Experimental analysis of the extension of the dorsal marginal zone in *Pleurodeles waltl* gastrulae

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Summary

The capacity for extension of the dorsal marginal zone (DMZ) in *Pleurodeles waltl* gastrulae was studied by scanning electron microscopy and grafting experiments. At the onset of gastrulation, the cells of the animal pole (AP) undergo important changes in shape and form a single layer. As gastrulation proceeds, the arrangement of cells also changes in the noninvoluting DMZ: radial intercalation leads to a single layer of cells. Grafting experiments involving either AP or DMZ explants were performed using a cell lineage tracer. When rotated 90° or 180°, grafted DMZ explants were able to involute normally and there was extension according to the animal–vegetal axis of the host. In contrast, neither single nor bilayered explants from AP involutes completely, and neither extends when grafted in place of the DMZ. Furthermore, when inside of the host, these AP grafts curl up and inhibit the closure of the blastopore. Once transplanted to the AP region, the DMZ showed no obvious autonomous extension. DMZs cultured in vitro showed little extension and this only from the late gastrula stage onward. Removal of blastocoel roof blocked involution to a varied extent, depending on the developmental stage of the embryos. From these results, it is argued that differences could well exist in the mechanism of gastrulation between anuran and urodele embryos. That migrating mesodermal cells play a major role in urodele gastrulation is discussed.

Key words: amphibian, cell lineage, dorsal marginal zone, extension, gastrulation, urodele, *Pleurodeles waltl*.

Introduction

Gastrulation of amphibian embryos involves a complex set of morphogenetic cell movements, such as the formation of bottle cells, convergent extension of the dorsal marginal zone (DMZ), epibolic movements of presumptive ectodermal cells and active migration of mesodermal cells using the animal pole (Holtfreter, 1943, 1944; Baker, 1965; Perry & Waddington, 1966; Nakatsuji, 1974, 1975; Keller, 1978, 1980, 1981, 1984; Keller & Shoenwolf, 1977; Keller, Danilchik, Gimlich & Shih, 1985; Nakatsuji & Johnson, 1984a, b). The underlying mechanisms controlling the gastrulation movements are presumably similar in the different species. However, there is now more evidence that, because of differences in the organization of cell layers at the blastula stage, anurans probably differ from urodeles in their gastrulation movements. For example, it is well established that superficial cells of *Xenopus laevis* blastulae do not contribute to the mesoderm, whereas those of the axolotl *Ambystoma mexicanum* do (Smith & Malačinski, 1983). In addition, recent experiments suggest that mesodermal cell migration may play a larger role in urodele gastrulation than in anuran (Lundmark, 1986).

Gastrulation in urodele amphibians has been studied by Vogt (1929). Using the vital-dye-staining method, he was able to establish the presumptive fate maps of the early gastrula. According to these fate maps, there exists a limit of invagination. Cells below this limit invaginate, whereas cells above it come to envelop the embryo. The formation of the bottle cells, which leads to a depression below the equatorial line, signifies the beginning of gastrulation. The
region between the limit of invagination and the blastopore is the dorsal marginal zone. During gastrulation, this region shows a convergent extension which is characterized by a stream of pigment.

Convergent extension brings the marginal cells vegetally. Next, these cells roll around the blastoporal lip and move inside the embryo. In Xenopus laevis, it is well documented that the convergent extension results from active intercalation of deep cells (Keller et al. 1985). The same author has shown that when DMZ is rotated 90° clockwise it extends and converges in the proper direction with respect to its own axis rather than the host axis and fails to involute (Keller, 1984). Recently, Lundmark (1986) has shown in Ambystoma mexicanum that radial intercalation of cells from the DMZ occurs as gastrulation proceeds.

The characteristics of DMZ have been studied in vitro, where it has been demonstrated that the cultured DMZs appear to show little extension until the late midgastrula stage (Holtfreter, 1939; Schechtman, 1942; Townes & Holtfreter, 1955; Ikushima & Maruyama, 1971; Cooke, 1975). The DMZ will not extend until a developmental age when normally it would have involuted. This suggests that the extension of the dorsal side of the embryo is due to a property of the DMZ after it has involuted to form the roof of the archenteron (Keller, 1984).

