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Heat-shock-induced grey crescent formation in axolotl eggs and oocytes: the role of gravity

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Summary

Axolotl eggs were heat shocked (36·8°C, 10 min) inside their jelly layers. Heat shock (HS) was shown to induce the precocious appearance of a grey crescent (GC) in a number of eggs immediately after fertilization (Benford & Namenwirth, 1974). It was also demonstrated that this phenomenon occurs in fertilized or artificially activated eggs only when they are shocked within 1½ h after spawning. The GC forms still later in heated unfertilized, nonactivated eggs. The role of the jelly layers is considered to be mechanical: a proportion of eggs is maintained in a tilted position until the egg is able to orient animal pole upwards under the influence of gravity as a late consequence of activation. The jelly layers are not essential if the eggs are artificially tilted or rotated during HS. GC formation can also be induced in *in vitro* maturing oocytes, provided they are tilted during HS. Gravity thus plays an essential role in the cytoplasmic rearrangements leading to HS-induced GC formation. Our results indicate a synergistic action between heat and gravity in this process. The cytological appearance of the GC formed in those experiments is that of a 'Born's crescent' with a conspicuous 'vitelline wall' (Pasteels, 1964).

When oocytes are enucleated before maturation, HS has no effect on GC formation. A nuclear factor is therefore essential, as has been demonstrated in early GC formation induced by inhibitors of protein synthesis.

Finally, incorporation of amino acids into oocyte proteins appears to be rapidly inhibited by HS (from 5 min). However, we cannot conclude that GC formation is in fact triggered by inhibition of protein synthesis. It is also likely that HS disrupts cytoskeletal structure, hence facilitating cytoplasmic rearrangements. Nevertheless, these results are in agreement with the scheme we recently proposed for GC formation in the rotated axolotl oocyte (Gautier & Beetschen, 1985).

Key words: oocyte, grey crescent, dorsoventral polarity, gravity, heat shock, protein synthesis, axolotl, *Ambystoma mexicanum*.

Introduction

Acquisition of dorsoventral polarity in amphibian eggs appears to be closely linked to the appearance of a particular cytoplasmic region, the grey crescent (GC), which appears in the fertilized egg before the first cleavage in various anuran species (*Rana*, *Bufo*, *Discoglossus*, *Xenopus*) and in the urodele axolotl (*Ambystoma mexicanum*). In eggs from this latter species in which fertilization is polyspermic, contrary to the monospermic anurans (Ancel & Vintemberger, 1948; Gerhart *et al.* 1981), there is no obvious link between the sperm entry point and the appearance of the GC on the opposite side of the egg. In normal fertilized eggs at 18°C, the first cleavage occurs between 6 and 7½ h after spawning (t_0) and GC forms at any time between 1½ h and 1 h before the first cleavage. Therefore, GC is not formed within the 4½ h after t_0. However, Benford & Namenwirth (1974) have shown that heat shock (34–37°C for 10 min) of freshly fertilized eggs can induce the appearance of GC several hours earlier than normal: a GC is visible in a high percentage of eggs immediately after a heat shock applied to eggs collected within 45 min of spawning. These authors reported that the induced GC appeared to have the same properties as that of a normal GC since gastrulation was observed to start at this point.

We repeated these experiments in order to define more accurately the period of reactivity of oocytes as well as that of fertilized and unfertilized eggs. A preliminary report (Beetschen, 1979) showed that

GC does not form when fertilized eggs, or unfertilized eggs activated by an electric shock, are submitted to heat shock more than 1½ h after spawning, GC is, however, observed in a significant percentage of nonactivated unfertilized eggs between 1½ and 3 h after spawning. Moreover, no fertilized egg was found to react if it had been dejellied before heat treatment. In the oviduct, the heated unfertilized oocytes form a GC after secretion of the jelly layers. Oocytes, matured *in vitro* by progesterone while still enveloped in the follicle wall during heat treatment, also display GCs in varying proportions. This posed the question of the exact role of the jelly layers in GC formation.

Previous research in this laboratory (Grinfeld & Beetschen, 1982; Gautier & Beetschen, 1983) has demonstrated that it is possible to induce early GC formation in matured oocytes *in vitro* by treating them with protein synthesis inhibitors (cycloheximide, diphtheria toxin). This occurs providing the nucleoplasm has interacted with cytoplasm after germinal vesicle breakdown (GVBD). In this case, GC formation is favoured by partial rotation of the oocyte and a three-step scheme was proposed for early GC formation in axolotl oocytes (Gautier & Beetschen, 1985).

In the present paper, it is demonstrated that GC formation consecutive to heat shock does not require the presence of the jelly layers if the oocytes are artificially tilted. The presence of the jelly layers before completion of activation produces a natural tilting effect, which also occurs in the *in vitro* maturing oocytes because of the follicle wall. This prevents the oocyte from rotating vegetal pole downwards under the influence of gravity. The jelly layers therefore appear to have a mechanical role. This enables gravity to exert essential effects on cytoplasmic rearrangements. In addition, we showed that amino acid incorporation into proteins is rapidly inhibited during heat shock.

Materials and methods

Eggs

Ambystoma mexicanum adult females from a white strain were bred in the laboratory and mated with sexually mature males. A high proportion (≥80 %) of control fertilized eggs from these females showed a normal GC 1 h before the first cleavage. Fertilized eggs were collected every 15 min and kept in water at 18°C before the heat shock. Nonfertilized eggs were obtained from virgin females following injection of a mixture of chorionic gonadotrophin (150 i.u.) and serum gonadotrophin (120 i.u.). When necessary, unfertilized eggs were activated by an electric shock (Signoret & Fagnier, 1962). The heat-shock procedure (36·8 ± 0·1°C) on jelly-coated eggs is described with the corresponding experiments. The heated eggs were scored for the presence of GC immediately after the heat shock. The GC was characterized by its grey colour, its regular shape and its curvilinear lower border along the nonpigmented yolk. These characteristics enabled it to be reliably discriminated from nonspecific disturbed pigmentation patterns. A few abnormally pigmented eggs were not taken into account. The subequatorial lightly pigmented area was considered as a GC only when it extended over an arc of more than 15°. As already mentioned by Benford & Namenwirth (1974), the dimensions of the crescent vary among eggs of a single spawning. Nevertheless, we observed very distinct and large GC in most cases (Fig. 1). It should be emphasized that the heat-shock technique was originally devised to obtain triploid larvae by preventing extrusion of the second polar body from the freshly fertilized egg (Fankhauser & Godwin, 1948). 90 % of the surviving larvae are actually triploid, but a high proportion of embryos die at prehatching stages. We chose a temperature of 36·8°C for our experiments because it induces a higher proportion of GC, but the consecutive embryonic mortality is higher than at lower temperatures (≥34°C). In this case, Benford & Namenwirth (1974) reported a greater than 60 % mortality. These authors also established that (1) the heat shock has no effect on the time at which the first cleavage occurs; (2) there is no correlation between the presence of a precocious GC and the viability of embryos, which is the same among crescent-bearing eggs and eggs that failed to show a precocious GC. Finally, it should be noted that in many axolotl spawnings from different females, 20–50 % of nontreated fertilized eggs may display an abnormal morphogenesis after the neurula stages, even when early development seemed normal. Against this background the importance of heat-shock-induced damage would appear to be minimized.

