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Cytoplasmic and cortical factors participating in cleavage furrow formation in eggs of three amphibian genera; *Ambystoma*, *Xenopus* and *Cynops*

By TSUYOSHI SAWAI[1]

From the Department of Biology, Faculty of General Education, Yamagata University, Japan

SUMMARY

A cytoplasmic factor and a cortical factor participating in cleavage furrow formation had been previously found in *Cynops* eggs (Sawai, 1972). These were investigated in the present study in *Ambystoma* and *Xenopus* eggs, by the method of cytoplasmic or cortical transplantation. Results were practically similar to those previously obtained with *Cynops*, indicating that in all the species of eggs during cleavage, the cytoplasm localizing along the cleavage furrow possessed furrow-inducing activity, and the cortex was competent to form the furrow in response to the activity of the cytoplasmic factor. In the present work, the species specificity of the two further factors was examined among *Cynops, Ambystoma* and *Xenopus* eggs, and the factors were found to act across species.

INTRODUCTION

The present author (1972) previously reported a cytoplasmic factor and a cortical factor involved in cleavage furrow formation in newt eggs. Preliminary results were also presented for the existence of these factors in frog eggs. The cytoplasmic factor was a transferable activity found only along the cleavage plane, and acting to induce the cortex to form the furrow. The cortical factor was defined by receptive capacity of the cortex to form the cleavage furrow in response to the cytoplasmic factor. The receptivity of the cortex first appeared at the animal pole region simultaneously with the onset of the first cleavage, advancing in a ring toward the vegetal pole with the leading tip of the furrow. In other words, in amphibian cleavage, the advance of the furrow from the animal toward the vegetal pole could be explained as the result of the propagation of both the cytoplasmic inductivity and the cortical receptivity hand in hand in the animal–vegetal direction.

The present experiments were aimed at examining whether such cytoplasmic

[1]*Author's address:* Department of Biology, Faculty of General Education, Yamagata University, Japan.

and cortical factors also exist in cleaving eggs of other amphibia, namely, the Mexican axolotl (urodele) and the African clawed frog (anura). Since the results were positive, the study was further directed to test the species specificity among eggs of the three amphibian species, including the Japanese newt (urodele).

MATERIALS AND METHODS

Preparation of egg

Spawning of fertilized eggs of *Ambystoma mexicanum* was stimulated by changing the temperature of culture water; the first stimulus being a sudden reduction of temperature to about 15 °C from usual rearing condition of about 22 °C and the next stimulus being gently returning temperature to about 22 °C. The first shock caused the male to discharge shuttlecock-shaped spermatophores, and concurrently the female to take up the floating spermatophore. After the completion of these breeding behaviours (which usually ended within about 24 h after the cold shock), the temperature was raised back to 22 °C. About 10 h later, the female began depositing fertilized eggs, and continued doing so for several hours.

Fertilized eggs of *Xenopus laevis* were obtained by hormonal stimulus. A male and a female frog were injected subcutaneously ca. 500 i.u. of chorionic gonadotoropin hormone in the dorsal side. After about 8 h, the female was clasped by the male and began laying fertilized eggs, continuing to do so for several hours.

Egg deposition of *Cynops pyrrhogaster* was also stimulated by the gonadotoropin. About 80 i.u. of the hormone was injected every other day into the abdomen of the female which had already taken up spermatophores in the fields. They began spawning after two to three injections, continuing to deposit eggs for about two weeks.

Removal of egg membrane

Ambystoma eggs were removed from the jelly coat with watchmaker's forceps, and then treated with about 0·1 % pronase E solution for one to two minutes to digest the vitelline membrane slightly. The weakened membrane was removed with two pairs of fine forceps. Jelly layers of *Xenopus* eggs were dissolved by treating them with about 2 % cysteine hydrochloride solution (pH 8·1 with NaOH) for several minutes. This treatment concurrently weakened the vitelline membrane, making its removal easy. The jelly capsule and the vitelline membrane of *Cynops* eggs were manually taken off with scissors and forceps, respectively.

