Autonomous mesoderm formation in blastocoelic roof explants from inverted *Xenopus* embryos

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ABSTRACT ***Xenopus* eggs, artificially fertilized, were prevented from undergoing equilibrium rotation by incubation in medium containing ficoll. Three orientations were selected: normal, with animal pole uppermost; inverted, with vegetal pole away from gravity; and an off-axis orientation, with embryos tilted approximately 90° from the animal-vegetal axis. At blastula stage 8, cells forming the blastocoelic roof were cultured in isolation as explants. These cells are normally fated to form epidermis ventrally and neural derivatives dorsally. Unexpectedly, in the fragments originating from inverted or 90°-off-axis embryos, axial structures were found: notochord, somites, neural cells, cement glands, and sometimes sensory organs. Inverted eggs could be exploited in studies of mesodermal specification.**

KEY WORDS: *Xenopus laevis, mesoderm formation, cytoplasmic localizations*

Introduction

Xenopus eggs are characterized by a marked polarity along the animal-vegetal axis of the cell. This polarity is established during oogenesis. It is reflected in the position of various organelles: for instance, the nucleus is located in the animal part, pigment granules are more concentrated in the superficial layer of the animal hemisphere, ribosomes, glycogen particles and lipid droplets decrease in number from the animal to the vegetal pole. Animal-vegetal polarity is also to be seen in the organization of the intermediate filaments of vimentin (Tang *et al.*, 1988) and those of cytokeratin (Klymkowsky *et al.*, 1987). Some maternal mRNAs exhibit a non-uniform distribution. These include an mRNA, called Vg1 encoding a protein, which belongs to the TGF-ß family (Weeks and Melton, 1987). Yolk platelets, finally, are larger and more abundant in the vegetal hemisphere than in the animal part. They are responsible for the orientation of the embryo: after a cortical reaction triggered by fertilization or activation, the egg is free to rotate so as to lie in its natural position with the heavy, yolk-laden vegetal pole downwards.

This initial animal-vegetal polarity correlates with the organization of the embryo. Ectoderm will form in the animal hemisphere, endoderm in the vegetal part, mesoderm in the marginal zone. The involution of mesoderm occurs towards the animal pole and determines the anterior-posterior axis of the embryo. However, the cells of the three germ layers retain more potentialities and until gastrulation can be diverted towards any pathway of differentiation.

One can raise the question whether the organization of the embryo depends on localized factors or whether the convergence of different factors inherent in egg polarity play a role in specification of cell fate.

When eggs are prevented from rotating or if they are turned upside down after fertilization, they undergo cytoplasmic rearrangements which can lead to reversal of polarity and modification of the fate map. Such inverted eggs have long fascinated embryologists (for review see Pasteels, 1938).

More recently, Neff *et al.* (1983, 1984) have published extensive studies on inverted *Xenopus* embryos or eggs prevented from undergoing rotation. Such eggs are maintained in an abnormal position from before fertilization. These authors show, for instance, that in inverted eggs the furrow begins to form at the upper pole, preceded by the movement of the nucleus to the upper part. Relocation of the furrow is favored when eggs are allowed to develop at low temperature. The embryos then display a reversal of the cleavage pattern: the small blastomeres develop in the original vegetal hemisphere of inverted eggs and the large blastomeres in the animal part. The anterior-posterior axis is also reversed. Neff *et al.* (1984) concluded that cell fate is completely reversed, as a proportion of these embryos develop normally at least until the tailbud stage.

We thought that inverted eggs might help to disentangle the various networks responsible for initial specification in development.

Abbreviations used in this paper: mRNA, messenger RNA; TGF, transforming growth factor; NT, Niu and Twitty's solution; preFO, prefertilization orientation.