Oocytes

Ovarian follicles were dissected out from anaesthetized females (MS222, 1 g l^{-1}; Sigma Chemical Co.) and separated manually from each other with scissors. Maturation of the intrafollicular oocytes was induced by 1 μM-progesterone (Sigma) in modified Barth's medium according to Merriam (1971), at pH 8·5 (Vilain, 1978). Maturation proceeded at 18 ± 0·1°C. Heat shock was applied at various times after progesterone treatment, from 10–20 h: oocytes were immersed in OR_2 medium, pH 7·6 (Wallace, Jared, Dumont & Sega, 1973), at 37 ± 0·1°C for 10–15 min. Orientation of intrafollicular oocytes was manually performed on a plastic plate in which cylindrical wells had been drilled, whose diameter was slightly smaller than that of the oocytes. The plastic plate was immersed in a Petri dish filled with warm OR_2 medium.

Intrafollicular oocytes could be enucleated in OR_2 medium. A simplified enucleation technique was adapted from Ford & Gurdon (1977) and has been described elsewhere (Gautier & Beetschen, 1985).

It must be emphasized that, in the axolotl, the follicular wall remains transparent and the progress of maturation (migration of the germinal vesicle, GVBD, formation of the pigmented 1st polar body in the clear maturation spot), as

well as GC appearance, can be easily checked under the dissecting microscope.

Cytological techniques

Fixation of intrafollicular oocytes and eggs was obtained by immersion in Bouin–Hollande fixative for 4–5 h. After rinsing and transfer for a short time in 35 % ethanol, the follicle cells were usually removed with fine forceps. The oocytes and eggs were dehydrated in alcohol, then kept in amylacetate for 2–3 weeks before embedding in paraffin wax, the last paraffin bath being performed in a vacuum oven. Serial sections (7 μm thick) were stained with azo-fuchsin–aniline blue–orange G (Ubbels, 1978).

Amino acid incorporation into proteins

[^{35}S]methionine (Amersham, 1100 or 1350 Ci mmole^{-1}) was used for incorporation into oocyte proteins. 1 μCi was dissolved in 100 nl modified Barth's medium, buffered with Hepes (MBS-H), pH 7·5 (88 mM-NaCl, 1 mM-KCl, 2·4 mM-$NaHCO_3$, 0·82 mM-$MgSO_4$, 0·33 mM-$Ca(NO_3)_2$, 0·41 mM-$CaCl_2$) and was injected into each oocyte. The oocyte (control or heat-shocked) was disrupted on a parafilm sheet in 50 μl lysis buffer (Tris 50 mM, NaCl 50 mM, NP40 2 %, PMSF 2 mM, aprotinin 0·15 i.u. ml^{-1}). Three oocytes were then pooled in an Eppendorf tube and incubated at 4 °C for 20 min. The tube was centrifuged at 4 °C (14 000 *g*, 10 min) and 50 μl supernatant were placed on a glass-fibre filter (ref. GF/C, Whatman). After precipitation by cold 20 % trichloracetic acid (20 min) and rinsing three times in 10 % and 5 % TCA, the filters were dried and radioactivity was measured in a Packard CSL 460 C liquid scintillation counter.

Results

Effects of heat shock on jelly-coated eggs

Three kinds of egg were used (1) normal fertilized eggs; (2) unfertilized eggs, obtained by gonadotrophin treatment; (3) unfertilized eggs submitted to artificial activation (electric shock) between 15 and 30 min after emission. Egg clutches were collected every 15 min, kept at 18 °C and placed inside nylon-grid baskets, immersed in a warm water bath (36·8 ± 0·1 °C). Heat shock lasted for 10 min and began between 20 min and 3 h (±15 min) after egg spawning. After the heat shock, eggs were placed in water at room temperature and immediately scored for the presence of GC (Fig. 1). The results are summarized in Table 1. 18 fertilized spawnings and 6 unfertilized spawnings were used.

The percentage of GC-forming eggs appears to be higher for unfertilized eggs, whether activated or not, than for fertilized eggs, during the first hour following egg deposition. During the next 30 min it is more markedly reduced in fertilized eggs than in nonactivated unfertilized eggs: in the latter case, the proportion of GC-forming eggs remains nearly at the same level as that observed in the first hour. After

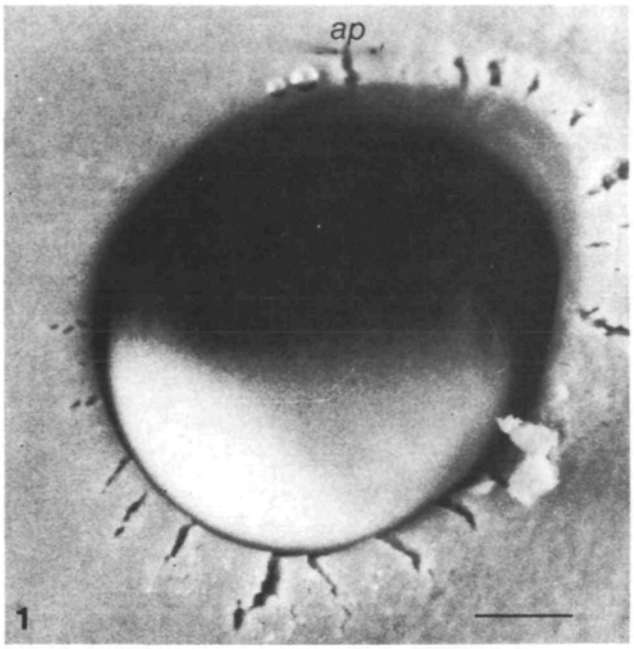


Fig. 1. Grey crescent in a heat-shocked fertilized egg (side view, i.e. left half of the egg). *ap*, animal pole. Bar, 0·5 mm.

1½ h, heat-shock-activated or fertilized eggs no longer form GC, while a significant percentage (38·4 %) of unfertilized eggs still show GC formation. The loss of sensitivity of the remaining unfertilized eggs might be due to 'spontaneous' activation, which occurs in some eggs after a few hours. An additional reason could be that, after the jelly coat has absorbed a certain amount of water, a number of unfertilized eggs can freely orientate animal pole upwards and no longer react to gravity. This is discussed below.

Therefore, when jelly-coated eggs are activated, either by normal fertilization or by an electric shock, their ability to form a GC seems to be progressively lost and is fully abolished after 1½ h. The ability to form a GC is not restored until the time at which normal GC formation occurs. This was demonstrated by two additional experiments made on two separate fertilized spawnings, which were heated between 3½ h and 5 h after fertilization (Table 2). In control eggs, the GCs did not appear before 5 h.

