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REVIEW ARTICLE

Establishment of the axis in chordates: facts and speculations

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Dedicated to the memory of Pieter D. Nieuwkoop, a beloved teacher and friend and a great but modest scientist

SUMMARY

A master plan for the early development of all chordates is proposed. The radial symmetry of the chordate ovum is changed at or after fertilization into a bilateral symmetry by an external signal. Until now two alternative triggers, sperm entry and gravity, have been demonstrated. It is suggested that a correlation exists between the amount of yolk stored in the egg and the mechanism used for axialization. The speed at which axialization of the embryo proper takes place depends on the translocation speed of maternal determinants from the vegetal pole towards the future dorsoposterior side of the embryo. On arrival at their destination, the activated determinants form, in all chordates, an induction center homologous to the amphibian 'Nieuwkoop center', which induces the formation of 'Spemann's organizer'. On the basis of the above general scenario, a revision is proposed of the staging of some embryonic types, as well as of the identification of germ layer and the spaces between them.

Key words: axis, chordate, radial symmetry, sperm entry, induction, Nieuwkoop center, Spemann's organizer, germ layer

INTRODUCTION

The development of a bilateral symmetric organism depends on the gradual formation of an orderly developmental pattern, where every section of the embryo acquires a specific identity. This process is initiated by a gradual cytoplasmic segregation, which proceeds in the different chordates at different paces, using a variety of mechanisms. However, a unitary plan can be drawn and speculation can be made on its evolution. The aim of the present paper is to compare information concerning the gross morphogenetic processes that take place in chordate eggs immediately after fertilization and during cleavage, morula, blastula and gastrula stages.

The main issue to be discussed is the process of cytoplasmic segregation, which dictates the axis. The idea proposed is that, in all chordates, dorsally migrating vegetal determinants are involved in the formation of a dorsoposterior induction center homologous to the amphibian 'Nieuwkoop's center'. In all chordates, the above center induces the formation of an organizer equal to Spemann's organizer. The line connecting the midline of the organizer with the animal pole will inevitably become the median plane of the future embryo, the head of which will therefore develop at, or towards, the animal pole (see also Wacker et al., 1994). The homology of the germ layers will be judged mainly on the basis of the function that they perform in development and will involve some revision of the identification of the germ layers as well as of nomenclature. Only a clear understanding of the morphogenetic homologies in a broad spectrum of chordate embryos can provide the basis for a comparative study of the location and role of developmental molecules in early embryogenesis.

THE STAGE AT WHICH THE EMBRYONIC AXIS IS DETERMINED IS RELATED TO THE AMOUNT OF YOLK

A common feature for all chordates is that the animal-vegetal axis is probably the only one determined during oogenesis so that, at ovulation, the egg is radially symmetric. The establishment of the axis of bilateral symmetry (axialization) is an event that takes place after fertilization, at a speed that seems (except for eutherian mammals) to be inversely related to the amount of yolk in the egg. In most primitive chordates, like Tunicata and Acrania, and in many anamniotic vertebrates with a low to medium content of yolk, the embryos undergo an holoblastic cleavage and usually, by the second cleavage, one can distinguish between the developmental potentials of the blastomeres. However, there is probably no obligatory correlation between the axialization of the cytoplasmic components of the zygote and the cleavage pattern of the embryo. The two above processes seem to be independent of one another (Nieuwkoop et al., 1985; Helde et al., 1994; etc.).

Two big groups among the Anamniota (teleosts and elasmobranchs) have eggs with relatively large amounts of yolk,

which enables the embryo to develop without going through a larval stage and metamorphosis. They have a meroblastic cleavage and a relatively late determination of the blastoderm's bilateral symmetry. The evolutionary trend leading to the amniotic terrestial vertebrates has again promoted the development of yolky eggs (reptiles, birds and Monotremata), meroblastic cleavage and a relatively late axialization of the blastoderm. Mammals have gone a few steps further in evolution. The more primitive Monotremata have yolky eggs; the Marsupialia have much less yolk and a new relationship between the developing embryo and the maternal uterus. The culmination of this trend is seen in the eutherians in which the embryo lacks yolk and depends on maternal supplies. However, there are some indications (to be discussed) that, despite the loss of yolk, eutherians still behave as far as axialization is concerned, like the telolecithal meroblastic amniotes.

