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Mechanisms of caudocephalic axis formation in the avian germ disc

Marc Callebaut, Emmy Van Nueten, Fernand Harrisson and Hilde Bortier

RUCA, UA, Laboratory of Human Anatomy and Embryology
Groenenborgerlaan 171, B-2020 Antwerpen (Belgium)

ABSTRACT. We studied some mechanisms preceding or accompanying caudocephalic axis formation in the young avian germ disc. In an initial group this was done using eggs extracted from the uterus before oviposition. The development of germ discs after incubation in ovo (obliquely positioned or not) was compared with development after culture in vitro: germ discs develop much better when incubated in ovo than when cultured in vitro. We previously demonstrated that in obliquely-oriented germ discs, localized in situ on their egg yolk ball, 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 overlying blastoderm. The observations in our present study suggest that at the moment of bilateral symmetrization, a "vertical" influence emanating from the central subgerminal ooplasmic layers on parallel parts of the overlying blastoderm takes place. The first sign of the development of bilateral symmetry (already seen on the surface of a germ disc after 4 h of oblique positioning) is the appearance of a broad anti-sickle in the future cranial half of the blastodisc. In a second group, by excision of parts of unincubated or incubated blastoderms, followed by culture in vitro, we tried to explain some phenomena occuring during early axis development. Whilst gastrulation phenomena can take place after in vitro culture of isolated cranial quadrants of unincubated chicken blastoderms (stage X-XI of Eyal-Giladi and Kochav: 1976), this is not the case in isolated anti-sickle regions. The actual presence of neither RAUBER's sickle material nor sickle endoblast is necessary for the formation of a primitive streak. Also the existence of a long-range secretion gradient of inducing factors derived from these structures after their removal can play an inducing role.

KEY WORDS: caudocephalic orientation, avian blastoderm, RAUBER's sickle, gastrulation, anti-sickle region.

INTRODUCTION

Recent studies (CALLEBAUT 1993a, b, c; CALLEBAUT & VAN NUETEN, 1994, 1995; CALLEBAUT *et al.*, 1998) yielded new data about the structure and developmental events in avian intrauterine germ discs after bilateral symmetrization and in unincubated eggs. To describe these events, we have adopted an unequivocal terminology of the structures involved. The words cranial and caudal are used instead of anterior and posterior, because, like humans, birds are bipeds in an erect position. The terminology used for the different components of an unincubated chicken blastoderm is represented in Fig. 1. The term RAUBER's sickle is used instead of KOLLER's sickle (1882) since RAUBER was the first to describe it (CALLEBAUT & VAN NUETEN, 1994). We use the term sickle endoblast because we have demonstrated that this part of the deep layer is directly derived from the RAUBER's sickle (CALLEBAUT & VAN NUETEN, 1994). The anti-sickle region (Fig. 1) was first described by CALLEBAUT (1993b) in gravitationally-oriented quail germ discs. In this anti-sickle region, an irreversible disruption takes place between the future cranial part of the germ and the underlying peripheral subgerminal ooplasm at the moment of bilateral symmetrization (CALLEBAUT, 1993a, b, 1994). The anti-sickle itself is formed by a sickle-shaped group of loose yolk masses and cells. In the anti-sickle region the local upper layer (UL) is also included. RAUBER's sickle divides the area pellucida into a peripheral caudal area marginalis (Fig. 1) and into an area centralis in which a more or less developed sheet of endophyll can be seen in the caudo-central region. We use the name endophyll, rather than primary hypoblast to distinguish the endophyll from RAUBER's sickle, also a deep layer component (CALLEBAUT 1993a, 1993c, 1994). When we speak about temporally-predisposed upper layer (UL)

Corresponding author: Marc Callebaut, e-mail: macal@ruca.ua.ac.be

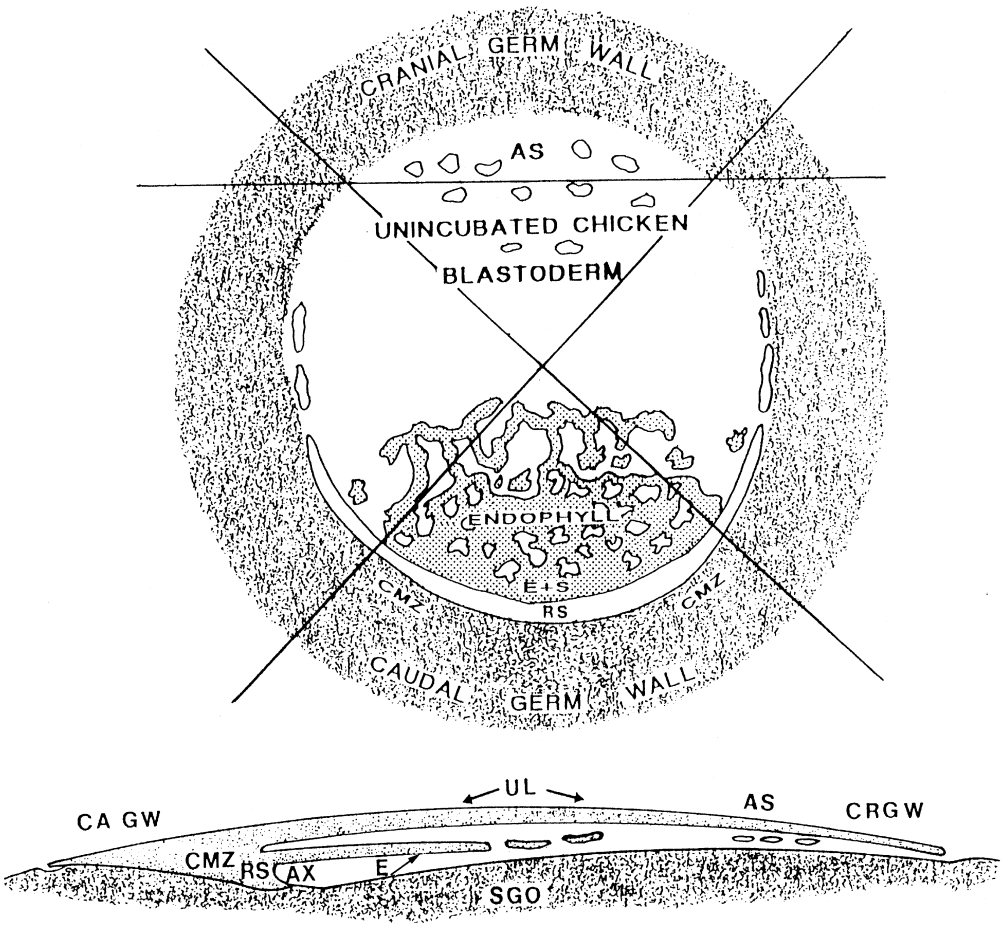


Fig.1. – Top: Schematic representation of the disposition of the components of the deep layer in the cranial, caudal and lateral quadrants of a freshly laid, unincubated chicken blastoderm seen from the ventral surface. In the unincubated chicken blastoderm the endophyll does not extend into the cranial quadrant.