When heated eggs do not form a GC during the heat shock, are they able to form it later, before first cleavage? Freshly laid eggs from two females were shocked at 35 °C only, for 10 min. This temperature induces a lower rate of GC formation than a 36·8 °C shock, and the overall number of surviving embryos and larvae is higher. Actually, the survival rate did not appear to be lower than the control one (Table 3), though morphological anomalies were more frequent in larvae developing from heated eggs, even when GC formation had not been precocious (Table 4).

Table 1. *Formation of a grey crescent after a heat shock in jelly-coated axolotl eggs*

Time of heat shock after emission	Number of eggs (N) and grey crescents (GC)*					
	Fertilized		Unfertilized		Unfertilized, activated	
	N	GC	N	GC	N	GC
≤ 1 h	657	281 (42·7 ± 5 %)	342	202 (59 ± 6·9 %)	150	120 (80 ± 8·4 %)
1 h $< t_0 \leq 1\frac{1}{2}$ h	450	158 (35·1 ± 5·8 %)	103	54 (52·4 ± 12·7 %)	—	—
$1\frac{1}{2}$ h $< t_0 \leq 3$ h	269	2 (0·7 ± 1·3 %)	239	82 (38·4 ± 8·1 %)	105	1 (1 ± 2·5 %)

* Percentages of oocytes forming GC are related to the number N of oocytes (100 %) ± S.E. GC were scored immediately after the heat shock.

Table 2. *Effect of a delayed heat shock on two fertilized spawnings*

	Time of heat shock after emission		
	≤ 1 h	$1\frac{1}{2}$–$2\frac{1}{2}$ h	$3\frac{1}{2}$–$5\frac{1}{2}$ h
Number of eggs	84	44	275
Number of GC	37	0	7*

* All obtained from one and the same female, whose control eggs began to show GC on some eggs from 5 h (see 'Introduction' for the timing of normal GC formation and first cleavage).

Most of the eggs that did not form a GC during the heat shock displayed a GC later, before first cleavage (Table 3). The eggs that had already formed a GC during the heat shock did not form a second GC later. In one case, it was observed that the size of an early-formed, but fairly narrow GC, increased after a few hours and was similar to the control size. Finally, a small proportion of the eggs that had formed a GC during the heat shock could develop into quite normal larvae (Table 4).

The cytoplasmic structure of the GC area is characterized by a conspicuous 'vitelline wall' (Pasteels, 1964; Ubbels, Hara, Koster & Kirschner, 1983). Large vegetal yolk platelets remain at the periphery, close to the plasma membrane, and form the 'vitelline wall', while animal pigmented cytoplasm, with smaller yolk platelets, is shifted towards the vegetal pole, under the wall (Fig. 2A,B; Fig. 3). In some eggs, the GC thus extends down to the vegetal pole itself. These cytoplasmic rearrangements are typical of a 'Born's crescent', which Born (1885) originally

Table 3. *Grey crescent formation during the heat shock (HS) or after the heat shock*

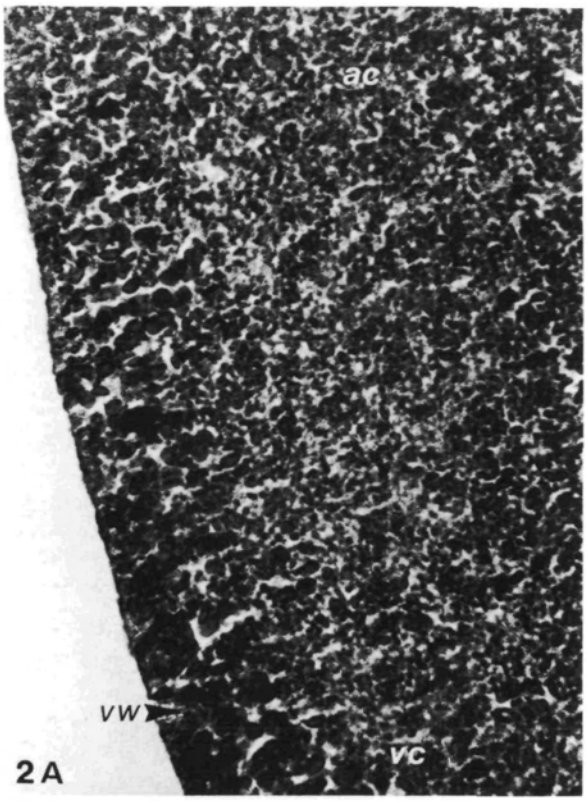
	A Total number of eggs (100 %)	B GC formation during HS	C GC formation only before 1st cleavage	D No GC formation	E Surviving embryos (tail-bud stages)
Heat-shocked	90	30 (33 %)	54 (60 %)	6 (7 %)	54* (60 %)
Control	32	—	25 (78 %)	7 (22 %)	17* (53 %)

* See Table 4 for more details.

Table 4. *Gross morphology of larvae obtained from heat-shocked and control eggs*

	Group*	Eggs	Larvae (hatching stage)		
			Normal	Abnormal	Total number
Heat-shocked	B	30	5	11	16
	C + D	60	26	12	38
Control		32	15	2	17

* Letters refer to columns in Table 3.



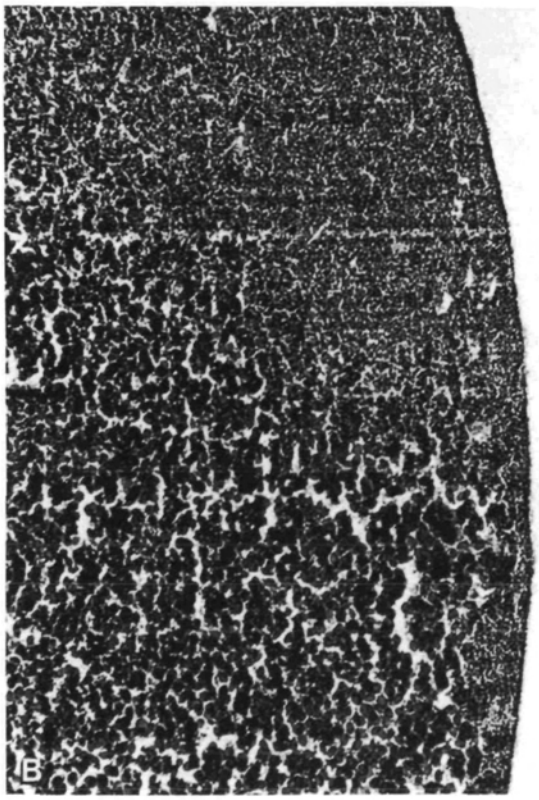


Fig. 2. Sagittal sections through a heat-shocked fertilized egg. (A) Grey crescent area (*dorsal side*) with vitelline wall (*vw*), animal cytoplasm (*ac*) and vegetal cytoplasm (*vc*). The structure is that of a Born's crescent.
(B) Section through the *ventral side*, opposite to the grey crescent area.

obtained in the frog egg after a 90° rotation. When induced later but still before first cleavage, such a crescent was shown to interfere with the normal GC, being able to induce the appearance of a supernumerary blastopore at the gastrula stage (Pasteels, 1948). The Born's crescent is therefore endowed with the same organizer properties as a normal GC. It was also demonstrated that such a Born's crescent, when it forms consecutive to a heat shock or to egg rotation several hours before the normal GC would appear in axolotl, plays the same role as this GC, since a normal blastopore is organized in this area (Benford & Namenwirth, 1974; Malacinski & Chung, 1981). Nevertheless, although the Born's crescent structure, with the vitelline wall, is easily recognized on sections, the normal GC structure is much less characteristic. Pasteels (1948) admitted that Born's crescent exaggerates the features of a normal GC, but further comparative studies are required.

