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Review

The original predisposed "extraembryonic" distribution of the surrounding α−y−δ−ooplasms regulates the patterning of the embryo proper (β−ooplasm)

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ABSTRACT

In the present report we describe the role of the three elementary tissues during early embryonic patterning. Not the absolute quantity, but the local spatial relationship, boundaries and contact zones of these elementary tissues play a determining role at the start of incubation *in vitro*. The formation of (hemi) primitive streaks or parallel asymmetric unequal hemi-primitive streaks in different hemi-sectioned (mediosagittal, oblique, transverse) or not sectioned avian blastoderms were compared and explained by a similar mechanism of strong sliding of upper layer cells in the concavity of the Rauber's sickle (RS). This occurs by induction on the upper layer of the Rauber's sickle material (positional information), without interference of the caudal marginal zone (in which the area vasculosa develops). There exists a caudo-centrally directed (from sickle-horn top to the central median part) induction polarity along side the internal border of the RS, explaining the so-called "polonaise movement" of upper layer cells. The caudo-centrally directed sliding of upper layer (UL) cells (which will transform in a half-PS and half-mesoblast mantle) in the concavity of Rauber's sickle (RS) at different levels is an extensive strong mechanical phenomenon. So, when unimpaired, these UL cells can cross the midline region. This indicates that in normally developing blastoderms a mechanical left-right equilibrium exists. When no RS-material is present, no sliding of UL cells in the neighboring area centralis occurs. There exists an obvious homology in avian blastoderms with the latero-lateral or caudo-cranial mosaic phenomena observed in other classes of chordates (Ascidia and Amphibia). Gastrulation (HOXB1 expressing domain) and neurulation (OTX2 expressing domain) can be explained by the presence of two different organ-group-organizing ooplasms: respectively y−ooplasm or δ−ooplasm−containing structures (Rauber's sickle or endophyll).

KEYWORDS: chick blastoderm, Rauber's sickle (RS), junctional endoblast, embryonic stem cells, endophyll, (hemi) primitive streak, (pre) neurulation, left-right asymmetry

INTRODUCTION

At the moment of laying, the unincubated chicken blastoderm presents a sickle-shaped apparent bilateral symmetry (Fig. 1A) with three elementary tissues [1]: the Rauber's sickle (RS), the primary organizer of gastrulation, containing y−ooplasm encircling the area centralis, the caudo-central endophyll with δ−ooplasm in the concavity of Rauber's sickle and the upper layer (UL) which give rise to the stem cells of the definitive embryo (with the classical germ layers) by inductory

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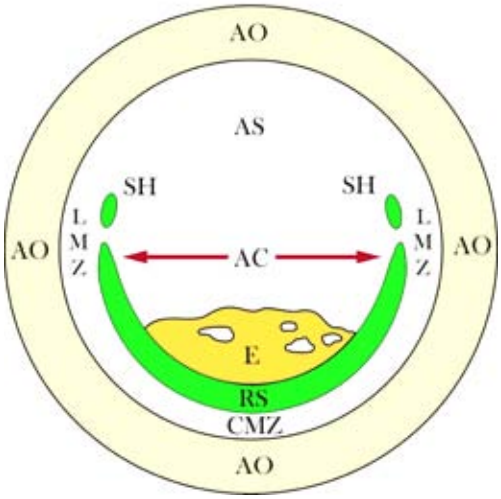

Fig. 1A. Schematic representation of the components of the unincubated avian blastoderm (ventral view): the three elementary tissues, Rauber's sickle (RS) and sickle horns (SH); endophyll (E) and the upper layer in the area centralis (AC) are seen; CMZ: caudal marginal zone; LMZ: lateral marginal zones; AS: anti-sickle; AO: area opaca.

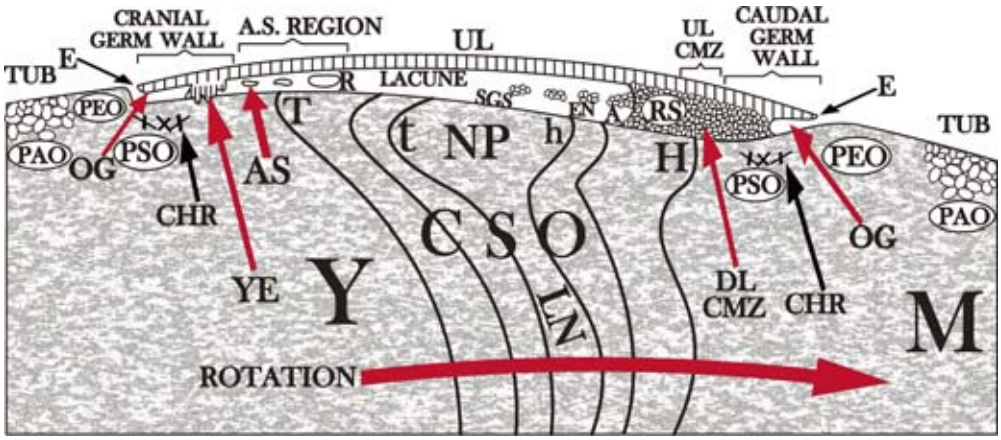

Fig. 1B. Schematic representation of a mediosagittal section through an unincubated blastoderm with surrounding ooplasms after fixation *in situ* on the egg yolk ball showing the three elementary tissues; UL: upper layer (β−ooplasm), EN: incomplete endophyll (δ−ooplasm) and RS (y−ooplasm); UL CMZ: upper layer from the caudal marginal zone; the caudal marginal zone being a more or less transparent part adherent to the caudal peripheral subgerminal ooplasm (PSO) via a deeper part (DL CMZ); SGS: subgerminal space forming a caudal pocket A (axilla shaped) and a cranial recess (R) in which free yolk masses or sometimes cells are found forming the anti-sickle (AS); E: edge of the blastoderm; OG: early overgrowth zone; YE: early development of the yolk endoblast, growing into the peripheral subgerminal ooplasm (PSO); CHR: chromosome clusters; PEO: perigerminal ooplasm; PAO: paragerminal ooplasm forming a tubulin-rich ring (TUB) at a distance from the edge of the blastoderm; YM: the voluminous yolk mass of the egg yolk ball in which the eccentricity of the successive yolk layers parallel to the eccentricity in the blastoderm is represented; CSO: central subgerminal ooplasm in which the central Nucleus of Pander (NP) is seen; t: toe-shaped and h: heel-shaped part of the Nucleus of Pander; T: toe-shaped and H: heel shaped part of the surrounding yolk layers as result of the rotation *in utero* (the arrow indicates the direction of rotation and compression of the yolk mass under the combined influence of gravity and egg rotation); LN: bent latebra neck. Note that in contrast to the caudal germ wall, the cranial germ wall is disrupted from the underlying peripheral subgerminal ooplasm: both form part of the area opaca.

influences emanating from Rauber's sickle or endophyll (Fig. 1B). The upper layer covers the whole blastoderm and contains mainly β−ooplasm. In the caudal half of the area centralis the upper layer stem cells form predisposed sickle-shaped embryonic Anlage fields which ingress via the primitive streak (PS) and form on both sides a mesoblast mantle below the upper layer [2, 3].

