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Original article

Activation of avian embryo formation by unfertilized quail germ discs: comparison with early amphibian development

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Abstract — In the present study we placed germ discs (or fragments containing the deep central part of it) from unfertilized laid or extracted quail eggs on the deep side of the upper layer of isolated anti-sickle regions from unincubated chicken blastoderms. After culture in vitro of associations where the central deep part of the germ discs was in contact with the deep side of the upper layer (UL), we observed in about 30% of the cases the onset of embryonic development. Both associated parts play a role in the final formation of an embryo. Our experimental results, suggest that the δ ooplasm of the nucleus of Pander influences the cranial upper layer to segregate an endophyll layer. The definitive embryonic structures i.e. mesoderm, epiblast and neural plate are derived from the chicken upper layer by respectively normal gastrulation and (pre)neurulation phenomena. Our experiments seem to have some homology with the association experiments of isolated cortices from various regions of unfertilized *Xenopus* eggs implanted into the ventroequatorial core of a recipient 8-cell Xenopus embryo.

avian blastoderm / unfertilized germ disc / nucleus of Pander / endophyll / Rauber's sickle

Résumé — **Activation de la formation de l'embryon d'oiseau par le disque germinatif non fertilisé de caille : comparaison avec le développement précoce d'amphibien.** En plaçant une partie de la zone centrale profonde (noyau de Pander) d'un disque germinatif de caille non-fertilisé sur la face interne de l'ectophylle cranial de blastoderme non incubé de poule, on peut observer, après culture, une activation de l'embryogenèse (dans à peu près 30 % des cas) qui peut aller jusqu'à la formation d'un embryon miniature complet. Les deux structures ainsi associées jouent chacune un rôle pendant son développement. L'entophylle inducteur ainsi que les structures gastruléennes définitives (mésoderme, épiblaste et plaque neurale) proviennent de l'ectophylle de poule qui doit être regardé comme potentiellement équivalent d'une jeune blastula. L'interprétation de nos résultats expérimentaux conduit à admettre que l'ooplasme provenant du noyau de Pander (ooplasme δ) exerce une influence sur l'ectophylle cranial qui lui-même, si isolé en culture, demeure indifférencié.

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Les résultats de nos expériences semblent présenter une homologie avec ceux d'expériences similaires effectuées chez les amphibiens. En effet, selon Kageura, on obtient aussi une activation du développement embryonnaire quand on place des fragments de cortex d'œufs non fertilisés de xénope dans la région équatoriale ventrale d'un œuf segmenté ayant atteint le stade 8 blastomères.

blastoderme d'oiseau / disque germinatif non fertilisé / noyau de Pander / entophylle / croissant de Rauber

1. INTRODUCTION

The terminology of the different components of an unincubated avian blastoderm has been described in earlier studies [5, 7, 8, 11, 12, 14, 15]. In the anti-sickle region, (which is used in the present experiment) an irreversible disruption has taken place between the future cranial part of the germ and the underlying subgerminal ooplasm at the moment of bilateral symmetrization [6, 7]. The boot-like aspect of the subgerminal ooplasmic layers surrounding the nucleus of Pander in natural conditions [4, 6] suggest that a compression occurred in a caudal and slightly upwards direction below and against the caudal upper layer. A similar interaction at the moment of cortical rotation between the cortex and core in the fertilized amphibian egg was already suspected long ago [18].