For operations these naked eggs were put on a shallow depression in an agar bed in the bottom of a dish filled with Holtfreter's saline.

Transplantation of cytoplasm

Cytoplasm was transplanted using a capillary as described in a previous work

(Sawai, 1972). The capillary was inserted into donor eggs and brought close to the surface of the opposite side, where subcortical cytoplasm was sucked into it. The loaded capillary was pulled out, and inserted anew into the recipient egg. The cytoplasm was deposited so as to line the cortex opposite the point of entry. The volume of cytoplasm injected was 50–100 nl.

Cortical grafting

A part of the cortex was cut out from recipient eggs with a fine glass needle. The wound was covered with a cortical piece taken from the donor egg and slightly larger than the wound of the host. The donor cortex could be fused by pressing it along the edge of the wound of the recipient cortex with a fine glass needle (Sawai, 1974).

All operations were performed freehand under the stereomicroscope at room temperature (18–23 °C) and the recipient eggs were observed through at least two cycles of cleavage after the operations.

RESULTS

Furrow-inducing cytoplasm in Ambystoma *and* Xenopus *eggs*

In *Ambystoma* and *Xenopus* eggs during the first cleavage, the subcortical cytoplasm was taken either from the bottom of the cleavage furrow or from a non-furrow region. The cytoplasm was injected beneath the cortex in the animal hemisphere of eggs of the respective species in the early first cleavage stage. In each recipient a furrow was restrictedly induced at the surface of the injection site of the furrow cytoplasm, but not at the site of injection of the non-furrow cytoplasm (Figs 1, 2. Table 1). The furrow induction was first recognized by a concentration of pigment 5–15 min after the deposition of cytoplasm (Fig. 1A, B), then by a dipping in of the surface (C) and finally by a flattening of the induced furrow 10 min later, exposing unpigmented pale surface like that around the normal furrow (D). The size and the direction of the induced furrow were variable, depending on the volume and the orientation of deposited cytoplasm. Ordinarily, such a furrow induction occurred only once; but occasionally, it was repeated again in the next cleavage stage (Fig. 1E). This phenomenon was newly observed in the present study, whereas other events were practically similar to those described previously (Sawai, 1972).

Receptivity of cortex

The receptivity of the cortex for the furrow-inducing cytoplasm (FIC) was analysed mainly in the *Ambystoma* egg, in terms of its appearance and disappearance during the cleavage cycle, as previously seen in the *Cynops* egg. First, to establish the time of first appearance of receptivity, FIC was transplanted to the animal pole region of eggs before the first cleavage, in three stages shown in