Bottom: Schematic mediosagittal section through an unincubated chicken blastoderm. Note that the caudal marginal zone and RAUBER's sickle are in permanent contact with the caudal subgerminal ooplasm, whilst in the anti-sickle region and at the cranial germ wall this is not the case.

parts of a blastoderm, we mean parts that are still not definitively committed and which, after further incubation and without any intervention, will differentiate into a particular kind of tissue as the result of their spatial disposition in the blastoderm and their mutual interactions (CALLEBAUT et al., 1996). In the central region of the subgerminal ooplasm we find the nucleus of PANDER (1817), which contains a special kind of yolk: primordial yolk (CALLEBAUT, 1974) localized in δ ooplasm (CALLEBAUT, 1983, 1987 referred to by BELLAIRS, 1991, 1998). At the moment of blastoderm formation, the superficial ooplasm and primordial yolk of the nucleus of PANDER (1817) settle mainly in the endophyll and in the primordial germ cells (CALLEBAUT 1983, 1984). The boot-shaped distortion of the nucleus of PANDER (1817) and latebra-neck (HAMILTON, 1965) was first seen in mediosagittal sections of naturally-developing germ discs after ^{3}H-tyrosine injection to the quail mother and autoradiography (CALLEBAUT, 1983). In the present study, in obliquely-oriented germ discs, the eccentric spatial aspect of the nucleus of PANDER (1817) and of the surrounding subgerminal ooplasmic layers was observed. Some mechanisms of caudocephalic axis formation, intervening in isolated parts of unincubated or incubated avian blastoderms, are described. In a previous study (CALLEBAUT & VAN NUETEN, 1995) we investigated only the developmental potencies in vitro of isolated cranial quadrants and anti-sickles in quail. In the present work we also studied the development of similar fragments from the chicken. There seems to be a difference in the developmental potencies after culture in vitro of cranial quadrants from unincubated quail blastoderms compared with cranial quadrants from unincubated chicken blastoderms. In the first group usually only preneurulation phenomena occurred, while in the second group often a small embryo developed. Together with observations from earlier experiments, our present results suggest that in the presence of upper layer and endophyll, a primitive streak (PS) develops from a region (eventually isolated) in the avian blastoderm where a maximum of RAUBER's sickle material or junctional endoblast is/or has been present.

MATERIAL AND METHODS

Experiments with extracted quail eggs

The quail eggs were extracted from the uterus at the beginning of calcification (approximately 10 h after oviposition of the previous egg, according to STEPINSKA and OLSZANSKA, 1983) to just before oviposition (25 h after oviposition of the previous egg). Some of the extracted eggs were incubated vertically at 39° C in a humid atmosphere, all or none by the air-bubble method (CALLEBAUT, 1993b). After 4-11 h of oblique positioning, the egg yolk balls were fixed in toto in calcium formalin overnight, followed by rinsing in tap water during one day. Shortly after the beginning of fixation, a linear charcoal mark was placed behind the caudal germ wall (behind the highest point of the blastoderm rim). Thereafter the egg yolk balls were dehydrated during approximately five days, successively in alcohol solutions of 30, 50, 70, 95, 100 percent. In the last liquid the germ discs (still fixed to their vitelline membrane) were excised from their egg yolk ball, and the nucleus of PANDER and surrounding yolk layers in the subgerminal ooplasm were progressively exposed using a gentle stream of absolute alcohol from a Pasteur pipette. Stereomicrographs were taken with a Sony color videoprinter (Mavigraph). Tissues were embedded in paraffin, and 8 µm-thick sections cut parallel with the caudocephalic axis (perpendicular to the linear charcoal mark applied behind the caudal germ wall). Sections were stained with Unna (after SILVERTON & ANDERSON, 1961). From other extracted uterine eggs the blastoderms were removed by excision from their egg yolk ball and cultured in vitro according to the method of NEW (1955).

Observations and/or experiments with unincubated chicken blastoderms or parts of them

We used unincubated chicken blastoderms presenting a sickle of RAUBER (1876) from eggs stored at 15-20° C for 1-2 days. After opening of the eggs and removal of the egg white, the egg yolk balls were placed in a chick RINGER solution. The vitelline membrane was sectioned all around the equator of the yolk ball and removed from the yolk by a slow movement in the direction of/and bending 180°C over the germ disc. In this manner the chicken blastoderm, still adhering to the vitelline membrane and underlying subgerminal ooplasm, could usually be separated from the yolk mass.

After excision of the chicken cranial quadrants or anti-sickle regions, the other blastoderm parts were removed and the former were cultured, still fixed on their own vitelline membrane, according to the method of NEW (1955).

Experiments with quail blastoderms, preincubated in ovo during 11-14 h (Stage 4, HAMBURGER & HAMILTON, 1951), containing junctional endoblast

The position and orientation of the differently-cultured germ discs or differently-cultured fragments of quail or chicken blastoderms are represented on a scheme accompanying the photomicrographs of the explants. The fixation of cultured blastoderms or fragments was performed in a modified Heidenhain's fixative (ROMEIS, 1948) containing 0.5g NaCl, 80ml water, 2g trichloracetic acid, 4ml acetic acid and 20ml formalin. The further histological procedures were as described in an earlier study (CALLEBAUT & VAN NUETEN, 1994).

Abbreviations used in the figures

AB:	air bubble
AO:	area opaca
AS:	anti-sickle region formed by a continuous upper layer part and an interrupted deep layer part, which contains no sickle material but numerous loose yolk masses and loose cells as a result of the disruption from the underlying ooplasm (CALLEBAUT, 1993 a, b, c)
AX:	axilla; arm-pit-like caudal part of the subgerminal cavity
CA:	caudal
CA GW:	caudal germ wall
CMZ:	caudal marginal zone, localized behind RAUBER's sickle
CR GW:	cranial germ wall
CR Q:	cranial quadrant
CSO:	central subgerminal ooplasm
E:	endophyll
EMBR:	embryo develops
E + S:	zone where sickle endoblast derived from RAUBER's sickle (CALLEBAUT & VAN NUETEN, 1994) penetrates into the endophyll (CALLEBAUT et al., 1997a)
IE ECT	intraembryonic ectoderm
JE:	junctional endoblast
LAT PLATE:	lateral plate
LGW:	lower germ wall
LN:	latebra neck (HAMILTON, 1965)
NO EMBR:	no embryo develops
NP:	nucleus of PANDER or neural plate
PNP:	preneural plate
PS:	primitive streak
RS:	RAUBER's sickle extends laterally and cranially as sickle horn fragments
S:	sickle endoblast
SC:	subgerminal cavity
SGO:	central subgerminal ooplasm
SH:	sickle horn
SOM:	somites
t:	cranial pointed end of nucleus of PANDER
UGR:	upper germ rim
UGW:	upper germ wall
UL:	upper layer
ULAS:	upper layer of anti-sickle
YE:	yolk endoblast

RESULTS

Formation of a caudocephalic axis in quail germ discs extracted from the uterus (n=25)

Only eggs with a hard but fragile calcareous white egg shell (approximately corresponding with a chicken stage VI-VII of EYAL-GILADI & KOCHAV, 1976) or with a more calcified shell, formed a caudocephalic axis (in 70% of the cases) and usually developed further normally when incubated in ovo. Similar germ discs removed from white-shelled eggs did not develop normally after culture in vitro. By contrast, blastoderms from extracted eggs presenting a pigmentation (beginning a few hours before oviposition and in which bilateral symmetrization had already taken place) developed normally when explanted in vitro.

