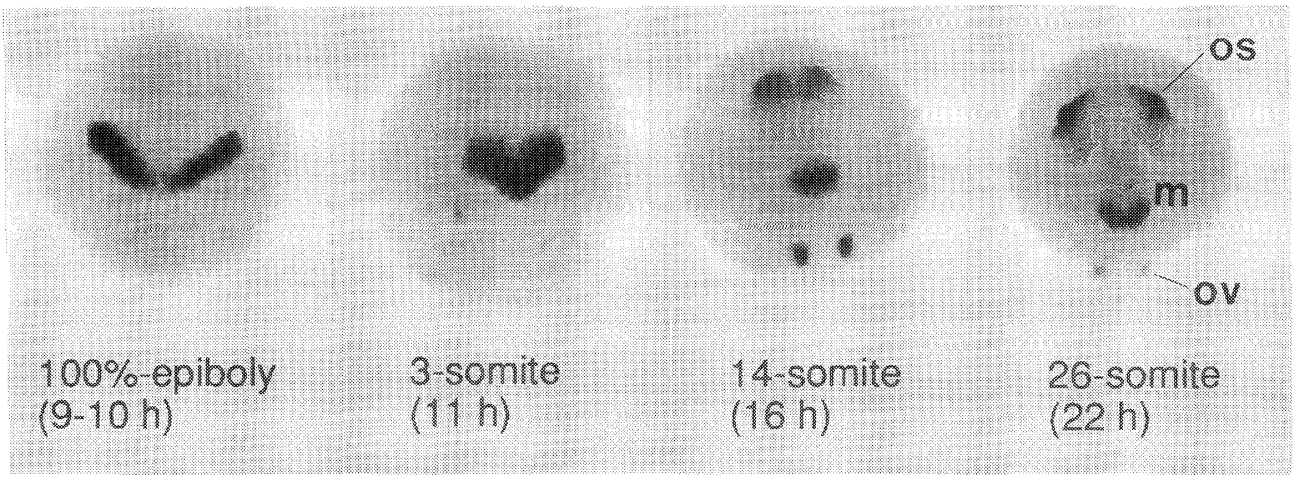

Fig. 7. Localization of *pax[b]* transcripts by in situ hybridization of whole-mount embryos at different developmental stages. Dorsal views are shown. The embryos are oriented with the anterior end to to the top. *Pax[b]* expression is first detected at 9–10 h of development (100%-epiboly) as two transverse bands at the presumptive hindbrain-midbrain boundary of the neuroepithelium. As neural tube formation proceeds, the two transverse bands join at the midline. In later development, the transcripts are also detected in the otic placode and optic stalk. Abbreviations: m, midbrain-hindbrain boundary; os, optic stalk; ov, otic vesicle.

commitment. Cells at or near the margin of the blastoderm involute during gastrulation to become the hypoblast layer, which gives rise to mesoderm and endodermal organs. Conversely, cells in the regions near the animal pole do not involute and always remain superficial to the hypoblast. These cells form the epiblast layer, which gives rise to ectodermal tissues such as the central nervous system (CNS) and epidermis.

Ho and Kimmel (1993) examined a commitment state of the involuted hypoblastic cells by a single-cell transplantation technique. They found that the hypoblastic cells remain pluripotent throughout the late-blastula and shield stages, and that they become committed to a hypoblast -derived fate during gastrulation (6.5–8 hpf). At the onset of gastrulation, single cells from the marginal region of labeled donor embryos were transplanted into the animal-pole region of unlabeled host embryos. Most of the transplanted cells, which normally would have expressed hypoblast-layer fates, gave rise to progeny typical of the epiblast, such as neurons or retina cells in the 24-h to 48-h host embryos. When similar transplantations were performed in early gastrula (6.5 h) or midgastrula (8 h) embryos, about one-third (6.5 h) to 100% (8 h) of the transplanted hypoblast cells retained the hypoblast fate, forming derivatives such as muscle and endothelial cells. Furthermore, it was revealed by time-lapse photographic analysis that committed hypoblast cells, when transplanted to new positions, can migrate along atypical pathway to reposition themselves within a more correct environment; they can migrate across the epiblast-hypoblast boundary and enter their original germ layer. This suggests that, as a part of the transition to the committed state, a cell may acquire new, layer-specific adhesive molecules on its surface.