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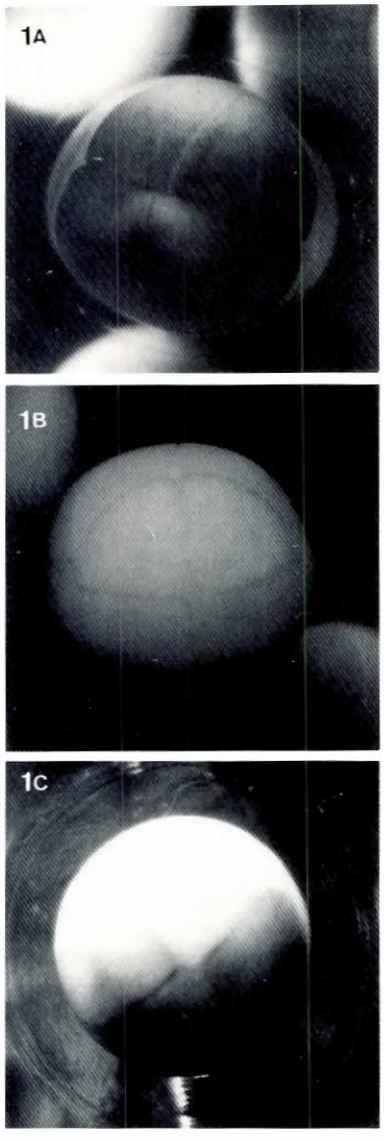

Fig. 1. 8-cell stage embryos maintained in ficoll since fertilization. (A) *0° preFO controls.* **(B)** *180° preFO.* **(C)** *90° off axis.*

In a search for phenotypic differences between animal and vegetal cells in connection with plasma-membrane proteins, we had planned a parallel investigation of inverted eggs. But we rapidly came across the finding that cells located above the blastocoel in inverted eggs do not behave like animal cells from normal blastulas when cultured as explants.

These observations are the starting point of the present work and will be described here.

Results

We have adopted the nomenclature proposed by Neff *et al.* (1983): prefertilization orientation (preFO) means that eggs were maintained in the same orientation in which they landed after stripping from the female. The orientations occur at random. 3 orientations were selected:

- preFO 0° (Fig 1A): controls, pigmented animal hemisphere upwards, lightly pigmented vegetal hemisphere downwards;
- preFO 180° (Fig 1B): inverted embryos, animal hemisphere (pigmented) downwards. The axis is inverted, *i.e.* tilted at about 180°, white cells are in the upper hemisphere;
- preFO 90° (Fig 1C): vertical axis forming an angle of about 90° with the animal-vegetal axis. The upper cells are half-pigmented and half-unpigmented.

The animal caps from preFO 0° embryos, composed of pigmented cells when explanted, formed round vesicles (Fig. 2C). They became folded and ciliated. After 48 h, the presence of vacuoles could be observed in some fragments. Sections of the fragments showed that they were composed of ectoderm. Indeed, some fragments contained a vacuole, sometimes filled with mesenchyme-like cells. In one case, we found a cement gland.

The corresponding fragments from preFO 180° embryos were lightly pigmented. In more than 90% of cases (Table 1), the fragments elongated after a few hours of culture (Fig. 2A). They sometimes remained very long and sometimes appeared shorter after 40 h in culture. Cement glands could sometimes be recognized (Fig. 2E). In sections (Table 2), most of the fragments were found to have formed notochord, somites and neural formation. In a few cases, an eye could be recognized. Mesenchyme and melanophores were found less frequently. Fig. 3 shows several sections from these embryoids.

Fragments taken from the blastocoelic roof of preFO 90° embryos were half-pigmented and half-unpigmented. They often elongated (Fig. 2B, Table 3). However, the elongated part was mostly composed of unpigmented cells, the pigmented part remaining round. They generally became less elongated after 44 h. Some fragments were more similar to controls. Fig. 2D shows a fragment with 2 cement glands in the pigmented part. Observations on the sections are summarized in Table 4. Fig. 4 shows several sections of these fragments. The notochord and somites, when present, are found in the unpigmented part, then come neural formations, further towards the pigmented part, which often forms a cement gland. This formation can also be hybrid (Fig. 4C) formed by pigmented and unpigmented cells. Sometimes the pigmented part remains ectodermic. Among the fragments which do not elongate, a cement gland or neural cells are often found.

Discussion

Specification of isolated fragments

Fragments composed of cells of the blastocoelic roof of normally oriented embryos generally form atypical epidermis when cultured as explants.