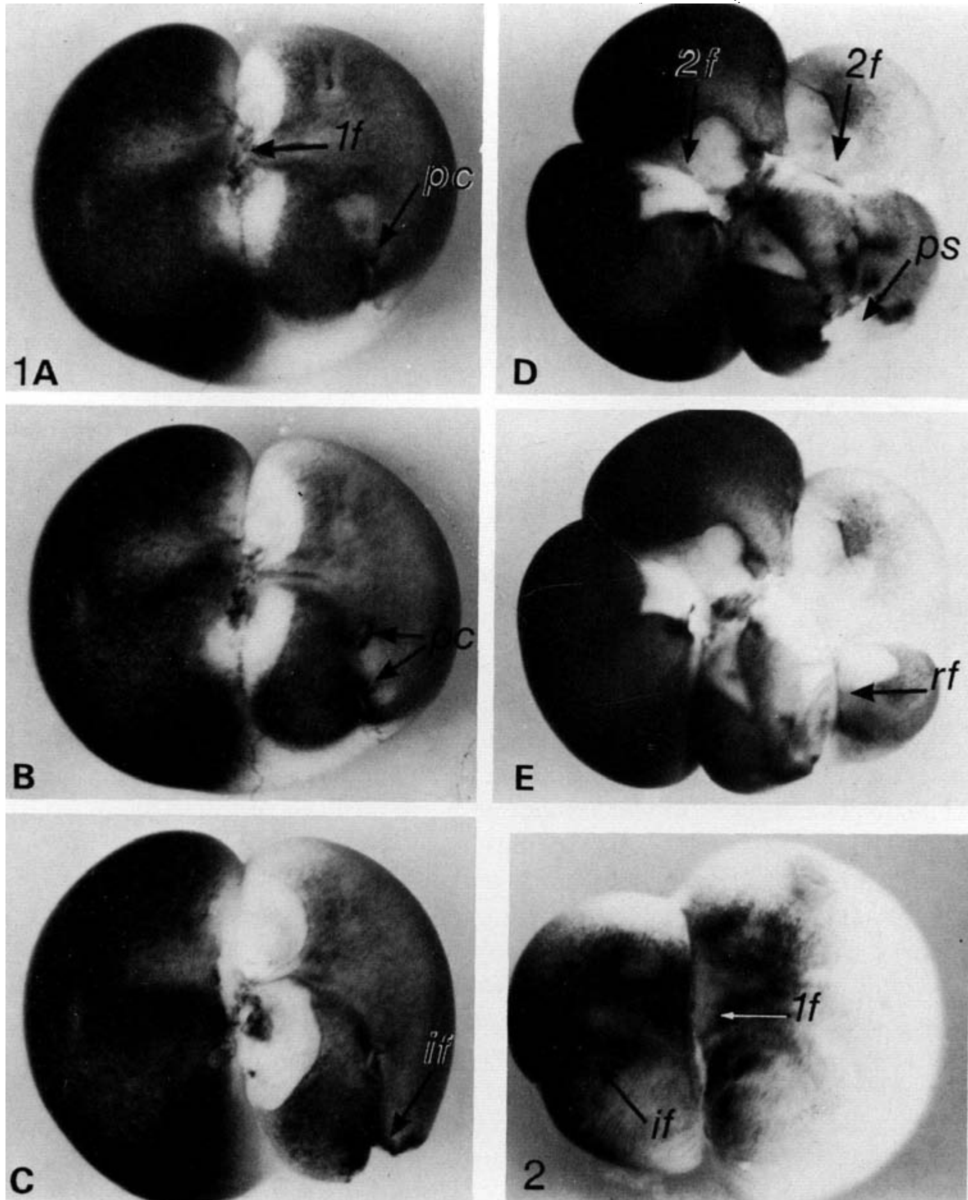

Fig. 1. Furrow induction in the *Ambystoma* egg by transplantation of the cytoplasm taken from the furrow bottom. The transplantation was made about 20 min after the onset of cleavage. Time (min) after the transplantation; (A) 15, (B) 25, (C) 50, (D) 110 (40 min after the start of the second cleavage) and (E) 135 min. 1f, 2f; the first and the second cleavage furrow. if; induced furrow. pc; pigment concentration. ps; pale surface. rf; re-induced furrow. about ×16.

Fig. 2. Furrow induction in the *Xenopus* egg. if, 1f; the same as in Fig. 1. about ×30.

Table 1. *Furrow-inducing activity of cytoplasm along the cleavage furrow toward the cortex in* Ambystoma *and* Xenopus *egg*

Genus	Kind of cytoplasm	Total no. of cases	Results		
			furrow induction	weak reaction*	no reaction
Ambystoma	furrow cytoplasm	29	20	1	8
	non-furrow cytoplasm	10	0	0	10
Xenopus	furrow cytoplasm	9	8	0	1
	non-furrow cytoplasm	9	0	0	9

* Pigment concentration occurred but no noticeable dent formation.