What about the commitment of the epiblast cells? We have been examining the potency of the epiblast cells in the neuroepithelial region, with a special interest in regionalization of CNS along the A-P axis (Miyagawa *et al.*, 1994). To address this, we use a special regional marker in the neuroectoderm, the *pax[b]* gene, which contains a paired box and is homologous to the murine *pax -2, -5,* and *-8* genes (Noll, 1993). As shown in Fig. 7, it is uniquely expressed at the midbrain-hindbrain boundary in the early neuroectoderm and is one of the earliest (late-gastrula stage) among the genes expressed in CNS (Krauss *et al.*, 1991). To ascertain when posterior midbrain cells become committed, cells from the presumptive midbrain of labeled donor embryos were transplanted anteriorly to a new position (presumptive forebrain region) of unlabeled host embryos at the same developmental stage. Thereafter, the cell-autonomous expression of the *pax[b]* gene in labeled donor cells was examined by in situ hybridization with antisense *pax[b]* probe, followed by avidin-peroxidase staining to visualize the biotinylated donor cells in the host (Fig. 8). This study revealed that the commitment of posterior midbrain cells occurred as early as at 55% -epiboly (6.5 h), at least 3 hours before the onset

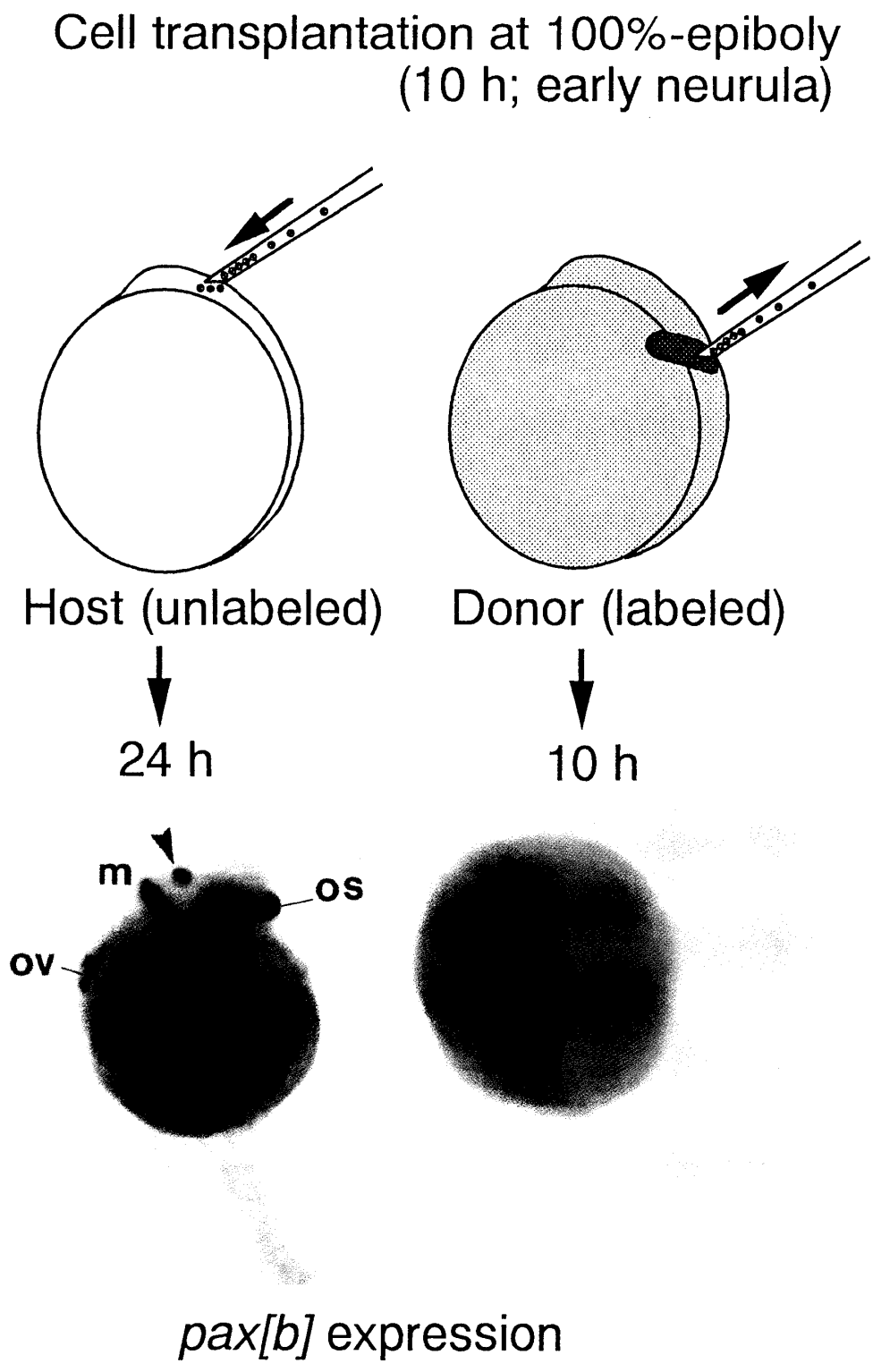

Fig. 8. Results of transplantation experiments of neuroepithelial cells at 100%-epiboly. At this stage, epiboly is just completed and the *pax[b]* gene expression is first detected in the presumptive midbrain-hindbrain region. The labeled (biotinylated) cells from the presumptive posterior midbrain region were transplanted anteriorly to the forebrain region of the unlabeled host embryo at the same developmental stage. After overnight incubation, the host embryo was processed for in situ hybridization with antisense probe for *pax[b]*. As indicated by an arrowhead, ectopic expression of *pax[b]* is observed in the host embryo. Avidin-peroxidase staining of the same embryo confirmed that the ectopic expression was confined to the donor cells. Therefore, the midbrain cells are already committed to *pax[b]* expression when the gene is activated. The donor embryo was also processed for in situ hybridization. The arrowhead in the donor embryo indicates a reduction in size of the *pax[b]* expression domain, which confirms that the donor cells were taken from the *pax[b]*-positive region and were transplanted into the host embryo shown at the left. The dark stippled area in the drawing of the donor embryo represents the *pax[b]*-positive area from which the donor cells were aspirated.

