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Original article

Spatial relationship between endophyll, primordial germ cells, sickle endoblast and upper layer in cultured avian blastoderms

M Callebaut *, E Van Nueten, H Bortier, F Harrisson, L Van Nassauw, A Schrevens

RUCA, UA, Laboratory of Human Anatomy and Embryology, 171 Groenenborgerlaan, B-2020 Antwerp, Belgium

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Summary — By isotopic quail-chicken chimera experiments of the Rauber's sickle or by radioactive labelling and isotopically replacing of the caudal endophyllic sheet in unincubated avian blastoderms, followed by culture, we demonstrated that the displacement of the endophyll by the cranially extending sickle endoblast is not exclusively a mechanical phenomenon, as suggested by earlier studies (Vakaet, 1962 a, b). Indeed, our study suggests that the sickle endoblast also migrates centripetally very soon (already after 5 h) in and through the caudal endophyll before ingression of upper layer cells takes place. We also describe the early spatial relationship between the three elementary tissues (endophyll, Rauber's sickle, upper layer) (Callebaut et al, 1996a) and the induction phenomena between quail sickle endoblast and chicken upper layer (UL) during the formation of the primitive groove. The latter already develops before ingression occurs. We found no evidence for an endophyllic origin of avian primordial germ cells.

avian blastoderm / Rauber's sickle / gastrulation / primordial germ cells / endophyll / quail-chicken chimera

Résumé — Relations entre l'entophylle, les cellules germinales primordiales, l'endoblaste provenant du croissant de Rauber et l'ectophylle dans le blastoderme d'oiseau. Par des xenogreffes de croissant de Rauber entre caille et poulet ou par marquage radioactif à la glucosamine tritiée de l'entophylle de blastodermes non incubés d'oiseau, nous avons démontré en culture que le déplacement de l'entophylle n'est pas exclusivement dû à un effect mécanique, mais est aussi le résultat d'une pénétration dans et d'une migration vers le centre de l'entophylle. Nous décrivons aussi les relations précoces entre les trois tissus élémentaires (entophylle, croissant de Rauber, ectophylle) du blastoderme non incubé d'oiseau, et plus particulièrement, le rôle inducteur de l'endoblaste prove-

* Correspondence and reprints
Tel: (32) 03 218 03 90; fax: (32) 03 218 03 98; e-mail: macal@nets.ruca.ua.ac.be

nant du croissant de Rauber sur l'ectophylle pendant la formation du sillon primitif avant l'invagination. Nous n'avons pas trouvé d'indications suggérant une origine entophyllique des cellules germinales primordiales.

blastoderme d'oiseau / croissant de Rauber / gastrulation / cellules germinales primordiales / entophylle / chimères caille-poulet