In this paper, we analysed the properties of the DMZ in a species of urodele amphibian Pleurodeles waltl using (1) a nondiffusible cell lineage tracer, fluorescein or rhodamine–lysine dextran (FLDx or RLDx), coupled with microsurgery and (2) scanning electron microscopic studies. Our present results support the view that mesodermal cell migration is a prerequisite for the extension of DMZ.

Materials and methods

Embryos

Eggs of Pleurodeles waltl were obtained by natural mating in the laboratory. They were reared at 18°C in tap water and staged according to Gallien & Durocher (1957). Jelly and vitelline membranes were removed manually with forceps. Embryos selected for experiments were washed twice and allowed to develop in 10% Steinberg’s solution (SS).

Cell labelling

Embryos to be used as donors were labelled at the 2-cell stage with either fluorescein–lysine dextran (FLDx) or rhodamine–lysine dextran (RLDx) which were prepared as described by Gimlich & Gerhart (1984). Each embryo was microinjected with 2 nl of the tracer at a concentration of 50 mg ml⁻¹ in distilled water. Injections were carried out with sharp needles shaped with a glass microelectrode puller (Narishige Sc. Inst. Lab.).

Microsurgery, grafting experiments and culture of explants

Microsurgery of embryos was carried out either on agarose-coated culture dishes or on modelling clay base, in sterile 100% SS without any antibiotics. Grafting experiments were performed using a tungsten needle and a platinum loop. After a period of 30 min, host embryos, bisected embryos and cultured explants were transferred in 10% SS and set in place on 2% agarose. The size of the grafted explant (0.6 mm in length, 0.4 mm in width) corresponds to approximately one third of the gastrula diameter. It was measured with a scale placed in the bottom of the agarose-coated culture dishes, and controlled with photographs taken just after healing.

Histological procedure

Grafted embryos were fixed overnight at 4°C in 3.7% formaldehyde in 10% SS. Fixed specimens were rinsed twice in 10% SS, dehydrated in successive baths of ethanol and embedded in PEG-400 distearate (Koch-Light, England). Sections were cut at 10 μm thickness and collected on 0.1% gelatine-coated slides. Slides were examined using a Leitz Dialux 20 microscope fitted with epifluorescence and photographed on Kodak Tri-X film.

Scanning electron microscopy (SEM)

Specimens were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 1% OsO₄ (same buffer). They were dehydrated in ethanol and critical-point dried with liquid CO₂. When desired, fractures were made with a sharp blade after critical-point drying. Dried specimens were coated with gold (20 nm) and examined in a JEOL JSM 35 at 15 kV.

Results

Arrangement of cells in the animal pole region and the dorsal marginal zone

The organization of cells in the animal pole (AP) region and the dorsal marginal zone (DMZ) was studied using scanning electron microscopy, concentrating on the pattern of changes in cell morphology occurring throughout gastrulation in regions involved in grafting experiments.

The arrangement of cells in the AP region changes between the late blastula (stage 7) and the early gastrula (stage 8a), when the blastopore becomes visible. At stage 7, the AP region is about 70 μm thick and consists of two layers of roughly cuboidal cells (Fig. 1A). Only very few cells extend through the entire thickness of the blastocoel wall. Later, just before the formation of the blastopore, cells become elongated, extending terminal lamelliform protrusions inward and outward. As gastrulation occurs,
nearly all cells are elongated and imbricated to form a single layer. Thus, at stage 8a, these AP cells are aligned with their long axis perpendicular to the surface of the blastocoel roof (Fig. 1B). In more peripheral regions the blastocoel roof remains bilayered.