THE FORCES INVOLVED IN AXIALIZATION

According to the above outline, the data collected from almost a century of publications will be discussed, to analyze how and when bilateral symmetry is brought about in the different chordates, which are held to be of a monophyletic origin.

Lower chordates

Nishida (1994) summarizes information concerning different steps of axis formation in ascidians (urochordates). Following fertilization, two waves of cytoplasmic segregation are generated in the initially radial symmetric egg (Fig. 1Ba). First, the subcortical cytoplasm, which contains 'maternal determinants', concentrates at the vegetal pole, the polarity still remaining radial (Fig. 1Bb); later on, the vegetally located cytoplasm moves towards the future dorsal pole (see also Sardet et al., 1989). Removal of a small portion of cytoplasm from the vegetal pole of *Styela clava*, or UV irradiation of the area, before the first wave of cytoplasmic segregation, has no effect on development. However, when the same manipulations are done after the subcortical cytoplasm with its determinants has concentrated in the vegetal pole of the zygote, axis formation is prevented (Bates and Jeffery, 1987; Jeffery, 1990). Similar results have been achieved when 8-15% of the cytoplasm of the posterior-vegetal region of the zygote of *Halocynthia roretzi* have been removed after the second phase of cytoplasmic segregation (Nishida, 1994). When posterior-vegetal cytoplasm (PVC) was transplanted into a PVC-deficient zygote, the axial deficiency was overcome, which means that the above cytoplasm contains some unique information involved in axis formation. Sardet et al. (1989) found that, after fertilization in *Phallusia*, the subcortical cytoplasm was first concentrated at the vegetal pole and then split into two fractions. The bulk of this cytoplasm moved together with the male pronucleus in a ventral direction to form the myoplasm,

