The fate of intramitochondrial paracrystalline inclusion bodies in germ line cells of water frogs (Amphibia, Anura)

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Received 22 February 1989; accepted 1 June 1989

Summary. Numerous intramitochondrial paracrystalline inclusion bodies (ICIB) were observed in the germinal plasm of a mid-blastula, and in primordial germ cells (PGCs) after their migration to the germinal ridges, in *Rana ridibunda*, *R. lessonae* and *R. esculenta*. In oogonia the number of ICIB decreases rapidly. Single ICIB are observed in the germ cells up to the leptotene stage; they have never been observed in pachytene oocytes. In diplotene oocytes that have reached a diameter of about 100 µm ICIB are visible again, and their number increases concomitantly with oocyte growth.
Key words. Intramitochondrial crystalline inclusions; primordial germ cell; germinal plasm; oogenesis.

Intramitochondrial paracrystalline inclusions were first described by Lanzavecchia and Le Coultre[1] in embryos of *Rana esculenta*. Ward[2] suggested that they were yolk precursors in *R. pipiens*. A great structural resemblance between yolk platelets and the intramitochondrial crystals led the authors to name the latter 'endomitochondrial yolk'[1] or 'intramitochondrial yolk'[2], and to formulate a hypothesis that the paracrystalline inclusions are the precursors of the yolk platelets. However, further cytological observations revealed some differences in the crystalline lattice of the two structures[3–6]. Studies of Kress and Spornitz[7] and Kress[8] led the authors to the conclusion that the term 'mitochondrial yolk' should be abandoned and replaced by the more general 'intramitochondrial paracrystalline inclusion bodies' (ICIB). The most recent biochemical investigations[9] revealed that the composition of proteins in yolk platelets and ICIB is quite different; the authors suggested also that the use of the term 'yolk', when referring to these crystals, should be postponed until their role in oogenesis and embryogenesis is clarified. Similarly, Hsü et al.[10] described a lack of yolk platelet formation in hypophysectomized tadpoles of *R. catesbeiana*, while ICIB were formed in a normal way. The results of the investigations just cited clearly show that ICIB are not yolk precursors and their coincidence with vitellogenesis is only spatio-temporal.

ICIB in amphibian oogenesis are known in the Ranidae and the closely related species *Rhacophorus maculatus*[7]. Their function and origin are unknown. ICIB have been described in growing and ripe diplotene oocytes[2, 6, 11, 12]. Mahowald and Hennen[13] and Kessel[14] observed ICIB in the germinal plasm in oocytes of *R. pipiens*. The germ plasm is a part of the cytoplasm containing germ determinants, in this case the germinal granules, responsible for the formation of germ line cells in the embryo, and – in consequence – for the future gametes[15, 16].

Observations of ICIB in the germinal plasm of ripe and growing diplotene oocytes of *R. pipiens* led Kessel[14] to suggest the possible continuity of these structures.

The aim of this study is to answer the question whether ICIB are present in all stages of the differentiation of germ line cells in frogs.

Material and methods

Blastulae, larvae, tadpoles and juveniles of *Rana ridibunda*, *R. lessonae* and their natural hybrid, *R. esculenta*[17, 18] were used in the study. Parental individuals of *R. esculenta* and *R. lessonae* were collected in the vicinity of Wrocław, Poland, while *R. ridibunda* came from the vicinity of Vienna, Austria.

The gonadal anlage and gonads of the tadpoles were dissected at Gosner[19] stages 25–40; 42–46 (metamorphosis), and before and after the first hibernation. The material was fixed in 2.5% glutaraldehyde and postfixed in OsO_4 as described elsewhere[20]. The mid-blastulae were fixed in 2.5% glutaraldehyde in toto, than the vegetal halves were cut off in 0.1 M phosphate buffer and postfixed in 1% OsO_4. Ultrathin sections were examined in the Tesla BS 500 electron microscope.

Results

In some blastomeres forming the blastocoel floor of a mid-blastula the 'patches' of germinal plasm were observed in one of the poles of the cell. Small yolk platelets, numerous mitochondria, vesicles, glycogen and germinal granules are characteristic of this region. In some of the mitochondria crystalline inclusions (ICIB) located in the outer mitochondrial compartment inside the cristae, or in the matrix, are visible (fig. 1). They are thin platelets, usually in a parallel arrangement in the same mitochondrion; however, sometimes their position is perpendicular. ICIB in the germinal plasm form groups. In the rest of the cytoplasm of the blastomere carrying the germinal plasm, as well as in other blastomeres of the vegetal half, mitochondria are rather rarely seen, and ICIB have never been observed. In the blastomeres of the animal hemi-