The corresponding fragments of inverted blastulas (preFO 180°), on the contrary, elongate and undergo morphogenesis. They form dorsal mesoderm often accompanied by neural structures. These explants behave like normal blastula animal cap explants treated with peptide growth factors or like dorsal marginal zones explanted from normal gastrulas. Let us recall that the cells forming the blastocoelic roof in inverted embryos are unpigmented and originate from the modified vegetal hemisphere.

In embryos 90° off axis, the blastocoelic roof is hybrid. It contains unpigmented cells from the original vegetal hemisphere and pigmented cells from the animal hemisphere. Their behavior is also composite. The unpigmented part gives rise to dorsal mesoderm, the pigmented part forms cement gland or epidermis. In between, neural cells of either animal or vegetal origin can be found. Cement gland can also be hybrid. In some cases, the fragments behave more like animal explants. However, pure ectodermal explants are

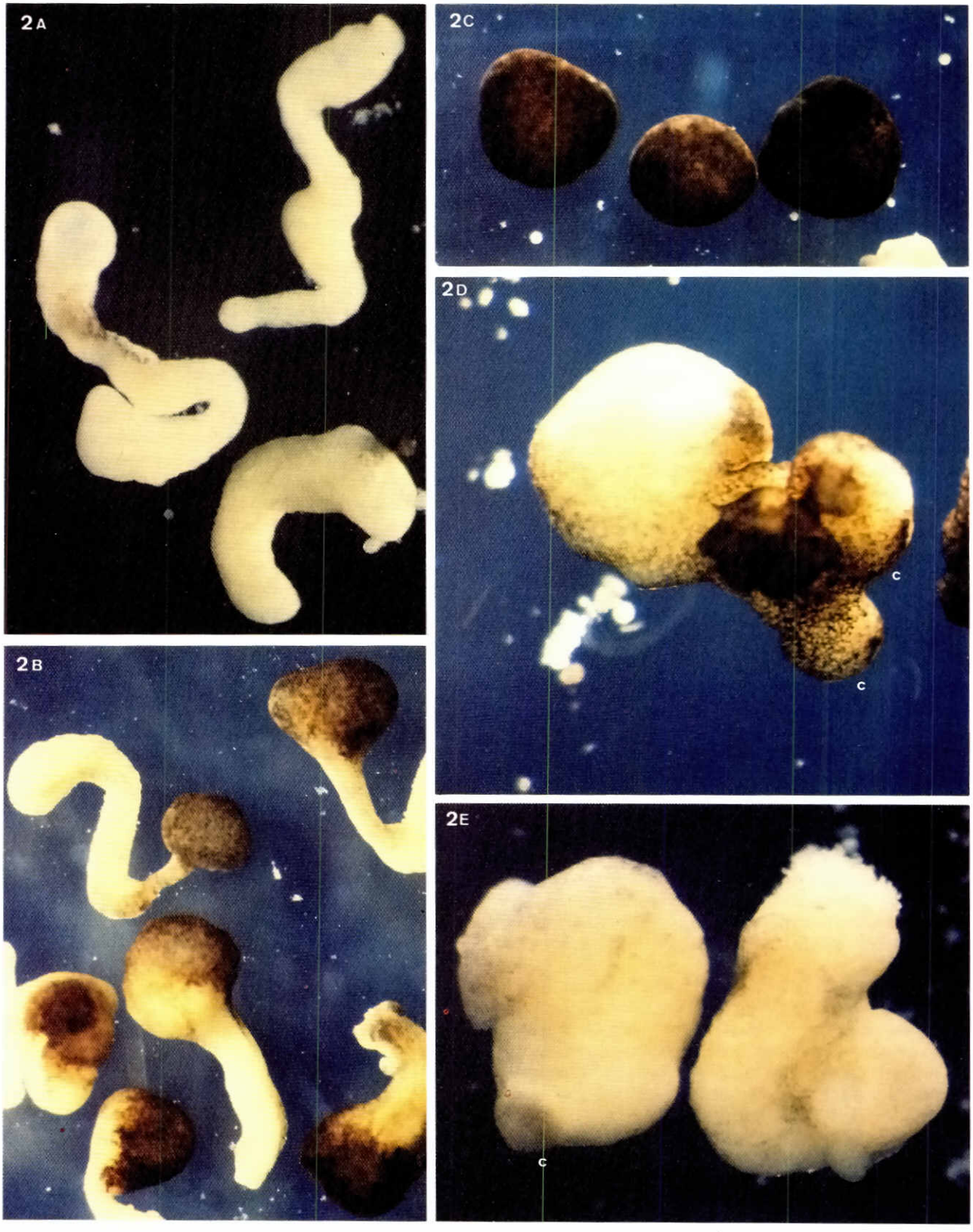

Fig. 2. Culture of blastocoelic roof fragments, (A) *from inverted blastulas - 24 h in culture.* **(B)** *from blastulas 90° off axis - 24 h in culture.* **(C)** *Control fragments - 24 h in culture.* **(D)** *Fragments from blastulas 90° off axis - 44 h in culture; c: cement gland.* **(E)** *Fragments of inverted blastulas - 44 h in culture.*

TABLE 1

BEHAVIOR OF EXPLANTS FORMED BY THE BLASTOCOELIC ROOF OF INVERTED EMBRYOS

Spawnings	Number of explants	Number of elongated explants
1	9	9
2	8	8
3	6	6
4	9	7
5	13	10
6	7	7
7	5	5
8	11	11
9	2	2
10	4	4
11	3	3
Total	77	72 (93%)

rare and generally include a cement gland and some neural cells. The structures formed are hybrids between preFO 0° and preFO 180° explants, modulated by reciprocal interactions.

Despite the fact that in intact embryos (preFO 0°, 90°, 180°) the cells forming the blastocoelic roof form epidermis on the ventral side and neural derivatives on the dorsal side, the observations described above lead to the conclusion that although gravity can cause fate map reversal in the intact embryo, some structures located in the vegetal region, important for dorsal mesoderm formation, escape the gravitational rearrangements.

Cytoplasmic zones not affected by gravity in inverted eggs

As the egg is not able to rotate when maintained in ficoll, gravity causes internal displacements.