By an oblique positioning of the egg yolk ball, a similar relative displacement between superficial and deep elements occurs in the avian germ disc [6, 7]. The deep part of the avian germ disc (with the nucleus Pander [34] in its centrum) turns temporally or definitively below the upper layer of the highest part of the blastoderm rim [14]. After this cellular-ooplasmic rearrangement the Rauber's sickle and the caudal marginal zone are formed by a cleavage-like mechanism of cell proliferation of blastoderm cells into the underlying eccentrically placed caudal ooplasm [7]. So (in contrast to the fertilized amphibian egg), the bilateral symmetrization takes place much later in the avian germ disc, which already contains thousands of blastomeres (approximately 6 h before laying: [16, 39]). In birds, recent experiments [10, 12] strongly suggest that Rauber's sickle [35] is homologous to the "Nieuwkoop centre" [32] (or vegetal dorsalizing cells) in amphibians. In amphibians it is generally accepted that cortical rotation determines the "Nieuwkoop center" that leads to the early organizer. The developmental fate of each region is determined by the subsequent mesoderm induction [17, 26, 32, 36] and neural induction (Spemann [37]). Kageura [25] described activation of dorsal development by contact between the cortical dorsal determinant and the equatorial core cytoplasm in eggs of *Xenopus laevis*: isolated cortices from various regions of unfertilized eggs and embryos were implanted into one and several positions of a recipient 8-cell embryo. In recent experiments [13–15] we found that central subgerminal ooplasm of fertilized, unincubated avian germ discs, placed on the deep side of the upper layer of avian anti-sickle regions has no embryo forming potencies if no Rauber's sickle is added. In the present study therefore we tried to find out if less influenced "younger" preembryonic ooplasm of unfertilized germ discs eventually contains inheredited oocytal determinants which can provoke the formation of an embryo in the upper layer of an isolated anti-sickle region.

2. MATERIALS AND METHODS

Unfertilized quail eggs were obtained by isolating a group of young laying females from males during at least 3 weeks. Further

we used unincubated chicken blastoderms presenting a Rauber's sickle. They came from fertilized chicken eggs (24–28 h after laying and stored at 15–20 °C). Chicken anti-sickle regions together with a neighbouring part of the area opaca were excised and explanted (ventral side upwards) in vitro. We used isolated anti-sickle regions and not lateral blastoderm quadrants, because reliably the first contain no Rauber's sickle material at all [14] and hence comparable results can be obtained. The chicken anti-sickle regions were either cultured alone as controls (n =10) or the deep side of an unfertilized quail germ disc was placed on their deep side. Also fragments were obtained by cutting the unfertilized quail germ discs through their central part (containing the nucleus of Pander) (Fig. 1) or parallel with this central part. The cut surface of these "thick" sections was then placed on the middle part of the chicken anti-sickle region. So a part of all the different areas of the unfertilized quail germ disc could theoretically come in contact with the upper layer of the chicken anti-sickle region. In another experiment after removal of the peripheral vacuolar part, the central superficial part of an unfertilized quail germ disc was placed on the deep side of an isolated chicken anti-sickle region and cultured (n = 11). After these interventions, the blastoderms fragments were cultured on egg white according to New [31]. Instead of Petri dishes, the culture vessels described by Gaillard [20] were used, on which an optically flat glass cover was sealed with hot paraffin. Stereomicroscope Polaroid photographs (with the culture vessels always oriented in the same direction) were taken at the beginning, during and at the end of the culture period. Fixation was performed in a solution containing 0,5 g NaCl, 80 mL water, 2 g trichloracetic acid, 4 mL acetic acid and 20 mL formalin. After 1 day of fixation, dehydration in an alcohol series and embedding in paraffin, the blastoderm fragments were sectioned perpendicularly to the already formed or presumed axis. The deparaffinized 8 µm thick sections were Feulgen stained to distinguish eventually quail nuclei from chicken nuclei [3, 28, 29]. The same histological procedure was used to study sections through unfertilized quail germ discs after fixation in situ on their egg yolk ball. Some of the sections were stained with Unna.

