Development 104, 415–422 (1988)
Printed in Great Britain © The Company of Biologists Limited 1988

Control of cell cycle length in amphibian eggs: evidence for a temporal relationship between the nucleus and egg cytoplasm

C. AIMAR

Laboratoire d'Immunologie comparée, UA 1135, Université P. & M. Curie, 4 Place Jussieu, 75230 Paris Cedex 05, France

Summary

The role of the cytoplasm and nucleus in the control of the length of the division cycle was investigated in *Pleurodeles waltl* eggs. Injection of spermine into enucleated eggs showed that the ability to cleave was not restricted just to the period of normal cytokinesis (T = 1·0) but was possible throughout most of the first egg cycle. The cytoplasmic components required for cytokinesis seem to increase progressively during the first division cycle.

Nuclear transfer experiments indicated that the timing of cleavage was normal only when the nucleus and egg cytoplasm were reassociated between T = 0·0 (activation time) and T = 0·50. Delayed associations, after T = 0·50, provoked an alteration in the chronology of first cleavage and led to abnormal embryonic development. In the absence of a nucleus, the egg cycle seemed to stop at T = 0·50. These different observations suggest that the normal timing of cleavage not only depends on a 'cytoplasmic clock' but is also determined by an isochronous nucleocytoplasmic relationship during the early phase of egg development.

Key words: cell cycle, nucleocytoplasmic relationship, amphibian egg, nucleus, *Pleurodeles waltl*, spermine, cytokinesis, cleavage, egg.

Introduction

After a relatively long period immediately following fertilization, the amphibian egg undergoes a series of rapid divisions with a defined rhythm. During each of these division cycles, characteristic changes in the egg cortex (Vacquier, 1981) occur with a precise chronology which is directly related to the timing of cleavage. Such changes include a series of surface contraction waves (Hara *et al.* 1980; Yoneda *et al.* 1982), and modifications in the rigidity of the egg cortex (Sawai & Yoneda, 1974; Yoneda *et al.* 1982).

At the end of the cell cycle, during the formation of the cleavage furrow (cytokinesis), there is a reorganization in the egg cortex structure. This includes a rearrangement of actin filaments giving rise to the contractile ring, originally described in sea urchin eggs by Schroeder (1981), and a concentration of membranous organelles essential for the constitution of the plasma membrane (Ohshima & Kubota, 1985; Byers & Armstrong, 1986).

The cyclical cortical patterns observed in the fertilized egg also occur in enucleated egg fragments (Sawai, 1979; Sakai & Kubota, 1981), but with a rhythm different from that observed in nucleated eggs (Sakai & Kubota, 1981; Sakai, 1982; Shinagawa, 1983). These observations suggest that the cyclic changes characteristic of the division cycle constitute an endogenous cytoplasmic activity regulated by a 'cell cycle oscillator' (Newport & Kirschner, 1984; Shinagawa, 1985) which can be influenced by the presence of nuclear elements.

In the case of cytokinesis it has not been clearly established whether the ability of the cortex to form a cleavage furrow is permanent or limited to a precise period of the cell cycle like the other cortical events that are regulated by cytoplasmic factors. In addition, the temporal relationships between the nucleus and cytoplasm and their influence on the timing of cleavage in fertilized eggs have yet to be fully investigated.

In the present work, I have studied the timing of cleavage and the development of activated enucleated *Pleurodeles waltl* eggs. Cell division was induced by either transferring a somatic nucleus into such eggs or injecting a polyamine (spermine). Polyamines can cause polymerization of actin (Oriol-Audit, 1978, 1980; Grant & Oriol-Audit, 1985).

These results show that, in enucleated eggs, the

capacity to cleave is established soon after the time of activation. Nevertheless, the formation of a furrow-like structure in response to the polyamine was most rapid when the injection time corresponded to the cleavage time of fertilized eggs. The nuclear transplantation experiments involving implantation of nuclei at different times of the first cycle demonstrated that the timing of cleavage is normal only when the nucleus and egg cytoplasm are reassociated between the activation time and the half time of the first division cycle. Later associations provoke delayed cleavages.

