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Short Contribution

Hereditary abnormal activation in *Pleurodeles waltl* oocytes

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ABSTRACT A mutation which affects the eggs was detected in a *Pleurodeles waltl* laboratory strain. About 4 to 5 hours after fertilization, i.e. before the first cleavage, mutant eggs exhibited one or more depigmented patches (DP) on the animal hemisphere. Later on, some eggs failed to cleave, or cleaved abnormally. However, if gastrulation was successful, further development occurred normally. Abnormal eggs showed a disorganization of the vitelline gradient as well as a large lens-like zone of hyaline cytoplasm lying under the DP and a heterogeneous distribution of actin and tubulin in the inner cytoplasm. This mutation was named *turbulent egg cytoplasm (tec).* The alterations described above seem to be related to modifications of the cytoskeleton. Since artificially activated *tec* ova also exhibited the mutant pattern, we may conclude that the *tec* phenotype is expressed as a maternal effect syndrome.

KEY WORDS: *maternal effect mutation, cytoskeleton, amphibian ova*

Mutations affecting cleavage are convenient tools for the studies of cell cycle regulation currently being conducted in amphibians. However, as opposed to what is observed in other zoologic groups, relatively few mutations of that type have been described in Amphibians (Carroll and van Deusen, 1973; Droin and Fischberg, 1984; Armstrong, 1985; Kubota *et al.*, 1991; Droin, 1992).

In the salamander *Pleurodeles waltl*, several mutations have been described (Gounon and Collenot, 1974, 1975; Collenot *et al.*, 1989; Darribère *et al.*, 1991). Only one of them, the recessive mutation «*léthal-mitotique* » *(lm)*, affected cell division in late tail bud stage embryos in which mitosis was blocked at metaphase.

A strain of *Pleurodeles waltl* named VA_{85} was bred by one of us with the aim of maintaining in the laboratory a lethal recessive mutation (not yet published). In the course of that work, a partial or complete spontaneous sex reversal was observed in genotypic males (Collenot *et al.*, 1992). In the meantime it was noticed that all the eggs issued from the same females exhibited an abnormal behavior, i.e. within the first few hours after being laid, these eggs exhibited white patches which appeared at, or close to, the animal pole. Later on, some eggs also failed to cleave or cleaved abnormally. Cytological and ultrastructural observations revealed intense alterations affecting the cytoplasmic compartment of the egg. In that way this abnormality was named *turbulent egg cytoplasm (tec)* and the abnormal eggs *tec* eggs. Immunolocalization studies in *tec* eggs showed that these alterations derived from alterations of the cytoskeleton. The experimental analysis indicates that this inheritable anomaly could be considered as a semi-lethal mutation with a maternal effect.

Developmental pattern and cytological features of tec eggs

Developmental pattern of tec eggs

In *Pleurodeles* as in most other amphibian species, a circular non pigmented area lies at the animal pole, on top of the pigmented animal hemisphere of the ovum (Fig. 1A). Initially, this so-called maturation spot covers 1% of the whole surface of the egg. It disappears progressively between 1.30 h and 5 h after activation (PA) or fertilization (PF). In *tec* eggs, between 4 to 5 h PF, a large circular depigmented patch (DP) appears in the animal hemisphere (Fig. 1A). This patch may be localized at the animal pole at the site of the maturation spot or it may occupy a more lateral location. Sometimes, instead of one single large DP there are several little DPs. In either case, the whole of the depigmented surface may cover up to 60% of the animal hemisphere surface.

Time-lapse video records showed that the pattern of the DP changed progressively during the first cycle of the egg cleavage, so that both the appearance and location of the DP were modified. Sometimes the DP remained visible during the cleavage period. The duration of the first five division cycles and the pattern of cell divisions were studied through video recording. Some *tec* eggs did not cleave. When cleavage did occur, in most cases the first division lasted 7 h, i.e. one hour late compared to control eggs. However,

Abbreviations used in this paper: DP, depigmented patch; MPF, mitosis promoting factor; MV, microvilli; PA, post-activation; PF, post-fertilization; PLP, paraformaldehyde lysin sodium periodate; *tec*, turbulent egg cytoplasm.

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the rhythm of the further divisions was normal — one hour per cycle. The late blastula stage was reached after about 24 h, as in control embryos.