3. RESULTS

Sections through an unfertilized quail germ disc are seen in Figures 2A and 2B. Three different regions are visible in the ooplasm: the peripheral ooplasm containing voluminous vacuoles surrounds the central superficial ooplasm and the central deeper nucleus of Pander containing primordial yolk (in continuity with the also primordial yolk containing latebra neck). On the Feulgen or Unna stained sections through unfertilized quail germ discs no chromatin elements were seen. A chicken anti-sickle region and an unfertilized quail germ disc are seen side by side in culture in Figure 3. The peripheral vacuolized ooplasm partially overlaps the sectioned rim of the anti-sickle region, whilst the central part of the quail germ disc is not in contact with it. After culture, such an association gives never a reaction in the upper layer or embryo formation. It is only when a stable contact between the central deep part of the unfertilized quail germ disc (or «thick» section of it) and the deep side of the upper layer of the chicken anti-sickle exists and remains during the culture period (Figs. 4A and 4B), that an embryo can form. Sections through the tissue association of Figure 4B, reveal (after 27 h of culture) the development of a complete miniature embryo with neural plate, notochord, mesoblast ingressing through the primitive streak and the formation of a subgerminal space (Figs. 4C and 4D). After stable placing of a "thick" section of an unfertilized quail germ disc on a chicken anti-sickle region and culture in vitro (n = 22) about 30% of the explants

present a reaction in their chicken upper layer. This reaction is more or less pronounced and varies from thickening and increase in height of the upper layer cells to the formation of a nearly complete miniature embryo. Intermediate formations with a short primitive streak and/or neural plate are also observed. The place where the

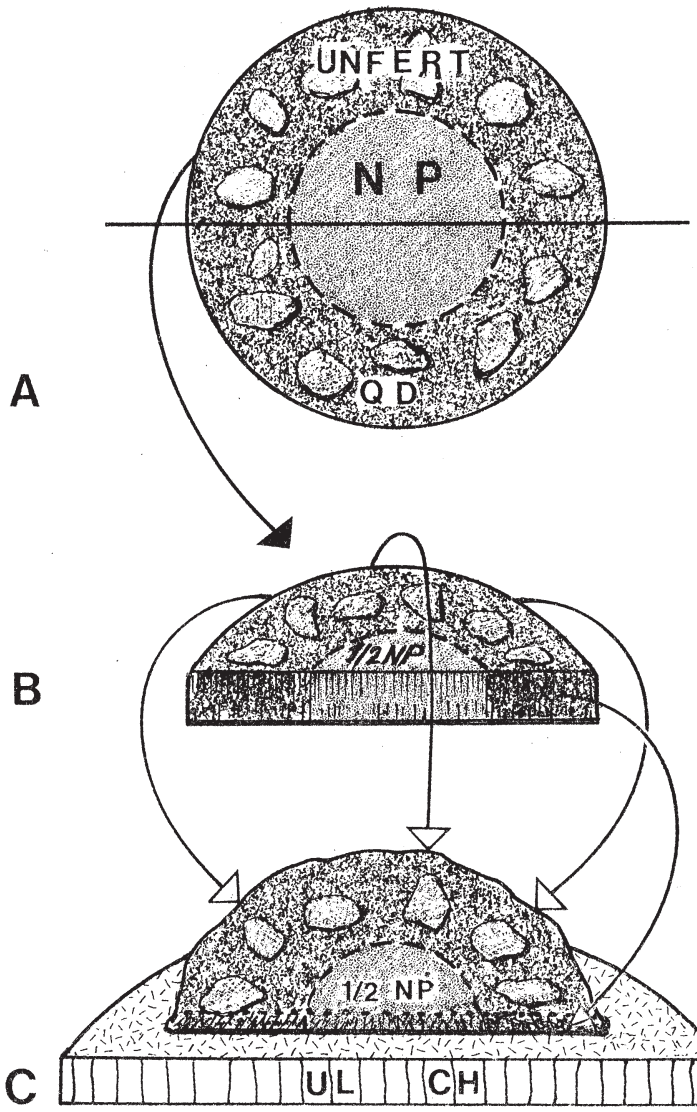


Figure 1. Schematic representation of the procedure of placing half or a "thick «section of an unfertilized quail germ disc (UNFERT QD) containing numerous vacuoles in its peripheral part, with its sectioned area on the deep side of the upper layer (UL CH) of an anti-sickle isolated from an unincubated chicken blastoderm. (**A**) Hemisection of the unfertilized quail germ disc; NP: nucleus of Pander. (**B**) Medial view of the so obtained hemisectioned germ disc; ½ NP: half of nucleus of Pander. (**C**) Placing of the sectioned area on the deep side of the upper layer (ULCH).