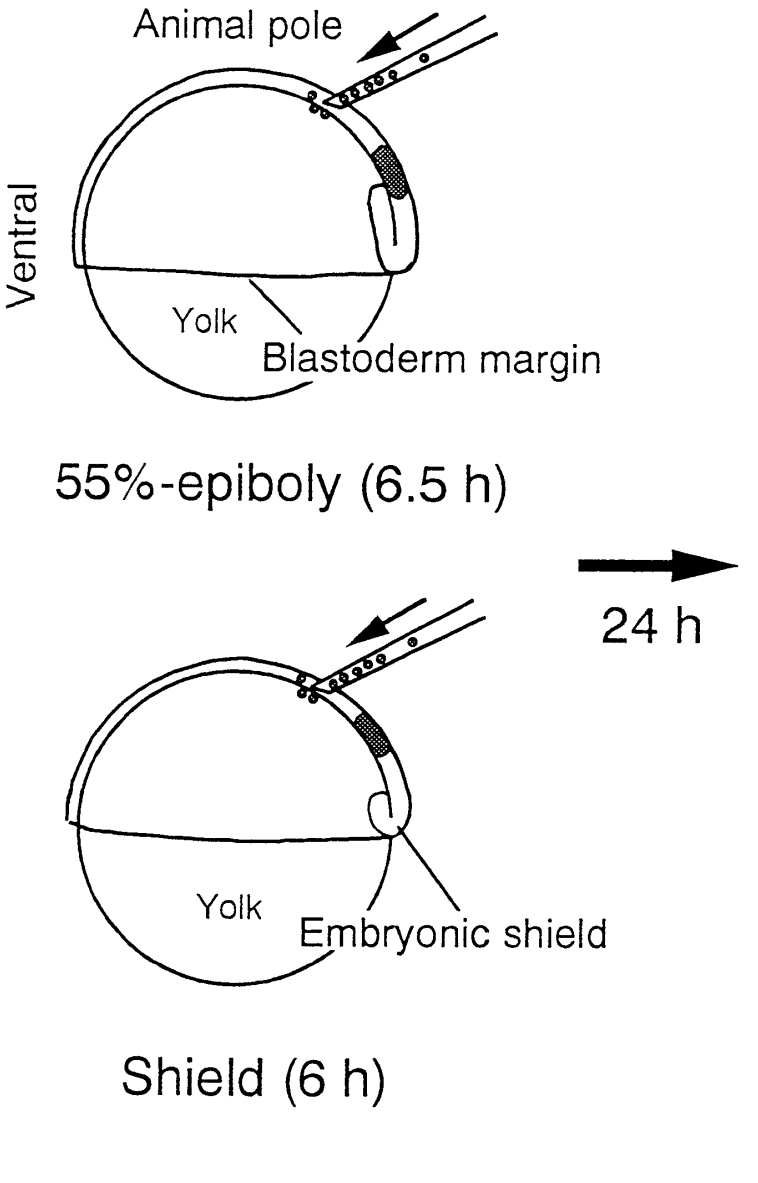

Fig. 9. Results obtained from transplantation experiments done at early stages. (A) When transplantation was performed at 55%-epiboly (6.5 h, early gastrula), the transplanted donor cells (stained brown by peroxidase reaction) were efficiently incorporated into the host neural tube and some were positive for *pax[b]* expression. The arrowhead indicates *pax[b]*-positive donor cells which are stained both brown and blue (by alkaline-phosphates reaction in the process of in situ hybridization). (B) By contrast, *pax[b]*-positive donor cells were undetectable in the host embryos when transplanted at the shield stage (6 hpf; 50%-epiboly), the stage when gastrulation had just started. Instead, the donor cells were widely distributed in the host head region, including the neural tube (NT), epidermis, neural retina (R) and head mesenchyme (asterisk). Endogenous expression of *pax[b]* can be seen in the host optic stalk (OS). The stippled areas in the drawings represent the presumptive *pax[b]*-positive areas from which the donor cells were aspirated.

of *pax[b]* expression (100%-epiboly) in normal development; when the cell transplantation was performed at 55%-epiboly, the transplanted cells were efficiently incorporated into the host neural tube and some of the donor cells were positive for the *pax[b]* transcripts (Fig. 9A). At this stage, morphological specification and specific gene expression in the neuroectoderm are not yet detectable. We also found that the epiblast cells still retain pluripotency at the onset of gastrulation (50%-epiboly or shield stage), 30 min earlier than 55%-epiboly. Those cells, when transplanted to the anterior region of the neuroectoderm, did not show expression of *pax[b]*; instead, they differentiated into many types of organs including neural tube, epidermis, and eye (Fig. 9B). This indicates that the presumptive neuroectodermal cells at the shield stage are not yet committed to a neuronal fate. Thus, the neuroepithelial cells acquire regional specificity alone the A-P axis almost simultaneously with the loss of pluripotency in the epiblast. Probably, neural induction and A-P patterning occur at the same time in a very short period of early gastrulation.

Taken together, it can be said that many early commitment events in both epiblast and hypoblast proceed simultaneously during early gastrulation in the zebrafish embryo. These findings again remind us of the famous words of Lewis Wolpert, "It is not birth, marriage, or death, but gastrulation, which is truly the most important time in your life."

Molecular analysis of early cell commitment in zebrafish

Commitment events could depend on the activity of relatively few specific regulatory genes. However, most of the vertebrate regulatory proteins isolated so far, such as those containing homeobox, paired box and helix-loop-helix structures, are expressed in the embryos after gastrulation, at which stage cell fates are already determined. Thus, the molecular mechanism of the maintenance of pluripotency and early cellular commitment is not fully understood at present.