Cleavage was sometimes aberrant and led to abnormalities in the orientation and the volume of the blastomeres. Three-cell embryos often appeared (Fig. 1A). As a consequence of the abnormal cleavages, some eggs exhibited a thin and transparent blastocoel roof (Fig. 1A). Moreover, groups of superficial necrotic cells were present which were progressively expelled into the perivitelline space.

The early development of *tec* eggs issued from two females (A and B) of the VA_{85} strain, all of which exhibited the DP, was examined. Among all of the embryos which cleaved, 60% cleaved and developed normally. Among the embryos which cleaved abnormally, only 34% were blocked at gastrulation. This observation indicates that abnormal cleavage is not necessarily lethal. Comparing the data, it appeared that the percentage of activated eggs which did not cleave varied according to the spawnings; 1.5% and 18% for females A and B respectively. Moreover, a similar difference was also observed both for eggs which cleaved abnormally (A: 26%; B: 63.4%) and for the abnormally-cleaving embryos which were blocked at the gastrula stage (A: 52.4%; B: 22.5%).

In addition, in 8 progeny exhibiting the DP and issued from 8 different females of the VA_{85} strain, the percentages of embryos that died at the gastrula stage varied from 9.7 % to 51% (M= 24.2%±13.5). They did not exceed 10% in standard spawnings.

In short, the abnormalities provoked lethality of embryos during the segmentation phase in variable proportions according to the different spawnings. However, when gastrulation had proceeded normally, regular development ensued. The *tec* mutation could be considered as semi-lethal.

Cytological features: modifications during the first cleavage cycle

Microvilli of the egg surface and membrane proteins

The surface of *tec* eggs was observed under SEM in order to detect possible anomalies at the site of the DP. Investigations were done at the time of the appearance of the mutant phenotype as well as at the time of fertilization and focused on the presumed location of the DP. In *Pleurodeles*, the plasma membrane of the fertilized eggs was covered with numerous microvilli (MV) as it is in *Xenopus* (Monroy and Baccetti, 1975; Oshima and Kubota, 1985). The appearance of the MV is a chronological marker for the transformation of the egg membrane (Aimar, 1991).

At fertilization, the plasma membranes in *tec* eggs and in control eggs have the same appearance, exhibiting conical and evenly distributed MV on the animal hemisphere. Between 4 and 5 h PF, the plasma membrane at the site of the DP was, for most of the *tec* eggs (70%), different when compared with the other parts of the animal hemisphere or with the whole animal hemisphere of control eggs. In some eggs the MV on the DP retained their conical appearance, typical of eggs at fertilization, while on the other parts of the egg surface they were organized in a network of convoluted ridges. In other eggs, the MV on the DP were still extended while elsewhere they were already regressed (Fig. 1B). These anomalies in the plasma membrane could be present in the depigmented blastomeres up to the blastula stage.

The plasma membrane of the *tec* egg surface was labeled at fertilization time by biotin in order to detect whether a neo-synthesis of plasma membrane occurred in the DP. These eggs were examined 4 h later when the DP appeared. In all cases, the plasma membrane was labeled as in control eggs, indicating the absence of plasma membrane neo-synthesis at the level of the DP.

Cortex and inner cytoplasm

Sections of *tec* eggs and control eggs were examined by light and electron microscopy at the time of egg laying and at the time of appearance of the DP. At egg laying, the plasma membrane of *tec* eggs was wrinkled, contrasting with the flat plasma membrane of control eggs. Also, the vitelline gradient seemed to be disorganized. Large vitelline platelets were often localized in the cortex of the ova. Moreover, they exhibited a heterogeneous distribution in the inner cytoplasm in opposition to their regular distribution in control eggs. In *tec* eggs, at 3 to 5 h PF, the plasma membrane was still wrinkled and the inner cytoplasm remained disorganized. In addition, a thick lens-like zone of hyaline cytoplasm, generally deprived of vitelline platelets, was observed underneath the DP (Fig. 1D). In the cortex of *tec* eggs, the peripheral hyaline layer exhibited a variable thickness contrasting with that in control eggs where it was constant. Here and there, this hyaline layer was invaded by vitelline platelets. What is more, it contained an increased number of annulate lamellae, organelles which are widely dispersed in amphibian oocytes (Kessel, 1985).