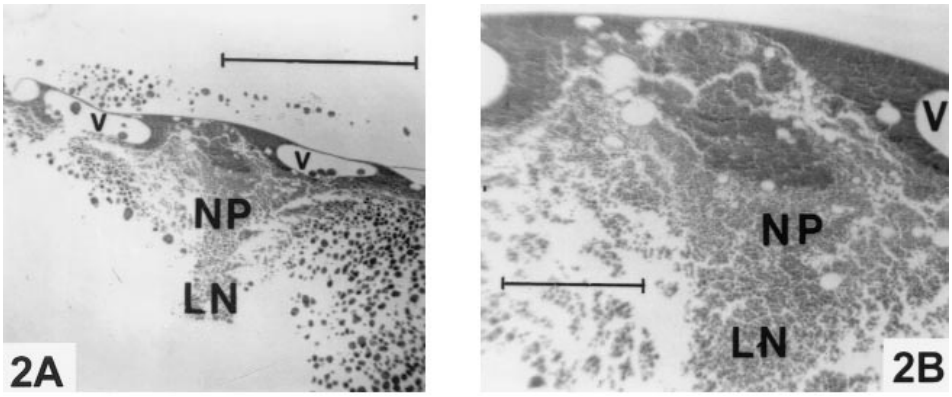


Figure 2. (**A**) Micrograph of an axial section through an unfertilized quail germ disc showing its different zones: the peripheral circular zone contains large vacuoles (V) and encircles the central zone in which we see from the depth to the surface, the latebra neck (LN), the central ooplasm with the nucleus of Pander (NP) and the most superficial part, containing irregular yolk masses; iron hematoxylin staining; bar: 1 mm. (**B**) Part of the central zone of (A) at higher magnification: the central ooplasm with nucleus of Pander (NP) and latebra neck (LN) containing primordial yolk spheres; V: vacuole of peripheral circular zone; bar: 200 μm.

embryo develops, corresponds to the central part of the apposed unfertilized quail germ disc or "thick" section, usually in the neighbourhood or immediately below the nucleus of Pander (still recognizable by the presence of primordial yolk spheres: [4]). Also the formed chicken endophyll is often seen in continuity laterally with the cells in the primordial yolk of the underlying nucleus of Pander (Fig. 5). Parallel with this endophyll layer and separated by a space, a (pre)neural plate Anlage is visible. The upper layer of isolated control anti-sickle regions of unincubated chicken blastoderms, still fixed on their cranial germ rim (n = 10), remained undifferentiated after culture in vitro. In the experiments (n = 11) where the central superficial part of an unfertilized quail germ disc was placed on the deep side of a chicken anti-sickle region, no embryo formation, was seen in the upper layer.

The remaining unfertilized eggs (n = 200) of the same quail group, who furnished the unfertilized quail germs in our association experiments, were incubated for 10 days. The eggs were then opened and controlled