INTRODUCTION

Rauber (1876) and later Koller (1882) were the first to describe a sickle (often visible from the exterior) in the future caudal part of the area pellucida of avian blastoderms. Rauber (1876) already considered that this structure was at the origin of a forward migration of cells, so that a progressively enlarging sheet of tissue expands in a caudo-cephalic direction below the upper layer (UL) of the area pellucida. Since the experimental work of Waddington (1932) demonstrating the inductor role of the endoblast (deep layer) on the upper layer in avian blastoderms, numerous investigations have been performed on the same topic. As far as we are aware, Vakaet (1962a, b) was the first to make a radical distinction between endophyll (first appearing caudocentral deep layer component) and the other endoblastic structures (fig 1). As shown by Vakaet (1962a, 1970) in the chicken and by Callebaut (1983, 1987) in the quail, the endophyll is localized in the cranial prolongation of the sickle of Rauber (1876) from which the *Anlage* already forms in vitro after symmetrization (Callebaut, 1993a, c). The caudo-cephalic sliding movement of the endophyll-sickle endoblast complex below the upper layer during early incubation, has been demonstrated by Vakaet (1962b, 1970) using labelling with carbon marks. Recent studies (Callebaut and Van Nueten, 1994, 1995) indicate that Rauber's sickle is the early gastrulation organizer in the avian blastoderm. In combination with endophyll, Rauber's sickle induces a normal embryo in the UL. Rauber's sickle cells seem to be homologous to the vegetal dorsalizing cells described by Nieuwkoop (1973) in amphibian blastulas (Callebaut et al, 1996b). We demonstrated that none of the three elementary tissues (Rauber's sickle, endophyll and upper layer) of the avian unincubated blastoderm present at that moment an irreversible functional polarity and that normal gastrulation and neurulation can take place even in the total absence of the marginal zone (Callebaut et al, 1997). The same study indicated that endophyll directs the movement of sickle material to form sickle endoblast (Callebaut and Van Nueten, 1994), which in turn induces a primitive streak in the upper layer. Until now, the earliest morphogenetic movements occurring at the onset of incubation of avian germs are not well known. Particularly the distinction and bounderies between the first two deep layer components (endophyll and sickle endoblast) is (without marking) difficult or impossible to make. In the present study, we demonstrate, by the use of isotopic quail-chicken chimeras in culture, that already very soon during early incubation, the sickle endoblast (starting from Rauber's sickle) migrates centripetally into the endophyll. At the same time, the sickle endoblast and junctional endoblast induce a thickening in the neighbouring upper layer. We describe also the spatial relationship of the early primitive groove and the neighbouring sickle endoblast before ingression through the primitive streak occurs. Finally by radioactive labelling, the localization of the endophyll and of the eventual accompanying primordial germ cells were screened in quail germs cultured for longer periods (early somite stages).

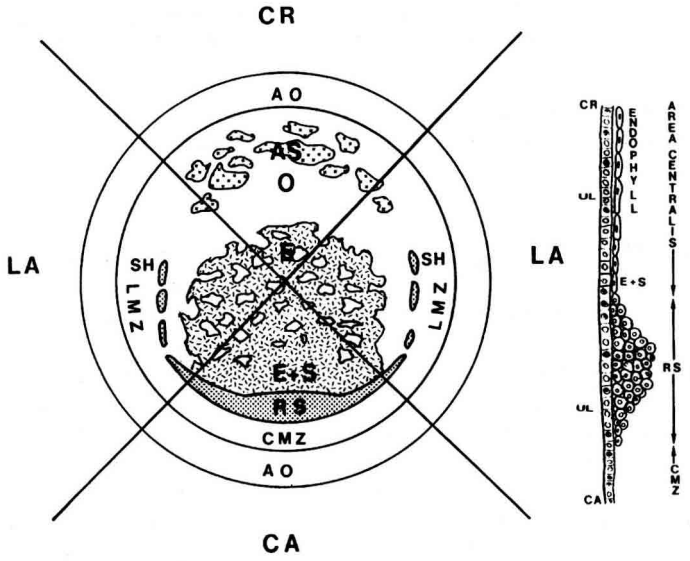


Fig 1 left. Schematic representation of the disposition of the components of the deep layer in the cranial (CR), caudal (CA) and lateral (LA) quadrants of a freshly laid unincubated quail blastoderm, seen from the ventral surface. AO: area opaca; CMZ: caudal marginal zone behind the middle part of Rauber's sickle (RS), extending laterally and cranially as sickle horn fragments (SH); E + S: zone where sickle endoblast (S), which is derived from Rauber's sickle, meets the endophyll; E: central, pure endophyll, which forms an incomplete layer; O: zone where the deep layer is absent; AS: anti-sickle region, which contains no sickle material, but here numerous yolk masses are present as the result of a disruption from the underlying ooplasm (Callebaut, 1993a, b, c). In the unincubated chicken blastoderm, Rauber's sickle forms only a narrow ridge adhering to the upper layer, and the endophyll does not extend into the cranial quadrant. The cranial and part of the lateral marginal zones (LMZ) are not defined because Rauber's sickle is absent there.