Cleine and Dixon (1985) suggest that the whole cytoplasm with yolk platelets moves as a whole, while Neff *et al.* (1984) propose a «density compartment model» and consider that cytoplasm is composed of separately moving compartments. One compartment containing large yolk platelets appears to shift to the animal hemisphere while the compartment composed of small yolk platelets is found in the upper, or original vegetal hemisphere. Smith *et al.* (1985) have also tracked 2 non-yolk proteins by means of specific antibodies. These proteins normally located in the animal hemisphere are found in the original vegetal hemisphere by 1st cleavage and seem to move independently of the yolk compartments.

Two zones do not appear to move: the animal and vegetal membranes and a layer lining these membranes. Furthermore, between the vegetal cortex and the small yolk platelets that have migrated upwards, a new zone can be distinguished where large yolk platelets were left behind when the large yolk mass or the whole cytoplasm shifted away from the original vegetal hemisphere (Neff *et al.*, 1984; Cleine and Dixon, 1985).

Although animal proteins have been shown to move to the upper part, the reverse might not be true for macromolecules from the vegetal part. It is known that maternal mRNA encoding a potential mesoderm inducer, Vg1, as well as the protein itself (Tannahill and Melton, 1989), are located in the vegetal hemisphere. Maternal Xsnail mRNA, a homologue of a *Drosophila* mesoderm gene (Sargent and Bennett, 1990), is located in the vegetal hemisphere. Messenger RNA of mix 1 (Rosa, 1989), another gene related to mesoderm formation, is also abundant in the vegetal hemisphere.

Mesoderm formation in normal development

This subject has been reviewed recently (Smith, 1989; New *et al.*, 1991). Here, we just want to focus on a few points which may be relevant to the present discussion.

It is generally accepted that mesoderm formation requires a signal emanating from endodermal cells. The model of Smith and Slack (1983) proposes that a signal from the vegetal pole will induce adjacent marginal cells to form ventral mesoderm. A signal emanating from the dorsal vegetal hemisphere will induce dorsal mesoderm which in turn will influence adjacent mesoderm to form lateral structures and will induce the dorsal epidermis to differentiate neural structures. Induction of mesoderm can be mimicked experimentally by various peptide growth factors, like bFGF (Kimelman *et al.*, 1988; Slack *et al.*, 1987) or activins (Asashima *et al.*, 1990; Smith *et al.*, 1990; Thomsen *et al.*, 1990; van den Eijnden-Van Raaij *et al.*, 1990). These exist in the embryo and very probably play a role in normal development.