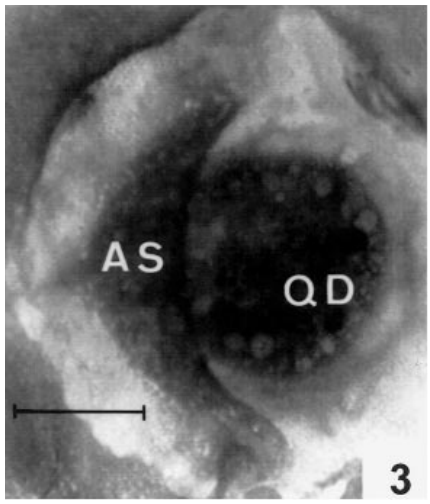
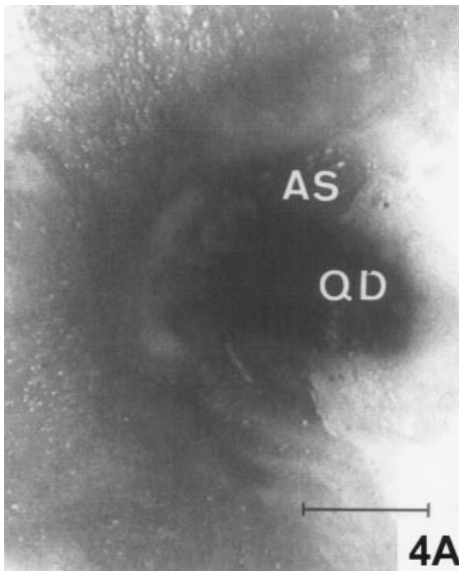
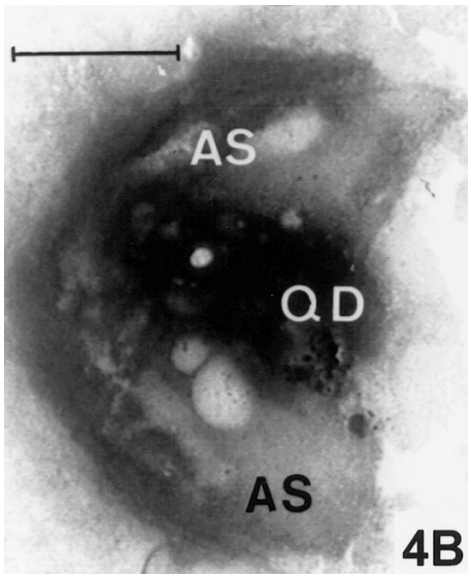
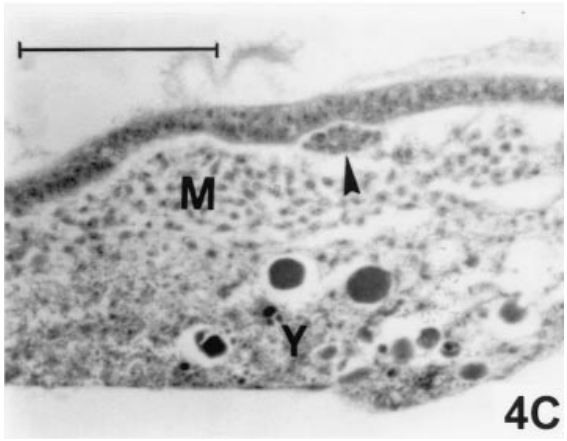


Figure 3. Stereomicrograph at the start of the culture of an unfertilized quail germ disc (QD) partially overlapping an anti-sickle region (AS) of unincubated chicken blastoderm. Since the central ooplasm of the quail germ disc is not in contact with the upper, layer of the anti-sickle region, no embryo will develop from this association; bar: 1 mm.