Dejellied eggs can form a grey crescent at any time, but only if they are rotated

It had been shown (Beetschen, 1979) that, if fertilized eggs are dejellied (with forceps) before they are heat shocked in a saline solution, they do not form a grey crescent. The role of the jelly layers was not exactly known, but it did not seem to be of a chemical nature. The viscous inner jelly layer was collected from freshly spawned eggs with a pipette and dejellied eggs were placed in the pooled heated jelly material: they did not form a GC (unpublished data). A mechanical role for the intact jelly layer was thus suspected.

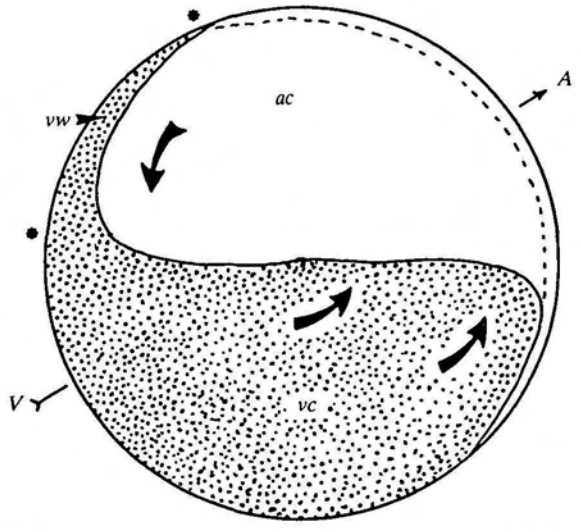


Fig. 3. Schematic sections through a rotated axolotl egg or oocyte, showing the main cytoplasmic rearrangements (movements indicated by arrows), leading to early GC formation ('Born's crescent'). The two asterisks mark the limit of the crescent. *A*, *V*, animal and vegetal poles; other abbreviations, the same as in Fig. 2.

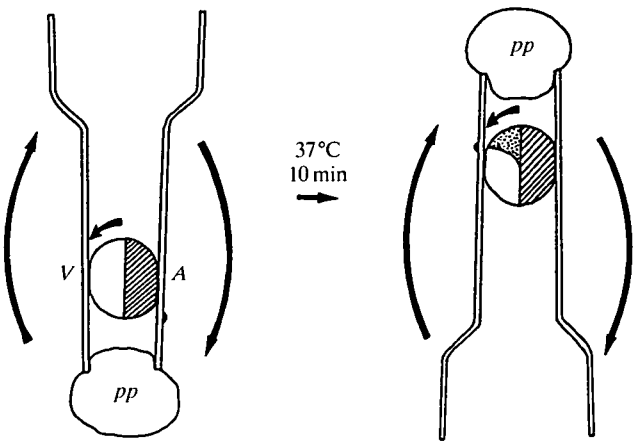


Fig. 4. Scheme of experiment involving a 180° rotation of a Pasteur pipette in which a 90°-rotated fertilized egg was introduced. The small arrow shows the direction in which the egg tends to move inside the pipette under the influence of gravity. This movement is balanced by the rotation of the pipette (large arrows). Grey crescent appears on the top side of the egg, constantly opposing the direction of gravity (see text). *A*, *V*, animal and vegetal poles; *pp*, plasticine plug.

It must be emphasized that activation, consecutive to fertilization, is slow in urodele eggs (Signoret & Fagnier, 1962). The vitelline membrane becomes detached from the egg surface and the egg is able to orientate under the influence of gravity with the animal pole upwards, inside the jelly layers, as in anurans (Ancel & Vintemberger, 1948). The role of the jelly coat in freshly fertilized eggs thus appears to be mechanical, maintaining the eggs in various orientations during heat shock. This interpretation is supported by two lines of evidence.

In the first set of experiments, an egg was dejellied 3 h after insemination, then was introduced and immobilized in a capillary glass pipette with the animal–vegetal axis perpendicular to the tube axis and to gravity (Fig. 4). The glass pipette was vertically immersed in warm water (37°C) and, since gravity tended to make the egg turn inside the tube with the vegetal pole downwards, the pipette was progressively rotated in the reverse direction, so that the animal–vegetal axis remained perpendicular to gravity. After 10 min, the glass tube had been rotated by 180° and a GC was conspicuous on the *upper* side of the egg, opposite to gravity. The experiment was repeated with ten different eggs and gave the same results.

In the second type of experiment, dejellied fertilized eggs were treated for 3 min with a 0·2 % papain solution to soften the vitelline membrane (Malackinski & Chung, 1981), then tilted in cylindrical wells, the diameter of which was slightly smaller than the eggs; the wells were drilled through a plastic plate, which was immersed into a Petri dish containing Ficoll 10 % in OR_2 medium pH 7·5 and then maintained at 37°C. The duration of the heat shock was 15 min and took account of the temporary heat loss due to orientation of the eggs. 11 eggs out of 14 formed a GC, 3 h after fertilization.

The following conclusions can be drawn from both experiments.

(1) When the jelly layers are present, GC forms earlier than normal in fertilized eggs, consecutive to heat shock; grey crescent formation does not take place after $1\frac{1}{2}$ h, even inside the jelly layers, when fully activated eggs are able to move and orientate according to gravity, with animal pole upwards.

(2) During the first 90 min, the jelly layers play a mechanical role, since they maintain a number of eggs in a tilted position during the heat shock. Dejellied eggs never form a GC when they are oriented animal pole upwards.

(3) Heat shock is still able to induce GC formation in a dejellied egg, at any time, if the egg is tilted (partial rotation).

(4) Early GC formation in heat-shocked eggs thus appears as a consequence, not only of heating (Benford & Namenwirth, 1974), but also of partial rotation of eggs. Therefore, gravity must be considered as a synergistic factor, essential for GC formation in heated eggs, but whose effect is only observed so quickly at a high temperature (≥33°C, Benford & Namenwirth, 1974).