Modifications during the further cleavage cycles

Cytological anomalies persisted during the first divisions of the *tec* eggs. The contiguous membranes of the animal blastomeres were smooth in control eggs. In *tec* eggs they were folded and bore protuberances, thereby diminishing the cellular cohesiveness (Fig. 1E). The peripheral pigment was condensed into several patches, leading, in some cases, to the formation of nodules of melanin, which were expelled into the intercellular space (Fig. 1E). The pigment was also present as tracts into the inner cytoplasm. Moreover, zones of hyaline cytoplasm deprived of vitelline platelets were contained in blastomeres of *tec* eggs.

Possible involvement of cytoskeleton proteins in tec mutation

Observation of the cytological abnormalities suggested a possible implication of the cytoskeleton in the *tec* phenotype. Actin and tubulin were studied in *tec* and control eggs, at the fertilization time and 4-5 h later.

Electrophoresis

No striking differences were detected in the panel of the soluble proteins. Moreover, no quantitative or qualitative modifications in actin and tubulin were detected in 2D slabs between *tec* eggs and their controls. Similar results were obtained in control and mutant ovarian oocytes.

Immunofluorescent staining

As early as fertilization, tubulin formed a continuous layer underlying the plasma membrane in the two types of eggs (Fig. 1F and G). The distribution of tubulin in the inner cytoplasm was more heterogeneous in *tec* eggs than in control eggs. At 5 h PF, the anti-tubulin antibody labeled the lens-like zone of hyaline cytoplasm differently when compared with the other parts of the egg (Fig. 1G). The appearance of the labeling in that zone suggested that tubulin might be in a non-polymerized state.

At fertilization, both in *tec* and control eggs, the anti-actin antibody strongly labeled the cortex and more slightly the inner