Table 2. *Furrow induction by FIC transplantation in three stages before the 1st cleavage, in* Ambystoma *egg*

Stage of recipient (min before 1st cleavage)	Total no. of cases	Results		
		furrow induction*	weak reaction	no reaction
within 30 min	12	9	1	2
over 30 but within 60	15	9	0	6
over 60	23	0	1	22

* In all cases the reaction occurred after the start of the 1st cleavage.

the extreme left column of Table 2. As shown in the table, when the transplantation was made within 60 min before the first cleavage (top and middle rows of data), furrow induction occurred, always after the onset of the first cleavage. However no induction occurred in the cases of transplantation more than 60 min before the first cleavage (bottom row). These results were essentially similar to the previous *Cynops* cases, indicating 1) that the cortical receptivity first appeared at the animal pole simultaneously with the initiation of first cleavage, and 2) that the FIC probably retained its activity for about 1 h at the injection site.

Next, to examine the change of the receptivity during the first cleavage cycle, FIC transplantation was made in each of three regions (a, e, v in Fig. 3) in each of three stages shown in Fig. 3A–C. The results (Table 3) indicate that the cortical area competent to respond to the FIC shifted from the animal pole toward the vegetal pole as the stage proceeded from A to C, i.e., with furrow

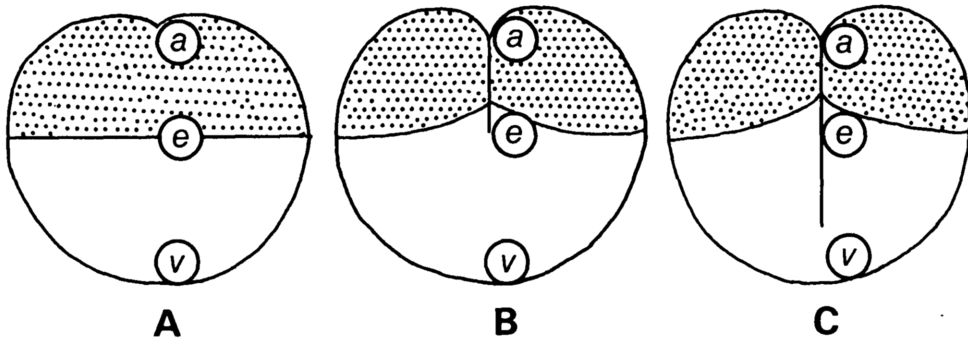

Fig. 3. Stages (A, B, C) and regions (a, e, v) in which the cortical receptivity was tested during the first cleavage in the *Ambystoma* egg. A; appearance of the furrow. B; furrow advance in about a half the egg circumference (about 30 min after A). C; shortly before the complete closure of the furrow (about 60 min after A, i.e., 40 min before the second cleavage). (a), animal pole; (e), equatorial; (v), vegetal pole region.

Table 3. *Propagation of cortical receptivity in the animal-vegetal direction in* Ambystoma *egg during the 1st cleavage*

			Results			
Stage of recipient	Regions tested	Total no. of cases	furrow induction	weak reaction	no reaction	% of positive
	animal	10	7	0	3	70
A	equator	23	17	1	5	78
	vegetal	10	0	0	10	0
	animal	10	1	1	8	20
B	equator	23	14	4	5	77
	vegetal	8	3	3	2	75
	animal	8	0(3*)	0	5	0(38*)
C	equator	10	0(2*)	0	8	0(20*)
	vegetal	10	5	1	4	60

* Cases reacted after the start of the 2nd cleavage.

advancement. Results at stage C further show that the same cycle is repeated in the second cleavage period. These changes in cortical receptivity in *Ambystoma* were almost identical to those in the *Cynops* egg.

Lastly, to investigate more precisely the propagation of the cortical change, the time of the furrow induction was compared at two regions of a single egg in the early first cleavage period, by simultaneously depositing FIC at two positions. The comparison was made in the meridional and the latitudinal directions (Fig. 4): in the former, in two sets, i.e., between the animal and the equatorial regions (Fig. 4-IA), and between the equatorial and the vegetal regions (IB); and in the latter direction, between two regions on the equator