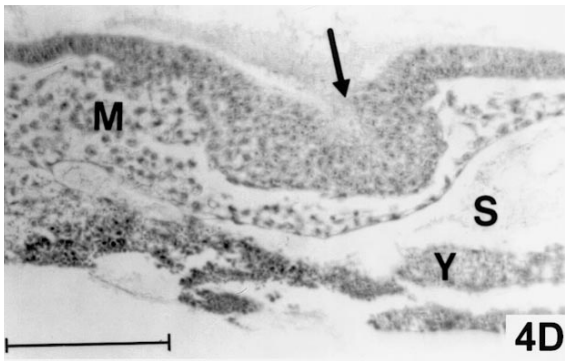


Figure 4. (**A**) Hemisectioned quail germ disc (QD) placed on an anti-sickle region (AS) of unincubated chicken blastoderm, so that the sectioned area comes in contact with the deep side of the chicken upper layer, at the start of the culture; bar: 1 mm. (**B**) The same association as in (A) after 27 h of culture: QD: fragment of unfertilized quail germ disc; AS: anti-sickle of chicken blastoderm; bar: 1 mm. (**C**) Transverse section through the chicken embryo formed in (B); a neural plate has developed in its cranial region: the chorda is indicated by an arrowhead; M: mesoblast; Y: yolk derived from the unfertilized quail germ disc; Feulgen staining; bar: 50 µm. (**D**) Transverse section through the primitive streak region of the chicken embryo of (B); primitive groove, indicated by arrow; M: mesoblast; S: subgerminal space; Y: partially digested yolk from unfertilized quail germ disc; Feulgen staining; bar: 50 µm.

for eventual parthenogenetic development. We concluded for the absence of parthenogenetic development in our unfertilized experimental quail group (criteria of Olsen [33]).

4. DISCUSSION

The present study indicates that the deep central part (containing the nucleus of Pander and surrounding ooplasmic layers) of an unfertilized quail germ disc, apposed on the deep side of the upper layer of an isolated anti-sickle region of an unincubated chicken blastoderm, can provoke in the latter, after culture, the development of a miniature embryo. Our experiments show that both associated parts intervene during this phenomenon. The appearance of endophyll in the here described cultured tissue association suggests the existence of an influence of the apposed central subgerminal ooplasm (nucleus of Pander and surrounding ooplasm) on the cranial upper layer. This can eventually been followed by the uptake of ooplasm form the quail nucleus of Pander (or surrounding ooplasm) by these chicken cells (see Fig. 5). In a previous study [13] we observed that (only in the presence of the upper layer a deep layer (containing endophyll) reformed during the migration of Rauber's sickle derived cells into the neighbouring central subgerminal ooplasm. We used anti-sickle regions as reactor tissue because they contain uncommitted (uninduced) upper layer and do not produce an embryo in culture if isolated from fresh unincubated chicken blastoderms. The isolation of the anti-sickle from the remainder of the blastoderm is indispensable since in the avian blastoderm a strong embryo inducing and dominating effect emanates from any Rauber's sickle material left in situ [14, 15] probably according to the concept of "positional information" [41]. Since the unfertilized quail germ discs present no chromatin elements, the observed embryo formation is not provoked by elements from quail nuclei. Moreover all the nuclei in the induced embryo are from chicken origin. How can the formation of an embryo in the

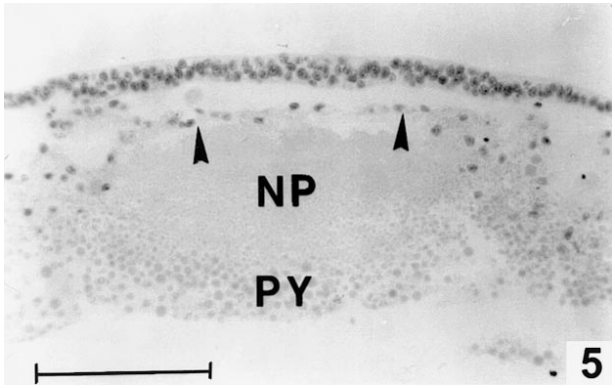


Figure 5. Section through the cranial part of an embryo developed after 26 h of culture in vitro under the influence of a "thick" section of an unfertilized quail germ disc apposed on an isolated chicken anti-sickle region: NP: centre of the nucleus of Pander from the unfertilized quail germ disc; note on top of the figure the thickened upper layer (preneural plate) and the endophyll layer (indicated by arrow heads) which is continuous with cells localized in the periphery of the nucleus of Pander, containing primordial yolk (PY) spheres; all the nuclei (both in the preneural plate as in the endophyll) are from chicken. Only above the endophyll, the upper layer shows a thickening with 3 rows of nuclei, whilst more peripherally the upper layer remains narrow (1 layer of nuclei); Feulgen staining; bar: 50 µm.