Materials and methods

Egg preparation

Animals (*P. waltl* and *X. laevis*) were supplied by SEREA (CNRS). Unfertilized eggs of *P. waltl* were obtained by stimulating adult females with an intracoelomic injection of pituitary gonadotrophin (Choay). Egg envelopes were removed enzymically with 2% (w/v) cysteine and 0·1% (w/v) papain in Steinberg medium (1957), pH 8·0. Unfertilized eggs were activated by an electric shock (70 V, 80 μF) in diluted Steinberg medium (1/10, v/v).

Enucleation of unfertilized eggs

Dejellied eggs were placed in Steinberg medium and enucleated within 1 h following activation. The location of the female nucleus can be identified by a small black mark present at the animal pole of the egg. The nuclei were removed manually with a fine tungsten needle. Following enucleation, eggs were left for 30 min in double-strength Steinberg medium to permit wound healing and then transferred to normal Steinberg medium.

The success of the enucleation operation was determined by examining the exovate for the presence of a nucleus. In addition, some enucleated eggs from each batch were kept aside to verify that no cleavage occurred within 10 h following enucleation. In control eggs, first cleavage furrow normally formed within 6 h after activation.

Injection protocol and nuclear transfers

Spermine tetrahydrochloride (Sigma Chemicals) was prepared as a stock solution of 40 mM in 100 mM-Trizma, pH 7·5. Injection of 100 nl of this solution into an egg corresponded to an intracellular spermine concentration of 2 mM, which is four times greater than the concentration necessary to induce precocious furrowing in *X. laevis* egg (Grant *et al.* 1984).

Injections were done in the animal hemisphere of enucleated eggs at a depth of 0·3–0·5 mm using a calibrated glass micropipette with an internal diameter of 30 μm.

The transplantation procedure was essentially that of Briggs & King (1952), as modified for Urodeles eggs (Signoret *et al.* 1962; Aimar, 1972). Grafted nuclei were obtained from blastula or neurula stage embryos of the newt *P. waltl* or the frog *X. laevis*. Embryonic cells were dissociated by incubating them in EDTA for 1 h. Nuclei were grafted using a glass micropipette with an internal tip diameter of 45 μm.

In these particular experiments, two important modifications were made to the original protocol. Recipient eggs were enucleated manually, as described above, and not by u.v. irradiation. As indicated in text and figures, nuclei were transferred into recipient eggs at variable times after activation.

Following spermine injections and nuclear transfers, eggs were incubated in Steinberg medium at 24°C.

Cytological examination

Eggs and embryos were fixed in Smith's fluid, dehydrated through an alcohol series, embedded in paraplast and then serially sectioned (7 μm sections). Nuclei were stained with Dapi (4,6-diamino 2-phenylindole dihydrochloride) and cytoplasmic structures were enhanced with light green dye.

Biological assays

For each experimental group, the development of eggs ($n = 25$) was checked every 15 min using a dissecting microscope and the time of the first cleavage scored for each egg. The timing of subsequent divisions was determined using a video camera connected to a time-lapse recorder (Panasonic).

The time of the first division for a group is given as the time required for 50% of the eggs to develop a cleavage furrow. The time of first division for activated unfertilized eggs, used as a control group (Tc), were compared with the time of first division for the corresponding experimental group (Te). Variations in the length of the first division cycle are expressed according to the following formula: $Tc-Te/Tc \times 100$, as previously described (Aimar *et al.* 1986). A positive value corresponds to an advance in the time of first cleavage relative to controls. To allow comparisons between batches of eggs for different experiments, actual times were normalized. The time of activation was set as $T = 0{\cdot}0$ and the time of first cleavage as $T = 1{\cdot}0$.

Results

(A) *Cytokinesis in enucleated* P. waltl *eggs injected with spermine*

(1) *Control eggs*

Activated enucleated eggs of *P. waltl* exhibited the same cortical modifications as fertilized eggs. These included the disappearance of maturation spots (Fig. 1A) and a modification in the cortical pigment pattern. However, like egg fragments of *X. laevis* (Hara *et al.* 1980), enucleated *P. waltl* eggs did not divide.

As in the case of *Rana pipiens* (Briggs & King, 1953), a few enucleated eggs were observed to fragment into noncohesive spheres of cytoplasm about 20 h after activation. At this time, control eggs were reaching the blastula stage.

(2) *Spermine-injected eggs*

To examine whether enucleated eggs maintained the

capacity to cleave, they were stimulated with spermine (18 experiments, 20–25 eggs/replicate).