Fig 1 right. Scheme representing the cellular organization in a mediosagittal section through the caudal part of an unincubated avian blastoderm with its three elementary tissues: endophyll, Rauber's sickle (RS) and upper layer (UL). E + S: mixed endophyll and sickle endoblast; CMZ: caudal marginal zone.

MATERIALS AND METHODS

We used unincubated chicken and quail blastoderms presenting a clearly visible not fragmented sickle of Rauber (1876) from eggs stored at 15–20 °C for 1 day after laying. After opening of the chicken eggs and removal of the egg white, the egg yolk balls were placed in a Ringer's 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° over the germ. In this manner, the chicken blastoderm, still adhering to the vitelline membrane and underlying subgerminal ooplasm, could usually be separated from the yolk. By contrast, in the quail, this procedure is only exceptionally successful since the unincubated quail blastoderm almost always comes loose of the vitelline membrane. Therefore to isolate unincubated quail blastoderms, the germ and surrounding yolk must be excised from the egg yolk ball. Subsequently, the yolk, the nucleus of Pander, the peripheral subgerminal, perigerminal and paragerminal ooplasm (ie, the extrablastodermic tissues of the egg) were progressively removed in order to expose the deep

side of the blastoderm. The Rauber's sickle was removed from the unincubated chicken blastoderms and isotopically replaced by the middle part of a Rauber's sickle from an unincubated quail, whilst the chicken endophyll was left intact. The so formed chimeras were cultured for 7–9 h. From unincubated quail blastoderms presenting a Rauber's sickle, the caudal part of the endophyll was removed as a sheet. This endophyll sheet was placed in a Ringer solution containing 33 μCi D-6-^{3}H glucosamine hydrochloride (18 Ci/mmol, Amersham) per mL at 39 °C for 15 min. After a rinse in Ringer solution, the endophyll was replaced isotopically in the quail blastoderm from which it was removed. The so reconstituted quail blastoderms were cultured for 26–28 h until two to five somites were visible. At this moment the primordial germ cells are distinctly visible on sections between the superficial cell layer and the endophyll in chicken (Dantschakoff, 1908; Swift, 1914) and in quail embryos (Callebaut and Vakaet, 1981). The blastoderms were cultured according to the technique of Spratt (1947). The culture medium was not pure egg white, as used by New (1955), but a mixture of 25 mL egg white and a gel made of 150 mg Bactoagar (Difco, Detroit, Mi) in 25 mL Ringer's solution. This semi-solid medium allowed microsurgery and further culturing on the same substratum. Instead of Petri dishes, the culture vessels described by Gaillard (1949), on which an optical flat glass cover was sealed with hot paraffin, were used. Stereomicroscopic Polaroid photographs were taken in the same direction at the beginning, during and at the end of the culture period. Fixation was performed overnight in a modified Heidenhain's fixative (Romeis, 1948) containing 0.5 g sodium chloride, 2 g trichloracetic acid, 4 mL acetic acid, 20 mL formalin and 80 mL water. After dehydration in a graded series of alcohol and embedding in paraffin, the chimeric blastoderms were sectioned perpendicularly to the visible or presumed axis. The deparaffinized, 8 μm-thick sections of quail-chicken chimeras were Feulgen stained after Demalsy and Callebaut (1967), in order to be able to identify the origin of the nuclei, using microscope objectives × 10 or × 25. This allowed us to observe the typical central or subcentral chromatin granule of the grafted quail cells (Callebaut, 1968; Koshida and Kosin, 1968; Le Douarin and Barq, 1969) as well as to overlook their distribution among the chicken cells. The radioactively labelled and some control sections were dipped in nuclear emulsion L4 (Ilford, England). After 5 weeks of exposure in the dark, the autoradiographs were developed according to Caro and Van Tubergen (1962). Thereafter, the autoradiographs were stained with iron hematoxylin and eosin.