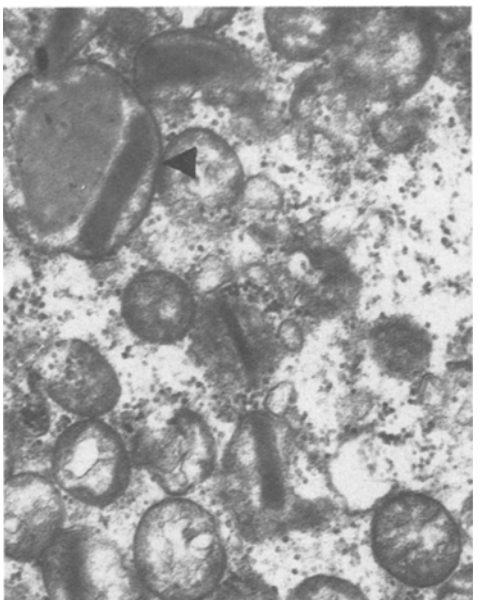

Figure 1. Mitochondria from the germinal plasm of mid-blastula of *R. esculenta*. Most of the ICIB are located in the outer mitochondrial compartment inside the cristae. The arrowhead points to a mitochondrion carrying two crystals situated perpendicularly. ×21,000.

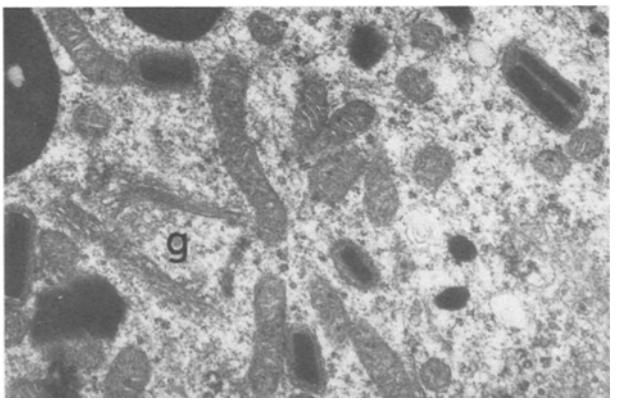

Figure 2. A group of ICIB in primordial germ cell of an undifferentiated gonad of *R. ridibunda*. g, Golgi complex. ×22,800.

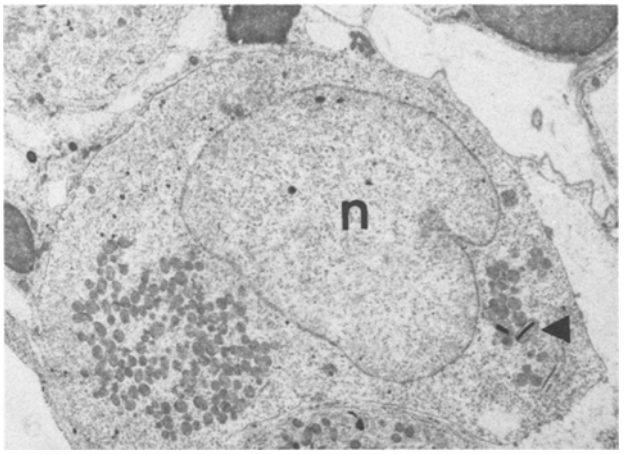

Figure 3. Low magnification of an oogonium from early ovary of *R. ridibunda*. Mitochondria form group lying near nucleus; ICIB are indicated with arrowhead.

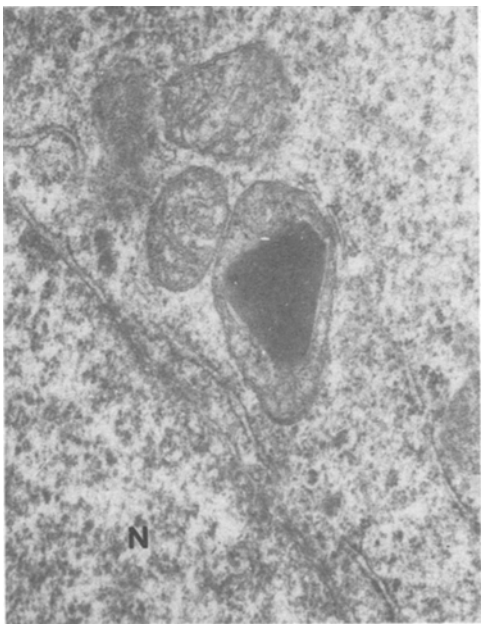

Figure 4. ICIB from leptotene oocyte from early ovary of *R. ridibunda*. N, nucleus. ×22,900.

sphere the mitochondria are more numerous, but ICIB are not visible.