When spermine was injected at T = 0·04, cleavage occurred after a delay of 150 min (Fig. 2). This delay increased to 180 min when injections were done at T = 0·2. Thereafter, it progressively decreased. At

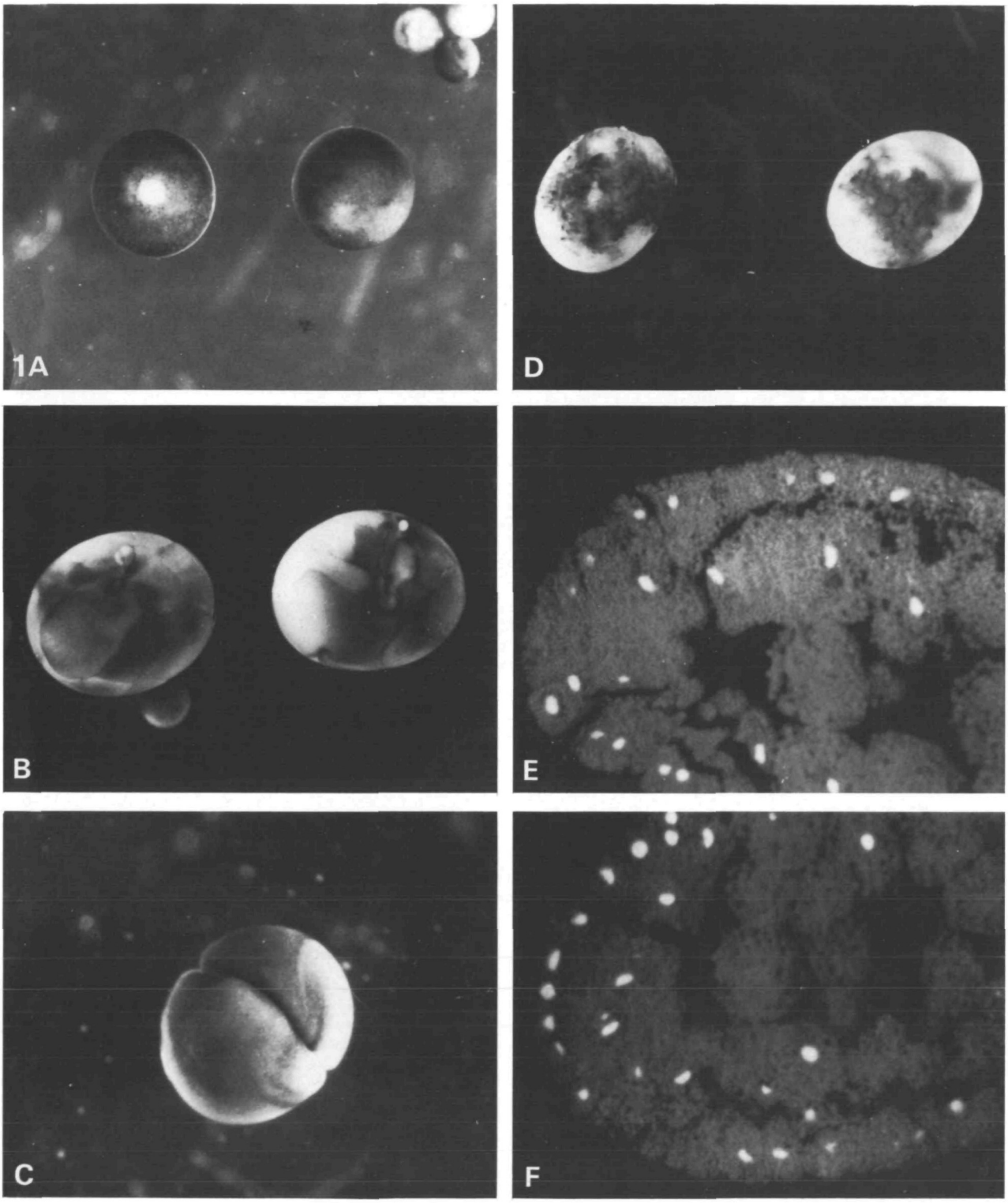

Fig. 1. Spermine injection and nuclear transfers in *P. waltl* enucleated eggs. (A) right: enucleated egg; left: control egg (×16). (B) Furrow formation after spermine injection (at T = 0·45) in enucleated eggs (×25). (C) First cleavage furrow after nuclear transplantation at T = 1·0 (×25). (D,E) Blastulae obtained from nuclear transplantation at T = 1·0. Note in E the abnormal number of nuclei. (F) Control blastulae (DAPI). (D: ×18; E and F: ×80).