After approximately one day of culture *in vitro*, the area vasculosa and associated coelomic vesicles develop in the caudal marginal zone under the inductive influence of the junctional endoblast (a massive V-shaped structure derived from Rauber's sickle) (Fig. 1C). An unidirectional outflow of precursors of mobile isolated blood islands sliding over the junctional endoblast occurs at the exterior side of the Rauber's sickle into the caudal marginal zone. The real role of the caudal marginal zone is not inducing a primitive streak, but forming a transition and harbour zone for blood islands induced by the neighboring Rauber's sickle material, so giving rise to the area vasculosa (Fig. 1C) [4]. After labeling of the caudal marginal zone with graphite particles and culture, only the area vasculosa presents labeling. Neither the area centralis, nor the area opaca were seen to be labeled, indicating that the primitive streak is

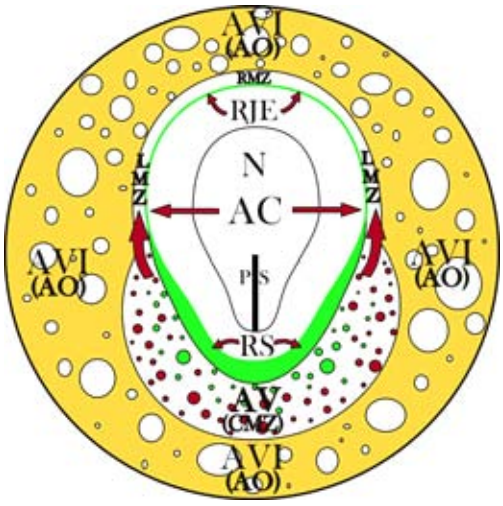

Fig. 1C. Schematic representation of the structures visible on the deep side of an avian blastoderm after 20 h *in vitro* incubation; Rauber's sickle (RS) in continuity with the rostral junctional endoblast (RJE), containing y−ooplasm, encircles the area centralis (AC); RMZ: rostral marginal zone in continuity with the lateral marginal zones (LMZ) and caudal marginal zone (CMZ) in which the area vasculosa (AV) develops; after further incubation the area vasculosa extends cranially in the lateral marginal zone; at the same time the area opaca (AO) extends spherically and differentiates in area vitellina interna (AVI) which typically contains rounded empty spaces; PS: primitive streak; N: neural plate.

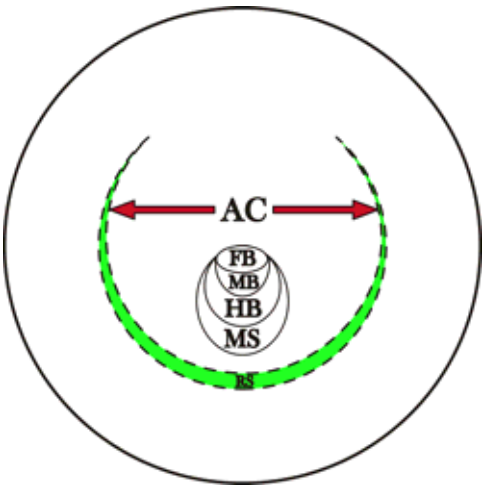

Fig. 1D. Earliest known disposition of the neural plate Anlage (and its subdivisions), on a dorsal view in the upper layer of the unincubated chicken blastoderm [2]; FB: forebrain Anlage; MB: midbrain Anlage; HB: hindbrain; MS: Anlage of the medulla spinalis; AC: area centralis enclosed by the deeply localized Rauber's sickle (RS), seen in transparency.

Fig. 1E. Schematic representation (surface view) of the spatial vertical relationship of (pre) neurulation phenomena induced in the overlying upper layer by δ−ooplasm−containing structures (endophyll and Nucleus of Pander) and gastrulation phenomena induced in the neighbouring upper layer of the area centralis by y−ooplasm−containing structures (RS and derived sickle endoblast), as the result of the eccentric tilting and nutation of these ooplasms and the Nucleus of Pander (NP), localized in the floor of the subgerminal cavity; the upper layer is represented as transparent over the whole surface.

exclusively formed by upper layer cells of the area centralis and not from the caudal marginal zone, as was supposed by Eyal-Giladi *et al.* [5]. Until now the exact pathway followed by the UL cells performing their "polonaise movement" [6] was not established. Here we demonstrate that this movement takes place in the concavity of RS (area centralis) and not in the caudal marginal zone. This was also confirmed in our study about the map of Anlagen in the area centralis [2]. Rauber's sickle forms a kind of "socle" by which the blastoderm is fixed to the underlying ooplasm and yolk. It also must be considered as the most vegetal part of the blastoderm. It contains Vg1. The formation of the primitive streak is also influenced by the cranially undergrowing sickle endoblast (derived from the Rauber's sickle) extending until below the Hensen's node which forms the secondary avian organizer [7]. The earliest known disposition of the shield-shaped neural plate Anlage (and its sickle-shaped subdivisions) corresponds approximately with the place where the deeper δ−ooplasm−containing, neural plate-inducing endophyll is localized (Fig. 1D). At the same time gastrulation phenomena (PS and hemi-PS formation) are induced by y−ooplasm−containing Raubers's sickle material (Fig. 1E).

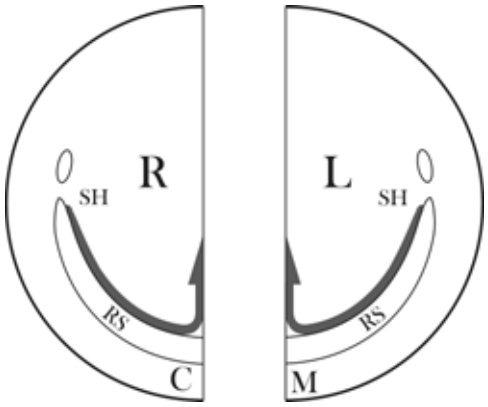

Fig. 2A. Schematic representation of a symmetrically hemi-sectioned unincubated chicken blastoderm; the curved arrows represent the caudo−central sliding of the UL cells in the area centralis ("polonaise movement") during early incubation, finally forming a left and right hemi-PS (half arrow) localized in the cut edge of the hemi-embryo. R: right side and L: left side.

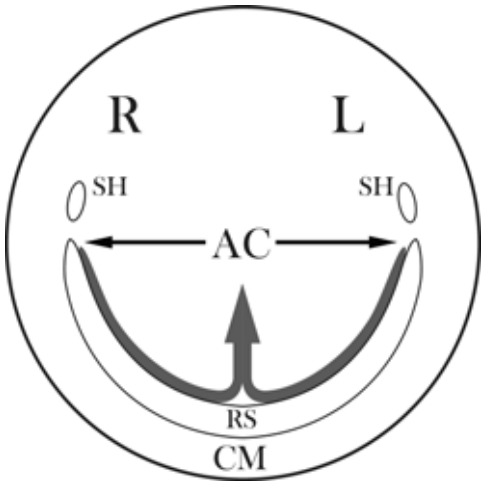

Fig. 2B. Schematic representation of a normal common PS in a normal blastoderm, during early incubation, by the unification in the midline of the area centralis of the right and left formed hemi-PS ("polonaise movement"). R: right side and L: left side.

