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Review article

Rearranging gastrulation in the name of yolk: evolution of gastrulation in yolk-rich amniote eggs

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Abstract

Gastrulating birds and mammals form a primitive streak in lieu of a circular blastopore, and a conspicuous underlying tissue layer, the hypoblast. In an attempt to understand the evolution of these amniote characteristics, pregastrula and gastrulation stages in selected amniotes are compared with the more ancestral situation in amphibians. At blastula/blastoderm stages, the overall fate maps and the arrangement of tissues around the organizer are rather similar, as is exemplified by a comparison of gene expression and fate maps in the frog and chick. Compared with amphibians, however, the eggs of reptiles, birds and monotreme mammals have a disproportionately large yolk that alters gastrulation morphology. During amphibian gastrulation, the organizer moves from anterior to posterior, to lay down the dorsal axis around the vegetal hemisphere (Arendt, D., Nübler-Jung, K., 1997. Dorsal or ventral: similarities in fate maps and gastrulation patterns in annelids, arthropods and chordates. Mech. Dev. 61, 1–15). In contrast, in amniote eggs, the large yolk impedes the organizer from moving around the entire vegetal hemisphere so that axis formation begins and ends at the same side of the egg. This has apparently provoked an evolutionary transformation of an amphibian-like blastopore, first into the 'blastoporal canal' of reptiles, and then into the birds' and mammals' primitive streak. The blastopore divides into two functionally divergent parts, one as the site of mesoderm internalization ('intraembryonic blastopore') and the other as the site of ectodermal epiboly ('extraembryonic blastopore'). The hypoblast is proposed to derive from the 'endodermal wedge' that is seen already in the amphibian gastrula. Hypoblast formation would then represent a special kind of gastrulation movement that also exists in the amphibians, and for which the term 'hypoboly' is introduced.

Keywords: Gastrulation; Comparative embryology; Hypoblast; Primitive streak; Endodermal wedge; Evolution; Chick

1. Introduction

Developmental studies in the vertebrates focus on few organisms, each of which has been chosen for specific advantages. For example, *Xenopus* development can be followed under the microsocope from the earliest stages onwards, and can be manipulated mechanically. The chick embryo with its very divergent early development as compared with *Xenopus*, is translucent and almost flat, yet also suitable for grafting experiments and lineage tracings. Beside the interest in their specific developmental modes, the study of vertebrate 'model organisms' also aims to elucidate general developmental principles, and to gain insight into the evolution of vertebrate development. The recent discovery that molecular mechanisms of embryogenesis are evolutionarily conserved to a large extent is very promising with respect to the comparative analysis of early vertebrate development (De Robertis et al., 1994; Tam and Quinlan, 1996). These molecular similarities allow a new synergism in developmental research: while exploiting the specific advantages of a given vertebrate model system one can hopefully extrapolate the results from another organism. A prerequisite for this, however, is to define a common morphological and temporal framework of vertebrate development.

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The gastrula stage has always been described as divergent between various vertebrate groups, in contrast to the later phylotypic stage, the pharyngula, considered the 'bottleneck' stage of vertebrate development (Elinson, 1987, see also Gilbert, 1994). In amphibians, midway during gastrulation, the blastopore is seen as a circular cleft between the animal and the vegetal hemispheres. Its amniote equivalent, the primitive streak, is a longitudinal thickening with a central furrow forming across the blastoderm. From a morphological point of view, blastopore and primitive streak thus appear rather dissimilar, except for the fact that both represent the site where cells internalize during gastrulation (Pasteels, 1936a; Pasteels, 1936b). A deeper level of analysis, however, reveals clear similarities. Hensen's node, a bulbous mass of cells at the anterior tip of the primitive streak, is considered the amniote equivalent of the amphibian organizer located at the dorsal blastopore lip (Leikola, 1976; see Gilbert, 1994). This view is substantiated by the common expression of several genes, for example *goosecoid* (Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993), *Xnot/Cnot* (in frog and chick; Von Dassow et al., 1993; Knezevic et al., 1995; Stein and Kessel, 1995), *noggin* (in frog and chick; Smith and Harland, 1992; Connolly et al., 1997), *nodal/Xnr1,2* (in mouse and frog; Zhou et al., 1993; Jones et al., 1995), and the sharing of strong axis-inducing properties upon transplantation (in frog, chick and mouse; Leikola, 1976; Beddington, 1994). Cell fate studies have revealed that also the overall temporal sequence in which groups of endomesodermal cells internalize along the frog blastopore (Keller, 1975) and amniote primitive streak (Lawson et al., 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996) are surprisingly similar: the first cells that involute around the amphibian blastopore lip in the organizer region, and that immigrate through Hensen's node, contribute to foregut endoderm and prechordal plate. Cells involuting further laterally in the blastopore, or entering via Hensen's node and the anterior primitive streak, contribute to gut, notochord and somites. Gastrulation then continues along the ventroposterior blastopore lip and posterior streak region, from where cells contribute to ventral and posterior mesoderm. Adding to this, *Brachyury* and *caudal* homologues are expressed circumferentially around the blastopore lips in the frog (Smith et al., 1991; Northrop and Kimelman, 1994), and along the primitive streak in chick (Frumkin et al., 1993; Kispert et al., 1995) and mouse (Beddington et al., 1992; Meyer and Gruss, 1993). This would suggest that, despite their different morphology, the amniote primitive streak and the amphibian blastopore are homologous structures (Eyal-Giladi et al., 1992), meaning that they have evolved from one and the same precursor structure by a continuous sequence of morphological modifications.

It is thus time to ask again some of the most exciting, classical questions of comparative vertebrate embryology. (1) What made the circular blastopore of a more primitive anamnian tetrapod 'transform' first into a pouch-like invagination, as found in extant reptiles, and then into a furrow along a streak in birds and mammals? (2) Where does the hypoblast come from, an endodermal cell sheet underlying the amniote blastoderm? To approach these questions, the ancestral mode of gastrulation as seen in amphibians is compared with the more derived mode in the yolk-rich reptiles, birds and monotreme mammals. During evolution, early tetrapod vertebrates gave rise to the extant Amphibia and to their sister-group, the Amniota. The now extinguished lower amniotes (*Cotylosauria*) then gave rise to the *Sauropsida* (reptiles and birds), and, in a divergent branch, to mammals (monotremes, marsupials and placentalians). Reptiles, birds and monotreme mammals have in common the ability to form a disc-shaped blastoderm on top of a huge mass of yolk, thus representing the ancestral amniote condition (the yolk is reduced secondarily in marsupials and placentalians). Given that a hypoblast is found in all amniotes, and that a primitive plate (the forerunner of the primitive streak) has evolved in the already rather yolk-rich reptiles, these traits appear to have evolved together with an increase of yolk. Morphological and temporal changes in gastrulation that accompany the immense accumulation of yolk during amniote evolution will thus be traced, to show how these might have provoked the evolution of hypoblast, primitive plate and streak. Particular emphasis will be laid upon the comparison of well-characterized model organisms such as *Xenopus* and *Gallus*, as representatives of the amphibians with moderate yolk (frog), and of yolk-rich amniotes (chick).

The further, far-reaching modifications of gastrulation in higher mammals (evolution of an inner cell mass, trophoblast, etc.) will not be discussed here. These are specialized traits that have accompanied the secondary reduction of yolk during mammalian evolution, an event out of scope of the present article.

2. Comparison of fate maps: similar blastula/blastoderm fate maps in frog and chick

2.1. Conserved patterns along the animal–vegetal axis

The majority of animals develop from a spherical egg with a single axis, the animal–vegetal (an–veg) axis. The *animal* half of the egg usually contains the nucleus of the oocyte, while the vegetal half of the egg is the preferred site for the storage of yolk. Eggs with an–veg polarity are considered ancestral for the vertebrates. Frogs, for example, have eggs with vegetally concentrated yolk and the nucleus located in the animal cytoplasm. The egg of the yolk-rich amniotes also exhibits an–veg polarity, albeit in strongly altered proportions. In reptiles (see Pasteels, 1936a), monotreme mammals (Flynn and Hill, 1939), and in birds (see Schoenwolf, 1991), the vegetal yolk makes up the bulk of the oocyte, with a small cytoplasmic disc (blastodisc) on top of the yolk mass. The oocyte nucleus lies in the center of the blastodisc at the animal pole of the egg. The yolk-rich avian

and amphibian eggs share a rather similar, radially symmetric cytoarchitecture. In the growing oocyte, mitochondria segregate into two populations, one forming clusters of mitochondria in a crown-like distribution around the nucleus at the animal pole, the other located more vegetally in the subcortical layer of the oocyte (Callebaut, 1972; Callebaut, 1983; for birds D'Herde et al., 1995; for frogs Tourte et al., 1984).

After fertilization, cleavage transforms the amphibian egg in its entirety into a blastula made of numerous blastomeres. In the yolk-rich amniotes, the vegetal yolk acts as an impediment to cleavage, allowing cleavage to occur only in the blastodisc cytoplasm around the animal pole. This discoidal cleavage produces a cellular *blastoderm*, separated from the uncleaved yolk by the *subgerminal cavity*, in reptiles (Peter, 1934; Pasteels, 1936a), monotreme mammals