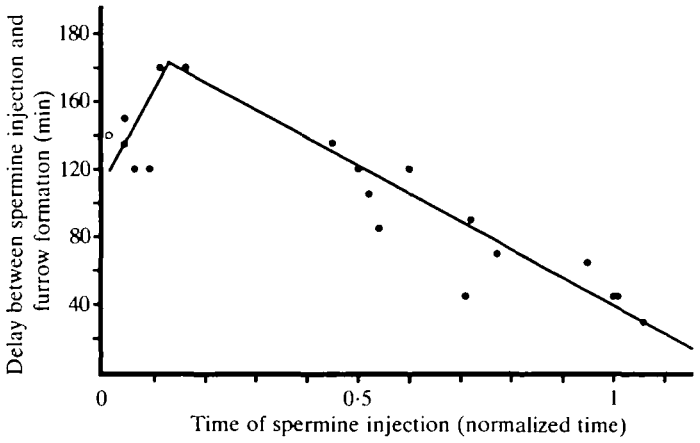

Fig. 2. Delay in time of cytokinesis following injections of spermine in *P. waltl* eggs. ●, Enucleated eggs; ○, nucleated eggs.

T = 0·5, the delay was 120 min. When enucleated eggs were injected at the time corresponding to cleavage in control eggs (T = 1·0), cleavage-like furrows formed about 30 min later.

Furthermore, the presence of the female pronucleus did not alter the time of furrow formation in spermine-injected eggs. In such eggs, injected before T = 0·1, the delay in furrow formation was the same as that observed in enucleated eggs (Fig. 2).

These results indicated that eggs are able to undergo furrowing activity even in the absence of nuclear elements. In addition, the rapidity at which an egg formed a furrow increased at a constant rate from T = 0·2 to T = 1·0.

The morphological aspect of the egg cortex changed depending on when the polyamine was injected. With early injections (T = 0·04 to T = 0·21), there was an apparent blebbing or folding of the egg surface immediately overlying the injection site in about 60 % of the eggs. Scanning electron microscopy revealed that this region contained clumps of elongated microvilli (data not shown). In the other cases, and in eggs injected later than T = 0·2, a fragmentation furrow formed (Fig. 1B). It appeared similar to that described for activated unfertilized eggs of amphibia (Briggs & King, 1953; Aimar *et al.* 1986).

Cytological examinations of fragmented eggs revealed that only the superficial regions of the animal hemisphere were involved in furrow formation. Such furrows rarely penetrated more than one third of the egg diameter and usually regressed over the next few hours. Centrioles or aster-like figures were never observed.

(B) *Role of the nucleus in the determination of the cleavage timing*

The regulation of cleavage time by the nucleus present in the egg at the beginning of development was tested in two ways. First, the ploidy of the nucleus was varied using isochronic nucleocytoplasmic associations and second, the temporal relationship between the nucleus and cytoplasm was altered by using heterochronous transfers.

(1) *Isochronous associations: effect of nuclear ploidy*

As shown in Table 1, six combinations (20 eggs per replicate) were prepared just after egg activation. In the first two experiments, used as control, eggs were enucleated. In the absence of any other procedure (exp. 1), no cleavage occurred and the eggs lysed by 20 h. However, as previously observed, in spermine-injected eggs, transient furrows formed about 2·5 h after injection and then regressed (exp. 2).