If the germ disc from an egg with a hard but fragile calcareous egg shell was placed in a permanently oblique position (n=15) (with reference to the vertical line) by injection of an air bubble at the topmost point of its egg yolk ball (air-bubble oriented method: CALLEBAUT 1991; 1993b) (see Fig. 2) then also a normal caudocephalic axis developed. If this oblique positioning was applied before symmetrization, a lower, broader (future cranial) germ wall and a higher, narrow (future caudal) germ wall appeared (Fig. 3). Also visible from the surface, even after a short time (e.g. after 4 h of oblique orientation) was the very obvious formation of a broad voluminous anti-sickle region. On sections, this anti-sickle region is initially seen to be formed by loose yolk masses and cells extending sometimes into the whole cranial half of the subgerminal space (Figs 4, 5, 6, 7, 8: paradoxical eccentricity: CALLEBAUT, 1993b)

The cells form 2-5 rows close to the vitelline membrane, whilst the large yolk masses are found in or at the bottom of the subgerminal space.

After 11 h of oblique positioning the germ disc usually takes the aspect of an unincubated blastoderm on sections: the cranial half is flatter than the caudal half, because in the latter endophyll, RAUBER's sickle and the caudal marginal zone have developed. During the gentle progressive removal of the yolk below the germ disc in the 100% alcohol solution (during dehydration), we were able to expose the general stereomicroscopic aspect and orientation (under influence of the oblique positioning) of the nucleus of PANDER and surrounding ooplasm (Fig. 9). Here the elongated, slightly oblong, coniform aspect of the nucleus of PANDER is seen bulging on the underside of the subgerminal ooplasm. The sharper end (toe-like) points in the direction of the cranial part of the blastoderm. The broader end (heel-like) is directed towards the caudal part of the blastoderm. The nucleus of PANDER has thus also an axis parallel with the caudocephalic axis of the overlying blastoderm. When we progressively removed the more peripheral ooplasmic layers of the nucleus of PANDER, we saw that they also have not a circular form but are ovoid, elongated, and parallel (Fig. 10) with the caudocephalic axis of the neighbouring blastoderm. The central core of the nucleus of PANDER usually also has a pointed end in a cranial direction. The ovoid layers surrounding the central core of the nucleus of

Legends to the figures (see opposite page)

Fig. 2. – Schematic representation of the localization of structures (eventually visible at the surface) in an extracted quail germ placed in an oblique orientation in situ on its egg yolk ball, by the air bubble method after in ovo incubation. A transition between the early eccentric stage and the freshly laid egg stage is represented: the RAUBER's sickle is still localized close to the upper germ rim; anti-sickle region indicated by stippled area; nucleus of PANDER and surrounding encircling ooplasmic layers together form an ovoid coherent mass.

Fig. 3. – Surface view of a similar germ disc as represented in Fig. 2 after 11 h of oblique orientation and in ovo incubation; several structures are visible by transparency; the lower germ wall is broader than the upper germ wall; the anti-sickle is localized in the cranial (lower) half of the subgerminal cavity; bar: 1 mm.

Fig. 4. – Mediosagittal section (seen at low magnification) through the germ disc from an extracted, white, thin-shelled quail egg after 4 h of oblique orientation and in ovo incubation; the future caudal part is at the right of the figure. Note the elongated form of the nucleus of PANDER with pointed end directed cranially; Unna staining; bar: 1mm.

Fig. 5. – Caudal part of the section of Fig. 4 at higher magnification: the number of cells in this region is lower than in the more cranial parts of the germ (usually only 1 row of cells is seen); bar: 200 µm.

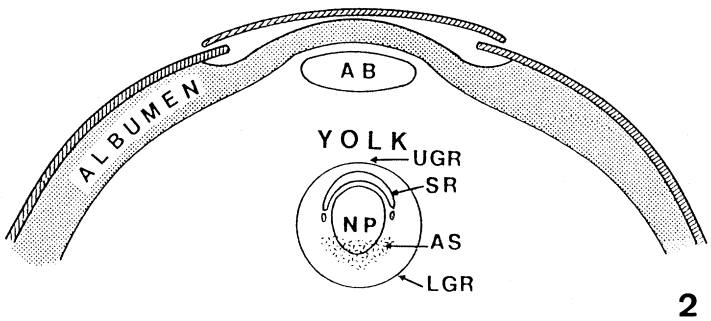
Fig. 6. – Central part of the section of Fig. 4 at higher magnification; note the increasing number of small round cells below the vitelline membrane (2-3 rows of cells are seen) and several large yolk masses in the subgerminal cavity above the nucleus of PANDER; bar: 200 µm.

Fig. 7. – More cranial part (anti-sickle region) of the section of Fig. 4 at a higher magnification; note the very numerous cells aggregated below the vitelline membrane (3-5 rows of round cells); bar: 200 µm.

Fig. 8. – Most cranial (lower) part of the section through the germ disc of Fig. 4 at higher magnification; near the cranial rim of the germ disc only 1 row of cells is seen; bar: 200 µm.

Fig. 9. – Surface view of coniform ovoid nucleus of PANDER with surrounding subgerminal ooplasmic layers, bulging at the deep side of the germinal disc after progressive removal of the uninvolved yolk. The germinal disc has been oriented obliquely in situ on its egg yolk ball during 11 h. Note the caudocranial orientation parallel with the caudocranial axis of the neighbouring blastoderm. Note the cranial pointed end and the caudal round larger end; bar: 1 mm.