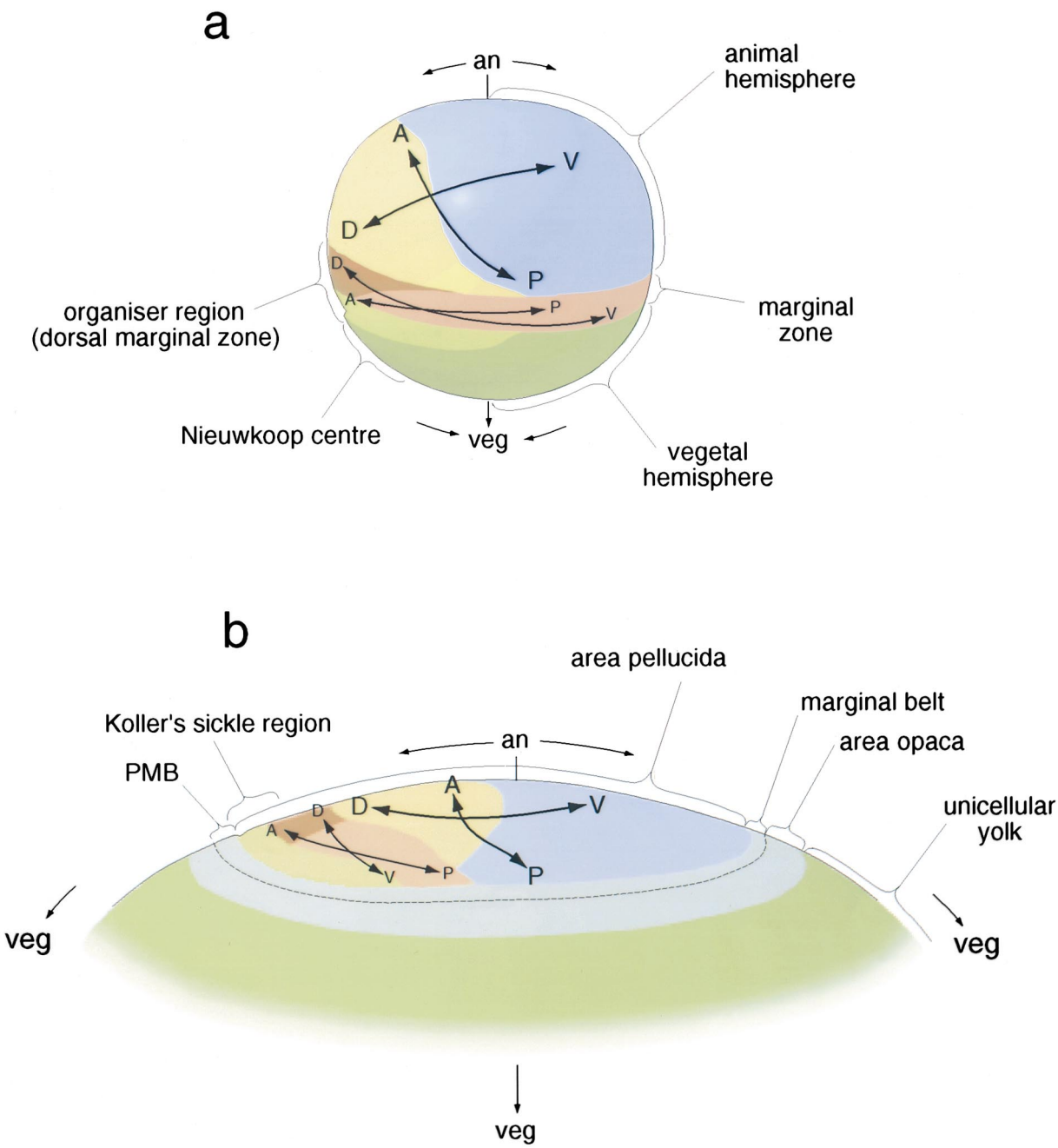

Fig. 1. Comparison of blastula/blastoderm fate maps. (a) Toad (*Bombina*; Vogt, 1929; Pasteels, 1940; cf. Keller, 1975) (b) Chick (*Gallus*; Schoenwolf and Alvarez, 1991; Hatada and Stern, 1994; Callebaut et al., 1996a). Yellow, neuroectoderm; blue, epidermal ectoderm; grey, extraembryonic ectoderm; red, mesoderm; brown, chordamesoderm; light-green, archenteron roof endoderm/definitive endoderm; dark green, nutritive endoderm. Arrows indicate orientation of the animal-vegetal axis (an–veg) and of the future A–P and D–V body axes. Note: the cellularized blastoderm in the chick consists of area pellucida, marginal belt, and area opaca. For clarity the anlagen in the chick fate map are drawn with sharp boundaries although in reality there is considerable overlap (compare the 'Modal map' concept of Vodicka and Gerhart, 1995).

(Flynn and Hill, 1939; Flynn and Hill, 1947) and in birds (Schoenwolf, 1991). The amniote blastoderm thus corresponds to only the more animal parts of the amphibian blastula. Note that discoidal cleavage is approximated also in the snake-like terrestrial amphibian *Gymnophiona* that form a disc of small micromeres at the animal pole, upon the large, yolky vegetal macromeres; Nelsen, 1953).

In birds, the an–veg polarity of the blastoderm is often described as running perpendicular to the blastoderm surface, with the 'animal' side attached to the vitelline membrane, and the 'vegetal' side facing the yolk via the subgerminal cavity (Schoenwolf, 1991; Khaner, 1992). Keeping in mind, however, that the amphibian, as well as the yolk-rich amniote egg, represent a sphere with one single axis, the polarity along this axis is manifest in two ways, straight *across* the egg (as described above) but also *on the surface* of the egg along any meridional line that runs from pole to pole. Accordingly, in the yolk-rich amniote egg an–veg polarity is also visible as a *concentric* arrangement of morphologically distinct regions on the egg surface, centred around the animal pole (Fig. 1).

How can this concentric arrangement of regions on the surface of the yolk-rich amniote egg be aligned with the different regions of the amphibian blastula? Conserved molecular markers with a specific an–veg distribution help to align 'animal' and 'vegetal' in the chick and frog. In the *Xenopus* blastula, *Otx2* transcripts are detected in the animal hemisphere and dorsal marginal zone (Pannese et al., 1995) The corresponding chick *c-otx2* is expressed in the area pellucida before streak formation (Bally-Cuif et al., 1995). *Otx2* is also expressed in the prestreak ectoderm of mice (Simeone et al., 1993; Ang et al., 1994), and in animal caps from zebrafish blastulae (Sagerström et al., 1996). This would suggest a correspondence of the avian area pellucida to the frog animal hemisphere (and marginal zone; Fig. 1). Avian area opaca and marginal belt show immunoreactivity against TGF-β1, the activity of which is known to mimick the mesoderm-inducing activity exerted by vegetal blastomeres of the amphibian blastula (Sanders et al., 1994). This would suggest that the more peripheral tissues of the chick blastoderm are of 'vegetal' character (corresponding to part of the vegetal hemisphere of the amphibian blastula).

Note that avian marginal belt (previously 'marginal zone', Eyal-Giladi, 1997) and amphibian marginal zone do not correspond, because, in comparison, the avian marginal belt is a more vegetal region than the amphibian marginal zone (Fig. 1; cf. Eyal-Giladi, 1997). Consequently, and as will be outlined below, the avian 'posterior marginal belt' (PMB) corresponds to amphibian Nieuwkoop center, and not to the (more animal) amphibian organizer region, the 'dorsal marginal zone' (Figs. 1 and 2).

Birds and frogs are also similar in that the 'vegetal' egg regions – uncleaved yolk in birds, vegetal-most blastomeres in frog – do not contribute to the definitive embryo, they are mainly nutritive in function and will later be absorbed (see below). These vegetal egg regions differ, however, in that in frogs they are internalized already during gastrulation, while in birds they remain external to the prospective embryonic body for a long time and are thus called *extra-embryonic*. In the course of amniote evolution the excessive storage of yolk has, thus, apparently interfered with an early internalization of vegetal blastomeres, so that the vegetal parts of the egg remained outside the embryo for an increasingly longer time period during embryogenesis (Bellairs, 1986; and see below).

2.2. Establishment of bilateral symmetry

In addition to the an–veg axis, early vertebrate embryos establish a second axis that is usually referred to as anterior–posterior (A–P) in the yolk-rich amniotes, and dorsal-ventral (D–V) in amphibians, and that has shown to be determined by gravity in the chick, and by the point of sperm entry in frog (Kochav and Eyal-Giladi, 1971; Gerhart et al., 1989; reviewed in Eyal-Giladi, 1997). Formation of this second axis establishes the organizer region on one side of the blastula/blastoderm and thereby impose bilateral symmetry on the vertebrate embryo (Spemann and Mangold, 1924). It has recently been shown that the establishment of bilateral symmetry similarly correlates with a sliding against each other of superficial against deep cytoplasm, in frogs ('cortical rotation', Gerhart et al., 1989), as well as in birds (Callebaut, 1994). Moreover, the establishment of bilateral symmetry involves the activity of conserved molecules. In *Xenopus*, the homeobox gene *goosecoid* is expressed shortly before gastrulation in the 'dorsal' part of the marginal zone, where the Spemann organizer is located (Cho et al., 1991; Vodicka and Gerhart, 1995). Expression of the avian *goosecoid* gene also starts before gastrulation (st.XI E.-G. and K.) in a few cells in the middle layer in the medial portion of Koller's sickle (Izpisúa-Belmonte et al., 1993), a crescent-shaped thickening at the 'posterior' edge of the area pellucida. This small population of cells localizes and initiates primitive streak formation, as suggested by grafting experiments, and it is overlain as in amphibians (Sokol et al., 1991) by cells expressing a Wnt-like signal (Hume and Dodd, 1993), indicating a common involvement of Wnt-like signal transduction in axis initiation (see Cooke et al., 1994). It has been suggested that these *goosecoid*-expressing cells in Koller's sickle of birds equal the *goosecoid*-expressing cells in the amphibian Spemann organizer (Izpisúa-Belmonte et al., 1993; Eyal-Giladi, 1997), in a way that the medial portion of Koller's sickle in birds would equate only part of the Spemann organizer in the dorsal marginal zone of amphibians (Figs. 1 and 2). Cells located towards the animal side of the *goosecoid* territory also form part of the organizer. They express *chordin* (encoding another axis-inducing, secreted factor) in *Xenopus* (Sasai et al., 1994) and in the chick (Streit et al., 1998).

Cells located vegetal to the *goosecoid*-expressing cells also have similar inducing capacities in birds and in amphi-