On the other hand, dorsal mesoderm formation depends on symmetrization, an event that takes place after fertilization. Extensive studies by Gerhart and his group (reviewed by Gerhart *et al.*, 1989) have confirmed and extended previous studies on this event by Ancel and Vintemberger (1948).

Fertilization triggers a reaction causing a 30° displacement of cytoplasm relative to the cortex. This brings vegetal cortex into the presence of animal cytoplasm in the marginal zone, on the future dorsal side. In *Xenopus*, dorsal structures normally form on the side opposite the sperm entry point. In normal development, the event occurring in the marginal zone has an incidence on the whole dorsal

TABLE 2

ANALYSIS OF HISTOLOGICAL SECTIONS. FRAGMENTS FROM INVERTED EMBRYOS - 44 HOURS IN CULTURE

spawnings	notochord	somites	neural cells	mesenchyme	cement gland
1	+	+			
	+	+	+	+	
	+	+	+		+
	+	+		+	
	+	+	+		
2	+	+	+	+	+
	+	+	+	+	
		+	+	+	+
	+	+	+		
		+		+	
	+	+	+		+
			+	+	+
			+	+	+
		+	+		+
	+		+	+	+
	+	+	+		+
3	+	+	+		+
	+	+	+		
	+	+			
	+	+			

These fragments were all elongated

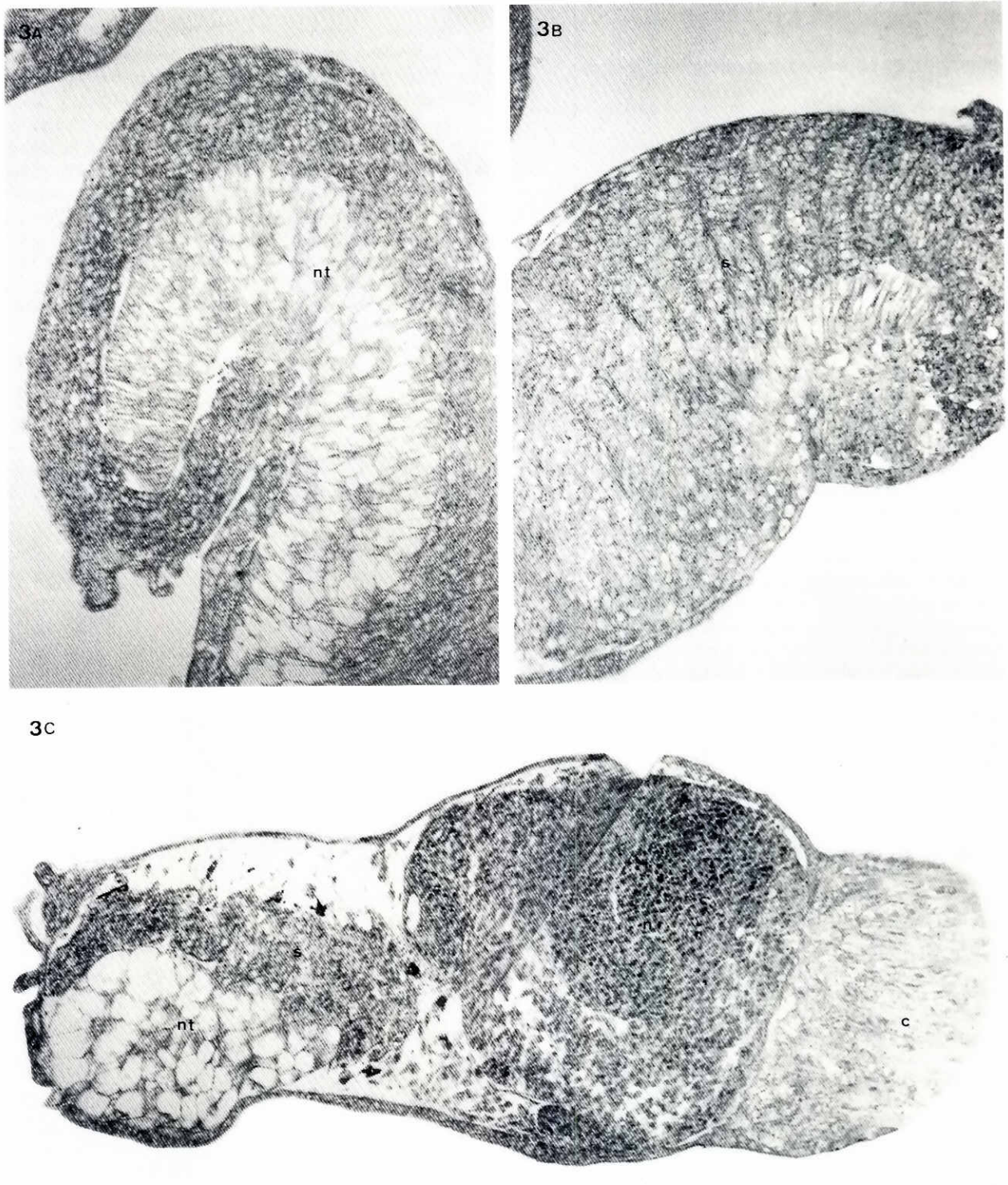

Fig. 3. Sections of explants from inverted blastulas - 44 h in culture. (A) *Notochord (nt).* **(B)** *Somites (s).* **(C)** *Explant containing notochord (nt), somites (s), neural cells (n), cement gland (c).*

side. It is reflected on the animal part, as shown recently by Sokol and Melton (1991), and also on the vegetal part which becomes Nieuwkoop's center (Boterenbrood and Nieuwkoop, 1973). It has been shown that the vegetal dorsal cells can induce dorsal mesoderm very early in development (Gimlich and Gerhart, 1984). What happens in the equatorial region is not clear. As endodermal and mesodermal cells do not separate early during cleavage, it is hard to tell whether cells in the marginal zone are endowed with an

TABLE 3

BEHAVIOR OF 90° OFF-AXIS BLASTULA EXPLANTS

Spawnings	Number of explants	Number of elongated explants
1	7	5
2	7	4
3	4	3
4	2	1
5	6	5
6	6	5
Total	32	23

autonomous capacity to form mesoderm. Nevertheless, the marginal zone will become the organizer, probably under the continued influence of the vegetal zone, and will have the capacity to reset polarization at gastrula stage.