Fig. 10. – After the peripheral yolk layers are progressively peeled away from the nucleus of PANDER, the ovoid ooplasmic layers surrounding the central core (arrowhead) can be visualized in surface view. Here also the caudal and cranial end of the layers converge behind the core; bar: 1 mm.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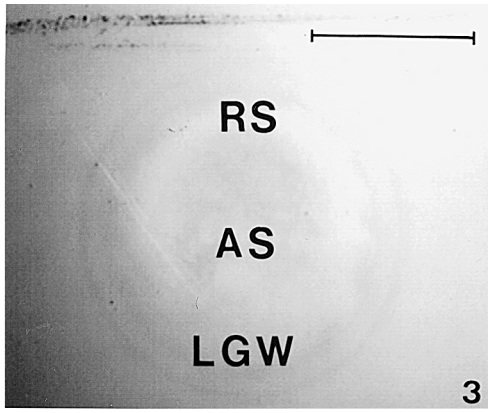
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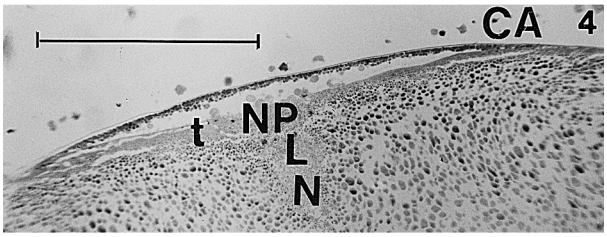
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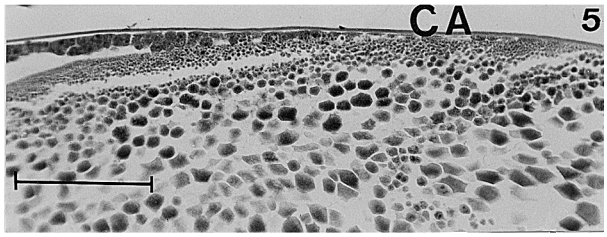
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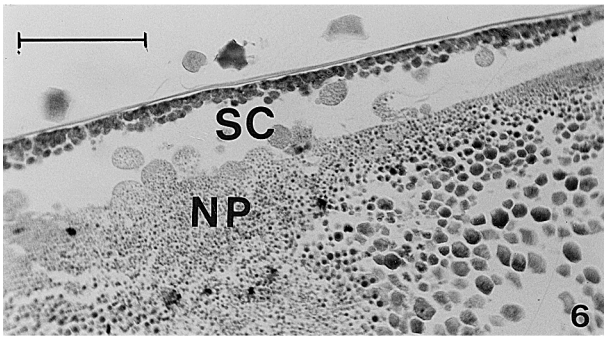
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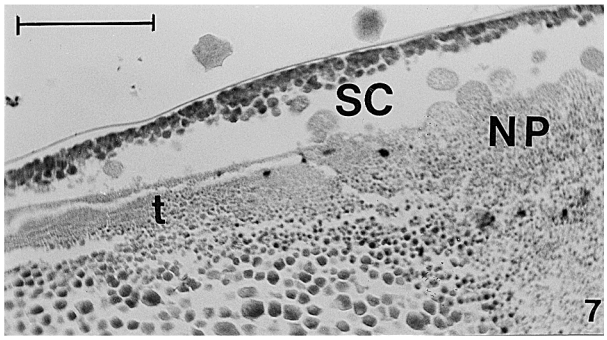
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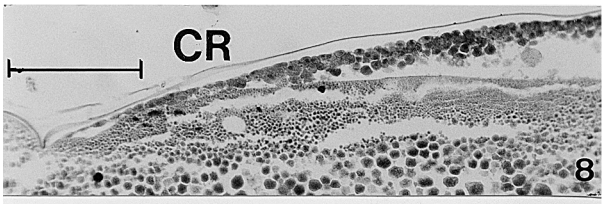
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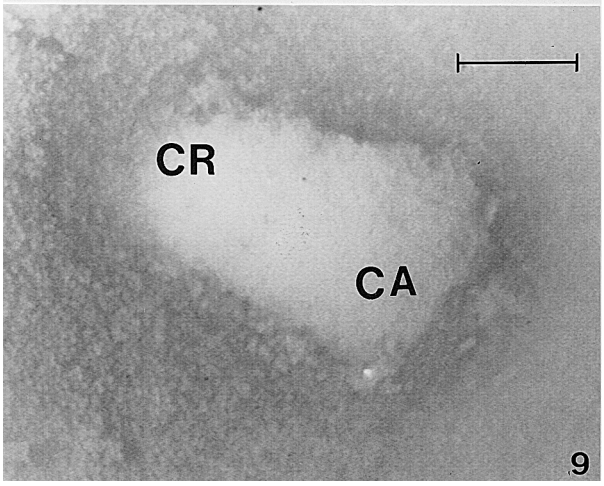
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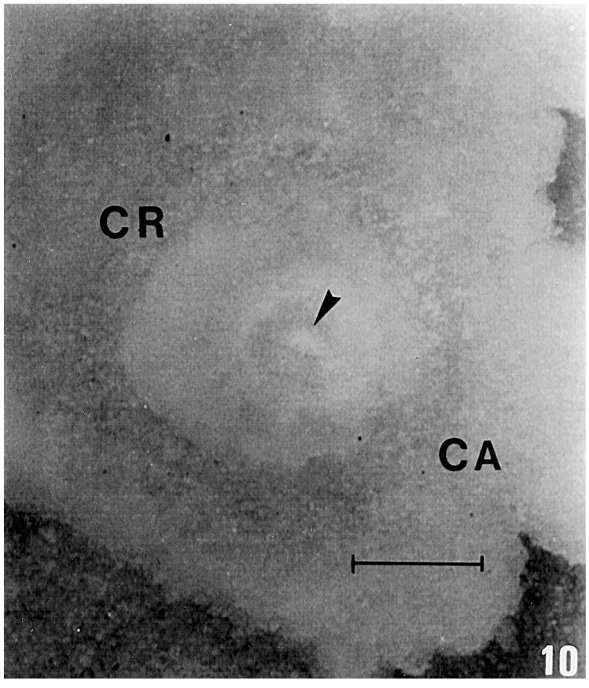
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PANDER are separated by a much larger distance cranially than caudally, where they are localized very close to, and just behind the central core. Let us note that the nucleus of PANDER together with the encircling subgerminal ooplasmic layers has a maximum length of circa 2-2.5 mm, which corresponds approximately with the extent of the area centralis of the overlying blastoderm. Since, during fixation, the orientation of the egg yolk ball and germ disc remains unchanged with reference to the vertical line (by the presence of the air bubble on top), the shape of the nucleus of PANDER and surrounding ooplasmic layers is unaltered (CALLEBAUT, 1991).

Development of axial structures in isolated cranial quadrants (n=11) or in isolated anti-sickle regions (n=12) from unincubated chicken blastoderms after culture in vitro.

In a stereomicrograph (Fig. 11A) the excised cranial quadrant, still fixed to the most cranial part of the germ wall of an unincubated chicken blastoderm, is visible at the start of the culture. After one day of culture (Fig. 11B) an embryo has developed with a centrally-oriented head region. The embryo is localized in a small area pellucida, clearly separated from a much more extended area opaca (epiboly). On sections, a PS and a neural plate are seen. In 50 % of the cultivated cranial chicken quadrants, a more or less developed embryo formed with PS and neural plate. In the remainder of the cases no PS was seen and only a broad preneural plate (Figs 12A, B) formed. Sections of the latter show a pronounced thickening of the UL above the segregated endophyll. On sections the thickened UL is separated from the endophyll by a large space, and presents localized "banding" of the nuclei (ENGLAND, 1973) indicating primary neural induction (ENGLAND & LAWSON, 1993) in the absence of chorda-mesoderm (CALLEBAUT & VAN NUETEN, 1995). The latter developmental pattern with only the formation of a preneural plate was also always seen after the culture of cranial quadrants from unincubated quail blastoderms (CALLEBAUT & VAN NUETEN, 1995). The reason for the different developmental behaviour was the presence (or proximity) or absence of RAUBER's sickle horns in the cranial quadrants (see also discussion).

Eventual development of axial structures in isolated cultured anti-sickle regions taken from unincubated chicken blastoderms (n=12).

Only one of these anti-sickle regions (Fig. 13A), removed from a seemingly unincubated chicken blastoderm (approximately stage XII – XIII of EYAL-GILADI and KOCHAV: 1976), as seen in Fig. 13B, presented, after one day of culture, a very small and short embryo (Fig. 13C) (confirmed by sectioning). The area opaca here again was very extended, whilst the surface of the area pellucida in which the rudimentary embryo formed, remained very small. In the 11 other cases (from genuinely unincubated blastoderms, i.e. stages X to XI of EYAL-GILADI and KOCHAV, 1976) no embryo developed and not even a preneural plate was observed after sectioning.

Development of new primitive streaks from lateral parts of intermediate streak quail blastoderms (Stage 3+ of HAMBURGER & HAMILTON, 1951 (n=5)).