In a tadpole beginning to feed (stage 25) the migration of PGCs from the endoderm to the germinal ridges has already been completed and undifferentiated gonads have been formed. Inside them the big PGCs full of yolk platelets are easily recognizable. Near the nucleus of PGC an aggregation of mitochondria is situated. Among them numerous ICIB are observed (fig. 2). Between some mitochondria lying close to the nuclear envelope the intermitochondrial cement is visible. ICIB are also found in the germ cells in which vitellolysis has already finished, now called the gonial cells (fig. 3). The number of ICIB decreases significantly when the gonial cells transform into oocytes, i.e. when meiosis starts. It can be first observed at tadpole stage 27/28 when the undifferentiated gonad differentiates into an ovary. At that time mitochondria do not form aggregations but are scattered in the cytoplasm, and their number seems to be relatively lower, as is visible on sections.

ICIB are sporadically observed in leptotene zygotene oocytes (fig. 4), but have never been found in pachytene or early diplotene cells. This phenomenon is observed only in tadpoles before stage 30. In older animals ICIB are not present although leptotene stages, as well as oogonia, are numerous.

In early diplotene oocytes, less than 100 μm in diameter, the mitochondria are still scattered in the cytoplasm and some of them lie close to the nuclear envelope, with intermitochondrial cement between them. After meta-

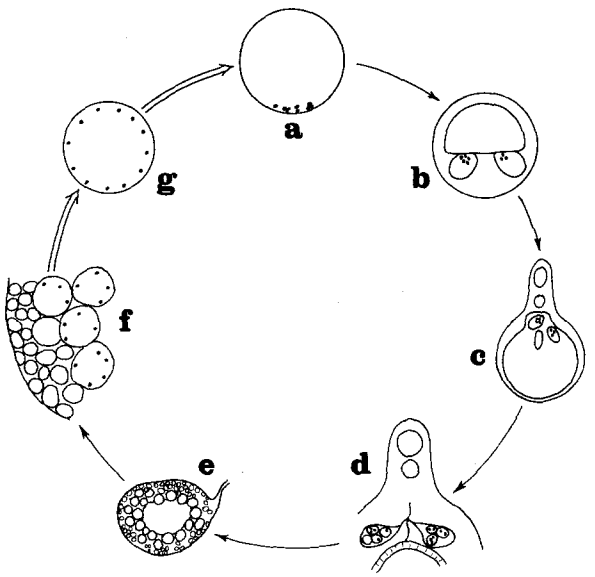

Figure 5. A diagram presenting cyclic formation of ICIB in germ line cells of water frogs. The black dots denote the presence of ICIB. The new generation of ICIB is indicated by double-line arrows. a, ripe egg; b, mid-blastula; c, migrating PGCs in endoderm of a larva; d, PGCs, oogonia and leptotene/zygotene oocytes in the earliest stages of ovary differentiation; e, no ICIB in early diplotene, pachytene, leptotene/zygotene oocytes and oogonia in a tadpole ovary before metamorphosis; f, a piece of a juvenile ovary from a frog after metamorphosis. ICIB are visible again in growing diplotene cells; g, previtellogenic oocyte with ICIB localized in a peripheral layer.

morphosis, in oocytes of more than 100 μm, most of the mitochondria form a peripheral layer in which ICIB are visible again. The number of ICIB, as well as the size of crystals, increases as the diplotene oocyte grows.

The observations of consecutive stages of the formation of germ line cells strongly suggest that ICIB in blastomeres, PGCs, oogonia and leptotene oocytes of the same embryo are the heritage of the ovum from which it has developed. In growing diplotene oocytes of the same animal after metamorphosis, ICIB are formed again, and will furnish the germ line cells of a new generation (fig. 5).

Discussion

The observations presented in this paper confirm the suggestion of the continuity of ICIB in germ line cells. In *R. pipiens* ICIB were observed in the germinal plasm of oocytes; sometimes they were also visible in the rest of the ooplasm[13]. Lanzavecchia[3] described the inclusions in a number of cells in developing embryos of *R. esculenta*; they disappeared at tadpole stages. In the present study ICIB were seen in the germ line cells only; almost all of them were located in the germinal plasm. Putting aside the insignificant distribution of ICIB to other cells as being only accidental, a conclusion can be drawn that the presence of ICIB is characteristic of ovum differentiation in the Ranidae with the exception of pachytene and early diplotene cells, which is clearly seen in early tadpole ovaries.