Maturing oocytes can form a grey crescent when they are rotated during heat shock

Preliminary experiments (Beetschen, 1979) showed that, inside the oviduct, late maturing oocytes can form a grey crescent when the whole oviductal segment is heated. GC formation only occurs in the middle and distal parts of the oviduct, when the jelly coat has been secreted. Very few oocytes react in the proximal segment of the oviduct, where secretion is not present. Naked coelomic oocytes, taken from the abdominal cavity, do not form a GC when heated in a salt solution, in which they orient animal pole upwards.

Here again, the presence of the jelly layers would appear to be required for GC formation, but gravity can still act on some oriented oocytes inside the very narrow proximal oviduct segment, although the jelly layers are more effective in maintaining oocyte orientation in the distal parts of the oviduct.

Intrafollicular oocytes maturing *in vitro* were therefore used to establish a timetable for ability to form GC.

As for the fertilized dejellied eggs, these oocytes were tilted in small wells in plastic plates covered with warm OR_2 medium (37°C, 15 min), then brought

back to room temperature in a Petri dish. They were then scored immediately for the presence of grey crescent (Fig. 5). Five different females were used and the maturing oocytes were grouped as indicated in Table 5, according to the stage of maturation. The youngest oocytes ($10\frac{1}{2}$–12 h after the start of progesterone treatment) displayed a large whitish maturation spot, following GVBD (Fig. 6A). This stage corresponds to the establishment of the metaphase I spindle. The GC formation had not yet occurred in most of those oocytes and was only observed in 6 out of the 22 older oocytes (12 h). Between 13 and 14 h, a fine dark circle was seen at the centre of the maturation spot, which corresponds to the presence of the maturation spindle (and telophase I): more than 50 % of the oocytes are now able to form a GC. Later, the fine circle becomes progressively thicker and turns to mark the extruding first polar body (Fig. 6C). The majority of 15–17 h oocytes form a GC and probably all of them can be symmetrized after 18 h. If oocytes are not tilted during the heat shock, with animal pole upright, no GC formation is observed. The cytological characteristics of the GC area in heat-shocked oocytes were quite similar to those observed in the eggs. On one side, pigmented animal cytoplasm replaced the inner yolk-laden cytoplasm which flowed downwards with gravity, leaving a conspicuous vitelline wall close to the egg surface.

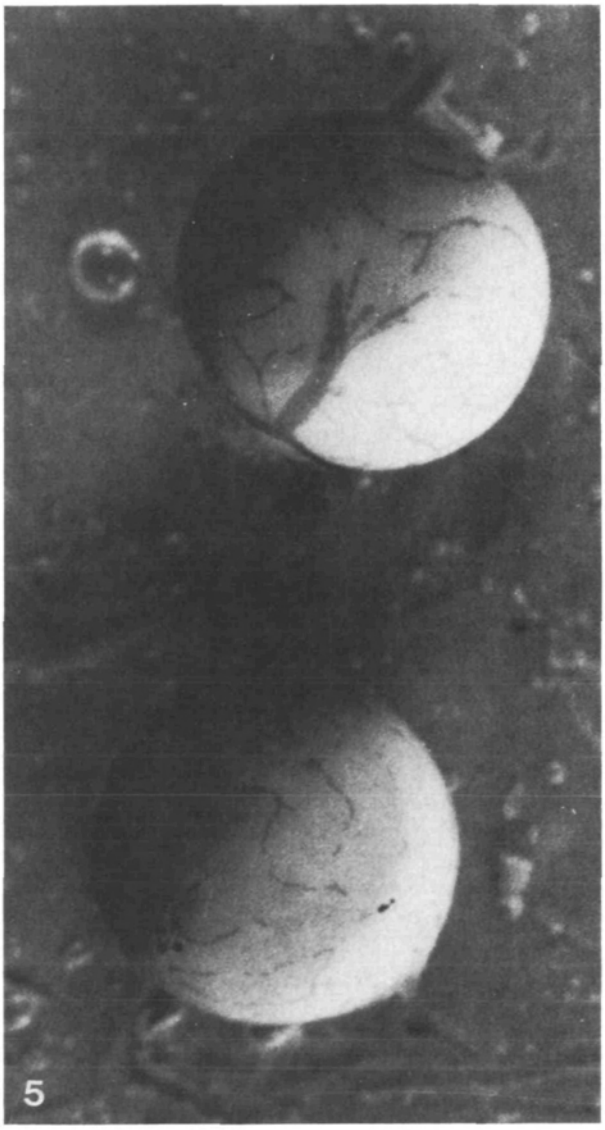


Fig. 5. Grey crescent in two heat-shocked intrafollicular oocytes.

Since enucleated oocytes are not able to form a GC unless nucleoplasm is reinjected (Gautier & Beetschen, 1985), the effect of preliminary enucleation on heat-shock-induced processes was investigated. We found that enucleated matured oocytes were not able to form a GC after heat shock at a time when nucleated normal oocytes can: no grey crescent was observed in 100 enucleated oocytes from two females, tilted and heat-shocked $15\frac{1}{2}$–$16\frac{1}{2}$ h after progesterone treatment.

The ability to form a GC following the synergistic action of heat and gravity therefore appears to be a late consequence of GVBD, which normally occurs 9 h after PG treatment: a few hours are still necessary before nucleoplasm and cytoplasm interact thoroughly. Since no microinjection was involved in the present experiments, it seems that the onset of GC formation is somewhat delayed, compared to GC formation after injection of a protein synthesis inhibitor into nucleated eggs or after a similar injection either followed or preceded by injection of nucleoplasm into enucleated eggs (Gautier & Beetschen, 1983, 1985).

Since inhibition of protein synthesis has been shown to trigger GC formation in a maturing oocyte and it is known that heat shock inhibits protein synthesis after 1 h in *Xenopus* oocytes (Bienz & Gurdon, 1982), it may be that rapid induction of GC by heat shock is linked to inhibition of protein synthesis. Heat may also have other specific effects on cytoplasmic structures.

Incorporation of [^{35}S]methionine into heat-shocked oocytes

[^{35}S]methionine was injected into maturing oocytes, $15\frac{1}{2}$–$16\frac{1}{2}$ h after progesterone treatment. Three oocytes were pooled as one batch. In the first series (control series), incorporation of [^{35}S]methionine into proteins lasted from 1 to 20 min and three measurements were made every minute. The control curve obtained (Fig. 7A) was linear: incorporation increased regularly with time from 2×10^4 to 2×10^5 disints min^{-1}/oocyte.

The effects of heat shock on [^{35}S]methionine incorporation were evaluated in three types of experiment.