Comparison of the effects of different ablation experiments of the three elementary tissues in unincubated chicken blastoderms

1. Mediosagittally sectioned avian blastoderms

In such isolated half blastoderms (left or right), after culture *in vitro*, a median hemi-primitive streak (Fig. 2A) and associated hemi-mesoblast mantle develops [8]. This median formation of a hemi-primitive streak can be explained by the pronounced uninterrupted caudo-central sliding of upper layer cells (embryonic stem cells) in the concavity of the corresponding half Rauber's sickle, since only the upper layer in the area centralis of one side is present and thus involved (Fig. 2A). This also suggests that a primitive streak during normal development is formed by parallel sliding of a left and right hemi-primitive streak (Fig. 2B). It is known that during normal development a majority of PS cells remains at one side [9] by a so-called "polonaise" movement [6] (Fig. 2C). Our experiments suggest that early in normal development each half blastoderm reacts first on its own, giving each a hemi-primitive streak (mosaicism) and only somewhat later a normal common primitive streak is formed by parallelization of the formed hemi-primitive streaks. A bilateral PS has also been described in Urodeles: superficial somitic, lateral and ventral mesoderm move into the deep layer during

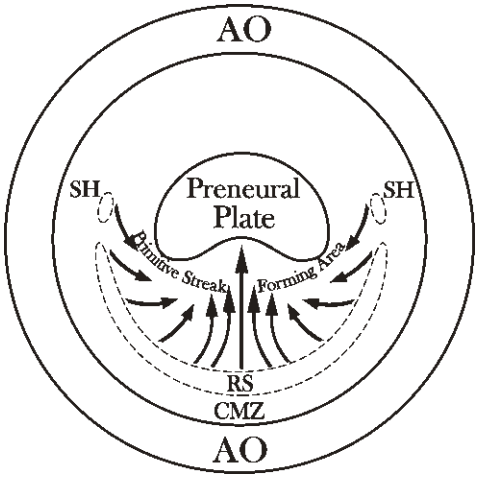

Fig. 2C. Schematic drawing representing the morphogenetic movements in the 2 main areas appearing in the UL of the area centralis, surrounded by Rauber's sickle (RS), during early incubation: 1. the primitive streak forming area (γ−ooplasm dependent H0XB1 expressing domain) and 2. the preneural plate area (δ−ooplasm dependent 0TX2 expressing domain). The arrows indicate the migration paths of UL cells, accumulating behind the preneural plate Anlage, which then fuse on the middle line, forming the PS. From there UL cells move in a cranial direction flattening and indenting the caudal border of the preneural plate Anlage in the zone where a "polonaise movement" has been described [9].

gastrulation, ingressing through a bilateral streak just inside the blastopore [10]. The limited left-right migration across the midline of gastrulating embryos [9] indicates that a real parallelism of hemi-primitive streaks exists in the common primitive streak. After further culture *in vitro* of isolated mediosagittally sectioned blastoderms a trunkally asymmetric hemi-embryo forms with strongly restricted development (derived from one single hemi-mesoblast mantle): only a unilateral rudimentary development occurs. More particularly at the incised side, the Rauber's sickle-dependent phenomena: gastrulation and coelomo-cardiovascular complex formation are absent or rudimentary. By contrast the head region and neural plate develop symmetrically after prolonged culture (Fig. 3A). The head region forms a medially directed head axis apparently developing according to the median centre of gravity-line going through the two cranially localized elementary tissues: halved endophyll and upper layer (Fig. 3B). Indeed in

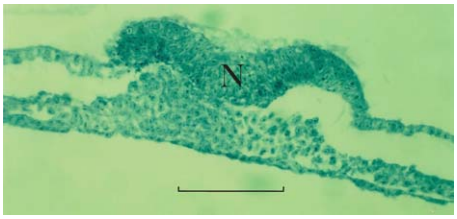

Fig. 3A. Section through a symmetrical neural plate Anlage (N) with neural groove, developed in a sagittally hemi-sectioned blastoderm after 2 days of culture. Bar = 100 µm.

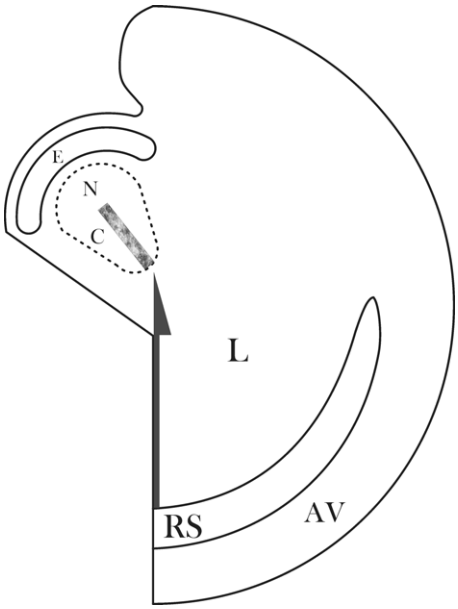

Fig. 3B. Schematic representation of the different structures found in a left hemi-blastoderm after 1 day of *in vitro* culture: the head region is bent over the original midline and contains a half endophyllic crescent; a central notochord (C) and a prechordal plate have formed below a symmetrical neural plate (N); the hemi-PS is indicated by a half arrow localized in the cut edge of the blastoderm. L: left side.

Fig. 3C. Section through a preneural plate (NP) developed under inductory effect of endophyll (E) in the absence of chorda mesoblast; N: preneural floor; G: preneural groove. Bar = 100 µm.

earlier studies we have demonstrated that endophyll induces and directs the head region and central nervous system [11]. Also endophyll (containing δ−ooplasm) on its own induces preneurulation phenomena [12, 13] in isolated cranial halves of avian blastoderms in contrast to the caudal halves where a PS and gastrulation phenomena take place under influence of Rauber's sickle (containing y−ooplasm) (Figs. 1D, 1E). In some mediosagittally hemi-sectioned blastoderms the caudal part of the blastoderm does not develop and thus no hemi-primitive streak forms. In contrast the head region with neural plate develops with or without the presence of a notochord (Fig. 3C). This observation suggests analogy with the experimental destruction of cranial versus caudal blastomeres by Conklin (1905) in Styela [14] which he also explains by the presence of different organ-territory-forming ooplasms (neurulation versus gastrulation), suggesting preformation.

2. Mediosagittally hemi-sectioned blastoderm (left or right side) from which the median part of the Rauber's sickle is scraped away

In Fig. 4A such an operated blastoderm fragment is seen at the start of the culture. After culture *in vitro* a rectilinear primitive streak with bilateral mesoblast mantle develops (Fig. 4B). No separated hemi-PS is seen in sections. The common PS not only contains mesoblast material from the caudo-centrally sliding upper layer cells in the concavity of the conserved Rauber's sickle fragment (forming a larger contribution) but also from upper layer cells found in the neighborhood of the cross-sectioned area of the Rauber's sickle (Fig. 4C). The developmental potencies of the latter hemi-blastoderms are thus much more pronounced when compared with those in purely mediosagittally sectioned blastoderms represented in Fig. 2A. Thus with the same quantity of upper layer

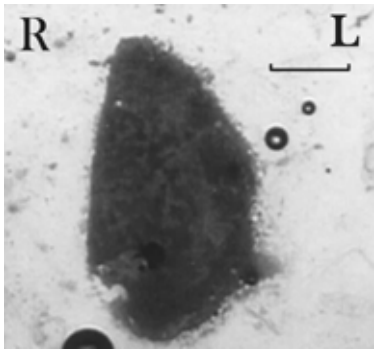

Fig. 4A. Mediosagittally hemi-sectioned blastoderm (left side) from which the median part of the RS is scraped away, at the start of the culture *in vitro*. Bar = 1 mm. R: right side and L: left side.

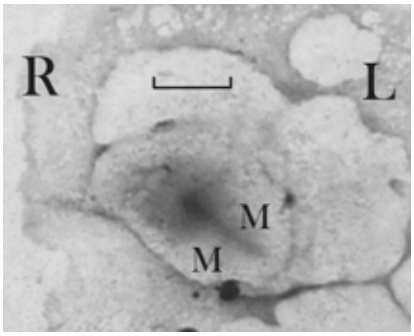

Fig. 4B. The same hemi-blastoderm after 27 h of culture: a rectilinear PS has developed with bilaterally developed mesoblast mantle (M). Neural plate and nodus are visible. Bar = 1 mm. R: right side and L: left side.