Fig. 1. SEM of *Pleurodeles waltl* embryo fractured midsagittally. (A) Stage 7, late blastula. The AP region consists of two layers of cells with few columnar cells (arrows) extending through the entire thickness of the blastocoel roof. Some cells are undergoing radial intercalation (arrow heads). (B) Stage 8a, early gastrula. The AP region contains one layer of cells with few intercalating cells (arrow heads). (C) Same stage as in B showing that the dorsal sector of the embryo remains bilayered with few columnar cells (arrow). (D) Stage 8b, advanced early gastrula. When gastrulation is occurring, cells of the dorsal sector intercalate to form a single layer. Note the pioneer mesodermal cells extending filopodia toward the inner surface of the blastocoel roof. *bc*, Bottle cells; *bl*, blastocoel; *mes*, mesodermal cells. Bars (A,B), 60 µm; (C,D), 100 µm.
As gastrulation proceeds, cells of the DMZ undergo important changes in shape. At the onset of gastrulation (stage 8a) the DMZ is a bilayered structure approximately 120 μm thick (Fig. 1C). It contains polygonal cells extending terminal filiform protrusions which connect the cells to one another. In rare cases a cell extends through the two layers and binds both inner and upper surfaces of the blastocoel wall (Fig. 1C). When the blastopore becomes crescent-shaped (stage 8b), the DMZ thins by about 60 μm. This decrease in thickness is caused by the radial intercalation of DMZ cells, which form a single layer of closely packed, roughly columnar cells (Fig. 1D). Just at the level of the blastopore, two or three layers of involuting cells are found. In contrast to the DMZ, there is no significant change in the arrangement of cells in the lateral and ventral marginal zones.

During gastrulation, the extension of DMZ toward the blastopore is associated with the formation of bottle cells. Interestingly, fractured sections show clearly that cells between these bottle cells and the noninvoluted DMZ cells undergo important changes in morphology (Fig. 1D). This is particularly the case of mesodermal cells that migrate ahead in the direction of the AP. They adhere to the inner surface of the blastocoel roof, extending numerous lamellipodia or filopodia. As shown in Fig. 1D, these pioneer mesodermal cells are loosely connected to one another by filiform protrusions.

Transplantation of nonrotated DMZ
To determine the behaviour of the DMZ in the normal orientation and position, a DMZ explant was removed from an early gastrula stage (stage 8a) and replaced by a nonrotated RLDx-labelled DMZ extirpated from a donor embryo at the same stage.

Histological examination at midgastrula stage 9, when the grafted DMZ is just turning over the blastoporal lip, showed about three layers of labelled cells above the blastopore (Fig. 2). At the site of involution, labelled DMZ cells elongated and adhered to the inner surface of the graft (Fig. 2). No labelled DMZ cells were found isolated in the nascent archenteric roof, beneath the grafted DMZ. From midgastrula stage onward, involuted labelled cells were loosely packed into the archenteric roof; some of them became scattered ahead.

Transplantation of rotated DMZ
The notion that extension of the DMZ is an autonomous process could be directly tested by grafting DMZ explants with various orientations and by analysing their evolution and changes in shape during the course of gastrulation (Fig. 3A,B).

DMZ explants were removed at early gastrula stages (8a and 8b). As shown above, at stage 8b the DMZ begins to involute. In these experiments, labelled DMZ explants were rotated either of 90° or 180°, with respect to the animal–vegetal axis and grafted into the dorsal midline of unlabelled host embryos at stage 8a.

DMZ 8a
Similar results were obtained whatever the rotation of the graft. In sagittal sections at the midgastrula stage, the labelled explant constitutes in part the nascent archenteric roof (Fig. 4A,B). At late neurula stage, labelled cells were scattered in the head mesoderm and aligned in the pharyngeal endoderm. Labelled DMZ cells were also seen in the anterior part of the notochord (Fig. 4C).
Extension of the dorsal marginal zone

Fig. 3. Schematic drawing of grafting experiments. (A) A DMZ explant is removed at early gastrula stage 8a or 8b in a RLDx-labelled donor, then rotated 90° or 180° with respect to its animal–vegetal axis and transplanted into an unlabelled early gastrula at stage 8a. (B) Combined grafts were made at stage 8a by grafting a RLDx-labelled DMZ explant rotated 90° side by side with another which is FLDx-labelled and rotated 180°.

Transplantation experiments also included 20 combined grafts of FLDx-labelled DMZ rotated 180° and RLDx-labelled DMZ rotated 90°. In these experiments, DMZ explants were removed and grafted side by side at stage 8a (Fig. 3B). Again, in all host embryos, the distribution of labelled cells at late neurula stage was essentially the same as that described for a single graft. As shown in Fig. 4D,E, both the green and red fluorescent cells were seen in the head mesoderm or the pharyngeal endoderm, and at the anterior level of the notochord. Moreover, because of the initial position of grafted DMZ on both sides of the plane of symmetry, numerous labelled cells were also found in somites, at the level where labelled cells were also integrated into notochord.