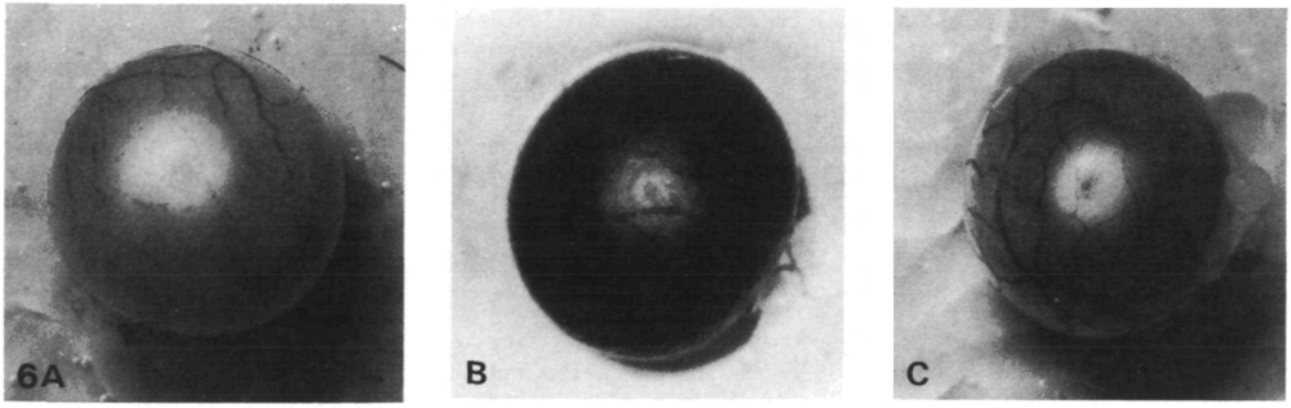


Fig. 6. Three stages of *in vitro* maturation of intrafollicular oocytes. (A) Maturation spot (10–12 h). (B) Presence of the first meiotic division spindle as indicated by a fine pigment ring (13–14 h). (C) Extrusion of 1st polar body (16 h).

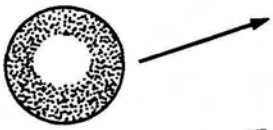
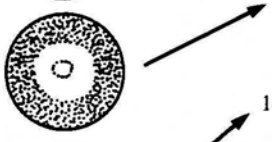
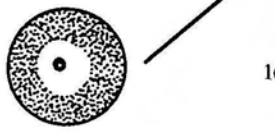
In the first case, oocytes were placed in the warm medium immediately (a few seconds) after methionine injection. Heat shock lasted from 1 to 10 min and then the oocytes were immediately lysed for analysis. Each point of the curve (Fig. 7B) corresponds to the mean value obtained from two batches of three oocytes (odd minutes) or from four batches of three oocytes (even minutes).

We found a statistically significant reduction in methionine incorporation in the heat-shocked batches from 8 min on, compared to control oocytes.

In the second group of experiments (Fig. 7C), oocytes were heat shocked 10 min after they had been injected with [^{35}S]methionine. The duration of the heat shock varied from 1 to 10 min. Three batches of three oocytes were taken each minute and the mean values obtained from each of the three batches were plotted (curve C, Fig. 7). In this case, after a slight increase between 1 and 4 min, above the control level for 10 min at room temperature, an early stabilization of incorporation was found after 5 min (i.e. 15 min after methionine injection and 5 min after the onset of heat shock). After 4 min, the values for the three batches were 1·36, 1·58 and 1·6 $\times 10^5$ at 5 min, and remained around 10^5 between 6 and 10 min.

Since the reduction in amino acid incorporation after a heat shock could have been due to a release of previously masked unlabelled methionine from an intracellular store, a third set of experiments was carried out. Methionine pool sizes in control and heated maturing oocytes were investigated. For both measurements, 1000 oocytes were homogenized and prepared for amino acid analysis according to Shih, O'Connor, Kèem & Smith (1978). Free methionine

Table 5. *Formation of a grey crescent after a heat shock in tilted maturing oocytes*

Maturation stage	Time*	Number of heat-shocked and rotated oocytes	Number of oocytes having formed a GC
	10–12 h	42	7† (16·6 %)
	13–14 h	81	44 (54·3 %)
	14 h 45–16 h	80	45 (56·2 %)
	16 h 15–17 h	43	29 (67·4 %)
	18½ h	6	6

* From start of progesterone treatment, at 18°C.
† Of which 6 out of 22 were 12 h old.

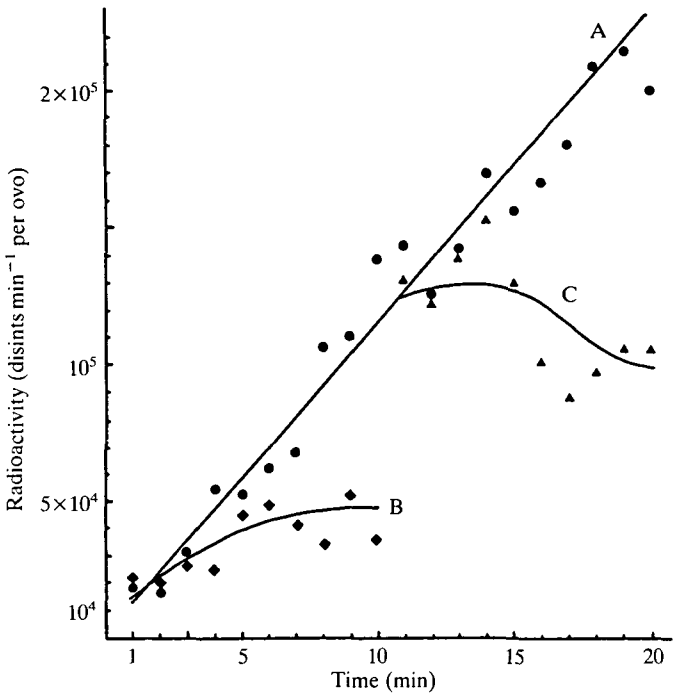


Fig. 7. Incorporation of [^{35}S]methionine in proteins of control (A) and heat-shocked (B,C) oocytes. A (●), control curve; B (◆), heat shock 1–10 min; C (▲), heat shock 11–20 min (first 10 min similar to controls and not represented). See text for details.

amounts were measured by a colorimetric assay using sodium nitroprussiate, according to McCarthy & Sullivan (1941). Methionine concentrations of 25 μg ml^{-1} and 22 μg ml^{-1} were found for control and heated oocytes, respectively. They correspond to an approximate pool size of 150 ± 10 pmoles/oocyte in both cases. Since no significant difference appeared between free methionine pool sizes, in heated and nonheated maturing oocytes, we believe that *de novo* protein synthesis is in fact inhibited during the heat shock.

The effect of heat shock is long lasting: methionine incorporation was measured in two batches of 15 oocytes 1 h after a 10 min heat shock followed by methionine injection. Incorporation was found to be only 20 % of that of nonheated control oocytes which were kept for 1 h after injection of the aminoacid.