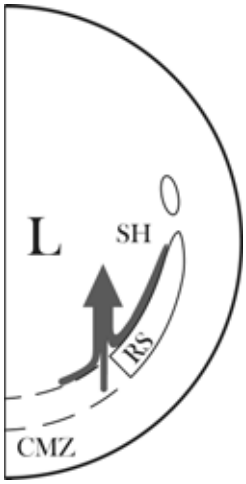

Fig. 4C. Schematic representation of mediosagittally sectioned half unincubated chicken blastoderm from which the median part of the RS is scraped away (indicated by double interrupted lines). After culture a common rectilinear PS has developed (starting from the sectioned part of the RS) composed of two parallel hemi-PS's: the major part and mesoblast mantle are formed in the concavity of the remaining RS material; the smaller part and mesoblast mantle are formed in the region of the cross-sectioned part of RS. L: left side.

material but with substantially lesser RS material, a full PS, flanked bilaterally with a mesoblast mantle develops. So quantitatively less RS material provides more developmental potencies when surrounded by upper layer cells at its cross-sectioned region. This experiment thus corresponds with the evolution after transplantation of an isolated RS fragment on the deep side of the central upper layer of an unincubated blastoderm [7, 15]. Our *in vitro* observed regulation phenomena are thus comparable with the regulation phenomena observed after the cleavage experiments *in ovo* of Lutz [16] and particularly with the *in ovo* cleavage with traction experiments, performed by Vakaet [17]. Vakaet observed different development scenarios according to the kind of traction procedure, probably due to the different reciprocal displacements of the deep layer with reference to the upper layer (more specifically at the rim of the blastoderm, where Rauber's sickle and endophyll are found) *in situ* on the egg yolk ball.

3. Unilateral removal of a half Rauber's sickle from a whole blastoderm

In Fig. 5 the removal of a half Rauber's sickle is schematically represented. After culture, a primitive streak starting from the incision point of the Rauber's sickle develops. Not only upper layer cells sliding in the concavity of the still present Rauber's sickle half form the corresponding part of the primitive streak (as is the case in Fig. 4C), but also upper layer cells from the neighborhood of the cross-sectioned Rauber's sickle are involved (Fig. 5). After fixation and staining with Unna of older embryos (42 h), the area vasculosa is seen to develop only at the unoperated side. The intraembryonic cavity is closed laterally in the operated side and usually (not always) only one heart tube develops in the unoperated side [8]. The eventual bilateral presence of the heart tube can probably be explained by the not-total removal of the sickle horn in the operated side. Indeed we have demonstrated [4] that the Rauber' sickle horn material, by direct induction on the splanchopleura mesoderm, gives rise bilaterally to the formation of the primary heart tubes, pericard and associated venae vitellinae.

4. A whole blastoderm in which more than half of the Rauber's sickle material is scraped away

In Fig. 6A such an operated blastoderm is seen at the start of the culture. After culture, a primitive

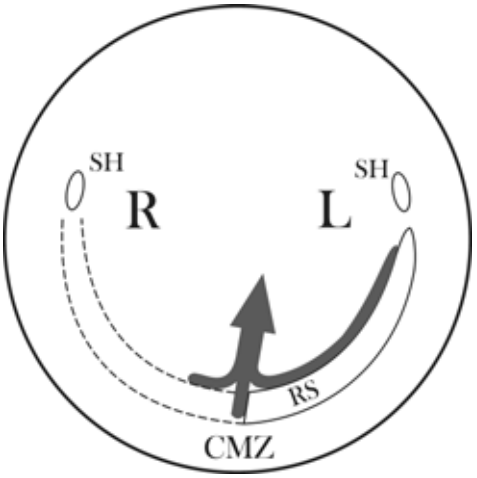

Fig. 5. Schematic representation of the unilateral removal of a half RS from a whole unincubated blastoderm. The development of a common PS (after culture) from two origins are indicated by curved arrows. R: right side and L: left side.

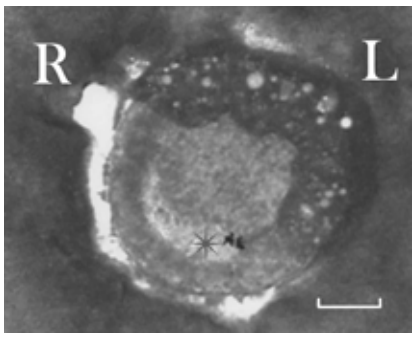

Fig. 6A. A whole unincubated blastoderm in which more than a half of the RS material is removed (*) at the start of the culture period; graphite particles are placed on the cross-sectioned part of RS. Bar = 1 mm. R: right side and L: left side.

streak starting from the incised part of the Rauber's sickle is observed. First the embryo has a racket-shaped form with a rectilinear PS starting from the incised part of RS. Because at the incision point of the RS, also freely UL cells are available, this region also forms a collateral part of the PS. Since UL cells are bilaterally taken up in the PS, there is no bending of the latter. The further development, noteworthy, is nearly normal, probably due to the proportional abundance of UL cells. After 33 h of culture a head fold embryo

has formed. After staining *in toto* with Unna, the mesoblast mantles on both sides are visible. The area vasculosa at the unoperated side is much more developed (Fig. 6B). This is of course due to the absence of Rauber's sickle material in the operated side. The composition of the formed PS is schematically represented in Fig. 6C. The main contribution to the rectilinear primitive streak and mesoblast mantles comes from the centro-caudal sliding of the upper layer cells in the concavity of the still present Rauber's sickle material. Also some induced upper layer cells in the neighborhood of the incised Rauber's sickle material are incorporated in the PS since some asymmetry is observed in the mesoblast mantles and areae vasculosae (Fig. 6B).

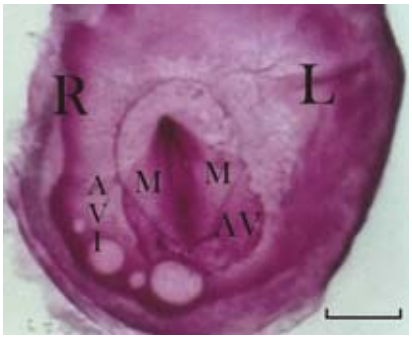

Fig. 6B. The same blastoderm after 33 h of culture, presents an apparently normal head fold embryo. There is asymmetry in the mesoblast mantles (M) and area vasculosae (AV); AVI: area vitellina interna, derived from the area opaca. Bar = 1 mm. R: right side and L: left side.

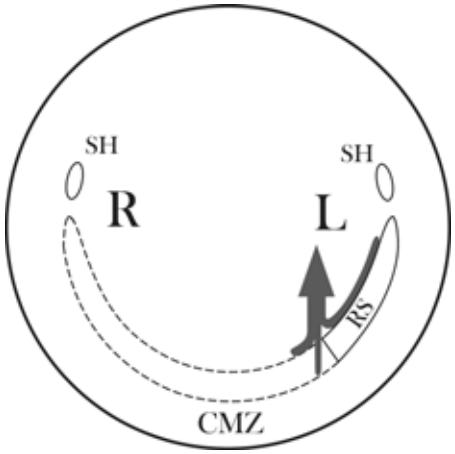

Fig. 6C. Schematic representation of the composition of the formed PS after removal of more than half of the RS material from an unincubated chicken blastoderm; one part is composed of UL cells sliding in the concavity of the remaining RS-horn, the other part is formed by UL cells induced in the cross-sectioned area of the RS. R: right side and L: left side.