DMZ 8b

In 30 host embryos bearing DMZ rotated 90°, results were virtually the same as those described above. In hosts, the extension of the labelled DMZ was visualized by direct in vivo observation under epifluorescence. As shown in Fig. 5A, 30 min after transplantation, the rotated DMZ had healed with no significant variation in size. At the midgastrula stage, the grafted DMZ extended toward the blastoporal lip and involution was in progression according to the animal–vegetal axis of the host embryo. There was no elongation of the grafted DMZ in its proper direction (Fig. 5B). In sagittal sections at the early neurula stage, the labelled DMZ formed the archenteric roof (Fig. 5C,D) and part of the somites. These results were also found with DMZ rotated 180°.

In most cases, the replacement of a DMZ by one rotated 90° at stage 8b had no effect on gastrulation, although there was some tendency for incomplete involution. This was particularly obvious in 12 host embryos grafted with DMZ rotated 180°. In such embryos at early neurula stage, labelled cells formed the archenteric roof except in the posterior region where a part of the graft remained uninvoluted.

Transplantation of AP to DMZ

To test whether the ability of grafts to extend is inherent to their position in the embryo, explants were dissected in the AP region and transplanted to the DMZ at the early gastrula stage (8a).

From the beginning of gastrulation, grafted AP explants were clearly apparent, due to the difference in pigmentation between AP and DMZ cells. At midgastrula stage, grafts moved to the blastoporal region but neither involuted nor extended. At the end of gastrulation, AP grafts became folded and persisted on the external surface of the embryo (Fig. 6A).
By the onset of neurulation the grafts moved inside. They stopped just under the blastoporal lip and the closure of the blastopore was blocked (Fig. 6B). After involution, the grafts became further folded with their inner faces pressed against each other. The grafted AP explant did not participate in the formation of the archenteric roof (Fig. 6C). However, if a half-sized AP explant (0.2-0.3 mm) was grafted to the DMZ region, it moved inside. It integrated into the archenteric roof but did not undergo extension.

The above results indicate two things. First, there is an important delay in the involution of the grafts. Second, once partially involuted, AP grafts curl up and do not extend toward the animal pole like grafted DMZ.

Since the grafted AP was single layered when removed from early gastrula, it could be argued that

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**Fig. 4.** Transplantation of rotated DMZ 8a. Distribution of labelled cells in host embryo. (A) Stage 10. At the midgastrula stage the RLDx-labelled explant rotated 90° moved inside and was perfectly integrated into the archenteric roof. (B) Phase contrast of the same section as A. (C) Stage 20. At late neurula stage labelled cells are scattered in the head mesoderm and aligned in the dorsal pharyngeal endoderm. Few cells are incorporated into the notochord (arrows). (D,E) Combined grafts, stage 20. The cells from FLDx-labelled DMZ rotated 180° are shown in D and those from RLDx-labelled DMZ rotated 90° are shown in E. At the late neurula stage they occupy the anterior and dorsal parts of the pharynx respectively. ar, Archenteric cavity; hm, head mesoderm; nt, notochord; ph, pharynx. Bars (A,B), 200 μm; (C), 250 μm; (D,E), 200 μm.
the graft has no opportunity to undergo extension by intercalation. To test this possibility, 30 transplantations were carried out in which bilayered AP explants were removed from late blastulae (stage 7) and grafted to the DMZ in early gastrulae (stage 8a). The morphological aspect of hosts was the same as previously described for grafting experiments of single-layered AP. When hosts were sectioned at the early neurula stage, it was clear that the grafts underwent no extension and that involution was incomplete. Consequently, the formation of the archenteron was affected (Fig. 7A,B). The failure of the AP graft to extend and to involute completely was therefore not linked to the rearrangement of cells in AP.