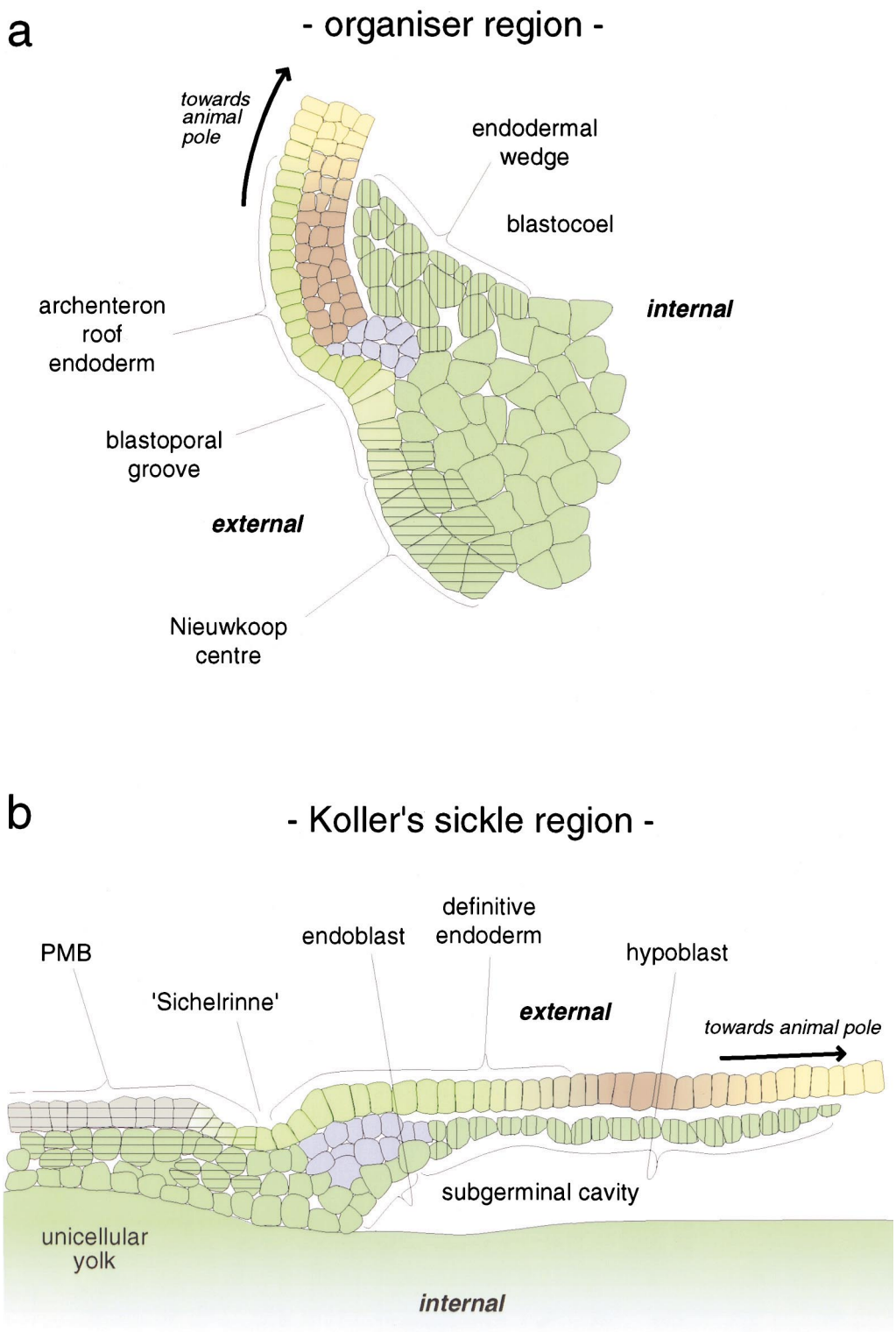

Fig. 2. Amphibian organizer region ((a), *Xenopus*, st.10, after Hausen and Riebesell, 1991; Nieuwkoop and Faber, 1967) and avian Koller's sickle region ((b), *Coturnix*, E.-G- and K.st.XI, after Callebaut et al., 1996b their Fig. 3), in schematic sagittal sections. Violet, prospective prechordal plate tissue; light-green, archenteron roof endoderm/definitive endoderm; dark-green, nutritive endoderm; brown, chordamesoderm; yellow, neuroectoderm; grey, extraembryonic ectoderm (and mesoderm). Horizontal stripes: cells with axis-inducing activity (Nieuwkoop center in amphibians, posterior marginal belt = PMB in birds). Vertical stripes: cells with anteriorizing capacities. Koller's sickle comprises the prospective prechordal plate (violet) and the adjacent endoblast cells (cf. Bachvarova et al., 1998; their Fig. 2a). 'Sichelrinne' is the equivalent of the amphibian blastoporal groove.

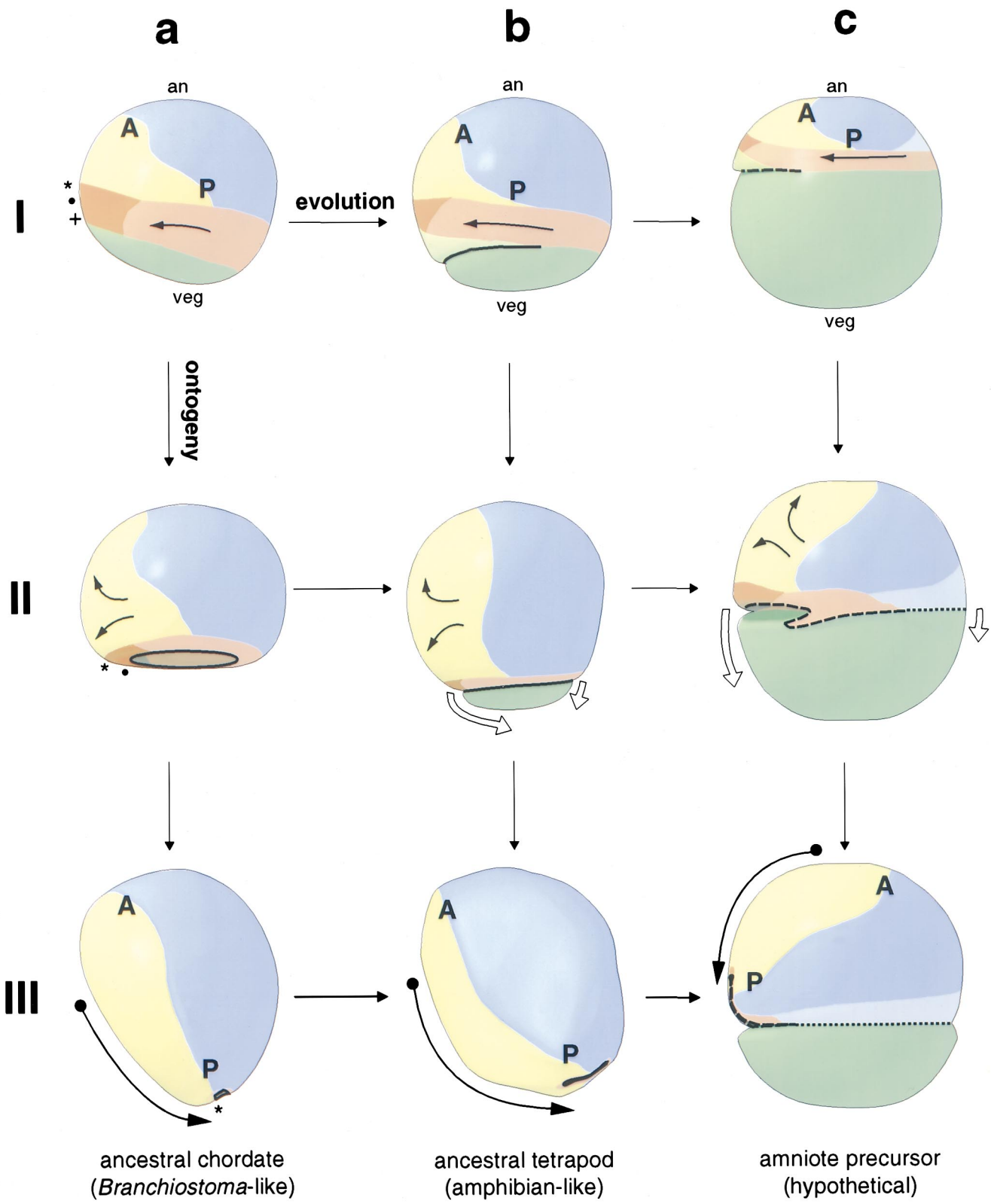
a
b
c
I
II
III
an
veg
A
P
evolution
ontogeny
ancestral chordate
(Branchiostoma-like)
ancestral tetrapod
(amphibian-like)
amniote precursor
(hypothetical)

Fig. 3(A–C)