The puzzling thing is that the initiation of symmetrization triggered by fertilization or activation can be controlled by gravity. When eggs are tilted at 90° for a short time, the dorsal side will form on at the side which was uppermost, the relation to the sperm entry point being erased. It is known that this displacement is driven by an array of microtubules which appear transiently at that time in the vegetal hemisphere (Elinson and Rowning, 1988). When the microtubules are destroyed, the displacement is inhibited. More or less ventralized embryos develop after treatment with U.V., cold, hydrostatic pressure. Ventralized embryos can be rescued by tilting the egg for a short time: the cytoplasmic displacement caused by gravity will substitute for normal dorsalization (Scharf and Gerhart, 1980). Moreover, two such displacements (possibly by centrifugation) will lead to double axis formation (Black and Gerhart, 1986).

Development of inverted eggs

Inverted eggs or eggs maintained off axis from before fertilization form mesoderm in the marginal zone. Neff *et al.* (1984) proposed that the direction of shift of the yolk compartment determines the dorso-ventral polarity of inverted eggs. In the preFO 180° orientation, a major percentage of embryos fail to gastrulate or are retarded and show abortive development. Neff *et al.* (1984) explained that eggs remaining in a 180° off-axis orientation display a symmetrical shift and fail to gastrulate. When a slight tilt occurs, as often happens, there is an asymmetric shift and development can be normal. Inverted eggs can also develop normally when the inversion is realized in two steps (Cleine and Dixon, 1985; Wakahara, 1989).

While in normal development the dorsal movement starts from the marginal zone, in inverted eggs the displacement of cytoplasm or the shift of the heavy yolk compartment starts in the apical part, either in the former equatorial region or at the original vegetal pole depending on whether the eggs are tilted 90° or 180° off axis. The organizer will nevertheless form in the marginal zone.