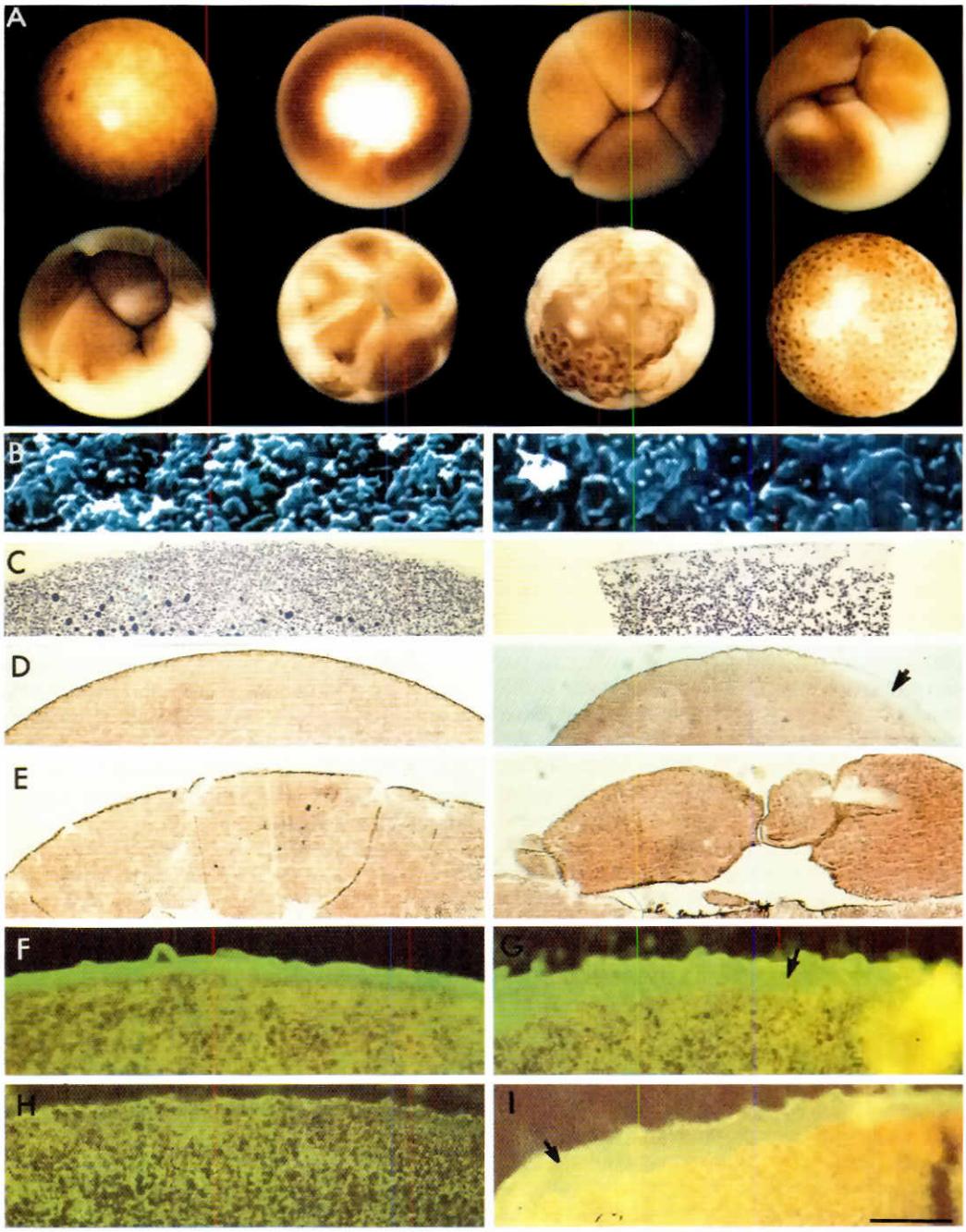

Fig. 1. Morphological and cytological features of the *tec* phenotype. (A) *Pattern of tec mutation on eggs from fertilization to blastula stage; (top, from left to right) tec ovum at fertilization time, exhibiting the white maturation spot and 2 dark spots (sites of sperm entry); tec egg at 4 h PF, a large depigmented area (DP) on the animal hemisphere characterizing this mutation; 4-cell stage control egg; 3-cell stage tec egg. (Bottom) different cleavage phases of tec eggs, from left to right: abnormal segmentation; morula (note the transparent blastocoel roof); partial blastula; 24 h-old blastula (some depigmented blastomeres still present on the animal hemisphere).* **(B)** *Scanning electron microscopy of a tec egg at 4 h PF. The MV pattern in the depigmented zone (left) is delayed as compared to another zone of the animal hemisphere (right).* **(C)** *Semi-thin sections of the animal hemisphere of a control egg (left) and a tec egg (right). Note the presence of yolk platelets in the cortex of the tec egg in contrast to the control egg.* **(D and E)** *Histological sections of the animal hemisphere of control egg (left) and tec egg (right), at 4 h PF* **(D)** *and at the cleavage stage* **(E)**. **(D)** *A lens-like yolk-free cytoplasm (arrowhead) was underneath the DP of the tec egg.* **(E)** *Note the irregular shape, the abnormal pigmentation and the extrusion of cytoplasm in blastomeres surrounding the blastocoel roof in the tec embryo.* **(F to I)** *Immunolabeling with antibodies against tubulin and actin of the animal hemisphere of tec eggs (histological sections).* **(F and G)** *Labeling of the microtubule network in tec eggs at time of fertilization* **(F)** *and at 5 h PF* **(G)**. *Note, in G, the lens-like structure (arrow).* **(H and I)** *Heterogeneous network of actin, at 4 h PF, in the cortex and in the inner cytoplasm* **(H)**; *in the lens-like cytoplasmic structure* **(I)** *(arrow). Magnifications: A x20; B x6000; C x450; D and E x250. Bars, F to I 20 μm.*

cytoplasm surrounding the vitelline platelets. At 4 to 5 h PF, the cortex of *tec* eggs was still uniformly labeled even in the DP. The inner cytoplasm contained small aggregates of actin microfilaments (Fig. 1H) contrasting with the homogeneous distribution of actin in control eggs. Actin was also detected in the yolk-free cytoplasm of the lens-like zone, but its labeling was more diffuse than in the other compartments of the egg (Fig. 1I).

Genetical aspect and inheritance of the tec anomaly

The tec anomaly as a maternal effect syndrome

The fact that cytoplasmic abnormalities were detected in *tec* eggs as early as the fertilization stage led us to hypothesize that the mutation might be a maternal-effect one. In order to test this hypothesis, ova from 9 virgin females of the VA_{85} strain were either *activated* or *fertilized* experimentally. Four females laid ova which expressed, in the two procedures, the *tec* phenotype, as do *tec* eggs issued from natural spawnings. The ova from the other 5 females were of the normal phenotype. As expected, the artificially activated ova from these 9 females degenerated after some rounds of atypical divisions.