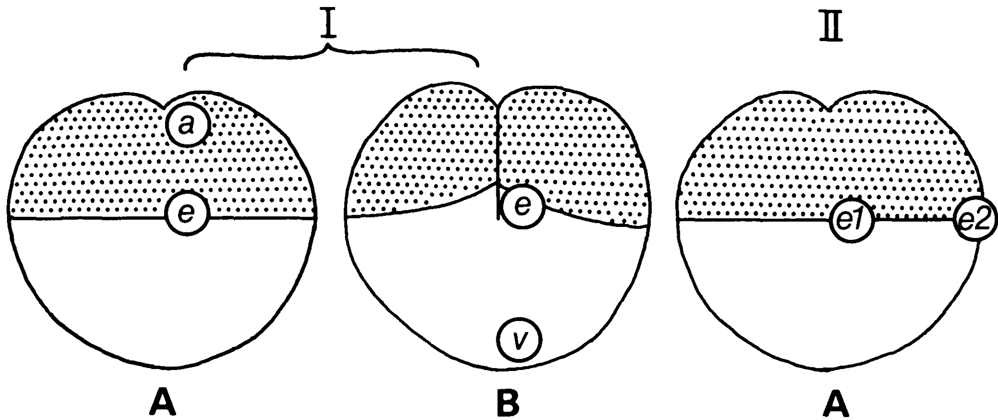

Fig. 4. The time of furrow induction was compared between two regions of one egg by simultaneous FIC injection, in the stage A and B defined in Fig. 3. I. Meridional comparison between the animal (a) and equatorial regions (e) in the stage A, and between the equatorial (e) and the vegetal regions (v) in the stage B. II. Latitudinal comparison between the equatorial region close to (e1) and most distant from the cleavage plane (e2) in the stage A.

Table 4. *Results in the comparison of the induction time between the two regions shown in the Fig. 4*

Stage of host	Two regions compared*	Total no. of experiment	Positive no. in both regions	Comparison of induction time		
		(I) *Meridional comparison*		Earlier in (a)	Simulta-neous	Earlier in (e)
A	(a) and (e)	24	15	12	3	0
				Earlier in (e)	Simulta-neous	Earlier in (v)
B	(e) and (v)	11	6	6	. 0	0
		(II) *Latitudinal comparison*		Earlier in (e1)	Simulta-neous	Earlier in (e2)
A	(e1) and (e2)	24	16	0	16	0

* (a), (e), (v); see Fig. 4.

(Fig. 4-II). Results of the meridional direction (Table 4-I) showed that the receptivity appeared in the order of animal, equatorial and vegetal regions, during the first cleavage. Results of the latitudinal direction (Table 4-II) showed that the reaction on the same level occurred at the same time.

With regard to the strict relationship between the time of the furrow induction by FIC and the advance of the cleavage furrow, it can be said that in all positive cases of above-mentioned experiments, the furrow induction by FIC always occurred slightly after the advancing tip of the normal furrow had traversed the same level as the transplant position, never before that time.

In *Xenopus* eggs, a detailed test of the cortical receptivity could not be completed, because the egg size (ca. 1 mm diameter) was much too small for the test and the process of division was too fast, as compared with the egg of the *Ambystoma* (2·2 mm) and the *Cynops* (1·8 mm). Therefore, the cortical