The experimental procedure is represented on the drawing (Fig. 14A): the original primitive streak with the whole median part of the quail blastoderm was removed. Thus, two parts were obtained, each of which contained a lateral part of the area pellucida and neighbouring junctional endoblast, yolk endoblast, and area opaca (Fig. 14B). Note that the three elementary tissues necessary for the start of an embryo formation (*i.e.* junctional endoblast, derived from RAUBER's sickle, upper layer and endophyll, still found in the cranial region) were present. After culture for 24-28 h (Fig. 14C) a small centrally-directed process appeared at the inner border of the explant. On the sections performed perpendicularly to the axis of this small process, a short neural plate and a primitive streak (presenting ingression phenomena) were seen. The development thus starts from the middle region of the remaining junctional endoblast as is the case during normal gastrulation. By the interaction between the three elementary tissues we can probably also explain the reconstitution of an embryo in avian lateral blastoderm isolates as observed by YUAN & SCHOENWOLF (1999), although they were unaware of the persistence of junctional endoblast in their isolates.

Legends to the figures (see opposite page)

Fig. 11A. – Stereomicrograph of an excised cranial quadrant of unincubated chicken blastoderm at the start of the culture; bar: 1 mm.

Fig. 11B. – The same explant as from Fig. 11A. after 24 h of culture, before fixation: an embryo has formed; bar: 1 mm.

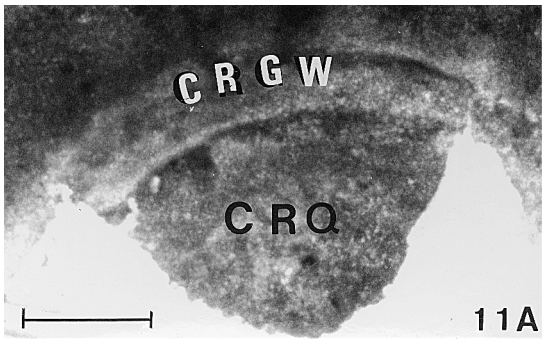
Fig. 12A. – Stereomicrograph of an excised cranial quadrant of unincubated chicken blastoderm at the start of the culture; bar: 1 mm.

Fig. 12B. – The same explant as from Fig. 12A. after 24 h of culture, before fixation; only a preneural plate has formed, no PS could be discerned (confirmed by sectioning); bar: 1 mm.

Fig. 13A. – Stereomicrograph of an anti-sickle region of chicken blastoderm, at the start of the culture; bar: 1 mm.

Fig. 13B. – Stereomicrograph of a seemingly unincubated chicken blastoderm from which the anti-sickle region was used for isolated culture in Fig. 13A; arrow indicates middle of RAUBER's sickle; arrowhead indicates far cranially-extending right sickle horn; bar: 1 mm.

Fig. 13C. – Stereomicrograph of the explant Fig. 13A. after 23 h of culture, alive, before fixation; arrowhead indicates a rudimentary embryo in a very narrow area pellucida; bar: 1 mm.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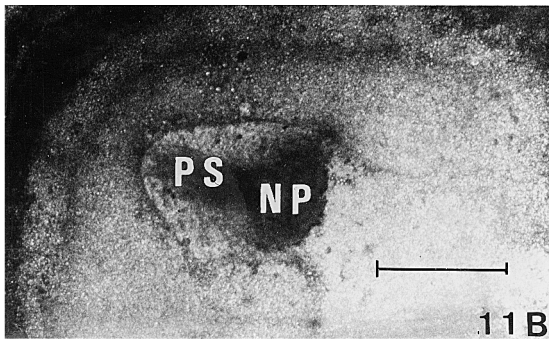
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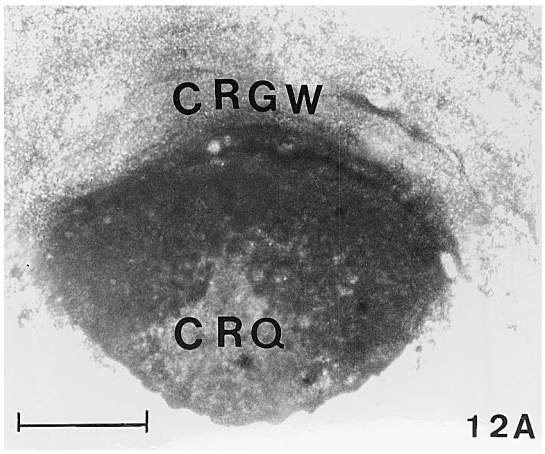
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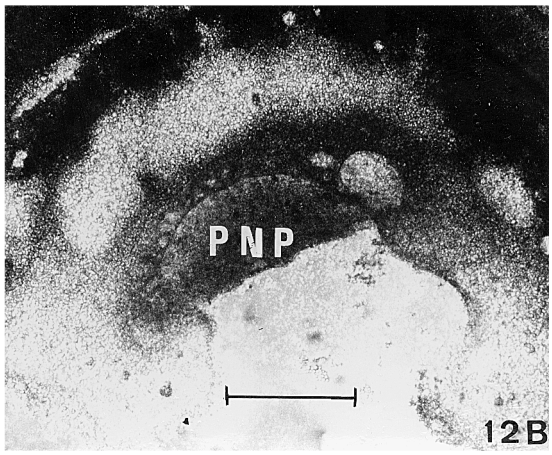
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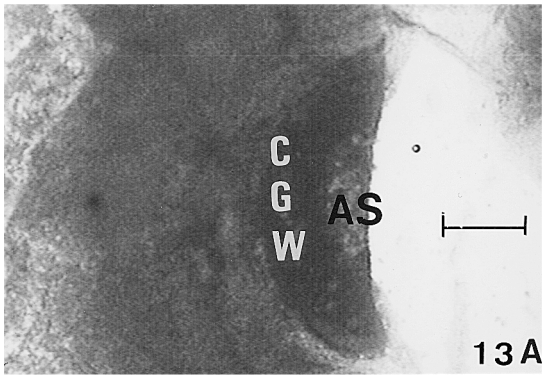
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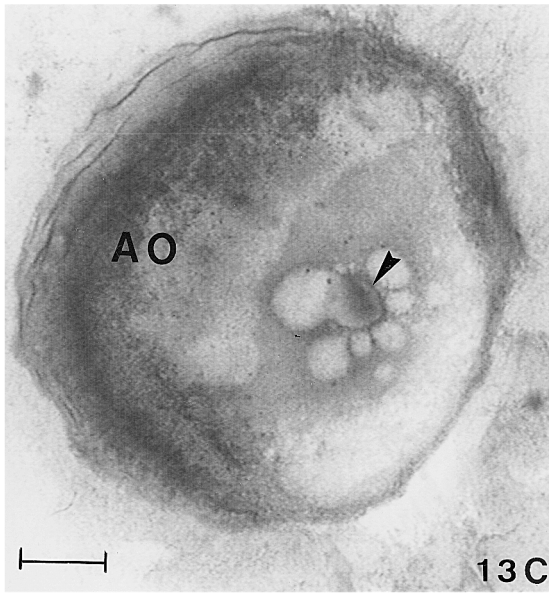
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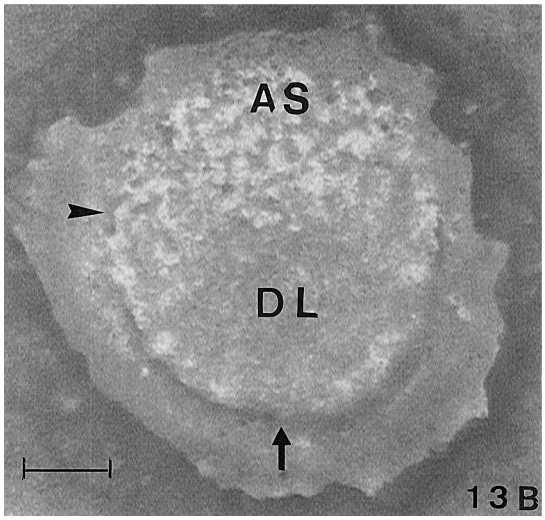
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DISCUSSION