Transplantation of DMZ to AP

To examine the ability of autonomous extension of the DMZ, labelled DMZs were grafted in the AP region which undergoes little extension in the course of gastrulation. In our experiments, labelled DMZs were removed from early gastrulae at stage 8a or 8b and transplanted to the AP region of host embryos at late blastula or early gastrula stages (7 or 8a respectively). After grafting, embryos were observed in vivo under epifluorescence, until they reached the early neurula stage. Then they were fixed for sectioning. External views of the behaviour of grafts are presented in Figs 8 and 9. They showed no obvious extension of grafted DMZ in their proper direction.
Moreover, by grafting labelled AP to AP as a control, the same results were obtained.

In sections at early neurula stage, labelled cells were detected migrating on the inner surface of the ectodermal layer, just at the periphery of the grafted DMZ (Fig. 10). Such migration of labelled cells was not found in control embryos bearing labelled AP explants (Fig. 11).

Fig. 6. Transplantation of single-layered AP explant to DMZ. (A) Stage 12. At the late gastrula stage the AP explants from stage 8a have reached the blastoporal lip and folded (arrows). (B) Stage 15. The AP explants have moved inside. Note that the host embryo has an exposed yolk plug. (C) Stage 13. Sagittal section made at the end of gastrulation. The folded AP explant does not participate in the formation of the archenteric roof. It is protruding into the archenteric cavity. ar, Archenteric cavity; yp, yolk plug. Bars (A,B), 1.5 mm; (C), 100 μm.

Fig. 7. Transplantation of bilayered AP explant to DMZ. (A) An AP explant from a blastula stage 7 has been grafted to the DMZ region of an early gastrula stage 8a. Involution is blocked. Only a limited archenteric cavity is present. The labelled cells form the posterior part of the neural plate. (B) Phase contrast corresponding to A. Arrow heads point to limits of the grafted AP explant. ar, Archenteric cavity; np, neural plate. Bar, 150 μm.
Culture of DMZ

In another set of experiments, the possibility of autonomous extension was studied by culturing in vitro the entire dorsal sector of early gastrulae (stage 8a) as described by Ikushima & Maruyama (1971), Keller et al. (1985). This experiment was carried out by culturing two explants with their inner surfaces against each other until neurulation occurs in control embryos. With regard to their prospective fate, two regions could be defined in the bisected dorsal sector: (1) noninvoluting dorsal ectoderm; (2) the DMZ which contained pharyngeal endoderm, head mesoderm, truncal and posterior chordomesoderm. An analysis of 60 cases was performed by direct observation. Fig. 12 illustrates the morphological aspect of explants at different times of culture. No obvious change in shape was noticed until the late gastrula stage (Fig. 12A,B). From this stage onward,

Fig. 8. Transplantation of DMZ 8a to AP region. DMZ explants were removed from RLDx-labelled early gastrulae (stage 8a) and transplanted to the AP region of host embryos at late blastula stage 7 (A,B) or early gastrula stage 8a (C,D). Grafted explant were observed under epifluorescence 30 min after healing (A,C) and at early neurula stage (B,D). Note that the morphology of grafted DMZ does not change significantly. Bar, 300 µm.

Fig. 9. Transplantation of DMZ 8b to AP region. RLDx-labelled DMZ excised at advanced early gastrula stage 8b were grafted into the AP region of host embryos at late blastula stage 7 (A,B) and early gastrula stage 8a (C,D). Epifluorescence. 30 min after healing (A,C) and at early neurula stage (B,D). There is no significant extension in grafted DMZ. Bar, 300 µm.

Fig. 10. Transplantation of DMZ 8a to AP region. Section through the grafted DMZ shown in Fig. 8B. (A) Epifluorescence. There are labelled mesodermal cells migrating beneath the ectodermal layer. (B) Phase contrast. Arrow heads point to superficial limits of the grafted DMZ. ec, Ectoderm; mes, mesodermal cells. Bar, 150 µm.
Fig. 11. Transplantation of AP to AP region. In this control experiment a RLDx-labelled AP explant was removed at early gastrula stage 8a and transplanted into the AP region of host embryos at the same stage. Section through the grafted AP explant at early neurula stage. (A) Epifluorescence. There is not any migration of labelled cells. (B) Phase contrast. Arrow heads indicate superficial limits of the grafted AP. ec, Ectoderm; en, endoderm. Bar, 150 μm.