Discussion

The present study has enabled more accurate definition of the conditions under which heat shock induces precocious GC formation, not only in the fertilized egg (Benford & Namenwirth, 1974), but also in the unfertilized egg and the maturing oocyte (Beetschen, 1979). It is shown that the jelly layers, whose presence is essential for the phenomenon to occur in eggs and oviductal oocytes, actually play a mechanical role, since they maintain many eggs more or less rotated to the animal–vegetal axis: yolk-laden vegetal cytoplasm flows downwards under the effect of gravity but leaves a 'vitelline wall' (Pasteels, 1964) which adheres to the plasma membrane. Animal cytoplasm flows under this wall when replacing the vegetal cytoplasm (Fig. 3) and the GC structure obtained is that of a 'Born's crescent' (Born, 1885) although it is endowed with the ability to organize subsequently a blastopore like a normal GC (Pasteels, 1948, 1964; Malacinski & Chung, 1981). Benford & Namenwirth (1974) have shown that the heat-induced GC also corresponds to blastopore formation in the axolotl egg, which is in agreement with the results of the above-mentioned studies and with our findings on the origin and cytoplasmic structure of this precocious GC. It can therefore be considered as a marker of potential dorsoventral polarity.

When activation has been completed either as a consequence of fertilization or following an electric shock, the egg surface becomes detached from the vitelline membrane and the jelly layers are no longer able to maintain the egg in a tilted position. The egg orientates freely under the influence of gravity and the cytoplasmic rearrangements, characteristic of a Born's crescent or of a normal GC, do not form after the heat shock. However, GC appears if the egg is artificially tilted during the heat shock. Conversely, a freshly fertilized egg does not form a GC if the jelly layers are removed before heating and the egg remains animal pole upwards in the warm bath. Therefore, gravity must be considered to be responsible for precocious GC formation, heat shock being a prerequisite but not a sufficient condition. The two physical factors appear to act synergistically.

Nevertheless, as shown for precocious GC formation induced by inhibition of protein synthesis (Gautier & Beetschen, 1983, 1985), oocyte maturation must first have proceeded to an advanced stage (10 h at 18°C). As a consequence of germinal vesicle breakdown, an interaction between a nuclear factor and cytoplasm is required before the necessary cytoplasmic rearrangements that lead to GC formation can take place. These conditions are also required for GC induction by heat shock, as shown by its failure to occur in previously enucleated oocytes. In nucleated oocytes, maturation must proceed for at least 12 h before GC formation can be induced by heat shock.

It has been shown in amphibian eggs and many eucaryotic cells that heat shock induces the synthesis of specific so-called 'heat-shock proteins', although normal protein synthesis is simultaneously inhibited. In *Xenopus* oocytes (Bienz & Gurdon, 1982), an immediate reduction in the rate of protein synthesis

was not directly demonstrated, since [^{35}S]methionine incorporation was only measured 1 h after a 20 min heat shock. Our studies have shown that methionine incorporation into proteins of a heat-shocked axolotl oocyte does not increase after the first 5 min and we interpret this as being due to precocious inhibition of protein synthesis. Nevertheless, this inhibition may not be essential for appearance of a GC within the next 5 min, at 37 °C, though this possibility should not be excluded. It is known that such a temperature on its own is sufficient for induction of tubulin polymerization and it could also disrupt the cytoskeletal structures (Ubbels *et al.* 1983) or change the viscosity of cytoplasm. This would allow dissociation of cortical cytoplasm and endoplasm, enabling the necessary rearrangements for GC formation under the effect of gravity to occur within a few minutes. In this respect, it is already known that the level of polymeric tubulin is high during the symmetry reaction in the *Xenopus* egg (Elinson, 1985).

Recently, the cytoplasmic rearrangements leading to GC formation in the *Xenopus* egg have been carefully investigated (Vincent, Oster & Gerhart, 1986). In a free-floating normal egg, the egg surface rotates 30° relative to the subcortical cytoplasm which remains stationary in a position of gravitational equilibrium. This is in agreement with the interpretation of Ancel & Vintemberger (1948) in *Rana* eggs. If the egg is embedded in gelatin, the surface is held fixed and two kinds of movements are demonstrated: a convergence movement of subcortical cytoplasm is followed by an overall rotation of the animal and vegetal subcortical cytoplasm, displacing the egg contents from 30° relative to the surface, irrespective of gravity. This rotation locates the future dorsal side of the embryo. Similar phenomena might occur in the axolotl egg, in which Banki (1929) produced some evidence for complex bidirectional movements leading to GC formation, using dye markers. This author also believed that gravity was not the primary factor for normal GC formation but could only act after active cytoplasmic displacements had taken place. Therefore, the properties of normal GC and those of precocious GC (Born's crescent) cannot readily be compared until the properties of normal GC are better established. It is not clear why the properties of both types of crescent appear to be similar although they form in apparently different ways and display cytological differences (preliminary observations have shown that the 'vitelline wall' is much less conspicuous in normal GC). A specific contact between animal and vegetal cytoplasms has been suggested to play a role (Banki, 1929; Pasteels, 1964) but further investigations will be required to clarify all these points.

Overall, the findings of the present study are in agreement with the three-step scheme that was proposed to define the preliminary conditions leading to GC formation in the rotated axolotl oocyte (Gautier & Beetschen, 1985). This scheme thus remains valid for the interpretation of precocious GC formation consecutive to a heat shock, in which rotation of the egg is a prerequisite.

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Résumé

Un choc thermique (36,8 °C, 10 min) a été appliqué à des oeufs d'axolotl revêtus de leur gangue. Il a été confirmé que les oeufs fraîchement inséminés peuvent alors réagir par la formation précoce du croissant gris (CG) pendant le choc thermique (Benford & Namenwirth, 1974) mais, en outre, il ressort que le phénomène n'est possible, sur des oeufs fécondés ou des oeufs vierges préalablement activés par une décharge électrique, que si le choc thermique est appliqué moins d'1 h 30 après la ponte. En revanche, le CG peut encore apparaître ultérieurement sur des oeufs vierges non activés. La présence des gangues ovulaires est indispensable à ce phénomène, lorsque les oeufs ne sont pas artificiellement inclinés pendant le choc. Mais un oeuf fécondé dégangué peut encore former un CG 3 h après la ponte s'il est incliné, l'axe animal–végétatif formant alors un angle (jusqu'à 90°) avec la direction de la pesanteur. On considère donc que le rôle de la gangue dans le phénomène est mécanique, un certain nombre d'oeufs étant ainsi maintenus en position inclinée aussi longtemps que l'oeuf ne peut subir la rotation d'orientation consécutive à l'activation. Il en résulte que la pesanteur doit jouer un rôle indispensable pour que les cytoplasmes animal et végétatif puissent s'écouler au cours du choc thermique et conduire à la formation du CG. Une action synergique des deux facteurs physiques est ainsi admise. L'aspect cytologique du CG obtenu est celui d'un 'croissant de Born', avec formation d'un 'mur vitellin' (Pasteels, 1964).