RESULTS

Quail-chicken chimeras (n = 6)

The quail Rauber's sickle fragment which at the moment of transplantation (fig 2A) forms a dense sausage-like mass, has flattened and extended centripetally after 5 h culture. It then forms a more or less circular vacuolar sheet (fig 2B), similar to what was described when Rauber's sickle was cultured in isolation (Callebaut, 1994). The ingrowth of the darker quail Rauber's sickle material at different points of the endophyll region of the embryonic shield is clearly seen. Notwithstanding the considerable distance between the transplanted quail Rauber's sickle and the chicken endophyll at the start of the culture (fig 2A), we see that after only 5 h of culture (fig 2B), the sickle material has not only bridged the gap between both deep layer components but has also migrated for approximately 1 mm in a cranial direction.

After 7–10 h, sometimes the denser quail sickle material is still visible in the caudal region with the stereomicroscope (fig 2C). On the successive sections through a similar chimera (seen at the start in figure 3A and after 8 h of culture in figure 3B with clearly visible groove), we can follow the rapid caudocranial migration (centripetally in the endophyll) of the quail sickle endoblast cells and/or the localization of the junctional endoblast. Most caudally in the region, where the quail Rauber's sickle was originally placed, only the massive yolk-rich, quail junctional endoblast close to a thickened chicken UL is seen (fig 3C). In more cranial sections we see laterally quail junctional endoblast (V shaped) continuous with

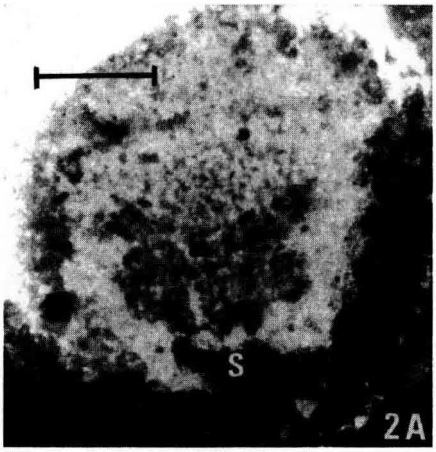


Fig 2A. Stereomicroscopic view of a living chicken blastoderm at the start of the culture: the chicken Rauber's sickle was removed and replaced isotopically by a large middle fragment of a quail Rauber's sickle (S); the latter forms a dense sausage-like mass; E: endophyll of chicken; bar: 1 mm.

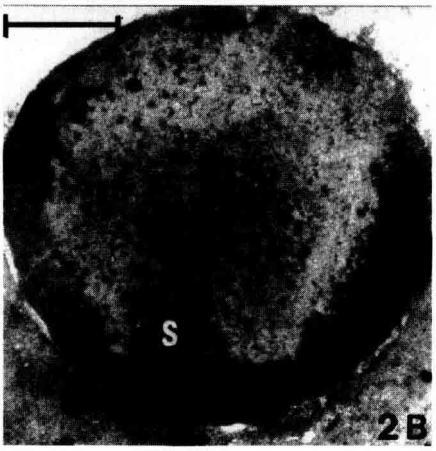


Fig 2B. Stereomicroscopic photograph of the same chimera as represented on figure 2A, after 5 h culture. Note the ingrowth of the darker vacuolar quail Rauber's sickle material (S) in different parts of the chicken endophyll region (E); bar: 1 mm.

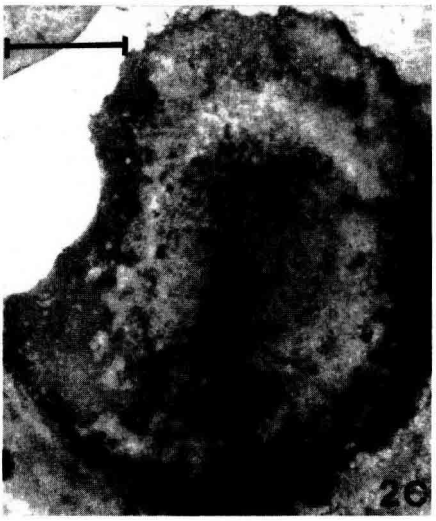


Fig 2C. Stereomicroscopic photograph of the same chimera as represented on figures 2A and 2B after 9 h culture, alive; a sector of darker vacuolar sickle material (S) is still visible in the caudal region; bar: 1 mm.