In summary, the expression of the *tec* phenotype is independent of zygote formation but is inherent in the ova and expressed as a maternal effect syndrome.

Inheritance of the tec anomaly

The abnormal *tec* phenotype was observed exclusively in the VA_{85} strain, itself issued from a single spawning which had been bred for the purpose of studying a mutation that is lethal at the hatching stage and provisionally referred to as *l* for lethal (not yet published). Inbreeding of adult newts, issued from the initial spawning, indicated first that the *l* mutation is recessive and secondly that the *l* mutation and the *tec* genotype are not related. Two successive progeny, obtained from heterozygotes for the *l* mutation (male 7409 and female 7454), exhibited the *tec* phenotype. Seventeen adult females issued from these spawnings were randomly tested for the expression, by their eggs, of *tec* phenotype; nine were *tec* (-) and eight were *tec* (+). It can be concluded that the gene(s) corresponding to the *tec* mutation is not on the W sex chromosome.

In the VA_{85} strain, the gonadal phenotype of genotypic males can be spontaneously reversed into functional ovaries, partially or completely (Collenot *et al.*, 1992). Five genotypic males (ZZ), i.e. an intersexual individual and four phenotypic females, laid *tec* eggs. This clearly illustrated that the production of *tec* eggs is not strictly dependent on the ZW female genotype. However, from all the results obtained until now, it cannot be specified which of the autosomes or the Z sex chromosome bears the gene(s) involved in the *tec* phenotype.

While most of the maternal effect mutations are lethal in *Xenopus* (Kubota *et al.*, 1991; review in Droin, 1992) and in axolotl (review in Armstrong, 1985), in *Pleurodeles waltl* the *tec* mutation exhibits a semi-lethal character as does the *ac* mutation (*ac* for *ascite caudale*, Beetschen and Jaylet, 1965). Among the lethal mutations, some prevent egg division and others lead to abnormal cleavage. In the *tec* mutation the alterations in ova cytoplasm lead to an arrest of early development at two distinct stages: either the first cleavage or the gastrula stage.

The *tec* phenotype comprises two main features which appear during the first cleavage cycle. The first one is an alteration of the cortex of the egg. The second one is a disorganization of the inner cytoplasm which provokes the formation of a lens-like zone of yolk-free hyaline cytoplasm. These cytological aspects and the time-lapse video records suggest that the cytoplasm has been altered by an endogenous stirring process. Movements of the cortical pigment are commonly observed during fertilization and cleavage of the amphibian egg (Picheral, 1977; Elinson, 1980). Consecutively to fertilization, the inner cytoplasm also undergoes important movements (Danilchik and Denegre, 1991). The *tec* phenotype does not seem to be the result of a stronger expression of these normal intracellular movements. We consider that the intracellular cytoplasmic flows are tightly dependent on the cytoskeleton (Dent and Klymkowsky, 1989). The distribution of actin and tubulin in the inner cytoplasm of *tec* eggs was strongly modified and could result in altered intracellular movements and in abnormal reorganization of the cytoplasm content. The delay in the transformation of the MV in the depigmented area of *tec* eggs might also be due to an alteration in the distribution and/or organization of the underlying actin, since cortical actin constitutes the core of the MV (Burgess and Schroeder, 1977) and is involved in the modifications of their features (Ezzell *et al.*, 1985; Aimar and Grant, 1992). The structural alterations observed in *tec* eggs affected the pre-formed plasma membrane of the ova, since these local alterations did not require neosynthesis of a plasma membrane

Cell surface and cytoskeleton constantly interact during the division process (Schroeder, 1981). The location of the cleavage furrow is directly dependent on the presence and the position of aster rays since the asters mediate the formation of the contractile ring (Schroeder, 1972; Devore *et al.*, 1989). In this respect, alterations of the cytoskeleton in the *tec* eggs, such as the formation of actin aggregates, might alter the extension of the aster rays and thus provoke either an absence of cleavage or a modification of the cleavage pattern.