De même, la formation du CG peut être induite dans les ovocytes en cours de maturation *in vitro*, à condition qu'ils soient maintenus en position inclinée pendant le chauffage. Le phénomène devient possible à partir de la 12ème heure de maturation (à 18 °C), c'est-à-dire au stade de la métaphase I. D'autre part,

des ovocytes préalablement énucléés, puis maturés à la progestérone, ne forment jamais de CG quand on les soumet ensuite au choc thermique. Comme dans le cas d'une symétrisation précoce induite par des inhibiteurs des synthèses protéiques, un facteur nucléaire est donc indispensable à la formation du CG dans l'ovocyte en cours de maturation.

Enfin, l'incorporation de [^{35}S]méthionine dans les protéines de l'ovocyte se révèle très rapidement inhibée (à partir de la 5ème minute) au cours du choc thermique. Il est impossible de décider si une telle inhibition précoce peut rendre compte à elle seule de la formation du CG comme dans les expériences faisant agir des inhibiteurs, ou bien si, plus probablement, la chaleur modifie directement les structures du cytosquelette, rendant possible un écoulement rapide du cytoplasme. Néanmoins, ces résultats s'accordent avec le schéma en trois étapes récemment proposé pour rendre compte de la formation du CG sous l'influence des inhibiteurs de synthèses protéiques dans l'ovocyte incliné d'axolotl (Gautier & Beetschen, 1985).

References

ANCEL, P. & VINTEMBERGER, P. (1948). Recherches sur le déterminisme de la symétrie bilatérale dans l'oeuf des Amphibiens. *Bull. Biol. Fr. Belg. Suppl.* **31**, 1–182.

BANKI, O. (1929). Die Entstehung der äusseren Zeichen der bilateralen Symmetrie am Axolotlei, nach Versuchen mit örtlicher Vitalfärburg. *Proc. 10th Intern. Congr. Zool.*, Budapest, 1927, 1ère part, p. 377–385.

BEETSCHEN, J. C. (1979). Recherches expérimentales sur la symétrisation de l'oocyte et de l'oeuf d'axolotl: facteurs conditionnant l'apparition précoce du croissant gris à la suite d'un choc thermique. *C.r. hebd. Séanc. Acad. Sci. Paris*, sér D, **288**, 643–646.

BENFORD, H. H. & NAMENWIRTH, M. (1974). Precocious appearance of the grey crescent in heat-shocked axolotl eggs. *Devl Biol.* **39**, 172–176.

BIENZ, M. & GURDON, J. B. (1982). The heat-shock response in *Xenopus* oocytes is controlled at the translational level. *Cell* **29**, 811–819.

BORN, G. (1885). Ueber den Einfluss der Schwere auf das Froschei. *Arch. mikrosk. Anat.* **24**, 475–545.

ELINSON, R. P. (1985). Changes in levels of polymeric tubulin associated with activation and dorso-ventral polarization of the frog egg. *Devl Biol.* **109**, 224–233.

FANKHAUSER, G. & GODWIN, D. (1948). The cytological mechanism of the triploidy-inducing effect of heat on eggs of the newt, *Triturus viridescens. Proc. natn. Acad. Sci. U.S.A.* **34**, 544–551.

FORD, C. C. & GURDON, J. B. (1977). A method for enucleating oocytes of *Xenopus laevis. J. Embryol. exp. Morph.* **37**, 203–209.

GAUTIER, J. & BEETSCHEN, J. C. (1983). Inhibition of protein synthesis elicits early grey crescent formation in the axolotl oocyte. *Wilhelm Roux' Arch. devl Biol.* **192**, 196–199.

GAUTIER, J. & BEETSCHEN, J. C. (1985). A three-step scheme for grey crescent formation in the rotated axolotl oocyte. *Devl Biol.* **110**, 192–199.

GERHART, J. (1980). Mechanisms regulating pattern formation in the amphibian egg and early embryo. In *Biological regulation and development*, vol. II (ed. R. F. Goldberger), pp. 133–316. New York: Plenum Press.

GERHART, J., UBBELS, G., BLACK, S., HARA, K. & KIRSCHNER, M. (1981). A reinvestigation of the role of the grey crescent in axis formation in *Xenopus laevis. Nature, Lond.* **292**, 511–516.

GRINFELD, S. & BEETSCHEN, J. C. (1982). Early grey crescent formation experimentally induced by cycloheximide in the axolotl oocyte. *Wilhelm Roux' Arch. devl Biol.* **191**, 215–221.

MCCARTHY, T. F. & SULLIVAN, M. X. (1941). A new and highly specific colorimetric test for methionine. *J. biol. Chem.* **141**, 871–876.

MALACINSKI, G. & CHUNG, H. M. (1981). Establishment of the site of involution at novel locations on the amphibian embryo. *J. Morph.* **169**, 149–159.

MERRIAM, R. W. (1971). Progesterone induced maturational events in oocytes of *Xenopus laevis*. II. Change in intracellular calcium and magnesium distribution at germinal vesicle breakdown. *Expl Cell Res.* **68**, 81–87.

PASTEELS, J. (1948). Les bases de la morphogenèse chez les Vertébrés anamniotes en fonction de la structure de l'oeuf. *Folia Biotheor.* **3**, 83–108.

PASTEELS, J. (1964). The morphogenetic role of the cortex of the Amphibian egg. *Adv. Morphogen.* **3**, 363–388.

SHIH, R. J., O'CONNOR, C. M., KEEM, K. & SMITH, L. D. (1978). Kinetic analysis of aminoacid pools and protein synthesis in Amphibian oocytes and embryos. *Devl Biol.* **66**, 172–182.

SIGNORET, J. & FAGNIER, J. (1962). Activation expérimentale de l'oeuf de pleurodèle. *C.r. hebd. Séanc. Acad. Sci. Paris* **254**, 4079–4080.

UBBELS, G. A. (1978). Symmetrization of the fertilized egg of *Xenopus laevis* (studied by cytological, cytochemical and ultrastructural methods). *Mem. Soc. Zool. France* **41**, 103–116.

UBBELS, G. A., HARA, K., KOSTER, C. H. & KIRSCHNER, M. W. (1983). Evidence for a functional role of the cytoskeleton in determination of the dorso-ventral axis in *Xenopus laevis* eggs. *J. Embryol. exp. Morph.* **77**, 15–37.

VILAIN, J. (1978). Maturation *in vitro* des ovocytes de *Pleurodeles waltlii* (Amphibien Urodèle). *Mem. Soc. Zool. France* **41**, 93–102.

VINCENT, J. P., OSTER, G. F. & GERHART, J. C. (1986). Kinematics of grey crescent formation in *Xenopus* eggs: the displacement of subcortical cytoplasm relative to the egg surface. *Devl Biol.* **113**, 484–500.

WALLACE, R. A., JARED, D. W., DUMONT, J. N. & SEGA, M. W. (1973). Protein incorporation by isolated amphibian oocytes: optimum incubation conditions. *J. exp. Zool.* **184**, 321–334.

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