Fig 3A. Stereomicroscopic photograph of a similar chimera as seen in figure 2A, at the start of the culture; S: middle fragment of transplanted quail Rauber's sickle; E endophyll of chicken; bar: 1 mm.

Fig 3B. The same chimera as in figure 3A after 8 h culture: a primitive groove has developed, visible in a denser area of vacuolar quail sickle material (S), which has penetrated centripetally into the chicken endophyll (E), 3 arrowheads indicate the borderlines between the clearer endophyll and the slightly denser sickle material; bar: 1 mm.

Fig 3C. Section through the most caudal part of the area pellucida of the chimera of figure 3B quail junctional endoblast (JE) in close contact with a thickened chicken UL; Feulgen staining; bar: 50 μm.

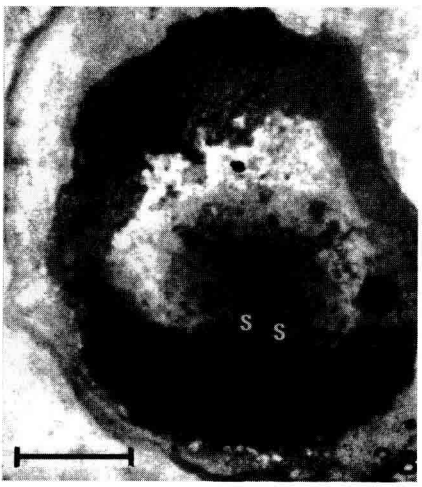
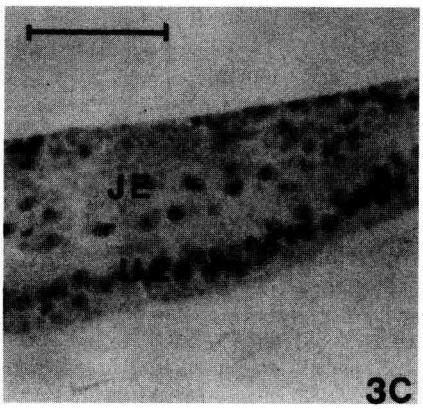
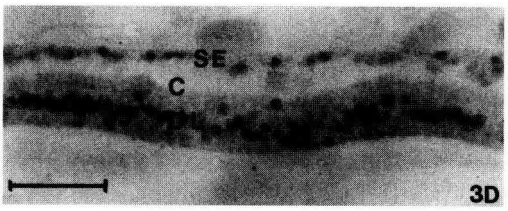
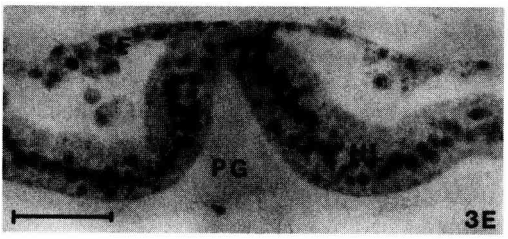


Fig 3D. More cranial section through the chimera of figure 3B, to show the quail sickle endoblast (SE) separated by a cavity (C) from the thickened chicken upper layer (UL); Feulgen staining; bar: 50 μm.

Fig 3E. Transverse section through the still more cranial primitive groove (PG) of the chimera of figure 3B. Note the intimate contact between the infolded thickened upper layer (UL) and the sickle endoblast on the middle line before a real primitive streak with ingressing UL cells has formed; in the cavities on each side of the primitive groove more or less loose quail cells are seen. Feulgen staining; bar: 50 μm.