If the egg cytoplasm was associated with a haploid

Table 1. *Summary of development of* P. waltl *eggs following different isochronous nucleocytoplasmic combinations*

Experiment number	Nucleo-cytoplasmic combinations	Characterization of eggs			
		Ploidy	Cleavage	Timing of the first cytokinesis (min)	Development
1	no nucleus	–	No	–	None
2	no nucleus + spermine (40 mM)	–	Pseudocleavage* (fragmentation)	150	abortive*
3	♀ pronucleus	N†	Pseudocleavage	360	abortive
4	♀ pronucleus + spermine (40 mM)	N	Pseudocleavage	150	abortive
5	Blastula nucleus	2N	Cleavage	360 Then every 60 min	Normal
6	Fertilization nucleus (control eggs)	2N	Cleavage	360 Then every 60 min	Normal

* Pseudocleavage; abortive development: see explanations in the text.
† N, haploid number of chromosomes.

nucleus (female pronucleus, exp. 3), a fragmentation furrow appeared about 6 h after activation, but such eggs did not develop. The time of this pseudocleavage corresponded to the first cleavage in fertilized eggs. In contrast, when eggs containing a haploid nucleus were injected with spermine, fragmentation occurred 2·5 h after the injection (exp. 4) as it did in enucleated eggs.

Recombination of egg cytoplasm with a diploid nucleus was performed either by transferring a diploid nucleus from a blastula cell into an enucleated egg or by natural fertilization. In both of these cases (exps 5 & 6), a cleavage furrow formed after 6 h and development progressed normally.

(2) *Heterochronous reassociations*

Experimental eggs were activated at the same time ($T < 0{\cdot}10$) as unfertilized control eggs, then enucleated and injected with a diploid nucleus. These nuclei were taken from endodermal cells of *P. waltl* embryos. Nuclear transplantations (227 operated eggs) were done in series ($n = 15$) from $T = 0{\cdot}04$ up to the time ($T = 1{\cdot}02$), when controls were at first pseudocleavage.

(a) *First division cycle.* In each series, about 65 % of the injected eggs divided. This percentage was independent of the time of the nuclear transfer or the origin of the nucleus (blastula or neurula embryos). A cleavage furrow even formed when the nucleus was reassociated with the egg cytoplasm as late as 6 h after activation (Fig. 1C,D).

Relative to controls, there was no change in the timing of the first division when the nucleocytoplasmic recombinations were made during the first half of the division cycle, from $T = 0{\cdot}04$ to $T = 0{\cdot}55$. In all of these cases, injected eggs cleaved at the same time ($T = 1{\cdot}0$) as controls. Similar results were obtained with nuclear transfers in eggs containing a haploid nucleus (Fig. 3A).

In contrast, nuclear graftings performed after $T = 0{\cdot}50$ produced first cleavages occurring at times significantly different from controls. The delay in cleavage was greater when injections were done later in the first division cycle. Nuclear transfers at $T = 0{\cdot}70$ and $T = 1{\cdot}0$ provoked, respectively, 18 % and 48 % increases in the duration of the first cycle.

To determine the biological significance of these delays, the period between the time of the nuclear transfer and that of first furrow formation was checked for each experiment (Fig. 3B). For experiments performed before $T = 0{\cdot}50$, this delay progressively decreased such that operated eggs always cleaved at the same time as controls. However, for all nucleocytoplasmic combinations performed after