In our search for zebrafish regulatory genes involved in early cell commitment events, we have focused on the POU-domain gene family. POU -domain proteins are a large family of transcription regulatory proteins, many of which are implicated in the control of gene expression during early development. This family contains a diverged homeodomain as part of a structure referred to as a POU domain (reviewed by Rosenfeld, 1991). The POU domain consists of a POU-specific domain and a POU homeodomain, both of which are required for high-affinity binding to the octamer motif. Several POU-domain genes have been identified as maternal transcripts and their expression is down-regulated during gastrulation. From their expression patterns, it is postulated that one of the possible roles of POU-domain genes may be to maintain cells in an undetermined state in preparation for future developmental signals (reviewed by Verrijzer and Van der Vliet, 1993).

Using the reverse transcriptase-PCR method followed by screening of a cDNA library of zebrafish, we have isolated several POU-domain genes (Matsuzaki *et al.*, 1992). Zebrafish *pou2* identified in this way is a novel POU-domain gene which is related to the murine *Oct-3* (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Schöler *et al.*, 1990). In addition to the structural similarity to *Oct-3*, the expression pattern of the *pou2* gene resembles that of *Oct-3* during mouse development (Fig. 10). Initially, *Oct-3* transcripts are uniformly distributed in mouse oocytes and early blastocysts. *Oct-3* expression subsequently declines in trophectoderm-derived cells and becomes progressively restricted first to the inner cell mass and then to the primitive ectoderm. By day 11, *Oct-3* transcripts are present only in primordial germ cells and the *Oct-3* expression in the adult is restricted to gonadal cells. Similarly, *pou2* is maternally expressed and the transcripts are present from the one-cell stage to the gastrula stage. In situ hybridization analyses revealed that the transcripts are present in all blastomeres until the midblastula stage, and thereafter the expression becomes restricted. Both the YSL and the EVL cells first lose the expression. As described above, early embryonic blastomeres in zebrafish embryos are pluripotent, and the YSL and EVL cells, characterized as 'extraembryonic', first become tissue-restricted lineage (Ho, 1992). Thus, the decrease in *pou2* expression seems to be correlated with the loss of pluripotency.

When gastrulation starts, the expression pattern of *pou2* is dramatically changed. While *pou2* expression becomes high in the epiblast, the hypoblastic cells show a rapid decrease in *pou2* expression soon after their involution (Fig. 11). Probably, the down-regulation of *pou2* expression is prerequisite for differentiation of the hypoblast cells. This notion is supported by results of overexpression experiments described below. The