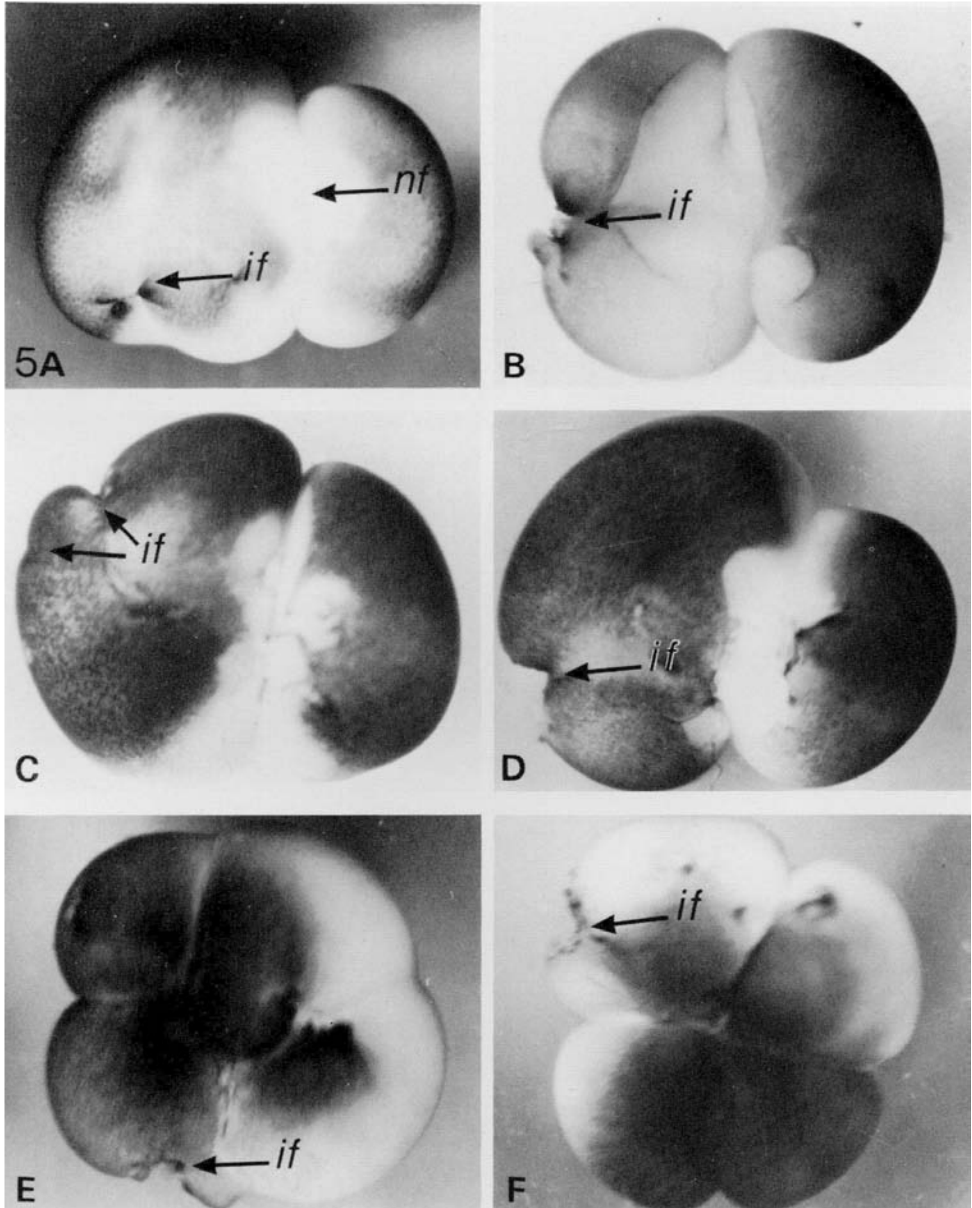

Fig. 5. Photographs showing the furrow induction by FIC of different species. Combinations of recipient-donor in each: *Cynops-Ambystoma* (A), *-Xenopus* (B); *Ambystoma-Cynops* (C), *-Xenopus* (D); *Xenopus-Cynops* (E), *-Ambystoma* (F). if; induced furrow, nf; normal furrow. A–D; about ×16. E, F; about ×25.

change in the *Xenopus* egg was examined only roughly, by comparing the time of the appearance of receptivity between the animal and the vegetal half, and between the two points at the equator. In the former comparison, six cases which were positive in both regions of a single egg were obtained; the reaction occurred earlier in the animal half in four cases and simultaneously in two cases. The comparison on the same level gave results that the reaction occurred simultaneously in all six cases positive in both regions. These results were practically the same as those in the *Ambystoma* egg.