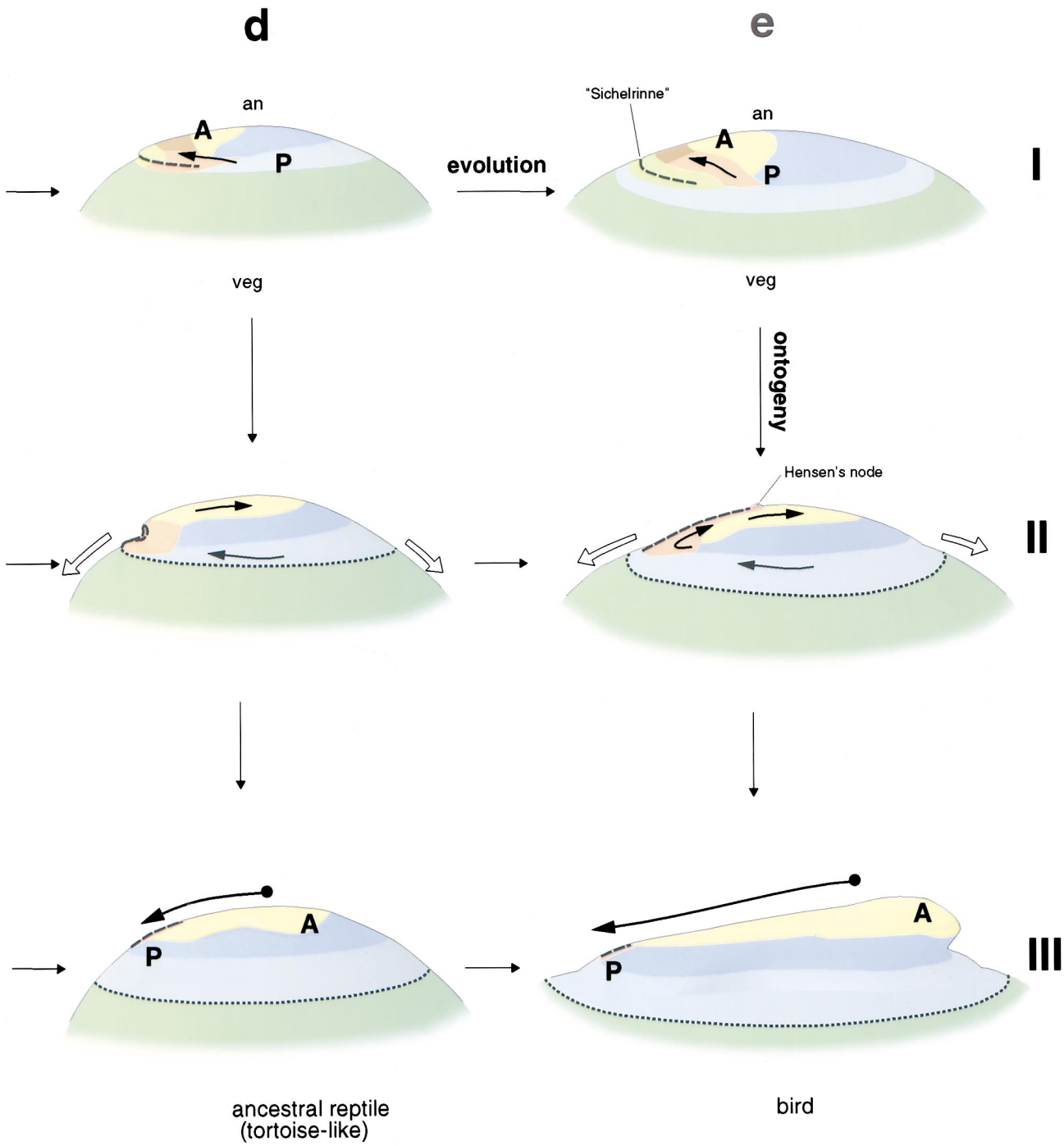

Fig. 3. Two-dimensional matrix for evolution and development of gastrulation. Ontogeny is represented from top to bottom, and its evolutionary transformation from left to right. The depicted ontogenies ((a) ancestral chordate, *Branchiostoma*, (b) ancestral tetrapod, *Bombina*, (d) ancestral reptile, *Chrysemis*, *Clemmys* (water-tortoises), (e) bird, *Gallus*, *Cotumix*) are meant to have existed on the evolutionary line leading from ancestral chordates to modern birds, but refer to extant animals chosen as typical for the respective groups. The ontogeny of an amniote precursor (c) is hypothetical. (I) Late blastula/blastoderm stages (II) stages halfway through gastrulation (III) early neurula stages. Thin arrows within embryos, convergence and extension movements; open arrows, epiboly; thin arrows outside of embryos starting with a dot, A–P proceeding of axis formation. Bold line, blastopore; bold dashed line, embryonic blastopore; bold dotted line, extraembryonic blastopore. The symbols (*,●,+) illustrate the sequential involution of the mesoderm in *Branchiostoma*. Colour code: yellow, neuroectoderm; blue, epidermal ectoderm; grey, extraembryonic ectoderm; red, mesoderm; brown, chordamesoderm; light-green, suprablastoporal/definitive endoderm; dark-green, nutritive endoderm. An–veg, animal–vegetal. Depicted after: aI, Conklin, 1932; Fig. 33; Tung et al., 1962; Fig. 13. aII: Conklin, 1932; Figs. 58–62; ibida, Figs. 69 and 73. bI–III: Vogt, 1929. dI: Nelsen 1953 Fig. 1 99a; Pasteels 1936a, Figs. 5 and 30a; Pasteels 1940, Fig. 9a. aII: Nelsen, 1953; Fig. 199b; Pasteels, 1936a, Fig 30b; Pasteels, 1940, Fig. 9b. dIII: Nelsen, 1953; Fig. 199c; Pasteels 1940, Fig. 9d. eI: Callebaut et al., 1996a, Fig 11b; Callebaut et al., 1996b, Fig. 3; Hatada and Stern, Fig. 2. eII: Schoenwolf, 1992; Garcia-Martinez et al., 1993; Fig. 11; see however Bortier and Vakaet, 1992; Fig. 2. eIII: Gilbert, 1994, Fig. 6.27.H. For further explanation see text.

bians. They form the Nieuwkoop center in the frog (Gerhart et al., 1989), and the 'posterior marginal belt' (PMB) in birds (Eyal-Giladi et al., 1994; Eyal-Giladi, 1997; Bachvarova et al., 1998) (Fig. 1; horizontal stripes in Fig. 2). These regions equally determine the position of the organizer and, after transplantation, can induce ectopic embryonic axes. Essentially, both frog Nieuwkoop center and avian PMB are vegetal egg regions that themselves do not contribute to the induced axial structures (Gimlich and Gerhart, 1984; Gimlich, 1986; Bachvarova et al., 1998; and see above). Their inducing activity can be mimicked by an exogenous source of activin (Asashima et al., 1990for frog; Mitrani and Shimoni, 1990; Cooke et al., 1994for chick). Beside activin, the *Vg1* protein is a good candidate molecule for the Nieuwkoop center activity in the frog (Thomsen and Melton, 1993), and correspondingly, the *cVg1* gene is expressed in the PMB in the chick, and its protein can initiate the formation of a morphologically complete primitive streak (Seleiro et al., 1996; Shah et al., 1997). This would suggest a correspondence of the avian PMB and the Nieuwkoop center of the amphibian blastula (Fig. 2; Callebaut and Van Nueten, 1994; Eyal-Giladi, 1997; Bachvarova et al., 1998).

Note that in chick, PMB grafts that include tissue internal to Koller's sickle are more potent to induce organizer and primitive streak than PMB grafts alone (Bachvarova et al., 1998). In keeping with this, the portion of Koller's sickle underlying the PMB in birds has been shown to possess strong axis inducing activity (Callebaut and Van Nueten, 1994; Callebaut and Van Nueten, 1995). However, PMB grafts including part of Koller's sickle have been shown to contribute to Hensen's node (and thus to the forming axis; Bachvarova et al., 1998) and therefore, probably form part of the organizer proper.

Why is Koller's sickle organizer said to lie 'posterior' in the chick, and the Spemann organizer to lie 'dorsal' in the frog, when both apparently represent homologous structures? Fig. 1 shows that prospective anterior and posterior, as well as dorsal and ventral body regions, locate to a similar area with respect to the an–veg axis in the animal hemisphere/area pellucida in amphibians and in birds (for frog: Vogt, 1929; Pasteels, 1940; Keller, 1975; Slack and Tannahill, 1992; for chick: Spratt, 1953; Hatada and Stern, 1994; Callebaut et al., 1996a). This holds true for the ectoderm, and as a mirror image, for the endomesoderm (where the dorsoventral orientation of tissues is reversed due to later involution/ingression). This arrangement of tissues seems to be phylogenetically ancestral for the vertebrates, since it also exists in teleost fish (Driever, 1995) and in the lower chordates (Conklin, 1905; Conklin, 1932; compare Arendt and Nübler-Jung, 1997). Notably, it is not before primitive streak formation that prospective 'anterior' and 'dorsal' tissues, located in the medial portion of Koller s sickle, are replaced by converging prospective 'posterior' tissues (Stern et al., 1992; and see below). Following the conventional usage, however, and for simplicity, the avian Koller's sickle region and the corresponding regions in reptiles and monotreme mammals will be referred to as 'posterior' also at pre-streak stages.

2.3. Archenteron roof endoderm, definitive endoderm and nutritive endoderm

Early bilateral symmetry is manifest in the blastula/blastoderm fate map where prospective axial tissues group around the organizer. The colour code in Fig. 1 relates the corresponding ectodermal, mesodermal and endodermal *anlagen* in the fate maps of frog and chick. However, while this relatedness is beyond doubt for most embryonic tissues – such as neuroectoderm, epidermal ectoderm, chordamesoderm – it is less obvious for the endoderm. Fig. 2 attempts to align endodermal tissues with special focus on the organizer region in amphibians and on Koller's sickle region in birds.

In the amphibian blastula, the superficial endodermal cells of the organizer region and of the vegetal hemisphere contribute to the lining of the archenteron after gastrulation (Vogt, 1929; Pasteels, 1940; Keller, 1975). Superficial marginal zone cells (light-green in Figs. 1 and 2a) give rise to the archenteron roof, and more vegetal superficial cells to the archenteron floor. There is a dramatic difference in the intensity of morphogenetic movements during gastrulation between the forming archenteron roof and floor, in that the roof shows strong convergence and extension integrated with rapid invagination, whereas the floor shows relatively little convergence and extension (Keller, 1975; and see below). In *Xenopus*, the later archenteron roof can be defined as the sheet of cells that overlies the (*goosecoid*-expressing) prospective prechordal plate cells (violet in Fig. 2a; Vodicka and Gerhart, 1995), and in molecular terms, as the sheet of cells that expresses the *Xenopus HNF-3β* gene (Ruiz i Altaba et al., 1993), *xNR3* (a *Xenopus* gene related to mouse nodal; Vodicka and Gerhart, 1995), and probably *XlHbox8* (Gamer and Wright, 1995). Differentiation of the archenteron roof requires the presence of mesoderm (Okada, 1957) and is, therefore, always closely associated with the mesoderm anlage, be it as a covering sheet (*Xenopus*; Fig. 2a; Keller, 1975), or lying in its immediate vegetal vicinity (e.g. *Bombina*; Figs 1, and 3b; Vogt, 1929). Prospective archenteron roof cells and mesodermal cells thus form a developmental unit, the *endomesoderm* (Jones et al., 1993).

Note that in the anuran amphibians *Bombina* (Vogt, 1929), *Discoglossus* (Pasteels, 1940) and *Xenopus* (Keller, 1975) the archenteron roof endoderm reaches farther laterally around the equator, while it is restricted to the organizer region in the urodelan amphibians *Triton* (Vogt, 1929) and *Axolotl* (Pasteels, 1940).

The archenteron roof endoderm in amphibians corresponds to the *definitive* endoderm in birds (light-green in Figs. 1b and 2b). As in amphibians, the anlage of the avian definitive endoderm exhibits strong morphogenetic movements during gastrulation, is closely associated with the mesoderm anlage and also expresses the *HNF-3β* gene (Ruiz i Altaba et al., 1995). In addition, the definitive endoderm anlage in the chick locates superficially along Koller's

sickle (Callebaut et al., 1996a), and overlies the deep (*goosecoid*-expressing) prospective prechordal plate cells in the middle layer of the sickle (Fig. 2b), just as is the case in the dorsal marginal zone of *Xenopus* (Fig. 2a). Note that while in birds the definitive endoderm is the sole source of gut epithelium, in amphibians the archenteron roof forms the dorsal gut lining only.

There is a second type of endoderm common to frog blastula and avian blastoderm. These are the more vegetal endodermal cells that are generally richer in yolk, so we refer to them as the *nutritive endoderm* (dark-green in Fig. 2; and see above). In frog, nutritive endoderm comprises superficial and deep cells of the vegetal hemisphere, including the floor of the blastocoel (Fig. 2a; Keller, 1975; and see below). In chick, the nutritive endoderm is equivalent to the extraembryonic endoderm and comprises the germ wall tissue of area opaca and marginal belt, the endoblast and the hypoblast, and the yolk (Fig. 2b; see Eyal-Giladi and Kochav, 1976; Bachvarova et al., 1998). Note that in amphibians as well as in birds, deep nutritive endodermal cells extending animally from the prospective prechordal plate cells (vertical stripes in Fig. 2) perform similar movements during gastrulation, and share anteriorizing capacities (as will be outlined below). These cells contribute to the lining of the blastocoel in amphibians, and to the hypoblast in birds. The vegetal-most yolk-rich regions of the amphibian blastula ('vegetal cap'; comprising large blastomeres heavily loaded with yolk; Uchiyama et al., 1994; Gamer and Wright, 1995) correspond to the avian uncleaved yolk mass and the peripheral germ wall of the area opaca (compare Hertwig, 1910p.134).

Difficulties arise when the avian yolk is considered as secondarily 'attached' to the egg during amniote evolution (see e.g. Pasteels, 1940; Waddington, 1952). It is then assumed that the cellular part of the avian endoderm anlage (including the hypoblast) corresponds to the amphibian endoderm as a whole. However, this latter concept neglects that the avian situation must have evolved *continuously* from an amphibian-like scenario, and it has lead to the bizarre assumption that 'to get a sauropsid from an amphibian egg, one has to remove the yolk from the endoderm anlage, to place it – considerably enlarged – in the middle of the epidermal ectoderm anlage' (Pasteels, 1936a; translation by the authors); or that the original blastula should be imagined as 'flattened out on the surface of a mass of yolk' (Waddington, 1952).