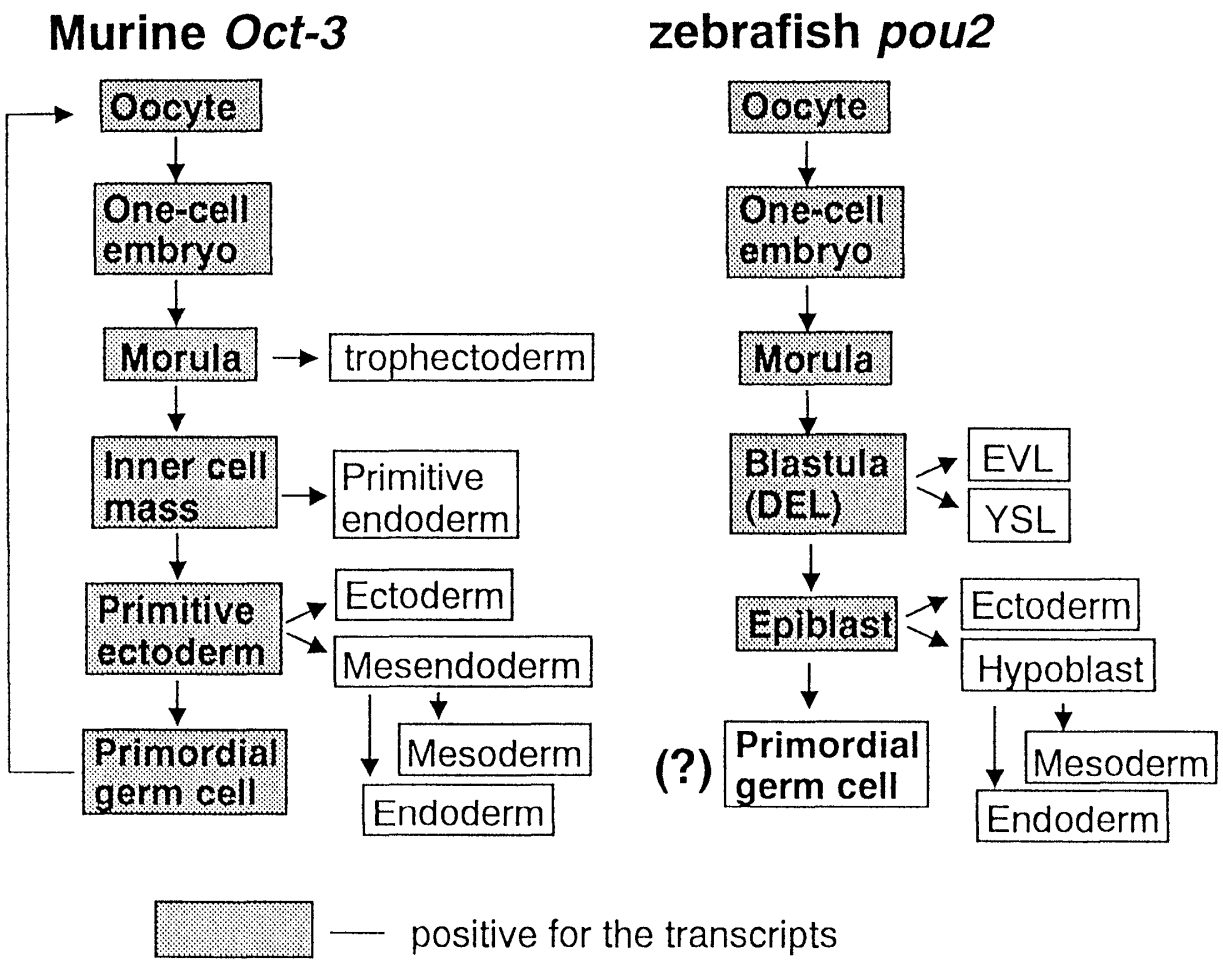

Fig. 10. Comparison of expression pattern of murine *Oct-3* (Schöler, 1991) and zebrafish *pou2* mRNAs during development. The shaded boxes indicate those stages or cell types that show expression of the transcripts. The unshaded boxes indicate those that show little or no expression of the transcripts.

pou2 expression in the epiblast finally disappears and the transcripts are undetectable in any embryonic tissues after 16 h of development. The germ-cell lineage of zebrafish has not yet been described, and the expression of *pou2* in this lineage is unknown at the moment. In the adult, *pou2* is expressed in the ovary but not in the testis.

We found that alternatively spliced transcripts, *t-pou2* RNAs, are also expressed in the embryos (up to 20% of the *pou2* RNAs). In contrast to the Pou2 product (472 amino acids), the t-Pou2 product (399 amino acids) lacks DNA-binding activity because of its incomplete POU-domain structure. To examine the roles of the Pou2 and t-Pou2 products, we increased their expression in the embryo by microinjection of synthetic *pou2* and *t-pou2* RNAs into the fertilized eggs at the one-cells stage. Most embryos that developed from the eggs injected with *pou2* RNA did not show any obvious developmental defects. In contrast, overexpression of the t-Pou2 product greatly affected the embryonic development. The defect was characterized by a disturbance in hypoblast formation which resulted in incompleted gastrulation. In those affected embryos, expression of the zebrafish *T* (*ntl*) gene, which is normally expressed in the hypoblast cells at the blastoderm margin, was greatly reduced (Fig. 12), confirming that the ectopic expression of t-Pou2 caused inhibitory effects on differentiation of the hypoblast cells. Probably, t-Pou2, which is a non-DNA -binding protein, could exert its effect through heteromeric interactions with other transcription factors. The temporal and spatial expression patterns and results of overexpression experiments are consistent with the idea that the *pou2* gene products are involved in early development of zebrafish embryos, such as in the proliferation of blastomeres in the undetermined state at the blastula stage and/or the early cell commitment event at the gastrula stage (Takeda *et al.*, 1994).