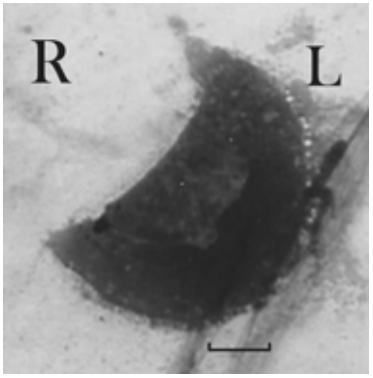

Fig. 7A. Caudal half of obliquely hemi-sectioned unincubated blastoderm at the start of the culture *in vitro*. Bar = 1 mm. R: right side and L: left side.

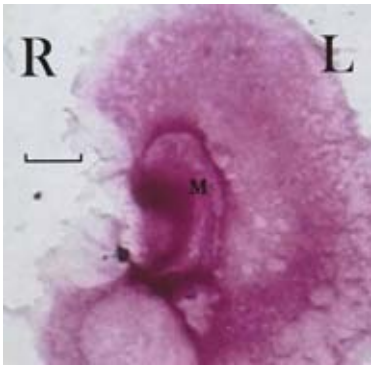

Fig. 7B. The same hemi-blastoderm after 34 h of culture: a curved embryo has developed with an unilateral mesoblast mantle (M). Bar = 1 mm. R: right side and L: left side.

Fig. 7C. Section through the trunkal region of the embryo of Fig. 7B: two unequal hemi-PS's are seen; C: unilateral intraembryonic cavity; arrow indicates primitive groove; E: cut edge of the blastoderm. Bar = 100 µm.

5. Caudal half of obliquely hemi-sectioned unincubated chicken blastoderms

In Fig. 7A the caudal part of such an incised unincubated blastoderm is seen at the start of culture. After culture a curved asymmetrical embryo develops. The asymmetrical trunkal region starts from the region which was originally labeled with graphite particles (cross-sectioned Rauber's sickle) (Fig. 7B). In sections through this trunkal region it is seen that the primitive streak is composed of two unequal parallel hemi-primitive streaks, separated by a primitive groove (Fig. 7C). This asymmetry of the hemi-PS's gives rise to the observed bending of the trunkal region. The major most voluminous hemi-primitive streak is formed by the sliding of upper layer cells in the caudal concavity of Rauber's sickle. This indicates that UL cells from one side of the blastoderm can cross the midline region in the concavity of the RS in a reversed direction, suggesting a mechanical disequilibrium between left and right side of the blastoderm (Fig. 7D). The small hemi-primitive streak is formed close to and parallel with the incision line (Figs. 7C, 7D).

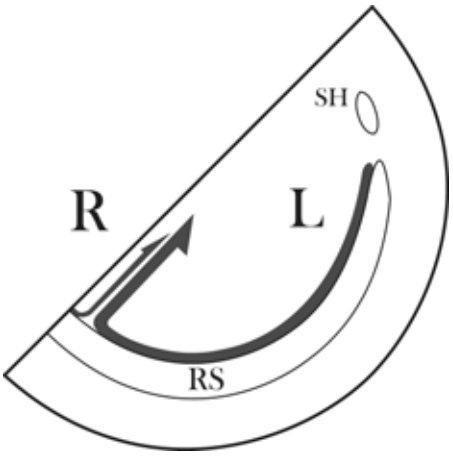

Fig. 7D. Schematic representation of the caudal half of an obliquely sectioned chicken hemi-blastoderm; after culture two parallel hemi-PS's have developed (indicated by two unequal curved arrows): the major hemi-PS is formed from UL cells sliding in the caudal concavity of the remaining RS (note that it exceptionally crosses the midline in a reversed direction as the result of a left-right disequilibrium; the minor hemi-PS is formed close to and parallel with the sectioned edge). R: right side and L: left side.

6. Cranial half of obliquely hemi-sectioned unincubated blastoderms

In Fig. 8A the cranial part of such an incised unincubated blastoderm is seen in culture. After culture a curved asymmetrical comma-shaped embryo develops (Fig. 8B). The neural plate is localized in a cranial direction while the trunkal region is narrow and curved in the direction of the incision edge. In sections through the trunkal region two parallel unequal hemi-primitive streaks, separated by a primitive groove are seen (Fig. 8C). The major most voluminous half primitive streak is derived from upper layer cells sliding in the concavity of the cranial remaining Rauber's sickle fragment, while the smaller half primitive streak is

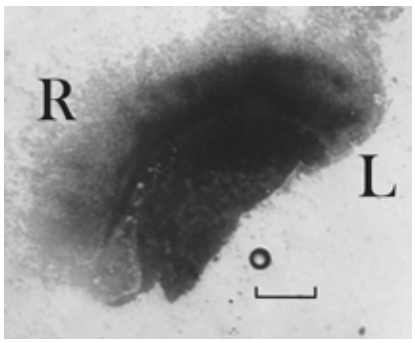

Fig. 8A. The cranial half of an obliquely hemi-sectioned unincubated chicken blastoderm at the start of the culture *in vitro*. Bar = 1 mm. R: right side and L: left side.

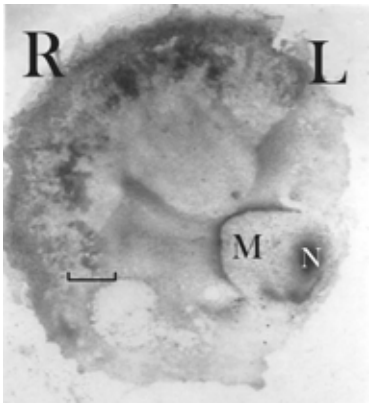

Fig. 8B. The same blastoderm fragment after 1 day of culture; note the comma-shaped aspect of the embryo; N: neural plate; M: unilateral mesoblast mantle. Bar = 1 mm. R: right side and L: left side.

formed with and close to the incision line (Figs. 8C, 8D). An intraembryonic cavity and mesoblast mantle was only seen at the unoperated side. The absence of an intraembryonic cavity in the operated side seems to inhibit the formation of a mesoblast mantle.

7. Oblique hemi-sectioning of the unincubated chicken blastoderm and unilateral removal of Rauber's sickle material and endophyll in the smallest side

The excision technique is schematically represented in Fig. 9. After culture *in vitro* a centrally directed primitive streak starting from the original middle of Rauber's sickle (incised part) is seen. The uptake of upper layer material from the operated side into a nearly symmetrically developing primitive streak is obvious. Here no visible separated hemi-primitive streaks develop (as is confirmed by histological processing). After prolonged culture a slightly asymmetrical embryo proper has developed. In the cranial region of the unoperated side a beating heart tube is observed [8]. It is remarkable that with a half UL surface and half quantity of RS material, after all a nearly normal development can be observed in contrast with the mediosagittally sectioned blastoderm (compare with experiment 1). After fixation and *in toto* staining with Unna an area vasculosa is seen to develop only at the unoperated side (mainly caudally) since in the operated side no RS material is present. Only at one side (unoperated) a hemi-coelomic cavity with blood islands is visible. If we compare the development in these blastoderms with the development seen when the Rauber's sickle material was left intact in the caudal half of obliquely hemi-sectioned blastoderms (experiment 5), we must conclude here again that when lesser Rauber's sickle material is present, the development occurs more symmetrically and completely. The sliding of the UL cells in the area centralis seems thus to be arrested where no Rauber's sickle material is present, even when the full extent of the caudal marginal zone remains *in situ*, demonstrating once again that the Rauber's sickle material is the true early gastrulation organizer [1, 2, 7] and blood island inductor.