3. Evolution of a primitive streak in yolk-rich amniotes

In an attempt to understand the series of events during the morphological transformation of an amphibian-like, circular blastopore into the longitudinal primitive streak in birds and mammals, the gastrulat ion pattern in reptiles is first deduced from the ancestral tetrapod situation. Ancestral reptiles were the evolutionary predecessors of birds and mammals, and since the gastrulation mode in reptiles is very similar in all species investigated, it can be taken as ancestral for the amniotes. It is then explained how a reptile-like pattern was subsequently modified to give rise to the primitive streak in the avian and mammalian lines of evolution. The evolution of amniote gastrulation is exemplified for the *Sauropsida* in a two-dimensional matrix for evolution and development (Fig. 3). It is assumed that the selective pressure for the evolution of early amniote gastrulation was the *ever-increasing storage of yolk*. A pivotal role for the yolk content in the evolution of amniote development has also been recently suggested with regard to the process of axis determination at pre-gastrula stages (Eyal-Giladi, 1997).

Despite all morphological dissimilarities, morphogenetic movements during gastrulation appear to be rather similar in amniotes as compared with the amphibians (cf. Gilbert, 1994). In brief, adding to the internalization of endoderm and mesoderm, the ectoderm expands over the vegetal yolk in both groups (*epiboly*). Endomesodermal and ectodermal cells from lateral regions of the blastula/blastoderm move towards the organizer region (*convergence*). The medial accumulation of cells is compensated for by a lengthening of the dorsoaxial tissues (*extension*).

The equivalence of morphogenetic movements during vertebrate gastrulation has long been recognized and is beyond dispute (see e.g. Pasteels, 1936b p. 475). Attempts to compare the divergent morphologies of the amphibian and the diverse amniote gastrulae, i.e. the *forms of gastrulation* have, however, remained controversial. While classical authors in the tradition of Haeckel's 'gastraea theory' (Haeckel, 1875) tended to homologize, for example, amphibian blastopore and amniote primitive streak (Hertwig, 1910 and references therein), their successors questioned these comparisons. They found nothing constant in vertebrate gastrulation except for the morphogenetic movements, the divergent chronology of which should bring about the rather divergent forms of gastrulation de novo, thus, apparently denying a continuity between forms (Pasteels, 1940 p.93; Ballard, 1981). We confirm the classical belief that there is indeed a continuity in the evolutionary modifications that have transformed an ancestral amphibian-like gastrula into the extant amniote forms.

3.1. Ground pattern of vertebrate gastrulation

Starting point for an evolutionary derivation of amniote gastrulation modes is the chordates' ancestral mode of *invagination* (as occurs, e.g. in *Branchiostoma*; Conklin, 1932) where the entire vegetal hemisphere bulges inwards such that the blastopore forms just one large opening (Fig. 3a). Already here is an amphibian-like 'involution' (see below) of the prospective mesodermal cells that turn inwards sequentially from the more 'vegetal' to the more 'animal', in a way that the actual blastopore lips comprise a changing population of cells (Fig. 3a).

Gastrulation in amphibians involves a modified form of invagination, where the vegetal blastomeres with their larger amounts of yolk do not bulge inwards. Instead, the subequatorial endomesodermal tissues turn inwards alongside the vegetal blastomeres (*involution*; see e.g. Gilbert, 1994), and the vegetal yolky blastomeres become internalized by

ectodermal epiboly. The blastopore now forms a circular cleft. According to early studies, the gastrulation of more ancestral bony fishes (*Acipenser*: Dean, 1895; *Amia*: Dean, 1896; Sobotta, 1896; Nelsen, 1953 *Lepidosiren*: Kerr, 1901), and of agnathan vertebrates (*Petromyzon*: Glaesner, 1910; *Lampetra*: Weissenberg, 1933; Pasteels, 1940) largely resemble amphibian gastrulation. On these grounds, we take a generalized amphibian pattern as ancestral for the tetrapods (Fig. 3b).

In amphibians (as in *Branchiostoma*), the cells start to internalize on the anterior/dorsal (A/D) side. The amphibian blastopore first appears as a sickle-shaped furrow (Fig. 3bI) to then elongate laterally. Halfway through gastrulation both ends of the furrow meet at the posterior/ventral (P/V) side, so that the blastoporal furrow completely encircles the vegetal blastomeres (Fig. 3bII). Amphibian gastrulation is highly asymmetric, since dorsal convergence and extension movements make involution and epiboly more pronounced on the A/D side of the gastrulating embryo. As a consequence, the A/D blastopore lip moves around almost the entire vegetal hemisphere during blastopore closure, while the P/V lip moves vegetally for only a very short distance (open arrows in Fig. 3bII; for detail see, Arendt and Nübler-Jung, 1997). Towards the end of gastrulation, the former A/D blastopore lip reaches the P/V lip on the opposite, now posterior side of the embryo (Fig. 3bIII). Being initially located at the A/D lip, the amphibian organizer also moves around the entire vegetal hemisphere, leaving in its wake the dorsally converging and extending mesoderm and neuroectoderm (Arendt and Nübler-Jung, 1997). Cells that sequentially emerge from the organizer region thereby become more and more posterior in character (Stern et al., 1992), until finally, the organizer forms part of the tailbud (Gont et al., 1993; Knezevic et al., 1995). The dorsal axis is thus, laid down sequentially *from anterior to posterior and around the vegetal hemisphere* during blastopore closure (Fig. 3b).

3.2. Separation of intraembryonic and extraembryonic blastopore in early amniotes

What if the vegetal yolk mass continues to increase as it did during amniote evolution? The comparison of ontogenetic sequences illustrates the resulting morphological modifications of gastrulation. While in the tetrapod ancestor (Fig. 3b) the forming axis completely encloses the vegetal yolk, in a hypothetical amniote precursor (Fig. 3c) the expanding yolk impedes the forming axis from engulfing the entire vegetal hemisphere. The A/D lip will thus no longer meet the P/V lip on the opposite side of the vegetal hemisphere. Instead, the A/D blastopore lip with the organizer moves over a more restricted equatorial section of the vegetal yolk mass only. *Axis formation begins and ends on the same the dorsal meridian of the egg*. As a consequence, the nutritive endoderm will temporarily remain outside of the embryo proper (*extraembryonic endoderm*), to only later be absorbed by the developing fetus. This in turn implies that the future gut epithelium will emerge in its entirety from the definitive endoderm (the former archenteron roof endoderm, see above).

Provided that the A/D lip with the organizer no longer moves around the vegetal hemisphere to meet the P/V lip, the P/V material will instead have to move towards the organizer to participate in embryo formation. Thus, while ancestrally the future mesoderm accompanies the blastopore along its entire circumference (bold line in Fig. 3bII), the blastopore now comprises two distinct portions, one in contact with the later mesoderm (dashed line in >Fig. 3cII) and another where the ectoderm directly faces the yolk (stippled line in Fig. 3cII). These two portions of the blastopore now become, respectively, the site of mesodermal internalization and the site of ectodermal epiboly. We propose that this functional subdivision of the blastopore foreshadows its physical subdivision into two structures with divergent functions, and that these structures be called ‘intraembryonic blastopore’ and ‘extraembryonic blastopore’.

Fig. 4 gives a possible evolutionary sequence of blastopore morphologies in the presence of an ever-increasing mass of yolk. In the tetrapod ground pattern, a blastoporal cleft forms inbetween the mesoderm and the vegetal yolky blastomeres, with the blastopore lips directly apposing the (cellularized) yolk (bold line in Fig. 4a). In the presumed amniote precursor, the converging P/V mesodermal cells from both sides form wing-shaped masses (‘mesodermal wings’, stars in Fig. 4b) that, in a subsequent step, fuse along the midline to physically divide the blastopore into two (Fig. 4c). This convergence movement of the mesodermal wings also translocates the extraembryonic ectoderm towards a medial position so that it finally forms a ring around the embryonic tissues. The single, amphibian-type blastopore is thus morphologically and functionally subdivided into an *intraembryonic blastopore* for the internalization of the mesoderm (dashed line in Fig. 4c), and an *extraembryonic blastopore* for ectodermal epiboly (stippled line in Fig. 4c). This outcome perfectly matches the actual reptile situation (see below).

The coalescence of mesodermal material ‘below’, i.e. vegetal to the A/D blastopore lip, is a characteristic feature of gastrulation in yolk-rich amniote eggs. Conceptually, this fusion of two initially separated cell sheets correlates with an evolutionary ‘switch’ from involution of the mesoderm (as in amphibians) to invagination (predominant in reptiles) and ingression (in reptiles, birds and mammals). Where the lips of the embryonic blastopore no longer enclose the vegetal yolk, an invagination cavity (such as the blastoporal canal in the reptiles) forms inbetween the inward-turning mesodermal layers (Fig. 4c; and see below). Where on the other hand, the right and left blastopore lips fuse along the midline (the suture of the ‘mesodermal wings’), the right and left inward-turning mesodermal layers lose their integrity at the line of fusion to the effect that single cells migrate