Studies of murine *Oct-3* and zebrafish *pou2* suggest the involvement of POU-domain genes in early commitment events of the vertebrate. At

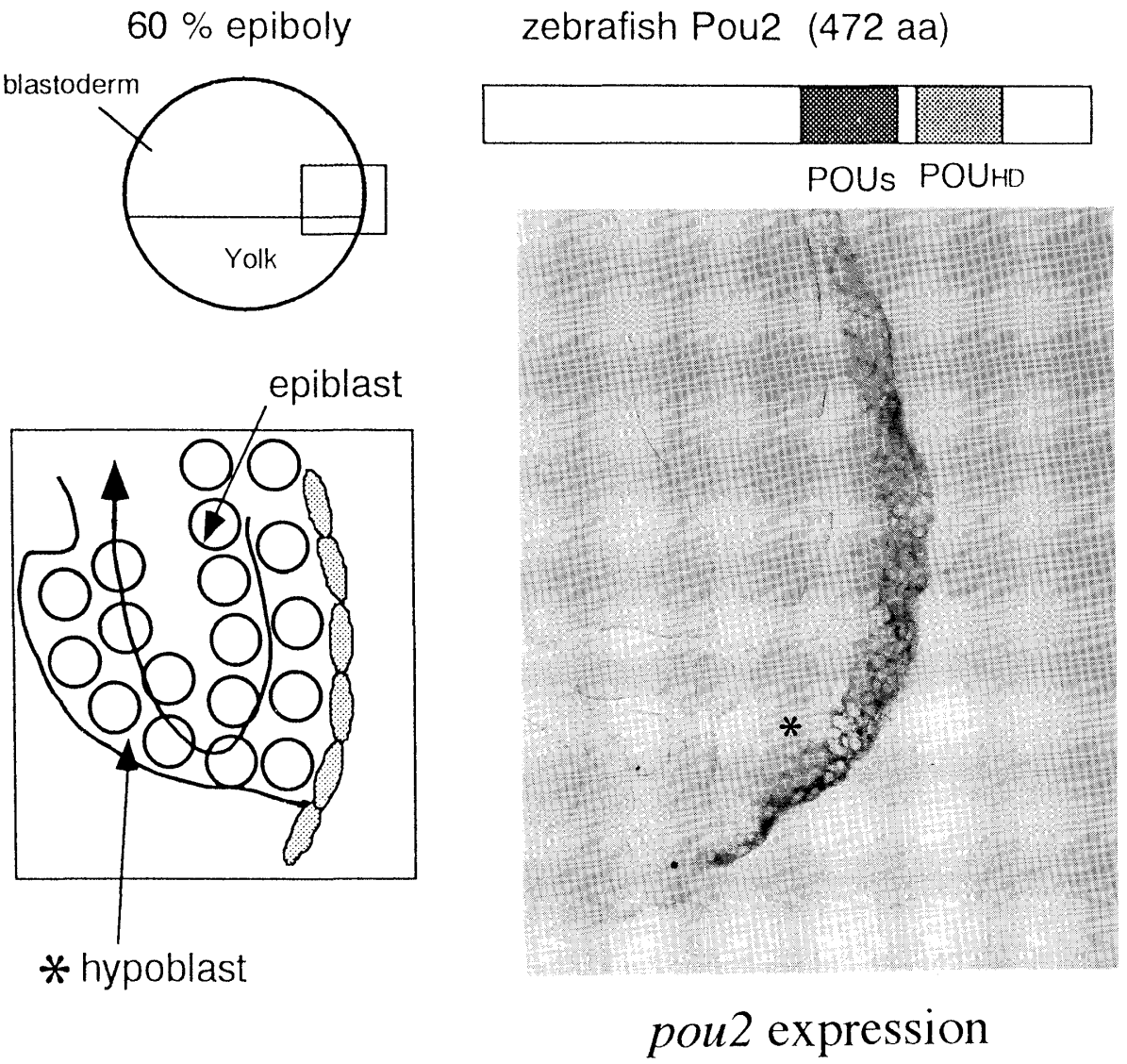

Fig. 11. Down-regulation of the zebrafish *pou2* gene during gastrulation. The photograph on the right shows a frozen section of blastoderm margin from a whole-mount in situ hybridization sample at 100%–600% epiboly. The line drawings on the left show schematic cross section of the blastoderm margin at this stage. As shown in the photograph, *pou2* expression rapidly decreases in the involuted cells (asterisk), while it remains high in the epiblast. The *pou2*-positive cells appear gray to black in this B/W photograph.

present, we have limited knowledge of the genes which are involved in the commitment process. Analysis of the commitment of embryonic blastomeres at a molecular level has just begun.