Fig. 12. Culture of DMZ. Two entire dorsal sectors of early gastrulae (stage 8a) were removed and sandwiched with their inner surfaces against each other. After healing (A), they were allowed to develop in culture through the midgastrula (B) and late gastrula (C) stages, to the early neurula stage (D). A slight autonomous extension is observed from the late gastrula stage onward (C, D). Bar, 500 μm.

the DMZ narrowed and there was a slight elongation of the explant. Finally, an ectodermal vesicle attached to a differentiating chordamesodermal structure was observed after 30 h of culture.

Removal of the blastocoel roof
To test the role of the mesodermal cell migration in DMZ extension, the blastocoel roof was removed and the gastrulation pattern observed. Experiments were carried out at three different stages: late blastula (stage 7), early gastrula (stage 8a), and advanced early gastrula (stage 8b). They consisted of removing the ectodermal part of the blastocoel roof by cutting along the pigment line. In such embryos, the marginal zone remained as an annulus whose upper region included part of the blastocoel roof.

Just after healing, the free edge of the marginal zone tended to cover the endodermal mass, especially in the dorsal sector of the embryo. Even after a prolonged culture time there was never a complete gastrulation. Thus, for 30 h of culture, in all 62 dissected embryos, a prominent yolk plug, surrounded by a circular blastopore, was always clearly apparent (Fig. 13).

The removal of the blastocoel roof at the blastula stage was later associated with only a shallow depression in the blastoporal region and involution did not occur (Fig. 13A). The most striking cases were those in which the blastocoel roof was removed at early gastrula stages (8a and 8b); for, as involution was proceeding in the dorsal sector, the free edge of the marginal zone was extending onto the endodermal mass (Fig. 13B, C). To study the behaviour of the DMZ in such cases, DMZ explants were exchanged between labelled and unlabelled early gastrulae at stage 8a before removing the blastocoel roof. As shown in Fig. 13D, it was clearly found that part of the DMZ turned inside at the blastoporal site and then interacted with the inner surface of the non-involuting part of the DMZ. Moreover, labelled cells always migrated ahead as far as the noninvoluting DMZ was available for adhesion. In each of these
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grafted specimens, involution was very limited both in lateral and ventral regions.

Discussion

The present studies confirm and extend the notion that mesodermal cell migration plays an essential role in the process of gastrulation in urodele.

The principal result is that grafted rotated DMZ explants involute and normally extend in accord with the host axis. This is contrary to the results of grafting experiments in *Xenopus* which have shown that the DMZ rotated 90° extends in the proper direction with respect to its own axis and fails to involute (Keller, 1984). The simplest explanation for these contradictory findings is that major differences exist between *Xenopus* and *Pleurodeles* in the cellular organization.

Fig. 13. Removal of blastocoel roof. Experiments were performed at late blastula (A), early gastrula (B), advanced early gastrula (C) stages, and results were analysed when control embryos had reached the early neurula stage. (A) Late blastula embryos developing without the blastocoel roof. Involution does not occur. (B,C) Early gastrula embryos allowed to develop without the animal cap. Involution and extension are proceeding. The blastopore does not close and the yolk plug remains prominent. (D) Same experiment as in (B) but, in addition, the DMZ has been replaced before removal of the blastocoel roof with another which is RLDx-labelled. Sectioned embryo at early neurula stage. Grafted DMZ involutes and labelled cells form the archenteric roof. Note that labelled cells are migrating ahead beneath the noninvoluted DMZ and stop just at the cutting site (arrow head). The reduced archenteric cavity is linked to the incomplete involution and extension of DMZ. ar, Archenteric cavity; yp, yolk plug. Bars (A,B,C), 1.5 mm; (D), 200 μm.
of the gastrula. Such an explanation is in agreement with studies on the initial arrangement of cells and the origin of the mesoderm in amphibian gastrulae. In *Xenopus*, the DMZ consists of a superficial epiblast and a deep layer of four to six cells thick. The mesoderm resides entirely in the deep layer (Keller, 1975, 1976; Smith & Malacinski, 1983). Keller (1984, 1985) recently reported that the DMZ in *Xenopus* undergoes convergent extension largely at the midgastrula stage. This convergent extension depends on cell rearrangement in the deep layer by the mechanism of intercalation (Keller et al. 1985). In urodele gastrula, by using vital dye staining, cell surface labelling procedures or fluorescent lineage tracers, it has been shown that superficial cells of the marginal zone contribute to mesodermal structures (Vogt, 1929; Smith & Malacinski, 1983; Lundmark, 1986).