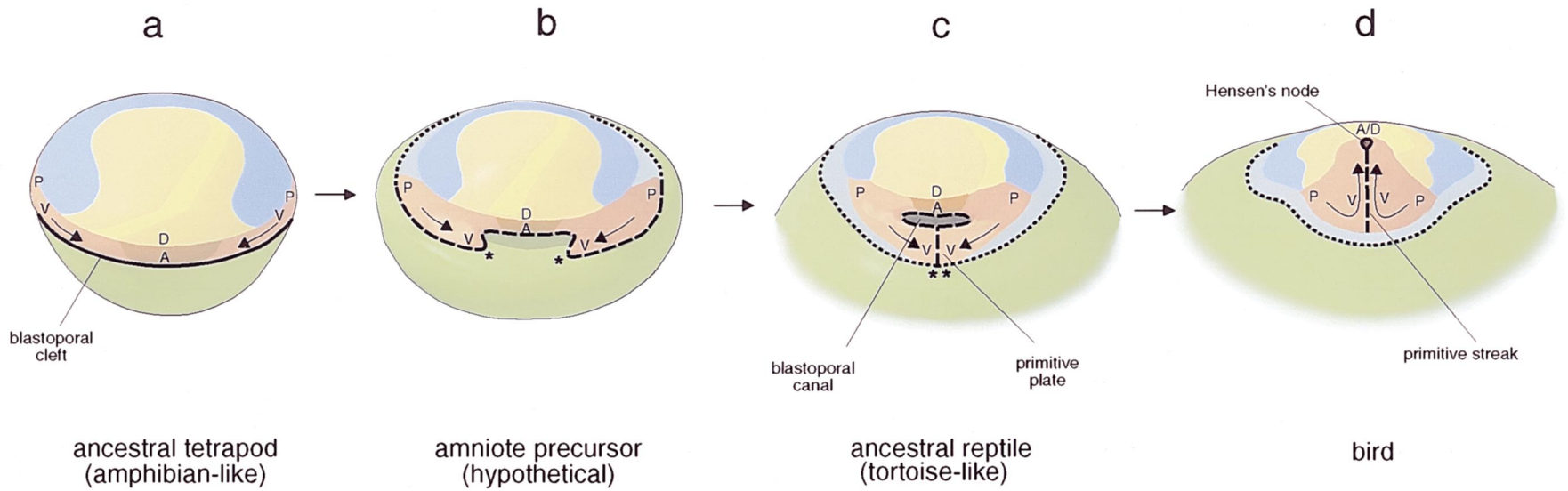

Fig. 4. Evolution of a separate embryonic and extraembryonic blastopore. The depicted sequence is a more detailed dorsal view of the evolutionary transition from an early frog gastrula (Fig. 3bII) to the corresponding stage in birds - Fig. 3eII. A, D, P, V: anterior, dorsal, posterior, ventral mesoderm. (a) Ancestral tetrapod. The blastopore (bold line) forms a groove between the involuting mesoderm and the vegetal yolky blastomeres. (b) Amniote precursor (hypothetical). Arrows: convergence of the mesoderm towards the organizer region in 'wings' (next to *) extending vegetally. (c) Ancestral reptile (e.g. *Clemmys*; Pasteels, 1936a). The converging wings of mesoderm fuse 'below' the A/D lip to form the primitive plate, thus effecting a physical separation of embryonic blastopore (dashed line) and extraembryonic blastopore (dotted line). The A/D and P/V blastopore lips no longer enclose the vegetal yolk and an invagination cavity forms in between them. (d) *Aves* (e.g. *Passer*; Hertwig, 1910). Arrows: 'polonaise' movements of mesodermal cells.

inwards, through the primitive plate in the reptiles (Fig. 4c), and along the primitive streak in birds and mammals (Fig. 4d).

With the physical separation of intraembryonic and extraembryonic blastopore *the site of endomesodermal invagination/ingression has become distinct from the advancing front of epiboly* (situated within, and at the outer margin of the blastoderm, respectively). This evolutionary derivation of an amniote 'double blastopore', as outlined here, sheds new light upon an old, and as yet unsettled question of comparative vertebrate embryology – how the spatial separation of mesodermal invagination and ectodermal epiboly might have evolved in the amniotes (see e.g Hertwig, 1910; Pasteels, 1940; Waddington, 1952). This separation was believed to require a 'saut brusque dans l'évolution' ('sudden evolutionary leap'; Pasteels, 1940). The sequence of evolutionary changes outlined above, however, is free of such a discontinuity and thus represents a possible solution for this old evolutionary enigma.

3.3. Intraembryonic blastopore: blastoporal canal, primitive plate and primitive streak

In line with the above scenario, in the reptile early gastrula the prospective mesoderm is located in and around the organizer region (Fig. 3dI). The mesoderm covers only about half of the circumference of the area pellucida, as contrasted to the ring-shaped mesoderm anlage in the amphibian-type blastula (compare Fig. 3bI; and see below; note that in the yolk-rich eggs of the shark *Scyliorhinus caniculus*, the mesoderm anlage is likewise restricted to the side where axis formation takes place, Vanderbroek, 1936). The internalization of cells starts with the formation of a marginal furrow extending laterally (Fig. 3dI), as described for ring-snake (Ballowitz, 1901), water-tortoise (Pasteels, 1936a; p.122), and lizard (Peter, 1938a). Following mesodermal convergence, the lateral ends of this furrow withdraw towards the midline, and the furrow forms the round opening of the so-called *blastoporal canal* (or 'chordamesodermal canal', 'notochordal canal'; Fig. 3dII) as described for gecko (Will, 1893), ring-snake (Ballowitz, 1901), water-tortoise (Pasteels, 1936a), and lizard (Peter, 1938a) (see also Hertwig, 1910p. 208 ff. and Nelsen, 1953p. 417 ff). The blastoporal canal corresponds to the invagination cavity of the intraembryonic blastopore as outlined in Fig. 4c. In the water-tortoise, this blastoporal canal is lined by invaginating mesodermal material, with cells from its 'upper' lip contributing to notochord, while cells from its 'lower' lip form somites and lateral plate (Pasteels, 1936a). Convergence and extension take place in the roof of the blastoporal canal and in the overlying neuroectoderm. Posterior to the opening of the blastoporal canal forms the thickened *primitive plate* (Will, 1893), composed of converging P/V mesodermal cells (e.g. in the water-tortoise; Pasteels, 1936a; Fig. 3dII). The primitive plate is continuous with the floor of the blastoporal canal, and is overgrown by the canal's 'upper lip' in the course of gastrulation (Fig. 3dIII). The more 'posterior'and 'ventral' mesodermal cells that do not participate in the invagination process, ingress through the primitive plate (Nelsen, 1953p. 419; and see above). Conceptually, the midline of the primitive plate demarcates the suture of the former 'mesodermal wings' (cf. Fig. 4c). As is also predicted by the evolutionary scenario seen in Fig. 4 the prospective extraembryonic ectoderm in the reptile blastoderm comes to surround the embryonic tissues (Fig. 3dI–III). At the posterior edge of the primitive plate, the epibolizing ectoderm associates with ingressing mesodermal cells that form the *extraembyronic mesoderm*. This is easily understood if one takes into account that these mesodermal cells lie, physically, closest to the extraembryonic ectoderm and thus are 'near at hand' to contribute to the extraembryonic material.

This gastrulation mode in reptiles represents an amniote ground pattern from which the situation in other yolk-rich amniotes can be easily derived. In the evolutionary lines leading to birds and lower mammals, the blastoporal canal diminishes in size to finally become a minute opening in Hensen's node. The primitive plate, on the other hand, is drawn out in length to form the primitive streak. Remarkably, this tendency is somewhat anticipated in the chameleon which, deviating from other reptiles, has only a rudimentary blastoporal canal, and the mesoderm essentially ingresses through the primitive plate (which is not, however, extended into a streak; Peter, 1935). In monotreme mammals a small blastoporal aperture behind a slight prominence (Hensen's node) opens into a flattened, cleft-like blastoporal canal (Wilson and Hill, 1907, 1915; Assheton, 1910). This aperture demarcates the anterior extremity of a primitive streak of considerable length (Wilson and Hill, 1907).

Avian gastrulation (Fig. 3e) is a similar modification of the reptile pattern. At the onset it resembles the reptile situation in that a marginal sulcus appears at the edge of the area pellucida along Koller's sickle, the so-called 'Sichelrinne' of Koller (1882) (Figs. 1b, 2b and 3eI). This sulcus subsequently converges into a furrow along the midline of the primitive streak, the site of mesodermal ingression. As described for various birds, the anterior end of this furrow terminates in a small invagination in Hensen's node that opens into a minute canal extending anteriorly. This canal is considered a remnant of the blastoporal canal in reptiles (Hertwig, 1910; p. 221 ff.). Notably, this narrow canal in the birds' node, as well as the reptile blastoporal canal, both end up as the neurenteric canal at late gastrula stages (Hertwig, 1910; p. 221 ff.; Pasteels, 1936a p. 148 ff).

The formation of a primitive streak in lieu of the (reptile) primitive plate can best be explained in terms of heterochrony, i.e. as a change in the chronology of developmental events (see Raff and Wray, 1989): while in reptiles, the mesoderm first invaginates to then converge and extend *internally* (inside of the blastoporal canal), in birds the mesoderm becomes internalized only after considerable

convergence and extension have taken place *externally* (Pasteels, 1940; p. 92; Bellairs, 1986; Stern, 1990; Stern, 1991; Eyal-Giladi et al., 1992). In other words mesodermal cells that converge towards Koller's sickle (Fig. 3eI) do not immediately internalize, but instead appear to make way by moving towards the center of the area pellucida such that the mesoderm anlage takes the shape of a streak ('polonaise movements'; arrows in Figs. 3eII and 4d; Gräper, 1929; Wetzel, 1931).

3.4. Reversal of A–P polarity in the area pellucida with respect to the yolk

During amphibian gastrulation the axis forms sequentially from anterior to posterior (Fig. 3bIII, and see above), and the same is true for the second phase of chick gastrulation when Hensen's node regresses from near the center of the area pellucida towards the posterior until it reaches the anal region (Fig. 3eIII; Selleck and Stern, 1991; Schoenwolf et al., 1992; Stern et al., 1992; Sausedo and Schoenwolf, 1993). Just as in amphibians, the prospective neural plate and the ingressing mesoderm undergo convergence and extension, in front of and lateral to the regressing node/organizer (Spratt, 1952; Bortier and Vakaet, 1992). In amphibians and birds, the node/organizer thus lays down dorsal axial structures of more and more posterior character and produces an anterior-to-posterior (A–P) gradient of developmental maturity. The same is true for reptiles, where the upper lip of the embryonic blastopore also moves posteriorly during gastrulation (Nelsen, 1953; p. 420).

In preparation of this movement from anterior to posterior, the 'polonaise movement' brings about a change in the relative position of prospective anterior and posterior tissues within the area pellucida in birds (Fig. 3eII; Gräper, 1929; reviewed in Stern, 1990; Eyal-Giladi et al., 1992) and, less pronounced, in reptiles (Fig. 3dII; Pasteels, 1940, p. 81 ff.). The presumptive forebrain (i.e. later 'anterior'), the head of the 'polonaise', gradually moves more to the center of the area pellucida, while the left and right tail primordia (i.e. later 'posterior') leave their lateral/marginal positions in order to converge and fuse medially, at the posterior margin of the area pellucida. With respect to the yolk, the developing embryo thus almost reverses its A–P polarity (compare panels in Fig. 3eI with 3eIII). These rearrangements are nicely traced by molecular markers. In the chick, the *goosecoid*-expressing cells initially locate to Koller's sickle and later appear displaced towards the center of the area pellucida where they give rise to Hensen's node (Izpisúa-Belmonte et al., 1993). At the same time, the formerly peripheral cells move towards the posterior end of the streak. Accordingly, expression of the chick *caudal* protein (CdxA) is initially found along most of the border of the area pellucida and in the PMB, to later extend into the posterior portion of the primitive streak (Frumkin et al., 1993).