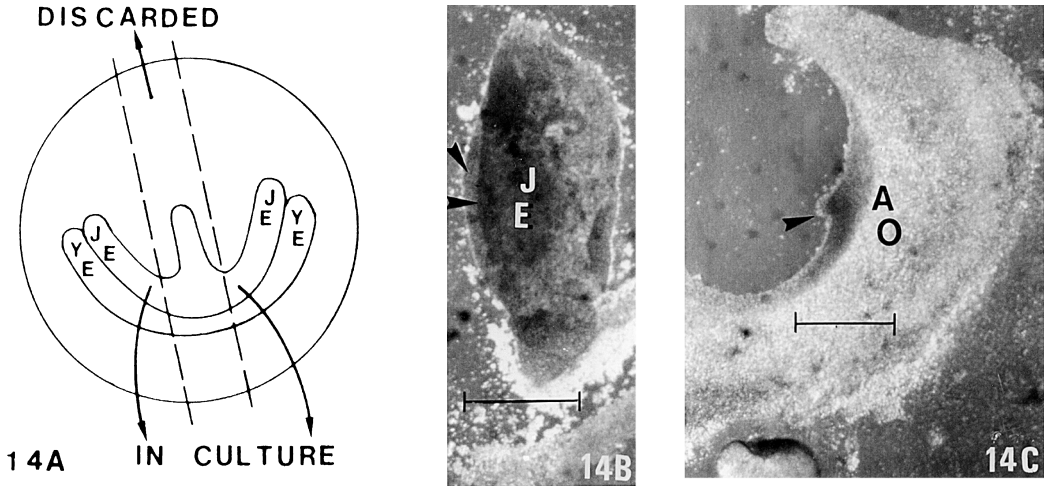
Our comparative study indicates that germ discs remaining on their egg yolk ball during in ovo culture (from eggs with a hard but fragile calcareous shell, extracted from the uterus) develop normally. By contrast, similar germ discs separated from their egg yolk ball and cultured in vitro, do not. The reason for this is probably the loss of normal contact of the extracted quail germ disc with its vitelline membrane and surrounding or underlying ooplasm. The eccentric shift of the subgerminal ooplasm with reference to the superficial part of the germ disc can probably not take place normally. Indeed after culture of young extracted germ discs there occurs no expansion at their rims. This is also in agreement with the experiments of OLSZANSKA et al. (1984), who cultured uterine quail germ discs (before area pellucida formation) still adhering on their own vitelline membranes with some subgerminal yolk still attached. The germ discs were stuck on a filter-paper ring and placed vertically or obliquely in egg albumen for culture. Thus they obtained, after prolonged culture, 50 % of quail embryos that developed a caudocephalic axis.

In the largely extended anti-sickle region of the cranial half of obliquely-oriented germ discs (before symmetrization), the rows of blastomeres always remain localized close to the vitelline membrane. We found no evidence that all these round cells, although loosely bound together (Figs. 6, 7, 8), fall into the sub-blastodermic cavity, where they, according to KOCHAV et al. (1980), should assemble beneath the future cranial part of the blastoderm. Moreover, by means of our labelling techniques using trypan blue (CALLEBAUT, 1987), or ^{3}H-leucine labelling (CALLEBAUT, 1994) we were able to demonstrate shedding, nor displacement (rolling over the bottom of the subgerminal cavity) of these cells in a cranial direction. Only the large yolk masses float in the fluid of the subgerminal cavity or are found on its bottom, where they detach from it. In our present experiments, after oblique positioning of the germ disc in situ, we have shown the eccentric parallelism existing between the aspect and localization of the nucleus of PANDER with its surrounding morphologically-visible layers in the subgerminal ooplasm on one side, and the predisposed Anlage fields in the UL of the avian unincubated blastoderm (CALLEBAUT et al., 1996: represented schematically in Fig. 15) on the other side. The general hemicircular disposition and form of these Anlage fields still seem to reflect, together with the RAUBER's sickle, the RNA-bound, original, ooplasmic, concentric and radial symmetry built up in the large oocytes (CALLEBAUT, 1972, D'HERDE et al., 1995). Moreover, there exists also a combination with the eccentricity of the deep layer components, which was observed during early symmetrization by gravitational orientation of the egg yolk (CALLEBAUT, 1993a,b).

Although the central subgerminal ooplasm is structurally completely disconnected from the area centralis of the blastoderm above it, there already exists a parallelism between both long before symmetrization has taken place. This eccentricity in the subgerminal ooplasmic layers is only visible in the latter uncleaved components as a result of the interaction of rotation and gravity on the egg yolk ball.

The caudal part of the deep ooplasm turns below the future caudal part of the blastoderm (CALLEBAUT, 1993b). This could explain the eccentric sickle-shaped effect on the distribution of the Anlage fields in the caudal half of the UL (CALLEBAUT *et al.*, 1996) if we accept a "vertical" influence of the subgerminal ooplasmic layers. The general ovoid shape of the nucleus of PANDER (containing δ ooplasm: CALLEBAUT, 1987) is parallel with the endophyll that also contains δ ooplasm. The latter induces a preneural plate Anlage with a very similar outline (CALLEBAUT et al., 1995; CALLEBAUT et al., 1996: see Fig. 15) in the neighbouring upper layer (CALLEBAUT et al., 1999a). In the presence of UL and RAUBER's sickle the nucleus of PANDER can give rise to endophyll, and an embryo forms (CALLEBAUT, 1994; CALLEBAUT et al., 1995; CALLEBAUT et al., 1998; CALLEBAUT et al., 1999c). On a comparative schematic drawing (Fig. 16) the tissue associations, which in earlier experiments did or did not produce endophyll (followed or not folowed by embryonic development) are represented. It is only in the presence of upper layer and RAUBER's sickle that endophyll is formed de novo, from subgerminal ooplasm, so that in particular cases the subgerminal ooplasm can be considered as a fourth elementary tissue. This association of germinal disc components (UL, RAUBER's sickle and subgerminal ooplasm) with the formation of a caudocranially- oriented embryo is found in natural conditions behind the caudal part of the subgerminal space (Figs. 4, 5) at the end of intrauterine development. Indeed, the nucleus of PANDER and surrounding ooplasms, during the oblique positioning of the egg yolk ball, shift temporally or definitively in a caudal direction (CALLEBAUT 1993b). So this mechanism can probably play a role during the determination of the future avian caudocephalic axis.