Mitochondria in previtellogenic diplotene oocytes of many amphibian species are arranged in a group often called the Balbiani body[21,22]. In *Xenopus laevis* the mitochondria of fast-growing diplotene oocytes are localized close to the nucleus and form a structure called the mitochondrial cloud[23], which corresponds to the Balbiani body. Further observations on the fate of the mitochondrial cloud in vitellogenic oocytes have revealed that this structure divides into two parts: one group of mitochondria stays close to the nucleus and shows mt-DNA replication activity; the other one migrates towards the vegetal pole into the germinal plasm. In this group the mt-DNA replication activity is arrested[24]. However, in the case of the frogs under study, as well as in *R. temporaria*[5], the mitochondria do not form a Balbiani body but are localized at the periphery of previtellogenic oocytes. Among the 'conventional' mitochondria of the subcortical layer numerous ICIB are visible. Later, in ripe oocytes, ICIB are observed almost exclusively in the germinal plasm. Thus it may be concluded that in *Rana* species the segregation of mitochondria in the fast growing diplotene oocytes also takes place. Maybe ICIB in the Ranidae are a homologue of that population of mitochondria in *Xenopus* in which the mt-DNA replication has been arrested.

Mitochondria containing inclusions were observed in various tissues of some animals species[25–28]. They often appear in human diseases[29,30]. Crystals have also been described in mitochondria of germ line cells in *Hydra*[31] and in mitochondrial derivative of snail sperm[32]. However, the crystal arrangement in these cases was not the same as in ICIB. Their function, as well as origin, are still uncertain. In general, the presence of various inclusions, also crystalloids, inside mitochondria is interpreted as an inactivation of these organelles[33].

Thus a hypothesis can be proposed that mitochondria in the germinal plasm are temporarily inactive until the PGC differentiates into an oogonium, when ICIB start to disappear. During early stages of meiotic prophase they would be activated, and the cycle would begin again in diplotene oocytes.

Acknowledgments. A part of this work was supported by the Polish Academy of Sciences, CPBP 01.04. Thanks are due to Ms B. Matkowska and Mr R. Adamski for their technical assistance.

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0014-4754/90/010098-04$1.50 + 0.20/0

Low allozyme and mtDNA variability in the island endemic species *Drosophila sechellia* (*D. melanogaster* complex)

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Received 13 March 1989; accepted 12 July 1989

Summary. Genetic variability of *D. sechellia* is investigated at both mitochondrial and nuclear levels. The results reveal the existence of a single main type of mtDNA with very few variants and a very low enzyme polymorphism. This situation is consistent with the small population size of this specialized species.
Key words. Genetic variability; mitochondria; allozymes; *Drosophila sechellia.*

Of the drosophilid species so far studied the great majority have high allozyme polymorphism, the average heterozygosity being above 0.10 and sometimes exceeding 0.20[1,2]. There is very little known about the restriction site variability of mitochondrial DNA (mtDNA); however, a large range of nucleotide diversity (an estimate of the average heterozygosity at the nucleotide level[3] (and see below) has been found in the few species studied; from 0.002 up to 0.020[4].

On theoretical grounds the level of polymorphism of both nuclear and mitochondrial genomes should be related to the effective population size, N_e. It is also expected that the population size should be much higher in generalist than in specialist species. The *D. melanogaster* complex, which comprises four species, appears to be a good model for testing such expectations. Two species, *D. melanogaster* and *D. simulans*, are cosmopolitan generalist species with huge populations, both in temperate and tropical regions[5]. The other two species are island endemics, *D. mauritiana* in Mauritius and Rodrigues and *D. sechellia* in the Seychelles. Their ecological niches, however, are different; *D. mauritiana* is a generalist and abundant species found in both natural and domestic habitats[6] while *D. sechellia* is specialized on a single resource, the fruit of *Morinda citrifolia* on a few tiny islands of the Seychelles archipelago about 1000 km northeast of Madagascar[7,8].

The allozyme polymorphism of the two cosmopolitan species has been extensively investigated[9,10] and some data on their mtDNA are also available[4,11,12]. In contrast, the two endemic species are still poorly known. In the present paper the genetic variability of *D. sechellia* is investigated at mitochondrial and nuclear levels and a very low polymorphism is found in both genomes.

MtDNA Restriction site variability. Two samples of *D. sechellia* were collected in the Seychelles in 1981 and 1985. From the first collection on Cousin Island, a mass culture was established. For the second sampling, 27 isofemale lines (26 from Cousin and 1 from Frigate) were established in the field and brought to the laboratory. The mtDNA was analyzed in 22 isofemale lines; 21 from the 1985 sample (20 Cousin and 1 Frigate lines) and one line derived from the 1981 mass culture. MtDNA was extracted from virgin eggs as described in Solignac et al.[13]. Since *D. sechellia* has a very low egg production[8], females were crossed to *D. mauritiana* males and the F1 fertile females were back-crossed each generation to *D. mauritiana* males. After a few generations, each line acquired the high fecundity of *D. mauritiana* although each kept the original *sechellia* mitochondrial genome. MtDNAs were digested with 19 restriction enzymes (see below). Restriction fragments were separated on vertical agarose 1% gels, stained with ethidium bromide and photographed under UV light. Alternatively, restriction