Species specificity of the cytoplasmic and the cortical factors

To test the species specificity of the cortical and the cytoplasmic factors, FIC transplantation was made by reciprocally exchanging the host among the three amphibian eggs, injected in the animal region in the early first or the second cleavage stages. The results were positive in all six combinations (Table 5, Fig. 5). A control experiment reciprocally transplanting non-furrow cytoplasm gave negative results in all the combinations (Table 5). As another trial for the same purpose, cortical grafting was made between different species, in a position in the future path of the furrow. This trial succeeded in six cases in only one combination, namely, between *Cynops* (donor) and *Ambystoma* eggs (host) (Fig. 6). In the successful cases, the graft was at first pulled toward the furrow tip of the host (Fig. 6B), after which time the host furrow passed through the graft in a complete division, in three cases (C, D). But a division of the graft was incomplete in two cases or not at all in one case, in which the host furrow once ceased to progress and a little later appeared on the median plane of the host

Table 5. *Species specificity of FIC*

Recipient	Donor	Kind of cytoplasm	No. of cases	Results: Furrow induction	Results: Weak reaction	Results: No reaction
Ambystoma	*Xenopus*	FIC	8	7	0	1
		non-FIC	8	0	0	8
	Cynops	FIC	18	11	2	5
		non-FIC	7	0	0	7
Xenopus	*Ambystoma*	FIC	5	5	0	0
		non-FIC	10	0	0	10
	Cynops	FIC*	9	7	0	2
		non-FIC	8	0	0	8
Cynops	*Ambystoma*	FIC	7	6	0	1
		non-FIC	15	0	0	15
	Xenopus	FIC*	5	5	0	0
		non-FIC	8	0	0	8

* A part of data in these combinations was previously reported (Sawai, 1972).