This rearrangement of the prospective A–P body axis in the chick area pellucida could explain why the apparent 'posterior-to-anterior' gradient of developmental maturity (at stages preceding primitive streak formation; Eyal-Giladi and Kochav, 1976) later changes into an 'anterior-to-posterior' gradient (see above) For example, the transition from a multilayered into a single-layered epithelium in the area pellucida is said to proceed from 'posterior-to-anterior', while axis formation later occurs in an 'anterior-to-posterior' gradient (Eyal-Giladi and Kochav, 1976) These apparently antidirectional gradients might thus be manifestations of one and the same gradient of developmental maturity that reverses its orientation with respect to the yolk, due to the 'polonaise' cell rearrangements that occurs during primitive streak formation.

3.5. Extraembryonic blastopore: the site of epiboly

The extraembryonic blastopore of yolk-rich amniotes is of rather uniform appearance. As described for the lizard *Lacerta agilis*, a marginal cytoplasmic zone surrounds the reptile blastoderm that, by structure and position, appears to correspond to the syncytial 'germ ring' in the monotreme egg (see Flynn and Hill, 1947) The latter is said to play an active role in the epiboly of the blastoderm (see Flynn and Hill, 1947). A similar syncytial zone of peripheral cytoplasm exists in birds ('marginal periblast', Blount, 1907; 'subgerminal ooplasm below the germ wall', Callebaut et al., 1996b) that contains tubulin immunoreactive threads. A second ring of dense tubulin immunoreactivity encircles the margin of the avian blastoderm at a larger distance ('paragerminal ooplasm'; Callebaut et al., 1996b). Notably, the situation in birds strikingly resembles the tubulin distribution in the zebrafish egg at the onset of epiboly (Solnica-Krezel and Driever, 1994; Callebaut et al., 1996a): The avian subgerminal ooplasm appears to correspond to the zebrafish external syncytial layer, and the avian paragerminal ooplasm to a circular cytoplasmic region from where microtubules radiate in the zebrafish egg. The zebrafish yolk syncytial layer has been shown to provide the major force in the vegetal spreading of the blastoderm in a number of teleost fishes (Trinkaus, 1951; see Solnica-Krezel and Driever, 1994, and references therein). In zebrafish embryos treated with a microtubule depolymerizing agent, microtubules are absent and epiboly of the yolk syncytial layer is blocked (Solnica-Krezel and Driever, 1994). Yolk-rich amniotes and teleost fishes, thus seem to share a syncytial cytoplasmic 'epiboly motor region' surrounding the blastoderm, that produces the driving force for the epiboly of the blastoderm. So, although the syncytial character of this region evolved independently in amniotes and teleost fishes – less derived bony fishes and amphibians lack an equatorial syncytium – the microtubular apparatus driving epiboly is considered a conserved feature of the vertebrates (Callebaut et al., 1996b). Interestingly, in *Xenopus*, an 'epiboly motor region' located vegetal of the blastopore lips has also been hypothesized (Keller, 1980). In addition to this marginal cytoplasm, the peripheral area opaca cells of

the avian blastoderm also play an active role in epiboly. These are considered a highly specialized population of actively migrating cells that pull along the blastoderm (see e.g. Gilbert, 1994). In this respect the avian area opaca resembles the cellular marginal rim of the zebrafish blastoderm that is also actively involved in the process of epiboly (see Solnica-Krezel and Driever, 1994).

It thus seems plausible that the extraembryonic blastopore at the blastoderrn margin of the yolk-rich amniote egg has inherited its epiboly machinery from the blastopore of the lower vertebrates. Owing to the physical separation of epiboly from the site of axis formation (see above), the proceeding of epiboly can be considerably accelerated. In the chameleon, for example, epiboly is almost complete before mesodermal ingression has even started (Peter, 1934). Equally, in the monotreme *Echidna*, epiboly is completed before the onset of primitive streak formation (Flynn and Hill, 1947). In the monotreme mammals, the acceleration of epiboly is of great developmental significance, as thereby the egg becomes converted very early into a blastodermic vesicle, or blastocyst, that is capable of absorbing nutritive fluid secreted by the maternal uterine glands (Flynn and Hill, 1947).

4. Evolution of the hypoblast

Besides ingression and epiboly there is a third kind of morphogenetic movement involved in amniote gastrulation, the formation of the hypoblast. In the developing yolk-rich amniote egg, hypoblast cells come to lie underneath the blastoderm, which thus becomes bi-layered with an upper epiblast and a lower hypoblast. Hypoblast formation in the reptiles has been a matter of debate: while Pasteels (e.g., 1936a), based on observation of the water-tortoise, claimed that the hypoblast of all reptiles should form by invagination from the posterior margin of the blastoderm, Peter (1934); Peter (1938b) and others maintained that the hypoblast should form by delamination from the blastoderm, at least in lizards, chameleon and snakes. While hypoblast formation by delamination in lizards has later been consented by Pasteels (1957), the notion of invagination has remained questionable (Nelsen, 1953; see however Pasteels, 1940; Pasteels, 1957). It has been agreed upon, however, that at least in the water-tortoise and ring-snake there is an intimate connection of hypoblast and overlying primitive plate (see Peter, 1938a). In monotreme mammals there appears to be an inward migration of scattered cells throughout the blastoderm that join secondarily to form a continuous hypoblast layer, reminiscent of reptile delamination (Wilson and Hill, 1907; Flynn and Hill, 1947). In birds, hypoblast formation has been described for the chick (see Eyal-Giladi and Kochav, 1976; Weinberger and Brick, 1982a; Weinberger and Brick, 1982b; Stern, 1990; Eyal-Giladi, 1991; Khaner, 1992; Eyal-Giladi et al., 1992; and references therein). The avian hypoblast develops from two sources: First, small groups of cells delaminate from the epiblast into the subgerminal cavity. Second, cells from the posterior margin of the area pellucida advance as a coherent sheet along the inner surface of the epiblast towards the center of the area pellucida. There are no morphological criteria that allow to distinguish between these two contributions, which very rapidly form a coherent lower layer (see Eyal-Giladi, 1991).

There are conflicting views concerning the posterior origin of hypoblast cells. Stern (1990) describes the hypoblast to form from the ‘deep (endodermal) region of the posterior marginal zone’. Deviating from this, Eyal-Giladi et al. (1992) view the hypoblast to form from superficial PMB cells, that ‘move into the hypoblast via Koller’s sickle’.

There is also some confusion in terminology (cf. Eyal-Giladi, 1991). Some authors refer to the delaminating isolated islands of hypoblast cells as ‘primary hypoblast’, and to the posterior sheet of cells as ‘secondary hypoblast’ (see e.g. Stern, 1990). Other refer to both populations together as ‘primary hypoblast’, and to the later forming endoblast portion of the lower layer as ‘secondary hypoblast’ (see e.g. Callebaut and Van Nueten, 1994). During primitive streak stages the endoblast (Fig. 2b) gives rise to a ‘new’ lower layer that displaces the ‘original’ hypoblast towards the anterior germinal crescent (Bachvarova et al., 1998). To avoid misunderstandings, hypoblast classification as ‘primary’ and ‘secondary’ should be abolished, and lower layer terminology be restricted to hypoblast (lower layer forming at pre-streak stages) and *endoblast* (lower layer forming at streak stages; cf. Bachvarova et al., 1998).

The avian situation with two distinct contributions to the hypoblast would suggest that the reptile hypoblast might likewise form from two sources, namely from scattered cells detaching from the blastoderm surface, and en bloc from the posterior primitive plate. However, it is an as yet unresolved question how the formation of the hypoblast in birds and reptiles can be compared at all to morphogenetic movements that occur during amphibian gastrulation.

4.1. Hypoblast as a modified amphibian ‘endodermal wedge’

The delamination from the epiblast of isolated islands of hypoblast cells finds no equivalent in amphibians and probably represents a derived feature. On the other hand, in amphibians the deep endoderm of the organizer region – topographically corresponding to the deep endoderm in Koller’s sickle, see Fig. 2 – undergoes changes in shape that are highly reminiscent of hypoblast formation as a coherent sheet of cells moving towards the animal pole. Hertwig (1910) has described, for a number of amphibian embryos, the formation of a ‘wedge’-shaped mass of deep endodermal cells that originate from the periphery of the blastocoel floor, and slide upwards along the inner surface of the blastocoel roof towards the animal pole (Fig. 5a; for *Rana fusca* compare Hertwig, 1910, his Fig. 115; for the agnathan *Petromyzon* see Glaesner, 1910, his Fig. P), in a manner comparable to hypoblast formation in reptiles (Fig. 5b) and in birds (Fig. 5c). This has also been observed in *Xenopus* by Keller (1975), and has recently been described as an involution of deep endodermal blastomeres ‘around an internal blastopore’ (Vodicka and Gerhart, 1995; see also Bauer et al., 1994 their Fig. 6E). The amphibian endodermal