Something at least remains imprinted in the apical part which is only expressed when cells are explanted, freed of the blastocoelic fluid, the perivitelline space, and the interactions with other embryo cells. Indeed, negative interactions in the intact embryo or the presence of an inhibitor, as proposed by Cooke *et al.* (1987) may explain the «normal» development of intact embryos. Cooke *et al.* (1987) observed that mesoderm-inducing factor injected into the blastocoel is far less active than on isolated animal caps.

Negative or suppressive interactions between embryo cells are frequently encountered. In sea urchin embryos, the potentialities of animal blastomeres are restricted by adjacent vegetal cells or by other animal blastomeres (Henry *et al.*, 1989; Khaner and Wilt, 1991). Vegetal cells can form spicules when separated from micromeres (Ettensohn and McClay, 1988). For *Xenopus*, similar results have been published. Gallagher *et al.* (1991) have shown that animal blastomere from the dorsal midline isolated at the *Xenopus* 32-cell stage has autonomous potentialities to develop dorsal mesoderm, while the whole animal cap of that stage will form ectoderm (Pierce and Brothers, 1988). In the same line, recent data published by Godsave and Slack (1991) show that isolated animal cells from an early blastula form neural cells. If the number of cells in the culture is increased, they form epiblast.

TABLE 4

ANALYSIS OF HISTOLOGICAL SECTIONS - FRAGMENTS EXPLANTED FROM BLASTULAS 90° OFF AXIS - 44 HOURS IN CULTURE

Spawn	Elongation	not	somites	neural cells	mes.	cement gland	ecto
1	5/7	-					+
		-				+	+
		+	+				+
		+	+	+		+	
		+	+	+		+	
		+	+	+		+	
		+	+	+		+	
2	4/7	-		+			+
		-			+		+
		-				+	+
		+	+	+			
		+	+	+			
		+	+	+			
		+	+	+	+		
3	3/4	+	+	+			+
		+	+	+	+	+	
		+	+	+			
		-					+
4	2/2	+	+	+	+	+	+
		-		+		+	+
5	5/6	+	+	+		+	+
		+	+	+		+	
		+	+	+		+	
		+	+		+		+
		+	+				+
		-					+

Besides the possible inhibition of mesoderm formation, one cannot exclude the fact that some ectopic mesoderm formation occurs in the intact embryo. Cell movement can mask ectopic formation if this formation is not too considerable. On the other hand, abnormal development often occurs in preFO 180° and might perhaps involve ectopic mesoderm formation.

In conclusion, the inverted vegetal parts have retained properties that are important in mesoderm formation. They deserve further