SEM observations presented here show that in *Pleurodeles*, the DMZ was two-layered in early gastrulae and became rapidly single-layered before the midgastrula stage. These observations would imply that the intercalation process plays a more limited role in the gastrulation of urodeles than in that of anurans. This could explain the absence of autonomous extension in grafted rotated DMZ.

Recently, Lundmark (1986) concluded that as presumptive somitic mesoderm moves over the blastoporal lip in early gastrulating *Ambystoma* embryo an important population of mesodermal cells was removed by ingestion. Once detached from the archenteric surface these cells adhere and migrate along the blastocoel wall; our results accord well with this aspect of her conclusion. In nonrotated DMZ grafting experiments, observations made when grafts begin to involute, always revealed that labelled cells were actively migrating along the inner surface of the blastocoel roof.

It also must be taken into account that the difference in results between *Xenopus* and *Pleurodeles* might be correlated to local difference in the disposition of prospective areas in DMZ. For example, the head mesoderm is found in the deep layers very early in *Xenopus* (Keller, 1976), whereas, as shown above, it migrates there later in *Pleurodeles*. Thus, here, variations in the state of commitment of DMZ cells could be a cause of the difference observed. On this basis, it can be predicted that in *Pleurodeles* a transplanted explant taken from the DMZ at a later time, i.e., when the head mesoderm is involuting, will extend according to its own axis. Indeed, our results with DMZ explants removed from a gastrula at crescent-shaped blastopore stage show that the ability to involute becomes reduced, particularly after a rotation of 180°. Such a difference in terms of involution might be indicative of changes in the state of commitment of DMZ cells. However, the fact that rotated DMZ already exhibits a significant involution according to host axis supports the idea that at this stage these changes have little influence on the mechanism of gastrulation.

Another important observation is that, once grafted into DMZ, AP patches block involution and do not extend. Although they have a tendency to curl up, AP explants move inside but then stop under the blastoporal lip so that involution is never complete. We have also shown that unfolded AP explants of half size remain cohesive and undergo no extension after involution. These facts are in agreement with the observation in *Xenopus* (Keller, 1984). Indeed, involution of grafted AP could be interpreted as a passive process in the sense that it depends on the movements generated in its environment. This interpretation could explain how the ability of AP cells to undergo intercalation, as tested by grafting either bilayered or single-layered explants, has no influence on involution.

Why grafted AP explants do not extend after involution is an open question. Interestingly, based on direct observation under epifluorescence, patches of DMZ show a very weak capacity for autonomous extension when transplanted into the AP region. However, detailed examination of sections in grafted areas reveals that whereas all AP cells remain regularly arranged, some DMZ cells undergo migration on the inner surface of the blastocoel roof, around the graft. These facts indicate that there are important differences in kinetic properties between AP and DMZ cells. They would imply that extension of DMZ may be directed by an active migration of cells.

When in *Pleurodeles* entire dorsal sectors of early gastrulae are cultured *in vitro*, no changes in morphology are noticed until the late gastrula stage. Elongation along the initial animal–vegetal axis and convergence of the dorsal chordamesoderm are observed from late gastrula stage onward. Thus, although extension and convergence occur, they appear delayed as compared to their timing in gastrulation. On the other hand, in *Xenopus* the DMZ begins its convergent extension at the midgastrula stage (Keller et al. 1985). From these observations, it seems likely that the intercalation process which is reported to generate the force underlying convergent extension in *Xenopus* is less important in *Pleurodeles*. In the latter, the extension of DMZ and the closure of the blastopore may be more dependent on mesodermal cell migration.