here described cultured tissue association been explained? It is possible that the deep central part of the unfertilized quail germ disc contains so called "vegetalizing factors". These factors were found in heterogeneous tissues (e.g. chick embryo extract) and have the ability to induce endoderm and mesoderm differentiation in newt animal cap explants [21, 22, 27]. These vegetalizing factors belong to the group of mesoderm inducing factors and mimic the action of the inducing signals from the Nieuwkoop centre (homologous with Rauber's sickle). According to Asashima et al. [1], activin could be the signalling molecule for endogenous mesoderm induction. Indeed in adult amphibia, activin is synthesized in follicle cells and transported into the growing oocyte where it remains stored until early cleavage stages when it serves as a signal which induces animal hemisphere cells to form mesoderm. Localized maternal determinants control the formation of dorsal axial structures in Xenopus embryos. UV irradiation of the vegetal surface of either prophase I oocytes or fertilized eggs, leads to the development of embryos that lack dorsal structures. Egg vegetal cortical cytoplasm is capable of restoring the dorsal axis of recipient embryos derived from UV-irradiated oocytes or fertilized eggs [23]. The maternal mRNAs, Vg1 and Xcat2 are localized to the oocyte vegetal pole [30], however their role, if any, in dorsal development remains unclear [40]. In a recent study [14] we observed that the nucleus of Pander and surrounding subgerminal ooplasmic layers present a spatial orientation parallel with Rauber's sickle and with the temporally-predisposed sickle-shaped Anlage fields in the upper layer of the unincubated overlaying blastoderm [10]. This parallelism suggests that at the moment of bilateral symmetrization i.e. approximately 6 h before laying [16], a long range "vertical" influence emanating through the subgerminal space from the central subgerminal ooplasm takes place. In view of the present observations this could perhaps explain the segregation of endophyll from the caudal upper layer under influence of the eccentrically displaced nucleus of Pander in undisturbed natural conditions. In our present study we assume that chicken cells from the anti-sickle region penetrate and proliferate into the neighbouring "young" quail ooplasm and form Rauber's sickle-like cells. The association of upper layer and subgerminal ooplasm (from fertilized, unincubated germs) can form endophyll in the presence of Rauber's sickle [9, 11]. This is followed by the development of a miniature embryo. That an embryo not always develops in every culture association of a chicken anti-sickle region and apposed unfertilized quail germ disc can be due to the loss of permanent contact between both. "Thick" sections through unfertilized quail germ discs are not very consistent and part of it can be lost during manipulations.

Comparison of the translocation studies of originally peripherally assembled yolk elements in avian oocytes and blastoderms [4, 5] with similar studies in amphibian oocytes and embryos [19] suggests that the nucleus of Pander is formed in an analogous way as the amphibian vegetal hemisphere. Also their ooplasms contain the oldest formed yolk [4, 5, 19]. A gene, named Brat is expressed maternally and its transcripts are localized to the vegetal hemisphere of the Xenopus egg. During early embryonic cleavage, Brat mRNA becomes partitioned primarly within the vegetal cells that are fated to form the endoderm and is essential for embryonic mesoderm formation [24]. Perhaps a similar phenomenon occurs in our avian experiments during the interaction of the upper layer with the central ooplasm of the unfertilized germ disc. Very recently, a germline-specific expression of chicken vasa homolog protein (homolog to the vasa gene which plays an essential role in germline formation in Drosophila) has been used for tracing the origin of avian primordial germ cells [38]. By this method the germline-specific expression was first found close to the depth

of the central cleavage furrows (which extend into the δ ooplasm of the nucleus of Pander [5] and later in the ventral ooplasm (also δ ooplasm) of 6–8 blastomeres in the center of the blastodiscs. This corresponds to the region where I have localized the germ cell yolk, which finally settles in the primordial germ cells [4, 5]. In Anura the "germ plasm" is localized in the depth of the vegetal hemisphere [2] which again suggests a similarity with birds.

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