8. Excision of the cranial quarter containing the sickle horn top of the unincubated blastoderm

In Fig. 10A two isolated cranial quadrants are seen *in vitro* at the start of the culture. After culture *in vitro* such blastoderm fragments present a comma-shaped embryo with centrally directed neural plate region (Fig. 10B) and a rudimentary narrow strongly curved trunkal region. In sections performed perpendicularly to the trunkal longitudinal axis, clearly, only unilaterally a half-PS is seen (Fig. 10C). Also an unilateral embryonic cavity, containing some rudimentary mesoblast mantle, can be observed. The hemi-PS formation of these embryos is schematically represented in Fig. 10D and the neurogastrulation phenomena in Fig. 10E. Here also it is clear that only one hemi-PS is formed by migration of UL cells at the side where they are present. The hemi-PS develops parallel with the free cut edge of the blastoderm flanked by a unilateral associated mesoblast mantle (Figs. 10B, 10E).

9. Asymmetrical displacement of Rauber's sickle material into the left cranial region of an unincubated blastoderm giving left/right inversion

Material from the median part of RS is displaced circularly in the prolongation of the left sickle horn in the cranial blastoderm quadrant, resulting in an asymmetrical disposition of the Rauber's sickle material (Fig. 11A). After approximately 41 h of culture (Fig. 11B) this provokes a left/right inversion (situs inversus cordis) [18]. The mechanism of inversion in this experiment seems to correspond to the teratological syndrome of duplicitas cranialis [19, 20].

This situation is comparable with the situation observed in conjoined twins where the situs of the right twin is inverted by the right side of the left twin [21] but without the formation of a twin.

GENERAL DISCUSSION

Obtaining of hemi-primitive streaks (right or left) by hemisectioning the avian unincubated blastoderm, in a plane going through the middle of its ooplasmic symmetry (Rauber's sickle material) is comparable with what was achieved in Ascidians [22, 14] or in Rana [23, 24] by destroying one of the first two blastomeres. These results were considered as evidence for the preformation against the epigenetic theory, where regulation was accepted [25]. In the first two cases, there already seems to exist a mosaic disposition at the beginning of cleavage together

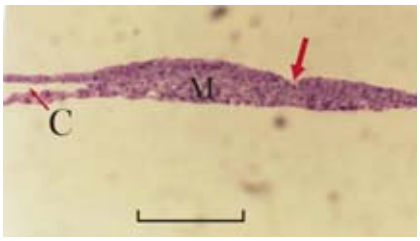

Fig. 8C. Section through the trunkal region of the embryo seen in Fig. 8B: two unequal hemi-PS's are seen with a unilateral mesoblast mantle (M) and intraembryonic cavity (C); arrow indicates primitive groove. Bar = 100 µm.

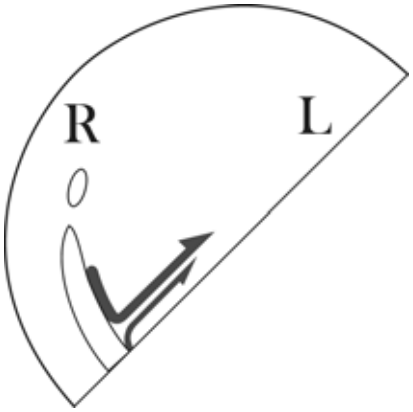

Fig. 8D. Schematic representation of the cranial half of obliquely hemi-sectioned unincubated blastoderms; after culture two unequal parallel hemi-PS's have developed (two curved arrows). R: right side and L: left side.

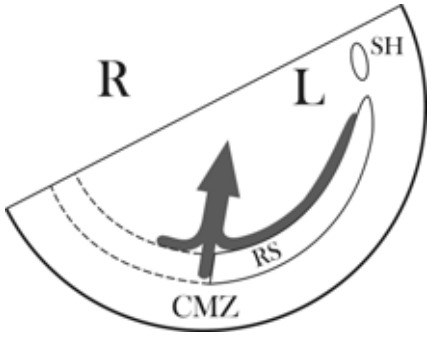

Fig. 9. Schematic representation of the oblique hemi-sectioning of an unincubated chicken blastoderm in which one sickle horn and corresponding half median part of RS and endophyll were removed; after culture *in vitro* an apparently normal PS develops. R: right side and L: left side.

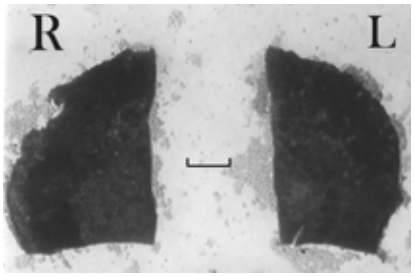

Fig. 10A. Ventral view of cranial quarters of unincubated chicken blastoderms at the start of the culture *in vitro*. Bar = 1 mm. R: right side and L: left side.

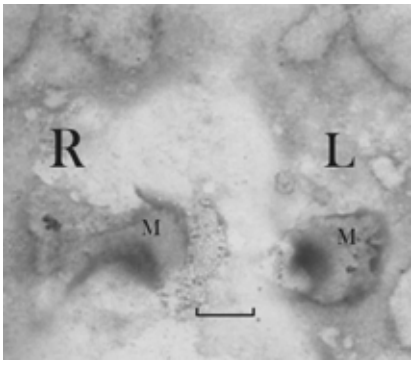

Fig. 10B. The same blastoderm parts after 30 h of culture; comma-shaped embryos with unilateral mesoblast mantle (M) have developed. Bar = 1 mm. R: right side and L: left side.

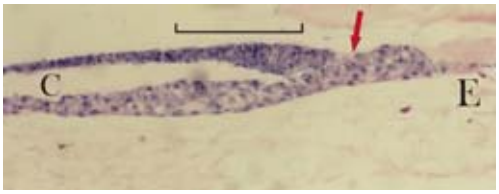

Fig. 10C. Section through a strongly curved trunkal region of Fig. 10B: only unilaterally a half PS and mesoblast mantle are seen; C: unilateral intraembryonic cavity; arrow indicates the hemi-primitive groove; E: cut edge of the blastoderm. Hematoxylin-Eosin. Bar = 100 µm.

with the appearance of bilateral symmetry in the ooplasm. In Rana, hemi-embryos were only obtained when the plane of the first cleavage coincided with the plane of ooplasmic symmetry going through the middle of the gray crescent [26, 27]. If the two first blastomeres were separated

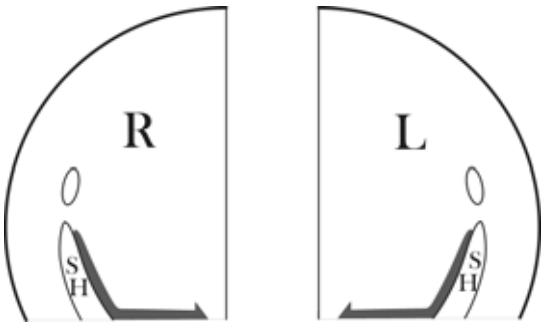

Fig. 10D. Schematic representation of excised left and right cranial blastoderm parts containing a sickle horn top: curved hemi-arrows represent the formation, after culture of hemi-PS's by caudocentral sliding of UL cells in the concavity of the sickle horns. R: right side and L: left side.