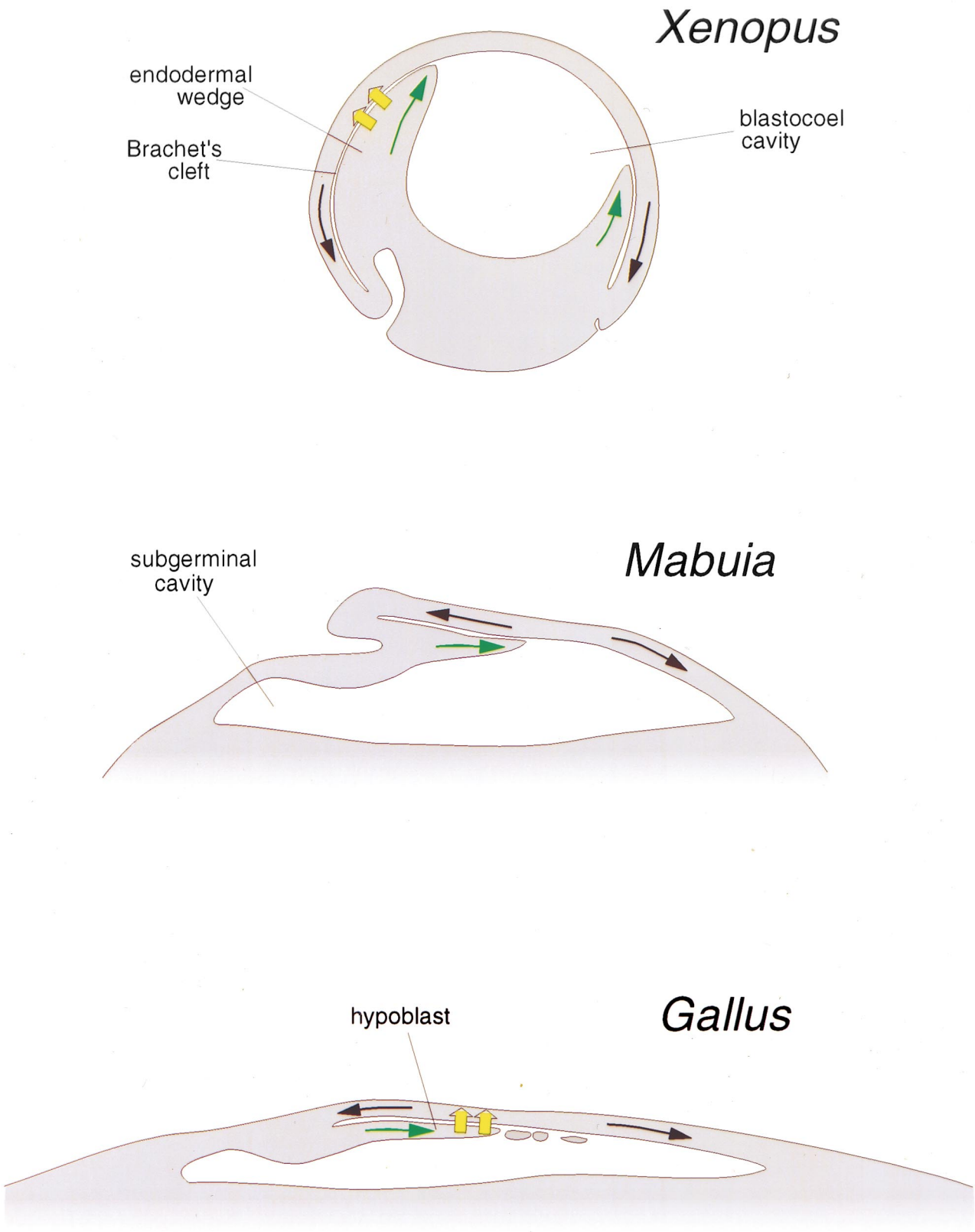

Fig. 5. Evolution of the amniote hypoblast. Comparison of the morphogenetic movements and of neural induction by deep endodermal cells during gastrulation in a prototype amphibian ((a) *Xenopus*), in a prototype reptile ((b) *Mabuia*; Pasteels, 1957) and in a bird ((c) *Gallus*). Black arrows: epiboly of the ectoderm. Green arrows, hypoboly of 'endodermal wedge'/ hypoblast; bold yellow arrows, anteriorizing activity.

wedge is separated from the overlying neuroectoderm by Brachet's cleft (Bouwmeester et al., 1996 and references therein). The endodermal wedge is most prominent dorsally, but also forms laterally and ventrally to finally form an 'endodermal bowl', or cylinder (Keller, 1975), surrounding most of the blastocoel (Hertwig, 1910).

Adding to the morphological similarities, recent molecular data also suggest a common role for the amphibian endodermal wedge and the amniote hypoblast in patterning the overlying anterior neuroectoderm (Fig. 5). The hypoblast triggers neuroectodermal differentiation in the overlying epiblast, as indicated by chick/quail transplantation experiments (Callebaut and Van Nueten, 1995). In *Xenopus*, the endodermal wedge expresses *cerberus*, a secreted factor that induces anterior neuroectodermal structures in the overlying cells (Bouwmeester et al., 1996). Another *Xenopus* gene expressed in the endodermal wedge at the beginning of gastrulation is *XANF-1* (Zaraisky et al., 1995; their Fig. 1b). *XANF-1* expression disappears from the wedge endoderm during gastrulation, but reappears in the overlying anteriormost neural anlagen, such as Rathke's pouch. In mice, genes related to *XANF-1* (*Hesx1*; Thomas and Beddington, 1996; Hermesz et al., 1996) and to *cerberus* (*cer-1*; Belo et al., 1997) have a remarkably similar expression pattern in the anterior primitive endoderm (which is the anterior hypoblast, see e.g. Bellairs, 1986; Gilbert, 1994). Moreover, removal of the anterior primitive endoderm in mouse disturbs molecular patterning in the rostralmost neuroectoderm, thus suggesting that the primitive endoderm/hypoblast is responsible for patterning the future prosencephalic neuroectoderm (Thomas and Beddington, 1996). On these grounds, it now appears well substantiated that the amphibian endodermal wedge and the amniote hypoblast are homologous structures, as previously suggested by Bonnet (1907), Hertwig (1910), Fahrenholz (1923) and Peter (1938b). Note that in this scenario the amphibian blastocoel (the cavity inside the endodermal bowl) corresponds to the subgerminal cavity in the yolk-rich amniotes, and that Brachet's cleft corresponds to the narrow cleft between the epiblast and the hypoblast (cf. Peter, 1938b). This stands in contrast to the idea that the amniote hypoblast is homologous to the entire vegetal hemisphere of the amphibian blastula, and that the amphibian blastocoel as a whole corresponds to only the narrow slit between epiblast and hypoblast (Pasteels, 1940; Khaner, 1992; Eyal-Giladi, 1997; and see above).

4.2. Hypoboly, a special kind of gastrulation movement

The avian hypoblast and the amphibian endodermal wedge both form similarly early during gastrulation, i.e. before the onset of ingression in birds (Eyal-Giladi and Kochav, 1976), and before the onset of involution in amphibians (Bouwmeester et al., 1996). This makes it likely that wedge formation in amphibians is an active movement of the deep endodermal cells, as is hypoblast formation in the *Sauropsida*. It is only after the onset of gastrulation that involution of the endomesoderm adds to the formation of the endodermal wedge (see Bauer et al., 1994). Formation of the endodermal wedge/hypoblast, thus appears to represent a special kind of gastrulation movements for which we propose the term 'hypoboly'. We define *hypoboly* as *the active spreading of the deep endodermal cells along the inner surface of the blastocoel roof towards the animal pole*. Hypoboly is thus a movement opposite to epiboly, which in turn is defined as the active spreading of the superficial ectodermal cells along the outer surface of the yolk towards the vegetal pole (cf. Gilbert, 1994). As depicted in Fig. 5, however, it is obvious that hypoboly and epiboly are interconnected movements. Both have in common, the sliding against each other of endodermal and ectodermal cell layers, thus transforming the an–veg sequence of germ layers in the blastula wall (ectoderm, animal; endoderm, vegetal) into the bi-layered arrangement in the gastrula (ectoderm, external; endoderm, internal). This is achieved *independently* of the turning inwards of endomesodermal cells around a blastopore/primitive streak (as is characteristic for involution/ingression).

There seems to be an inverse relationship in the relative contributions of involution and hypoboly to the internalization of endodermal tissues. Formation of the endodermal wedge in amphibians is more pronounced in the yolk-rich blastulae (e.g. frogs) as compared with the blastulae with less yolk (e.g., *Triturus*, Hertwig, 1910; and see below). In the yolk-rich *Xenopus* embryo the dorsal, lateral and ventral endodermal cells even meet under the blastocoel roof so that the endodermal collar closes up to form an endodermal lining underneath the animal cap (see Hausen and Riebesell, 1991). Therefore, the higher the amount of yolk in the vegetal hemisphere, the more pronounced is the contribution of hypoboly to the formation of the inner cell layer during gastrulation, and the less pronounced is the contribution of involution to this process. This rule applies for amphibians, when ordered with respect to their increasing content of yolk, for example: *Triturus*, *Xenopus*, *Salamandra maculate*, Gymnophiona (Hertwig, 1910p. 177), and it can probably be extended to reptiles and birds as well, suggesting that the formation of the hypoblast is an outcome of the ever-increasing, extensive storage of yolk during the evolution of the amniotes.

5. Conclusions

Comparative molecular and embryological data are utilized to define a common morphological framework for the early development in amphibians and yolk-rich amniotes, and to trace the evolution of gastrulation in the yolk-rich amniote condition.

Avian and amphibian eggs are similarly organized along the an–veg axis, except that in birds the vegetal yolk-containing region is disproportionately large. After establish-

ment of bilateral symmetry there is a similar arrangement of the prospective tissue qualities (fate map/anlagenplan) in frog and chick (Fig. 1). Homology of *goosecoid*-expressing cells in the amphibian organizer and in the medial portion of the avian Koller's sickle would imply that what is usually called 'posterior' in the early avian blastoderm corresponds to 'dorsal'/'anterior' in the amphibian blastula.

Endodermal subregions are compared in frog blastula and chick blastoderm (Fig. 2). In the frog, the prospective archenteron roof endoderm locates equatorially to the organizer region. In the chick, the corresponding 'definitive endoderm' locates to Koller's sickle. Both express similar genes, exhibit strong morphogenetic movements during gastrulation and are thus considered as homologous. The '*nutritive endoderm*' comprises the vegetalmost, yolk-rich blastomeres in amphibians, and the uncleaved yolk plus peripheral blastoderm cells in birds. An equatorial/marginal portion of the dorsal nutritive endoderm generates the axis-inducing signals (Nieuwkoop center/posterior marginal belt).

The evolution of gastrulation in the yolk-rich amniotes is depicted in a two-dimensional matrix of evolution and development (Fig. 3). In the amphibian-like, ancestral tetrapod situation, the anterior/dorsal blastopore lip with the organizer moves around the vegetal hemisphere during gastrulation, leaving in its wake the dorsal axial tissues. In early amniotes, this movement around the vegetal hemisphere is no longer possible in conjunction with early axis formation due to the enormous amount of yolk. Here, the organizer moves meridionally over just a small section of the vegetal yolk mass, so that axis formation begins and ends on the same side in the animal region of the egg. It is suggested that, in consequence, the single amphibian-like blastopore divides into two morphologically and functionally separate parts, an *intraembryonic blastopore* for the involution of endomesoderm and an *extraembryonic blastopore* for ectodermal epiboly (Fig. 4). This explains why in the yolk-rich amniotes the site of mesoderm internalization is distinct from the advancing front of epiboly. Changes in the shape of the intraembryonic blastopore have accompanied the evolutionary transition from involution in amphibians to invagination and ingression in reptiles and birds (Fig. 4). A continuous sequence of morphological alterations is described that helps to understand how the primitive streak and Hensen's node (of birds and mammals) evolved from the primitive plate and blastoporal canal (in reptiles), and how the latter structures had evolved from a circumferential, cleft-shaped, amphibian-like blastopore. As shown for birds, formation of the primitive streak leads to a seeming reversal of A–P polarity with respect to the underlying yolk.

Finally, it is outlined that the amniote hypoblast corresponds to the amphibian 'endodermal wedge' (a wedge-shaped mass of deep endodermal cells lining the blastocoel cavity; Fig. 5). We show that amniote hypoblast and amphibian endodermal wedge are similarly involved in patterning the anteriormost neuroectoderm, and that they undergo similar morphogenetic movements during gastrulation. These movements are referred to as *hypoboly*, which, as a counterpart to epiboly, is the active spreading of deep endodermal cells along the inner surface of the animal ectoderm towards the animal pole.

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