In one case out of 12 cultured chicken anti-sickles a very small rudimentary embryo developed (Figs. 13A, 13B, 13C). The reason for this exception seems obvious: the deep layer of that particular chicken blastoderm (seemingly unincubated) (Fig. 13B) extended much more cranially than in the other genuinely unincubated chicken blastoderms (Fig. 18), probably through incubation having, in fact, begun. So in contrast to the general belief that there still exists a radial symmetry in unincubated chicken blastoderms, we were able to demonstrate that this is not the case in quail as in chicken. How can we explain that in cultured isolated cranial quadrants from unincubated quail blastoderms usually only a preneural plate develops (CALLEBAUT & VAN NUETEN, 1995) whilst in the present study in similar cranial quadrants (but from chicken) small embryos (presenting both a PS and a neural plate) also formed? We think that this can be explained by the



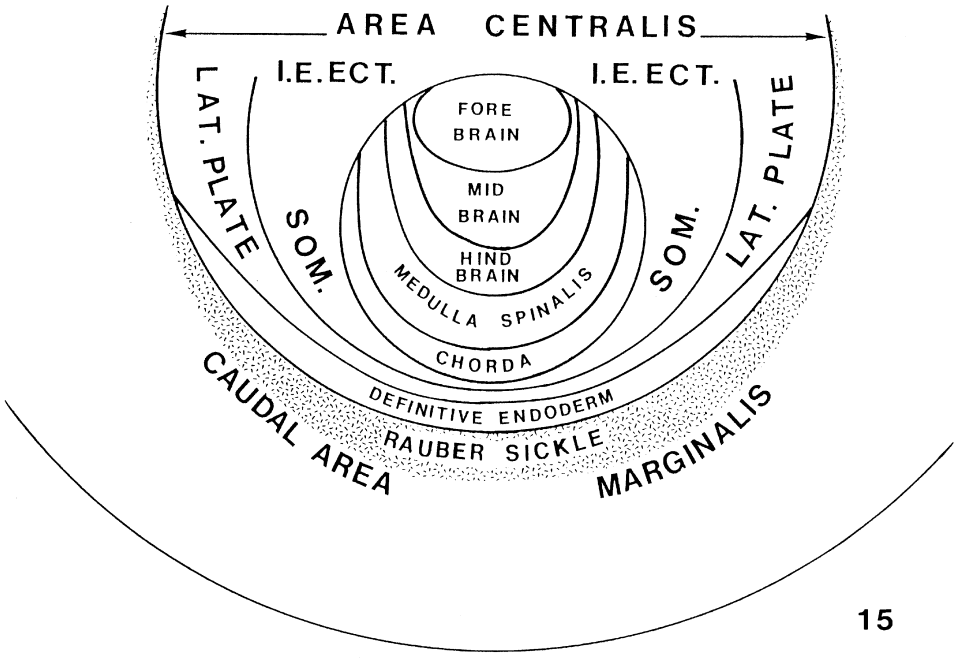


Fig. 14A. – Schematic representation of a quail blastoderm preincubated during 12-14 h (stage 3+ of HAMBURGER & HAMILTON, 1951); showing the experimental procedure for culture of the lateral parts: the original PS with the whole median part of the area pellucida of the quail blastoderm was discarded. The two lateral parts containing the lateral parts of the area pellucida, junctional endoblast, yolk endoblast and area opaca were cultured for 24-28 h.

Fig. 14B. – Stereomicrograph of a lateral part of a quail blastoderm (as obtained according to the experimental procedure of Fig. 14A.), at the start of the cultural period. The upper arrowhead indicates the incision line of the upper layer of the area pellucida; the lower (horizontal) arrowhead indicates the retracted incision line of the deep layer (mainly sickle endoblast) of the area pellucida; JE: junctional endoblast; bar: 1 mm.

Fig. 14C. – The same explant as represented in Fig. 14B, after 25 h of culture: a small centrally-directed process (arrowhead) containing a miniature embryo has appeared at the inner border of the explant.

Fig. 15. – Schematic representation of the mean localization of the predisposed (not definitively committed) Anlage fields (in good order but with possible partial overlapping of neighbouring parts) in the upper layer of a chicken unincubated blastoderm (slightly simplified after CALLEBAUT et al., 1996). Note the general eccentric sickle-shaped aspect of the Anlage fields in the area centralis. There is an obvious parallelism between the shape of the Anlage fields in the UL and the ovoid central subgerminal ooplasmic layers.

difference in structure and aspect between unincubated quail blastoderms (Fig. 17) and unincubated chicken blastoderms (Fig. 18) at the start of the culture period. Indeed most unincubated quail blastoderms have a massive RAUBER's sickle which, however, does not extend far in the cranial half of the blastoderm. By contrast, the RAUBER's sickle of the chicken is usually very narrow but the sickle horns often extend into the cranial quadrant.

Even if RAUBER's sickle material is not actually present in the cranial quadrants, a long-range gradient of diffusible factors emanating from neighbouring more caudal parts of RAUBER's sickle could induce a PS even after removal of these parts. The existence of such long-range gradients of diffusible factors can be deduced from other experiments:

1/ In a recent study (CALLEBAUT et al., 1999b) we demonstrated the strong embryo-inducing and often dominating effect over the autochthonous RAUBER's sickle, of a fragment of junctional endoblast placed on the diametrically-opposed anti-sickle region of an unincubated chicken blastoderm.

2/ After the culture of the central part of the area centralis of unincubated chicken blastoderms (in the total absence of RAUBER's sickle) a normal embryo with a preneural plate and PS develops in approximately 20% of cases, with a caudocephalic orientation according to the original localization of the RAUBER's sickle (CALLEBAUT et al., 1997b). If, on such a central part of a chicken area centralis, we place a quail RAUBER's sickle fragment, then a normal embryo always develops in the upper layer, with a caudocephalic axis directed from this apposed RAUBER's sickle fragment towards the nearby endophyll. Thus the original "imprinted" caudocephalic axis imposed by the removed autochthonous RAUBER's sickle will be totally ignored. Also this phenomenon can be explained by the dominating effect of the apposed RAUBER's sickle, probably as a result of its more pronounced secretion gradient.

The study of BACHVAROVA et al., (1998) seems to indicate that the caudal marginal zone isolated from RAUBER's sickle can induce a primitive streak. However, this observation can perhaps also be explained by the diffusion of substances secreted by RAUBER's sickle into the caudal marginal zone. The latter can then induce a PS in tissues placed in the neighbourhood after the secondary release of these substances. The presence, during gastrulation, of many diffusible proteins with signalling function has been described:

- Mesoderm-inducing fibroblast growth factor in the frog (KIMELMAN et al., 1988).
- The growth factor Vg1 from the TGF- family gives an induction of dorsal mesoderm and should be the real inductor found in the NIEUWKOOP center of amphibian blastulas (WEEKS & MELTON, 1987). SHAH et al., (1997) found expression of chick Vg1 in the UL region above RAUBER's sickle and above the cranially-growing sickle endoblast (which they call the "marginal zone of the area pellucida"). This seems to indicate that the sickle endoblast and the RAUBER's sickle from which it is derived (CALLEBAUT & VAN NUETEN, 1994) influence the overlying UL to express the chick Vg1.
- Activin (also a TGF- family member) is expressed in the deep layer of the chick blastoderm and can induce an axial structure (MITRANI et al., 1990).
- Noggin, follistatin and chordin are proteins that are expressed in the dorsal mesoderm during gastrulation, as appropriate for the formation of neurectoderm with a cranial character (HEMMATI-BRIVANLOU et al., 1994).

Activin and follistatin (an activin antagonist) (CONNOLY et al., 1995) encode signalling proteins that have been implicated in fundamental events in early vertebrate embryogenesis.