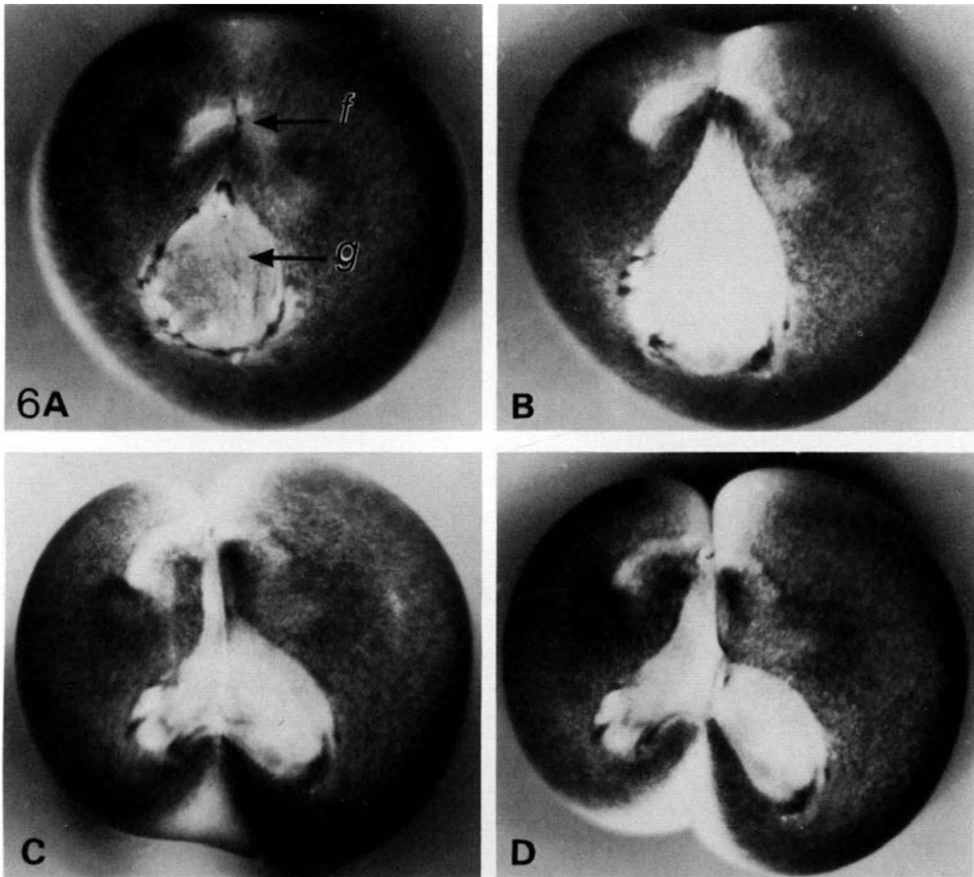

Fig. 6. Cortical grafting between *Ambystoma* (host) and *Cynops* (donor), and furrow formation in the graft. A, just after grafting; B, 15 min after A, transplant was pulled toward the host furrow tip; C, 30; and D, 35 min after A, host furrow travelled on transplant. f; host furrow. g; graft. about ×17.

beyond a part or the whole of the graft. In other combinations, surgery failed mainly because of the difference in elastic properties of the cortex between the different species.

DISCUSSION

In the cleavage of amphibian eggs, Dan & Kojima (1963) reported that in the cortex certain preparations necessary for furrow formation preceded the advancing tip of the visible furrow, in *Cynops pyrrhogaster*. Furthermore, Kubota (1969) found that the furrow plane was determined by some special endoplasm underlying the cortical layer, in *Rana nigromaculata*. Sawai, Kubota & Kojima (1969) and Sawai (1972) further investigated and established that the furrow arose by an interaction of the cytoplasmic cleavage factor with the cortical one.

The present study additionally demonstrates the existence of the same factors in eggs of *Ambystoma* and *Xenopus*, and further demonstrates that the two factors interact across three amphibian genera.

Concerning the cytoplasmic factor, although a similar factor had also been suggested in echinoderm eggs (Rappaport & Conrad, 1963; Rappaport & Ebstein, 1965), the properties in a biochemical sense have scarcely been analysed in either echinoderm or amphibian eggs. However, it has been found that the factor is restrictively localized in the cleavage plane. Considering this localization in light of the general fact that the division plane of animal cells is determined by the location of the mitotic apparatus, the mitotic apparatus may play a role in distributing the cytoplasmic factor along the prospective cleavage plane.

Concerning the cortical factor, its appearance and propagation are roughly synchronized with other cortical changes such as the second surface contraction wave (Hara, 1971; Hara, Tydeman & Hengst, 1977; Yoneda, Kobayakawa, Kubota & Sakai, 1982; Sawai, 1982) or the increase in stiffness (Selman & Waddington, 1955; Sawai & Yoneda, 1974). These changes must reflect some cortical preparation indispensable for furrow formation. Such changes also occur cyclically in non-nucleated egg fragments, roughly synchronized with the division cycle of the nucleated half (Sawai, 1979; Hara, Tydeman & Kirschner, 1980; Sakai & Kubota, 1981). This cyclic behaviour by the non-nucleated fragment implies that the cortex may be given a competence to form the furrow by a rhythmic change in some endoplasmic activity, independent of the nucleus. Only after this modification, may the cortex be able to form the actual cleavage furrow on receiving a stimulus from the cytoplasmic factor which is distributed along the cleavage plane by the mitotic apparatus.

On the other hand, electron microscopic observations have shown that a bundle of actin-like filaments directly takes part in furrow formation (Bluemink, 1970, 1971; Selman & Perry, 1970; Kalt, 1971; Perry, John & Thomas, 1971; Singal & Sanders, 1974; Kubota, 1979). In an effort to relate this fact to the above-mentioned cortical changes, the present author considers it plausible that the cortical change in question reflects some structural modification of the cortical layer, such as an establishment of a network of microfilaments, whereas the arrangement of such filaments in a definite direction may depend on the cytoplasmic factor. This idea is supported by electron microscopic observations in sea urchin eggs, in which the egg surface is underlined by a network of filaments at approximately the time of the increase of cortical stiffness, that is, just before the start of cleavage. Subsequently, the filaments become arranged in the cleavage plane as a bundle parallel to it (Usui & Yoneda, 1982), seemingly as a later event.

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