Cell division cycle was shown to be governed by the mitosis promoting factor (MPF, Masui and Markert, 1971; Gerhart *et al.*, 1984). In *tec* eggs, the duration of most of the division cycles remained unchanged, proving an unaltered MPF oscillatory system. Thus, the process of cytokinesis seems to be the sole event to be affected by the *tec* syndrome.

The fact that most of the *tec* eggs, even those with severe cleavage abnormalities, developed illustrates the intervention of regulative processes which could progressively compensate for the initial alteration and allow a normal embryonic development.

Experimental Procedures

Biological material

The strain VA_{85} of *Pleurodeles waltl* was introduced in 1985 into the Laboratory of Developmental Biology (Paris). The abnormality, as yet, has never been observed in eggs issued from other strains. *Pleurodeles waltl* were bred as usual at the ambient temperature. Spawnings were obtained either through natural mating or through hormonal stimulation of virgin females, followed or not by experimental fertilization or by artificial activation. In the latter case, manually dejellied eggs were activated by an electric shock (70V, 80 mF) in the normal amphibian medium (NAM, Slack and Forman, 1980) diluted to 1/10. Eggs were raised in 1/10 NAM at 20°C. Oocytes were obtained from pieces of ovaries excised by laparotomy from different adult females.

Chronology of embryo development

During the 6 h following activation (post-activation, PA) or fertilization (post-fertilization, PF), the period during which the mutant phenotype is expressed, the development of the eggs was recorded through photographs

taken every 15 min or through video recording (Panasonic), with a time-lapse sequence of 24 sec.

Biotinylation of egg surface proteins

Within 2 h of being laid, the eggs were manually dejellied and incubated in biotin (NHS-biotin, PIERCE) diluted to 0.5 mg/ml in 1/10 NAM. At relevant times, the eggs were extensively washed with 1/10 NAM, then fixed in paraformaldehyde-lysine sodium periodate (PLP) for 30 min, according to Gendelman *et al.* (1983). Small pieces of the egg surface were cut off. They included the area of the depigmented patch in the mutant eggs and the corresponding zone in control eggs. For immunofluorescent staining, these cortical mounts were labeled with streptavidin conjugated-fluorochrome (FITC, Amersham).

Electron microscopy

Several batches of eggs were prepared at the time of activation or fertilization as well as when the *tec* phenotype appeared. Manually dejellied eggs were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate at pH 7.4 for 1 h at ambient temperature and then for 12 h at 8°C. They were post-fixed in 0.1 M sodium cacodylate, 1% osmium tetroxide at pH 7.4 and finally dehydrated in ethanol.

The samples chosen for scanning electron microscopy (SEM) were critical-point dried. After coating with gold they were observed in a Jeol JSM 840 A operating at 15 or 25 Kv. The selected images were recorded using an image processing system (Quantel). The samples selected for transmission electron microscopy (TEM) were fixed in osmic acid, dehydrated and then embedded in resin Spurs (Fluka). Ultrathin sections at 60 nm, post-contrasted with uranylacetate and lead citrate, were examined in a Philips EM 400 T. After staining, semi-thin sections were observed with a light microscope.

Cytological examination

Control and *tec* eggs were fixed in PLP, then dehydrated in ethanol and embedded in paraplast; sections, 7 mm thick, were stained with safranine methyl blue orange G.

Antibodies binding for fluorescence microscopy

Eggs were fixed overnight in ethanol with 1% acetic acid, then stored in ethanol 50%. After embedding in gelatin, they were cut with a cryostat (10 mm sections). Sections, previously stained by indirect labeling using monoclonal anti-actin antibody (Amersham) and polyclonal anti-tubulin antibody (gift of Dr. D. Huchon, Univ. Paris VI) and FITC labeled secondary antibodies, were then observed in epifluorescence.

Electrophoresis (mono and bidimensional)

Enzymatically defollicularized ovarian oocytes and manually dejellied eggs were washed in PBS, crushed and centrifuged at 10500 rpm. The hyaline fraction of each sample was boiled in sample buffer. Samples were loaded onto a 12% polyacrylamid gel using the discontinuous system of Laemmli (1970) for mono-dimensional electrophoresis. Bi-dimensional electrophoresis was effected according to O'Farrel's technique (1975). The gels were stained with silver nitrate and dried.

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