the more medial quail sickle endoblast (fig 3D). A space exists between the quail sickle endoblast and the thickened chicken UL. On more cranial sections, one can observe that the flat quail sickle endoblast cells are in intimate contact with the chicken UL cells, forming the primitive groove (fig 3E). In the spaces, on both sides between the thickened UL and the sickle endoblast layer, more or less loose quails cells are seen. Their biological marking indicates that these cells are derived from the quail Rauber's sickle material and not from invaginated UL cells. Still more cranially, cranial to the primitive groove quail sickle endoblast cells are mixed with chicken endophyll cells. Apart from a thickening of the chicken UL (preneural plate) above the cranial chicken endophyll (Callebaut and Van Nueten, 1995), no indication of a neural plate is observed, since the incubation time is too short. In the present short time study, we found the same correlation between sickle derived material (quail sickle endoblast and quail junctional endoblast) and the thickening of the neighbouring UL, as observed after long culture periods (Callebaut and Van Nueten, 1994). This indicates that the sickle derived material has already an inductory influence on the UL after a short exposure time.

Culture of unincubated quail blastoderms with radioactively labelled endophyll ($n = 8$)

At the start of the culture (fig 4A), the Rauber's sickle and the somewhat more centrally placed, loose, radioactively labelled endophyll sheet is seen. After 18 h of culture, a normal quail germ has developed (fig 4B). The axis of the germ is directed from the original middle of Rauber's sickle to the centre of the replaced endophyll. In figure 4C we see the same normal embryo after 27 h of culture (five somites), alive just before fixation. As a consequence, the interruption of the connections and the eventual direct contact between Rauber's sickle, endophyll and UL have not impaired the reorganization of a normal germ. We know that this reorganization no longer occurs when, in isolated caudal blastoderm quadrants, the endophyll is removed (Callebaut and Van Nueten, 1995). In the perpendicular sections through the blastoderm of figure 4C, only radioactively labelled tissue is seen, at some distance and laterally from the head region, symmetrically on each side (figs 4 D, E). The superficial layer (epiblast) in the neighbourhood is not labelled. We found no labelled primordial germ cells in the space between the endophyll and this superficial layer. Also the endophyll and primordial germ cells localized in front of the head region are not labelled.

DISCUSSION

In a previous study (Callebaut and Van Nueten, 1994), we described the gastrulation organizing effect of heterotopically placed Rauber's sickle fragments on the deep side of the UL of unincubated avian blastoderms after longer culture periods. In the present study, by isotopic quail-chicken exchange experiments of the Rauber's sickle, we could demonstrate the early relationship between the sickle derived structures (ie, junctional endoblast and sickle endoblast) and the UL and endophyll during shorter in vitro culture periods. By this technique, the three elementary interacting tissues (Rauber's sickle, endophyll and UL) of the avian unincubated blastoderm remain in their original position. By heterotopic transplantation experiments at any point of the area pellucida (Callebaut et al, 1997), we have unequivocally demonstrated that the growth direction of the sickle endoblast (starting from Rauber's sickle) is determined by the localization of the endophyll. Indeed the caudocephalic direction of the induced primitive streak is always oriented from Rauber's

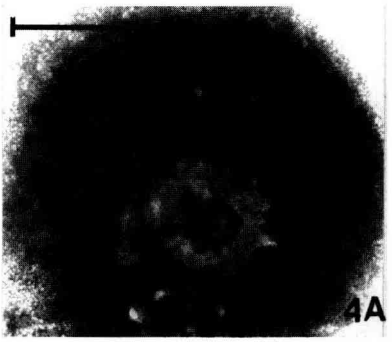
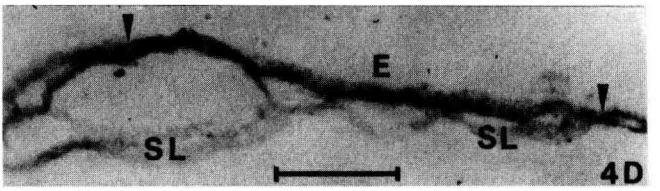


Fig 4A. Stereomicroscopic view of an unincubated quail blastoderm from which the endophyll sheet (E) was separately labelled with $_3$H glucosamine, at the start of the culture. Note the interruption of contact (indicated by an arrowhead) between the loose endophyll and the Rauber's sickle (S); bar: 1 mm.