Legends to the figures (see opposite page)

Fig. 16. – Comparative schematic drawing of the interactions in culture between avian central subgerminal ooplasm and RAUBER's sickle or upper layer or both and the ability or inability to still form an embryo; the vertical black ovals indicate the place of sectioning and removal of the neighbouring tissue;

A) the upper part of the drawing represents a surface view of the caudal quadrant, and the lower part of the drawing is a mediosagittal section of the same; the central subgerminal ooplasm is in contact with both RAUBER's sickle and upper layer; nothwithstanding that all the endophyll of the caudal quadrant is removed, there occurs a restoration de novo of endophyll and an embryo forms (CALLEBAUT et al., 1995).

B) The same disposition as in A but a fragment of upper layer of the anti-sickle is used; here again endophyll forms de novo; followed by development of an embryo (CALLEBAUT et al., 1999c).

C) The same disposition but in the total absence of upper layer and endophyll: no embryo forms (CALLEBAUT et al., 1999c).

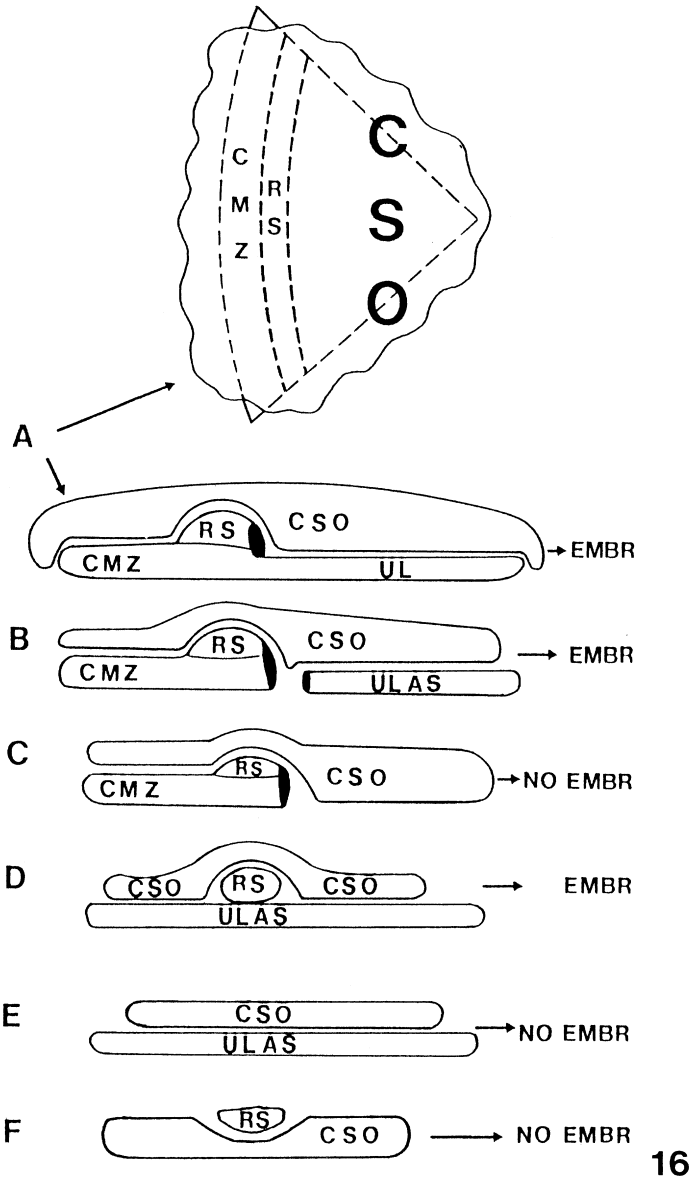
D) On the deep side of the upper layer of the anti-sickle region a RAUBER's sickle fragment is placed, covered by central subgerminal ooplasm: endophyll forms de novo and an embryo develops (CALLEBAUT et al., 1998).

E) The same as in D but in the absence of RAUBER's sickle: neither endophyll nor an embryo forms.

F) A RAUBER's sickle fragment is placed on a mass of central subgerminal ooplasm: no endophyll and no embryo develops (CALLEBAUT, 1994), nothwithstanding the migration of RAUBER's sickle cells in the central subgerminal ooplasm. In every case where a RAUBER's sickle fragment comes in contact with central subgerminal ooplasm, its nuclei penetrate and migrate over a long distance in it. However, only when an upper layer fragment is also present in the immediate neighbourhood, does endophyll form and an embryo develop.

Fig. 17. - Stereomicrograph of a living unincubated quail blastoderm; arrowhead indicates a heavy RAUBER's sickle, typical for quail; bar: 1 mm.

Fig. 18. – Stereomicrograph of a living unincubated chicken blastoderm; arrowhead indicates the middle part of a very narrow RAUBER's sickle, which, however, extends into the cranial quadrant, visible as a sickle horn (indicated by arrow) at the right side of the blastoderm; bar: 1 mm.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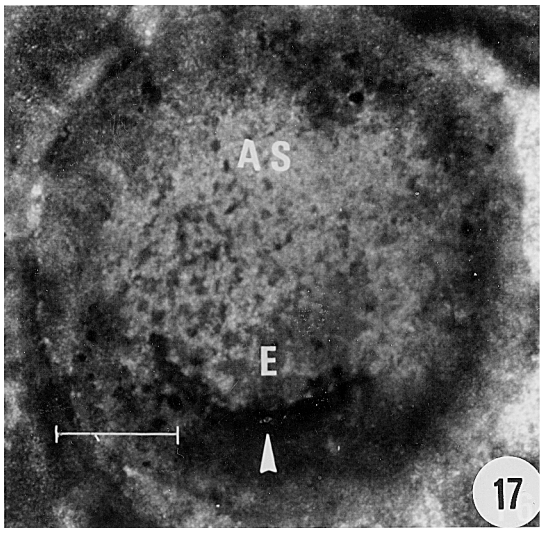
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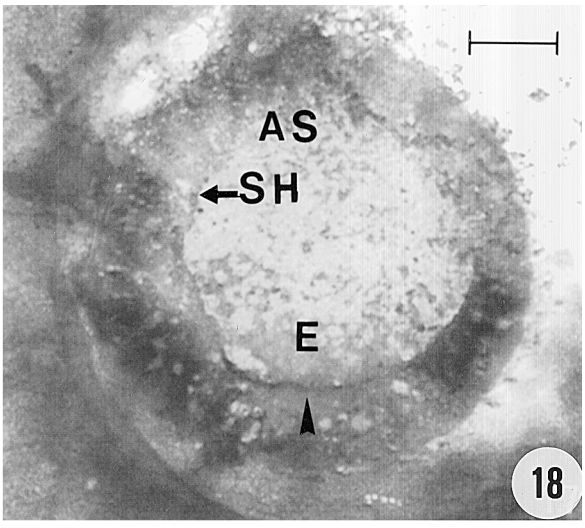
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In the present study we isolated the lateral parts of a mid-streak blastoderm from their median primitive streak region (Fig. 14A). However, after their isolation these lateral parts still contain part of the three elementary tissues (junctional endoblast derived from the RAUBER's sickle, upper layer and endophyll, still present in the cranial region). The presence of the three elementary tissues at one point of the avian blastoderm starts the development of an embryo at that point (CALLEBAUT & VAN NUETEN, 1996). These experiments confirm the strong embryo-inducing potencies of junctional endoblast after transplantation in the anti-sickle region of unincubated avian blastoderms (CALLEBAUT et al., 1999b). By the excision and isolation of the lateral part of the original blastoderm, the orientation of the new PS starting from the middle region of the lateral junctional endoblast forms approximately perpendicularly to the orientation of the original PS.

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