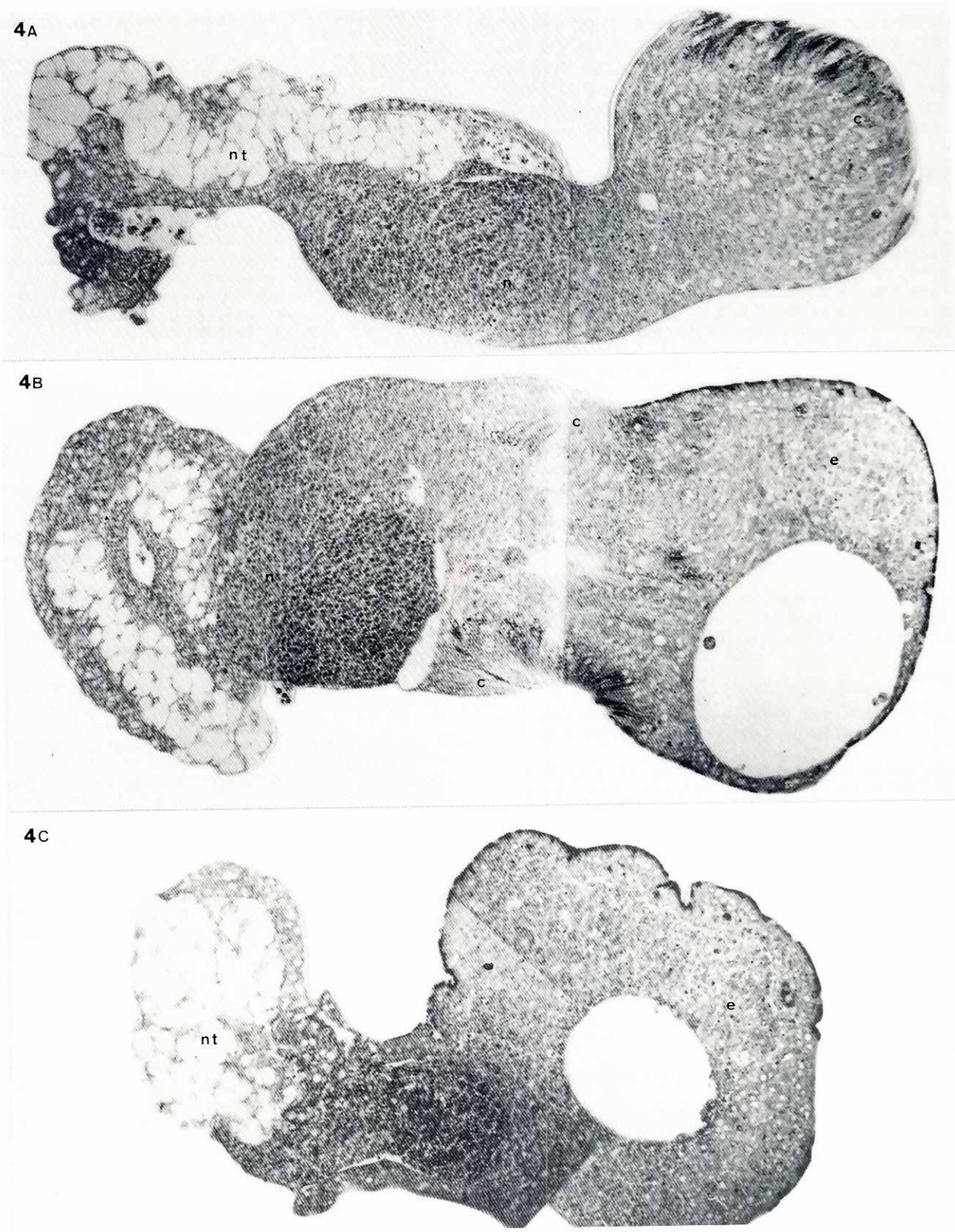

Fig. 4. Sections of 90° off-axis blastula explants 44 h in culture. **(A)** *Notochord - neural cells - cement gland.* **(B)** *Notochord - neural cells - hybrid cement glands - ectoderm (e).* **(C)** *Notochord - neural cells - ectoderm.*

scrutiny with available tools and probes. Such studies might indicate whether the properties favoring mesoderm formation are related to localized factors such as peptide growth factors (Vg1, activins), early gene products involved in mesoderm formation (snail, mix 1) or whether the mesoderm-forming capacity originates in local activation linked with the initiation of the cytoplasmic shift and resembling the events that occur in normal development.

Materials and Methods

Xenopus laevis eggs were obtained from hormone-stimulated females and inseminated with a concentrated sperm suspension in half-strength Niu and Twitty's solution (NT/2): 58 mM NaCl, 0.67 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 0.77 mM Na_2HPO_4, 0.14 mM KH_2PO_4 (Niu and Twitty, 1953). Within 5-10 min., the eggs were covered with 20% ficoll in NT/2 (w/v) and incubated at 14°C.

Embryos were selected at the 8-cell stage ormore often at blastula stage 8 (Nieuwkoop and Faber, 1969).

At blastula stage 8, the embryos were dejellied by immersion in NT containing 1% mercaptoethanol adjusted to pH 8-8.5 (modified from Wolf and Hedrick, 1971). Vitelline envelopes were removed with watchmaker's forceps and a cap of cells forming the blastocoelic roof were excised with iridectomy scissors.

The fragments were cultured in NT containing gentamycin (50 µg/ml) in small Petri dishes coated with agar 1% in NT.

Several spawnings were analyzed. In some experiments, explants were fixed with Zenker's fixative, dehydrated and embedded in paraffin. 10 micron sections were stained with methyl green/pyronin.

Acknowledgments

We are grateful to A. Styns for assistance in the histological preparations and to D. Franckx for the photographic work. This work was supported by a grant from the European Communities (Contract ST2J 0324- C(TT).

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