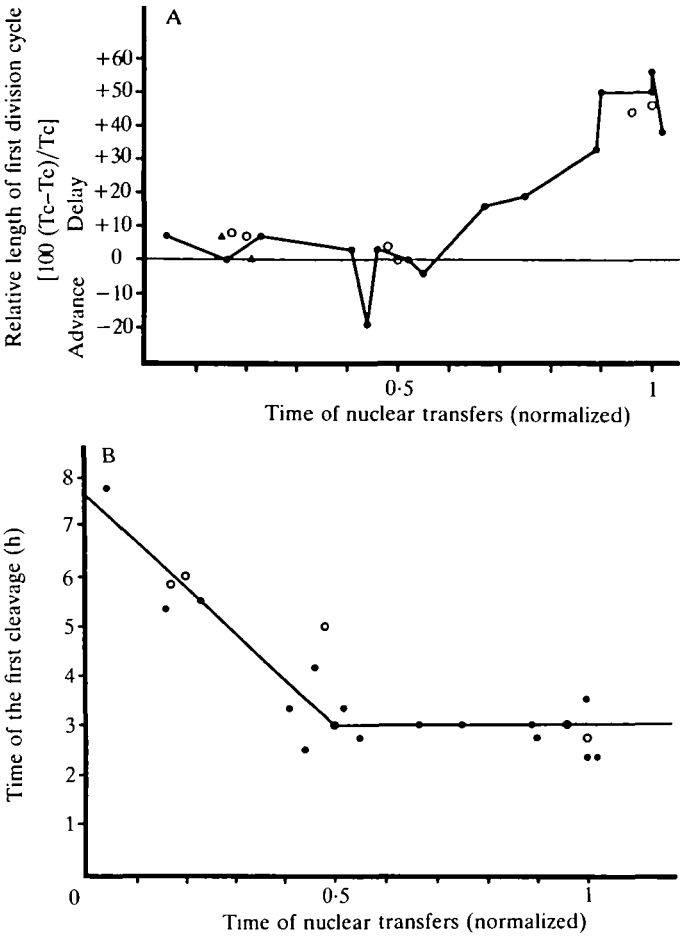

Fig. 3. (A) Variations in length of the first division cycle following nuclear transfers into enucleated *P. waltl* eggs at different times after activation. These variations are expressed as 100(Tc–Te)/Tc (see methods); positive and negative values correspond respectively to an advance and a delay in cleavage time relative to controls. The line at the 0 of the ordinate corresponds to cleavage time of controls. ●, *P. waltl* nuclei; ○, *X. laevis* nuclei; ▲, *P. waltl* nuclei in non-enucleated eggs. (B) Delay in time of first furrow formation following nuclear transfers in *P. waltl* eggs. ●, *P. waltl* nuclei; ○, *X. laevis* nuclei.

$T = 0{\cdot}50$, this period was relatively constant (180 min).

In addition, species specificity of the grafted nuclei was examined using heterospecific transfers of *X. laevis* nuclei into *P. waltl* eggs. The data obtained with heterospecific transfers were similar to those described above for homospecific transplantations suggesting no species specificity (Fig. 3A,B).

(b) *Segmentation phase.* Using a time-lapse recorder, the timing of later cleavage cycles in operated eggs was checked. The length of the cycle varied between 30 and 150 min for the second cycle and 45 and 90 min for the third and fourth cycles. The duration of these divisions was identical to that of

Table 2. *Development of embryos issued from nuclear transplantations*

Experiment	Time of nuclear transplantation normalized	Number of eggs	Developmental stages reached (% of injected eggs)			
			Blastula	Gastrula	Neurula	Postneurula
I	0·22	31	58	29	6	6
II	0·31	44	32	18	4	4
III	0·42	25	64	36	12	8
IV	0·88	80	57	7	5	0
V	0·94	49	59	6	4	0
VI	1·04–1·11	98	81	19	0	0

controls and not related to the time of nuclear transfer. Moreover, when injections were done after T = 0·50, about half of the eggs passed through a 3-cell stage. Irrespective of the segmentation pattern, all eggs reached the blastula stage about 30 h after activation.

(c) *Postblastular development.* Subsequent development of eggs depended on the time at which the nuclear grafts were made. In a total of six experiments (327 eggs), gastrulation was observed in more than 50 % of the eggs operated on before T = 0·50, but in only 20 % of eggs operated on after T = 0·50. Arrested blastulas, resulting from late transfers, exhibited severe abnormalities. The nuclei were distributed irregularly and were often only present in ectodermal cells (Fig. 1E).

On average, 25 % of the gastrulas, whatever their origin, developed into neurulas. Nevertheless, embryos from late nuclear graftings were lethal at this stage, whilst 80 % of the embryos from early nuclear transplantations continued to develop (Table 2).

Discussion & Conclusions

(A) *Furrowing activity in enucleated eggs*

In amphibians, the ability of eggs to cleave depends on nuclear material present in the germinal vesicle of oocytes (Smith & Ecker, 1969; Heidemann & Kirschner, 1975*b*). After oocyte maturation, this nuclear material becomes dispersed in the egg cytoplasm (Imoh, 1984). However, if nonfertilized eggs are enucleated (ablation of the female pronucleus) no division occurs (Briggs & King, 1953). In these eggs, as in activated eggs lacking the material that originates from the germinal vesicle, some endogenous cyclic activities persist such as oscillations of the so-called 'Maturation Promoting Factor' (MPF) and kinase activities (Hara *et al.* 1980; Dabauvalle *et al.* 1988).