Fig 4B. The same blastoderm as in figure 4A has developed normally after 18 h culture; bar: 1 mm.

Fig 4C. The same embryo as in figure 4B after 27 h culture, has developed normally and presents five somites; bar: 1 mm.



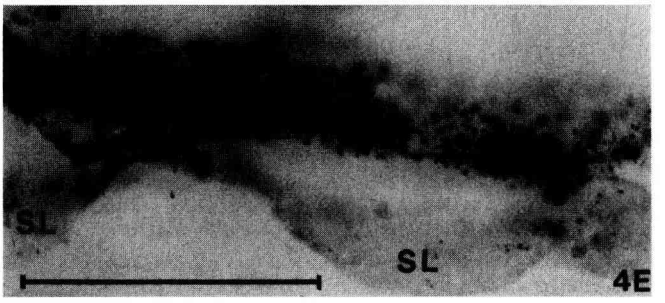


Fig 4D. Autoradiograph from a section laterally from the head region of the embryo of figure 4C. The borderlines of the labelled part of the deep layer, derived from the incorporated endophyll (E) are indicated by two arrowheads; SL: unlabelled superficial layer; bar: 100 μm.

Fig 4E. Higher magnification of part of figure 4D to show the intense labelling of the endophyll (E) and the absence of labelling of the superficial layer (SL); bar: 50 μm.

sickle to endophyll. Our present study indicates that the sickle endoblast not only moves in the direction of the endophyll but also that it penetrates in it. Indeed if there was only a mechanical effect of the sickle endoblast, then the endophyll would only be displaced as a whole. The combination of the observations with the stereomicroscope and on the sectioned material, demonstrates also that penetration in the endophyll occurs. The present study moreover suggests that the sickle endoblast, moving in the direction of and in the endophyll, first influences the overlying UL to increase in thickness and later to fold in its middle region. Still later, an intimate contact occurs between the quail sickle endoblast and the developing primitive groove in the chicken UL. The presence of loose quail cells in the space on both sides between the thickened UL and the sickle endoblast indicates that these cells are not derived from eventually invaginated UL cells. By contrast to Spratt and Haas (1965) who refused to accept invagination, Rosenquist (1966), and Vakaet (1971) have indeed unequivocally demonstrated that invagination movements occur through the primitive streak. So our study indicates that infolding with formation of a primitive groove occurs before invagination of UL cells. This is in contrast with common belief (Houillon, 1968) that invagination of UL cells already begins after 5–6 h of incubation. That the contact between sickle-derived material and endophyll is indispensable for a primitive streak to form, has been shown in isolated caudal blastoderm quadrants (Callebaut and Van Nueten, 1995). In any way, both the present short-time experiments with quail-chicken chimeras and the present long-time experiments (with labelled quail endophyll), indicate that the sickle endoblast has not only a mechanical influence on the endophyll by pushing it in a cranial direction (Vakaet, 1962a, b), but also that it penetrates centripetally into the endophyll. It can finally bissect a caudal sheet of endophyll. Its rapid extension possibilities seem to be in agreement with the observations of Sanders et al (1978) that in hanging drop cultures, the sickle endoblast produces large ruffling membranes and moves rapidly, with a tendency to break away from its original location. This tendency of the sickle endoblast to migrate centripetally can perhaps explain why the tip of the primitive streak (which develops under its inductory influence: Callebaut and Van Nueten, 1994) becomes localized in the original central part of the area pellucida where also the definitive endoderm appears (as represented by Vakaet, 1970). The rapid centripetal ingrowth of the sickle endoblast bissecting the caudal endophyll after only a few hours of incubation, probably explains why the endophyll can be easily and completely removed from the UL in caudal prestreak blastoderm quadrants (Callebaut and Van Nueten, 1995). The tissue labelling used in the present study (exclusively Rauber's sickle cells or exclusively caudal endophyll) was much more selective than the tissue labelling performed by Eyal-Giladi et al (1992). Indeed these authors labelled whole caudal parts of chicken blastoderms with the fluorescent dye rhodamine dextran-lysine. These caudal parts not only contained part of Rauber's sickle but also a part of all its surrounding tissues, ie, upper layer from the area centralis and from the caudal marginal zone, area opaca and some sickle endoblast and/or endophyll. As early as 1981, Callebaut and Vakaet demonstrated that the ooplasm which will be incorporated in the quail primordial germ cells (germinal ooplasm or germinal yolk) derives only from the original deep central region of the oocytal germinal disc. By trypan blue labelling of the yolk protein, the germinal ooplasm can be traced successively in the deep central (paraxial) region of the oocytal germinal disc, in the central region of the unincubated blastoderm, mainly in the endophyll but also in the upper layer (during the early primitive streak stage) and finally in the primordial germ cells of the germinal