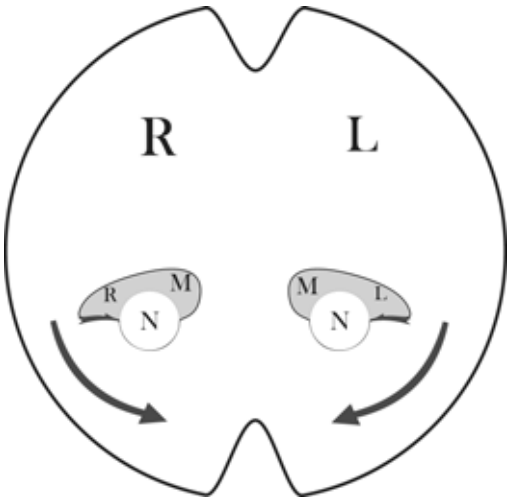

Fig. 10E. Schematic representation of the neurogastrulation phenomena seen in Fig. 10B; M: mesoblast mantle; N: neural plate; R and L indicate the chiral asymmetry of the formed hemi-embryos. R: right side and L: left side.

by an oblique cleavage furrow, not going through the ooplasmic middle, more or less deficient embryos were obtained [28]. We have demonstrated that, in the chicken blastoderm, both mosaic or regulation phenomena can be obtained, only by the different geometric distribution of Rauber's sickle material in the isolated blastoderm fragment [29]. Rauber's sickle material which contains y−ooplasm [30, 3], regulates early embryonic patterning (a.o. gastrulation) by its inducing effect on the upper layer (containing β−ooplasm) which

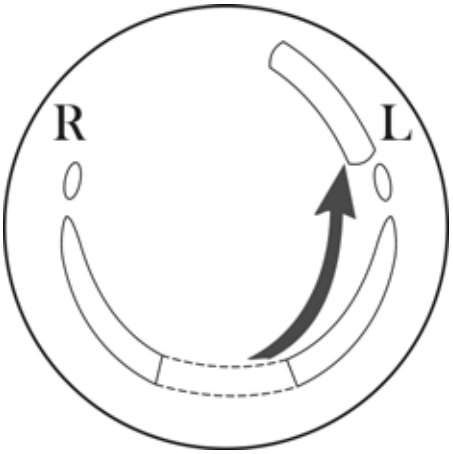

Fig. 11A. Schematic representation of an unincubated chicken blastoderm in which the central median part of the RS is ectopically displaced in the prolongation of the left sickle horn so giving an asymmetrical distribution of RS material. R: right side and L: left side.

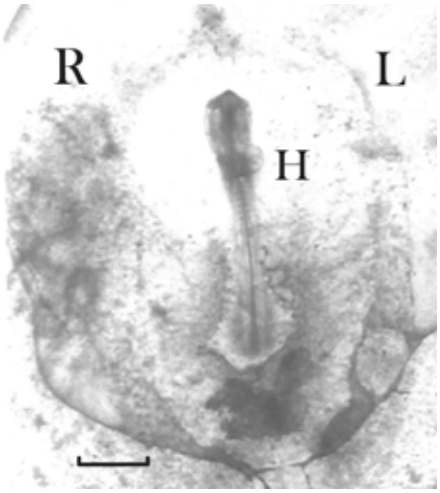

Fig. 11B. Ventral view of a similar blastoderm (as represented in Fig. 11A) after 40 h of culture: the embryo presents situs inversus cordis with ventricular looping to the left (H). Bar = 1 mm. R: right side and L: left side.

will form embryonic stem cells by early nucleization. In birds, the bilateral ooplasmic symmetry (recognizable by the appearance of the Rauber's sickle) occurs much later than in Anurans, already visible by the appearance of the grey crescent before the first cleavage takes place. The reason seems that the gastrulation-inducing y−ooplasm is localized at the periphery of the flat

avian germ disc [30, 3] and comes in contact with the embryo proper only when the latter is formed by ten thousands of cells (before laying). In Amphibians, induction of mesoderm and the formation of Spemann's organizer [31] occur much earlier during the cleavage period and are dependent on the activity of an earlier vegetal signaling site, the Nieuwkoop center [32], which is directly dependent on oocytal gene activity, and is homologous to the Rauber's sickle and derived sickle endoblast in birds [2]. The Ascidian egg has been regarded as a typical mosaic egg also during the early cleavage period which shows a highly determinate mode of development [33, 34, 35]. The localization and function of Rauber's sickle present a strong similarity with the localization and function of the Ascidian Wnt gene, Hr Wnt-5 from Halocynthia roretzi [36]. Indeed, Hr Wnt-5 m RNA is first present in the vegetal cortex of unfertilized eggs. After fertilization, Hr Wnt-5 moves to the equatorial region (similar to the y−ooplasm in birds) to form a sickle-shaped structure. Afterwards, this mRNA is concentrated in the most caudal region of the two-cell embryo, which already presents a left-right asymmetry visualized by the natural mediosagittal cleavage plane through this caudal sickle-shaped Wnt gene-expressing zone [36] homologous to the avian Rauber's sickle. It has recently been demonstrated that Vg1 is present in the avian Rauber's sickle material (inclusive of the sickle horns) and not in the caudal marginal zone [37]. In Ascidians and Amphibia at one hand and birds on the other hand, similar ooplasms seem to be involved but the degree of early "nucleization" is different [29]. Both an equal quantity of upper layer (half the surface of a blastoderm) and an equal and isotopical quantity of Rauber's sickle material are present in experiments 1 and 7 (Fig. 12). A mediosagittal hemi-sectioning (in experiment 1) thus makes a world of difference for further embryonic development. The fact that upper layer cells in the concavity of both halves of Rauber's sickle are needed for normal primitive streak formation can also be explained by the experiments of Lepori [20]. He observed the existence in the upper layer of a centripetal, chiral, non-mirror symmetric counter clock wise movements, which results in an asymmetric ingression (left side earlier and more pronounced and directed to the right and to the depth) during early gastrulation [38, 39, 40, 41]. There seems to be homology with the situation at the moment of bilateral symmetrization in Amphibia. Indeed the normal first cleavage in the Xenopus egg is also chiral and not mirror-image symmetric. Indeed there occurs a slight counter clock-wise torsion in the first two blastomeres during the first cleavage furrow

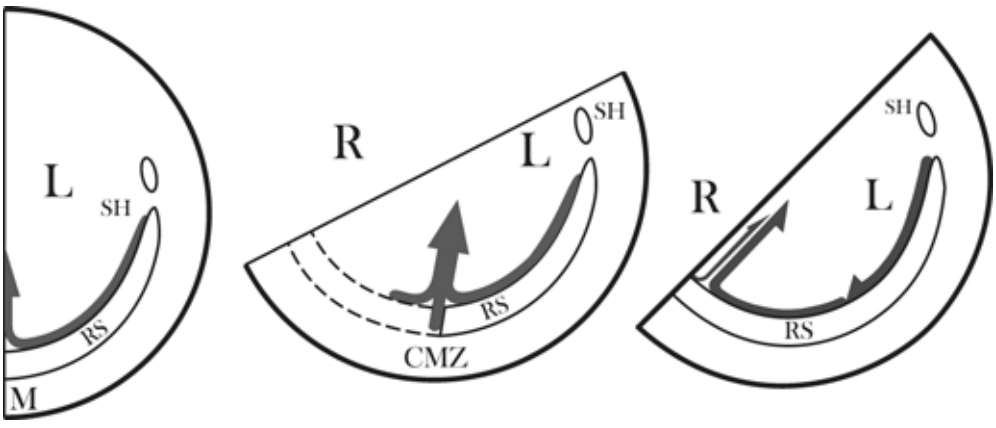

Fig. 12. The different kinds of formation of (hemi) primitive streaks and early embryonic patterning in differently hemi-sectioned avian blastoderms during early *in vitro* culture depends on the local spatial relationship of RS material with reference to the neighbouring upper layer cells in the area centralis (stem cells of the embryo), sliding caudo-centrally in the concavity of the RS (comparison of figures 2A, 9, 7D); only after intervention-9 - a nearly normal development occurs. R: right side and L: left side.

advance [42]. The study of these authors suggests that the ooplasmic cytoskeleton provides a peripheral unambiguous directional cue that serves to redistribute ooplasmic components of the left-right pathway unidirectionaly along the mediolateral dimension during or shortly after the establishment of bilateral symmetry. Researchers studying left-right asymmetry by laterality genes [43, 44, 45] have concluded from their study of asymmetrically expressed genes that the left and right side in the chicken blastoderm can be viewed as distinct and autonomous fields. Both blastoderm flanks are required but also the blastoderm must be intact.