Future prospects

The fish embryo has long been a favorite system for experimental embryology. As described in this article, detailed knowledge of cell movements and the state of cell commitment during early development has been accumulated. However, the molecular analysis of these early events has just started. A growing number of investigators are beginning to study the zebrafish embryo, and molecular characterization of genes involed in embryogenesis is now underway. Results of these studies seem to indicate that the embryonic organization of the overall body plan of all vertebrates is very similar, as can be seen from the fate maps and the structures and expression patterns of homologous genes in fish, frogs and mice. Furthermore, the recent establishment of genetic methods in zebrafish will permit systematic isolation and analysis of mutants defective in early development. In the near future, many mutants showing defects in early embryogenesis such as a defect in gastrulation will be isolated and characterized. The accessibility of the zebrafish embryo for observations and manipulations, great similarities between its development and that of

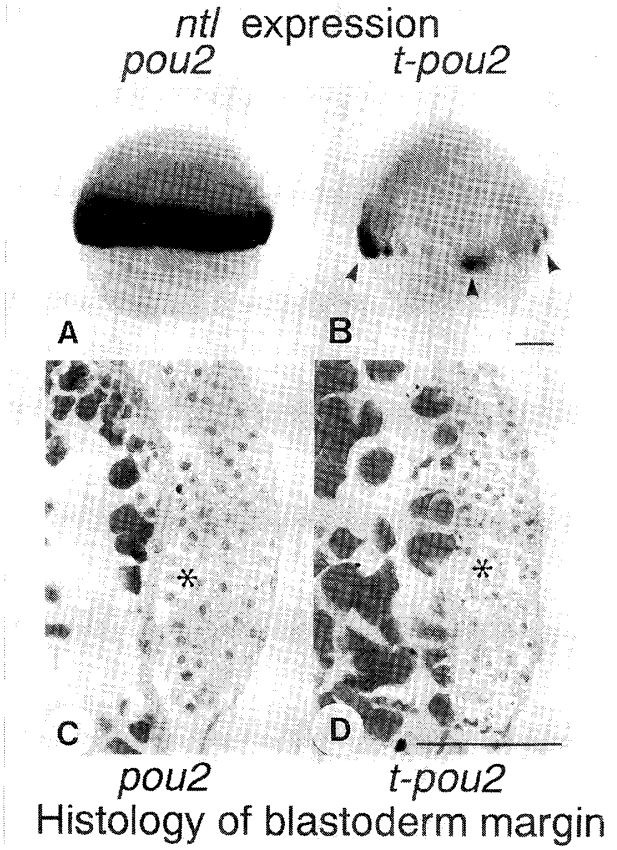

Fig. 12. Phenotype caused by injection of *t-pou2* RNA. The RNAs (0.15 μg/μl, 300 pl/embryo) were injected into embryos at the one-cell stage, and the injected embryos were allowed to develop until the midgastrula stage. (A and B) Whole-mount in situ hybridization of zebrafish *ntl* (*T, Branchyury*) of the embryo which was injected with *pou2* RNA (A) or *t-pou2* RNA (B). Arrowheads indicate the blastoderm margin. The expression of *ntl* in the *t-pou2*-injected embryo was greatly reduced as compared with the *pou2*-injected embryo. In mice and zebrafish, mutations in the *T* gene have phenotypic effects upon the axial mesoderm (Herrmann *et al.*, 1990; Schulte-Merker *et al.*, 1994). (C and D) Histology of the blastoderm margin of the embryo which was injected with *pou2* RNA (C) or *t-pou2* RNA (D). Asterisks indicate the newly formed hypoblastic layer. Note that hypoblast formation is greatly disturbed in the *t-pou2*-injected embryo (D). All scale bars = 100 μm.

other vertebrates, and the availability of mutants will continue to make it an attractive species for studying the mechanisms underlying early vertebrate development.

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