Further support for this suggestion was provided by removal of the blastocoel roof as in *Ambystoma* (Lewis, 1952) and more recently in *Xenopus* (Keller et al. 1985). If the animal cap was removed at the late blastula stage, i.e., when pioneer mesodermal cells have not yet undergone migration on the blastocoel
roof, involution and extension of DMZ remained extremely limited. The same experiment performed at the onset of gastrulation gave similar results, except that involution and extension of DMZ were more advanced. The observation that there was active migration of mesodermal cells was of particular interest. Moreover, as shown by grafting labelled DMZ explants, the migration of mesodermal cells proceeded to the free edge of the gastrula wall, and was blocked where no substratum was available. These observations agree with the previous data from Lewis (1952) showing that when the animal hemisphere was cut off in Ambystoma, gastrulation continued. However, it should be pointed out that in Pleurodeles the involution and extension of DMZ were never complete. Furthermore, the fact that in Xenopus the removal of the blastocoel roof did not inhibit the closure of the blastopore supports the view that, in relation to the contribution of mesodermal cell migration to gastrulation movements, differences exist between anurans and urodeles.

In order to place the above data in perspective, it is useful to distinguish (Lundmark, 1986) three populations of mesodermal cells in DMZ after involution: the spreading head mesoderm, the somitic mesoderm undergoing ingression and the chordamesoderm, which displays extension and convergence. The evidence presented here that mesodermal cell migration is required for extension and to a certain extent for involution has important implications with respect to the mechanisms underlying gastrulation. In particular, with regard to the chordamesoderm, our evidence implies that, as far as elongation and spatial distribution are concerned, both cell rearrangements and cell interaction with extracellular components should occur. At the onset of gastrulation, it is conceivable that once turned over the blastoporal lip or ingressed, mesodermal cells require interactions with specific extracellular constituents of the matrix to undergo active migration and to direct their movements. Such a possibility is in agreement with several lines of evidence showing that mesodermal cell migration is indeed dependent on the presence of suitable substrates.

First, gastrulation implies the formation of an extracellular fibrillar network in the inner surface of the ectodermal cap. Mesodermal cells use this network as an adequate substratum for adhesion and movement, but also as an important contact guidance system (Nakatsuji, Gould & Johnson, 1982; Nakatsuji & Johnson, 1983a,b, 1984b). Second, immuno-labelling studies demonstrate that the fibrils covering the inner surface of the blastocoel roof contain fibronectin (Boucaut & Darribère, 1983a,b; Lee, Hynes & Kirschner, 1984; Darribère, Boulekbache, Shi & Boucaut, 1985; Nakatsuji, Smolira & Wylie, 1985a) and laminin-related polypeptides (Nakatsuji, Hashimato & Hayashi, 1985b; Darribère, Riou, Shi, Delarue & Boucaut, 1986). When mesodermal cell migration is inhibited by using either monovalent antibodies to fibronectin or synthetic peptides of the cell binding site of fibronectin, gastrulation movements fail to occur (Boucaut, Darribère, Boulekbache & Thiery, 1984a; Boucaut, Darribère, Poole, Aoyama, Yamada & Thiery, 1984b). Third, gastrula mesodermal cells adhere and actively move in vitro on artificial substrata coated with the extracellular matrix or with fibronectin alone. On both substrata, mesodermal cells are migrating preferentially along the aligned fibrils by the mechanism of contact guidance (Nakatsuji & Johnson, 1983a, 1984b; Nakatsuji, 1986). Finally, in arrested interspecific hybrid embryos, as well as in hybrids obtained by nuclear transplantation whose development is arrested at early gastrula stage, the extracellular fibrils are either absent or disorganized and could not promote cell migration (Nakatsuji & Johnson, 1984a; Delarue, Darribère, Aimar & Boucaut, 1985; Johnson, 1986).

It is clear that further investigations such as local modifications of extracellular constituents coupled with studies of cell lineage are required to increase our understanding of the involution and extension of DMZ in urodeles. In addition, antibodies specific to extracellular components and membrane proteins might be useful in clarifying the cellular mechanisms underlying the migratory processes during amphibian gastrulation.

The authors are most grateful to Dr G. Ville for preparing the labelled dextrans and to Dr K. E. Johnson for a critical reading of the manuscript. They also would like to thank H. Boulekbache and F. Meury for their contribution to SEM observations; P. Groué and C. Montmory for technical assistance; J. Derosiers for illustrations; and A. Besicovitch for typing. This work was supported by grants from CNRS, MEN and ARC.

References


Extension of the dorsal marginal zone


(Accepted 13 January 1987)