Our data show that the stimulation of an enucleated egg by spermine can initiate the formation of a cleavage-like furrow. This pseudocleavage is comparable to that induced by calcium (Hollinger & Schultz, 1976), polycations and other polyamines (Gingell, 1970; Grant *et al.* 1984). Thus, it appears that the cleavage capacity depends solely on the contractile system present in the egg cortex (Franke *et al.* 1976; Vacquier, 1981; Merriam & Sauterer, 1983).

The furrows formed after stimulating the enucleated egg with spermine were transient and abnormal. Stabilization of furrows seems to require the presence of functional centrioles. Karyoplasts free of centrosomes are unable to induce cytokinesis in *X. laevis* eggs (Karsenti *et al.* 1984). In contrast, free centrosomes (Heidemann & Kirschner, 1975*a*; Sluder, 1979; Karsenti *et al.* 1984) or centrosomes associated with somatic nuclei or male pronuclei (Table 1) can induce egg division. Thus, although the capacity of the egg to cleave appears to be an autonomous characteristic of the cortex, development of the normal cleavage pattern and temporal stabilization of cleavage furrows depend on the participation of both the nucleus and centriolar or astral material. Such a requirement is in agreement with the hypothesis that a cleavage message travels along the astral rays and induces cleavage furrow formation (Rappaport, 1971; Inoue, 1981; Mitchison, 1984).

Since cytokinesis occurs at the end of cell division, one might expect that the cortex's capacity to furrow would be limited to this phase. As the present study demonstrates, the capacity of the egg to cleave is established a short time after activation and then persists during the entire length of the first division cycle. Nevertheless, this capacity is somewhat variable, showing a minimal response at the time of the second polar body extrusion, which marks the end of meiosis at T = 0·25, and a maximal response at the time of first cleavage. This observation suggests that there is a gradual accumulation of cytoplasmic components required for cleavage. This may be explained by variations in polyamine levels influencing the time of cleavage (Kusunoki & Yasumasu, 1976). Since the polyamines can induce polymerization of actin *in vitro* (Oriol-Audit, 1978, 1980; Grant *et al.* 1983), they may play a direct role in cytokinesis in amphibian eggs by provoking the reorganization of cortical actin in the contractile ring (Oriol-Audit, 1980; Grant *et al.*

1984; Grant & Oriol-Audit, 1985). Furthermore, the concentration of polyamines reaches a maximum at the time of first cleavage in *P. waltl* (Oriol-Audit *et al.* unpublished data).

(B) *Nucleocytoplasmic relationship and the timing of cleavage*

The present data indicate that cleavage and development proceed normally after the transfer of somatic nuclei into enucleated eggs between T = 0·0 and T = 0·50. Similar observations have been made in *Rana pipiens* eggs (Subtelny, 1969). In addition, we demonstrate that nuclear transplantations performed after T = 0·50 and as late as the second division cycle, i.e. 6 h after activation, also support cleavage in *P. waltl* eggs.

However, there are differences in the timing of division. Eggs operated on before T = 0·50 cleaved at the same time as controls, thereby providing further evidence that a cytoplasmic clock operating during this period regulates the timing of amphibian egg development (Newport & Kirschner, 1984).

In the case of late recombinations (T > 0·50), irrespective of the origin (*P. waltl* or *X. laevis*) of the nuclei, the cleavage response occurred, but with a constant delay of 3 h. Interestingly this time corresponds to the half-time of the first division cycle in *P. waltl* eggs. This suggests that, in activated eggs, the cleavage process (cytokinesis) stops at T = 0·50 in the absence of a nucleus. Only the addition of a nucleus, or just a centrosome (Karsenti *et al.* 1984), into these eggs permits the cleavage process to continue, leading to furrow formation 3 h later. This T = 0·50 point in the egg cycle may correspond to the 'restriction point' described in mammalian cell cycle (Pardee, 1974).

In the amphibian egg, this switch-point, which marks the end of DNA synthesis, also corresponds to changes in cytoplasmic consistency (Elinson, 1983; Aimar *et al.* 1986). Such cytoplasmic changes may alter the morphogenetic capacities of totipotent blastula nuclei (Briggs & King, 1952; Aimar, 1972) and thus lead to abortive development in the case of late nuclear transplantations.

This research was supported by grants from INSERM (961/296) and CNRS (ATP no. 960100).

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(Accepted 24 June 1988)