Levin and Mercola [46] have observed that introducing discontinuities in the circumferential path by making peripheral slits in the chick blastoderm likewise affects left-right asymmetry. Here in the present study we have seen that not only the distribution of Rauber's sickle material, but also the spreading of the neighboring upper layer plays a role in further development of the embryo. It is important to note that Rauber's sickle material also presents a circumferential spreading around the area centralis and, thus, Rauber's sickle material together with the upper layer can be isolated into separate parts by peripheral slits resulting in a different behavior (as is shown here). A pronounced asymmetric development is started from the beginning of incubation in the mediosagittaly sectioned hemi-blastoderm indicating a strong caudo-central sliding of the upper layer cells under the strong influence of the half median part of Rauber's sickle-endophyll complex, even so by the absence of the upper layer cells of the contra-lateral removed side which normally are in mechanical equilibrium. The only possibility for the upper layer cells in the remaining half of the blastoderm is to slide medially over the basement membrane [47] into the direction of the cut edge of the half blastoderm, which results in the formation of a half primitive streak and mosaic development with an asymmetric embryo. We think that during normal development after a few hours of incubation, both halves of the blastoderm develop more or less independently as parts of a mosaic under the influence of the corresponding neighboring halves of Rauber's sickle. Somewhat later, the interaction between the left and right halves of the formed primitive streak becomes more pronounced and is indispensable for further normal embryonic development. The mechanical interaction between left and right halves of the blastoderm seems to be concentrated mainly in the midline region, at the contact zone between left and right lip of the top of the primitive streak, where the first morphological asymmetry is observed [48, 49]. More recently Lepori [20] explains the asymmetry in the node by stressing on the earlier ingrowth (directed to the right) of the left lip. An asymmetrical strength of the caudo-centrally oriented sliding of the UL cells in the concavity of the left versus right RS half could give rise to asymmetrically growing PS's at the origin of the asymmetry in the nodus. If only regulation phenomena would strictly exist in any part of the blastoderm (so-called totipotency), as propounded by Lutz [16, 50], a symmetrical primitive streak growing from a more lateral part of the remaining Rauber's sickle half in sagittally hemi-sectioned blastoderms, could be expected. Our experiments suggest that a strong predetermined induction polarity exists along the Rauber's sickle from the top of the sickle horn into the direction of the median part. Another fact that must be taken into account is that, centro-medially in the concavity of Rauber's sickle a fenestrated sheet of endophyll is present, while this is not the case at the top of the sickle horn region. Indeed endophyll is known to direct the growth of the sickle endoblast (derived from Rauber's sickle) which forms the basis on which the primitive streak region will be built [11].

Comparison of our experiments with earlier observations of spontaneously developed or artificially obtained polyembryony

Apart from embryonic cleavage or fusion theories [51, 52, 53], the radial disposition theory was propounded by Rauber [54], i.e. the embryonic heads in spontaneous polyembryonic groups are usually directed to the center of the germ disc (always the one in the original caudal region) with head against head and not tail against tail orientation. The cleavage experiments performed *in ovo* with unincubated Peking duck blastoderms indicated, indeed, the existence of some early radial symmetry, suggesting totipotency of the isolated parts and regulation phenomena [50, 16, 17, 55]. This radial symmetrical disposition seems to be the remainder

of the original mitochondrial RNA-bound ooplasmic radial symmetry visible in the premature avian oocyte [56, 57]. In contrast, the results obtained *in vitro* culture by Spratt and Haas [58] in the unincubated chicken blastoderm after excision of different parts, suggest a caudocephalic gradient of development. The external aspect of the unincubated duck blastoderm presents no differentiation; it has a homogenous whitish aspect (no area opaca and no area pellucida). It is usually only after some hours of incubation that Rauber's sickle appears in ducks [16, 17]. In most unincubated chicken blastoderms *in situ* on the egg yolk, an area centralis is visible, with eventually a caudocephalic density gradient and sometimes a Rauber's sickle. This radial symmetry in young duck blastoderms can be explained by an original circular localization of the y−ooplasm, which will finally give rise caudo-laterally to Rauber's sickle by oblique formation of the subgerminal space [59, 60]. It is remarkable that in the case of twin formation *in ovo* by mediosagittal sectioning [50, 16, 17, 55] or *in vitro* [58] mosaic development in birds was not observed. One reason is that in their experimental procedure the exact orientation of the presumed caudocephalic axis was not precisely known, due to the orientation according to Von Baer's rule [61]. Formerly only regulation phenomena and no mosaic development were accepted to exist in birds. In view of our present *in vitro* experimental study we can explain more particularly the *in ovo* cleavage and/or traction experiments of Vakaet [17] giving rise to well developing and regulated twins or even triplets by local displacement and loosing of contact between Rauber's sickle material and UL cells in the neighborhood of the incision rim (*in situ* on the egg yolk ball) as in the case in some of our *in vitro* conditions. The production of avian hemi-embryos can perhaps be useful for the early determination by laterality genes of left-right asymmetry since the left hemi-embryo is induced point by point, by positional information of the left half of Rauber's sickle and associated sickle horn [62] via the left half primitive streak. The same phenomena occur at the right side. By mediosagittal hemi-sectioning of the unincubated chicken blastoderm, some midline structures are destroyed in our study, so in some cases only (pre) neurulation phenomena are conserved. Indeed it has been propounded that the midline structures play a role in the regulation of left-right sidedness [63]. The idea that the midline acts as a barrier during left-right specification was first proposed by Danos and Yost [64] to explain the results of experiments where the midline was experimentally manipulated or excised. According to Levin *et al.* [44] there is evidence that Hensen's node forms asymmetrically very early (in HH stage 3 embryo; 12 h incubation). Also, it has been shown that morphological left-right asymmetry of Hensen's node precedes the asymmetric expression of Shh and Fgf 8 in the chick embryo [65]. In our case this could be explained by a slightly asymmetric parallel growth of the hemi-primitive streaks. Our experiments indicate that not only left-right asymmetric expression of genes plays a role in early morphogenesis but that also epigenetic mechanical equilibrium or disequilibrium must be considered (so called "cellularmechanik" as propounded by Rauber) [66]. Based on fate and gene expression the first aspects of the avian gastrulation organizer [7] can be recognized in Rauber-Koller's sickle [67]. The latter authors demonstrated by whole mount *in situ* hybridization of chicken embryos, the expression of OTX2 in neural plate derived structures and of HOXB1 in mesoblast derived structures (implicated in the cranio-caudal segmentation of the body trunkal region). Our observations strongly suggest that these two expression domains are localized in regions where originally δ−ooplasm (endophyll) or y−ooplasm (RS) containing structures were functional.

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