crescent region (Callebaut, 1983). Moreover, Callebaut (1984) demonstrated that avian primordial germ cells contain ooplasm (yolk) from the superficial part of the oocytal nucleus of Pander (1817). This central localization of primordial germ cells in the avian blastoderm was more recently confirmed by the culture of central discs of unincubated chicken blastoderms, irrespective of the embryo forming process (Ginsburg and Eyal-Giladi, 1987). Cuminge and Dubois (1989) found by culture of unincubated quail blastoderms in which the morphogenetic movements were mechanically inhibited, a mainly centrocranial distribution of the primordial germ cells. This localization seems to correspond to the region O, localized cranially to the endophyll (fig 1), where only the upper layer is present. In the most cranial part of the area pellucida (corresponding to the anti-sickle region, fig 1), these authors found no or a very small number of primordial germ cells. So by contrast to the older theory of an endophyllic origin of avian primordial germ cells, the results of more recent studies localize the primordial germ cells in the upper layer of the avian blastoderm (Eyal-Giladi et al, 1981, Cuminge and Dubois, 1989, 1992). This also seems to be the case in the turtle *Emys orbicularis* (Cuminge and Dubois, 1987; Dubois and Cuminge, 1990) and probably also in mammals (Gardner and Rossant, 1976). So in the germs of all the amniotes there would be a stage during which the primordial germ cells are localized in the external (upper) layer. Even if the recent theory of the upper layer origin of primordial germ cells in birds is exact, this means in no way that their ooplasm was originally localized in the surface of the young germ. Indeed Callebaut (1983, 1987), by trypan blue labelling, has unequivocally shown that a vertical mixing of deep and superficial ooplasm occurs during the cleavage stage and so original deep central germinal ooplasm becomes localized superficially. Although Eyal-Giladi et al (1981) claimed that already at the early somite stages it would be possible to distinguish quail primordial germ cells from chicken primordial germ cells, there has been doubt on this possibility (Callebaut, 1973; Tachinante, 1974; Nakamura et al, 1992). Therefore in the present study we used radioactively labelled endophyll and not the quail-chicken chimera system, to trace or to exclude the eventual origin of the primordial germ cells from the endophyll (Vakaet, 1962a, b; 1970). That we found no radioactively labelled primordial germ cells can perhaps be explained by the fact that we only transplanted the caudal part of the endophyll (already present as a sheet) whilst according to Cuminge and Dubois (1989) primordial germ cells originate preferentially from the centrocranial part of the quail blastoderm. So it could also be possible that the segregation of more cranial endophyll and eventual primordial germ cells occurs later from more cranial parts of the primitive UL (region O, fig 1). Indeed, originally the primitive upper layer was equivalent to a morula. As such, it contains the entire range of embryonic and extraembryonic potentialities, as well as the germ line (Cuminge and Dubois, 1992). Our present results seem to indicate, as can also been concluded from the study of Cuminge and Dubois (1989), that the original distribution area of the primordial germ cell precursors in the quail blastoderm does not coincide with the whole endophyll area.

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