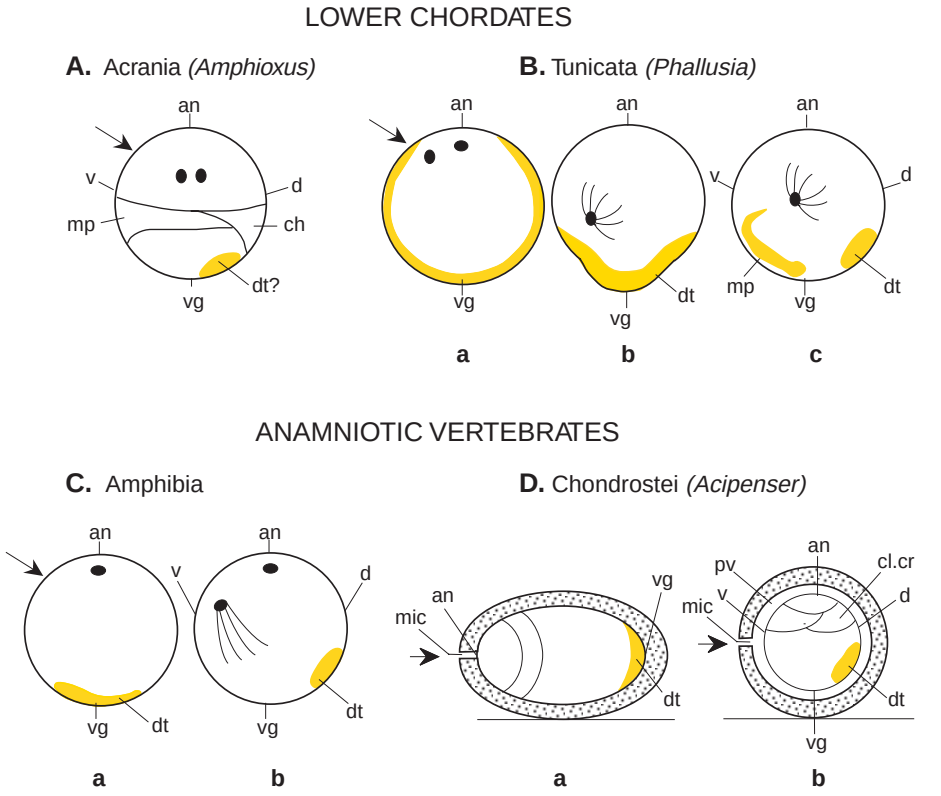

Fig. 1. Chordates with holoblastic cleavage. Following fertilization, reorganization of vegetal maternal determinants involved in axis determination takes place. While types A,B,C react to sperm entry (arrow), type D reacts to its spatial position. (A) *Amphioxus* (Acrania): cytoplasmic segregation following fertilization (according to Conklin 1932). (B) *Phallusia* (Tunicata). Interpretation of the results of Sardet (1989). (a) Sperm entry; (b,d) two successive stages of segregation of the determinants. (C) *Xenopus* (Amphibia). Interpretation of Gerhart et al 1989. (a) Sperm entry; (b) shift of vegetal determinants by microtubules of the aster. (D) *Acipenser* (Chondrostei – fish). Interpretation of the results of Detlaff (1954). (a) The radial symmetric egg prior to fertilization; (b) reorganization of the cytoplasm into a bilateral symmetric pattern following the egg's rotation after fertilization. *Abbreviations for all figures:* a, anterior; adt, activated vegetal determinants (orange); al, albumin; an, animal pole; a.o, area opaca; arch. can, archenteric canal; blast., blastocoele (blue); bl. ca, blastocystic cavity; blp, blastopore; cl. cr, clear crescent; DEL, deep layer; d, dorsal; dt, vegetal determinants (yellow); EVL, enveloping layer; ep, epiblast; epc, ectoplacental cone; E-YSL, external yolk syncytial layer; H.n., Hansen's node (green); hyp, hypoblast; ICM, internal cell mass; I-YSL, internal yolk syncytial layer; m.b, marginal belt; mesend, mesendoderm (pink); mic, micropyle; mp, myoplasm; m.z, marginal zone; N.C, Nieuwkoop's center (orange); p, posterior; pv, perivitteline space; PS, primitive streak; s. ca, sub-blastodermic cavity; s. cy, subcortical cytoplasm; sh, egg shell; sh.m, shell membrane; Sp. or, Spemann's organizer (green); t, trophoblast; u.v, uterine wall; v, ventral; vg, vegetal pole; yk, yolk; z.p, zona pellucida.

while a smaller part moved to the vegetal dorsal side of the zygote (Fig. 1Bc). The dorsal fraction might be involved later on, between the 32- and 64-cell stage, in the induction of the notochord as described by Nakatani and Nishida (1994). Information on the Acrania (Amphioxus) is quite scarce. However, the morphologic observations indicate that fertilization causes a cytoplasmic segregation very similar to that in tunicates (Waddington, 1956; Nieuwkoop et al., 1985), which dictates the establishment of bilateral symmetry already in the zygote (Fig. 1A).

Vertebrates

Anamniota

Among the lower vertebrates, in all the holoblastic eggs (with a low to medium content of yolk) such as: Cyclostomata, Dipnoi, Holostei, Chondrostei and Amphibia, axialization is believed to be accomplished in the zygote before cleavage (Clavert, 1962) by one of two mechanisms: the sperm entry point (SEP) and gravity. In most of the above groups, the SEP does not coincide with either the animal or vegetal pole and thus forms a third reference point for the future axis. An array of asymmetric vegetal microtubules formed by the centrosome is essential for both the cortical rotation and the translocation of the vegetal determinants to the future dorsal side of the zygote (Gerhart et al., 1989) (Fig. 1Ca,b). An alternative mechanism is applied in those fish eggs that have a micropyle situated exactly above the animal pole and determines the SEP. In the absence of a third reference point to dictate the axis, gravity takes over and the turning of the zygote inside its envelopes is responsible for the rearrangement of the zygotic cytoplasmic fractions. Ginsburg (1968) and Dettlaff and Ginsburg (1954) observed that the somewhat oval, unfertilized egg of the sturgeon (Acipenser), always lies with its animal-vegetal axis parallel to the substrate to which it is attached (Fig. 1Da). Immediately after fertilization and the cortical reaction, the zygote is released from the tight grip of the envelope, becomes round and rotates by 90° so that the animal pole is brought to the highest point. A rearrangement of the previously radially distributed cytoplasm follows, and a ‘clear crescent’ appears on the descending side of the vegetal pole (Fig. 1Db). If the zygote is not disturbed, the middle point of the clear crescent, together with the animal and vegetal poles, will indicate the plane of bilateral symmetry. Even in those anamniotes (like amphibians) in which the normal trigger for axialization is the SEP (Ancel and Vintemberger, 1948), turning the zygote before the first cleavage causes a redistribution of the cytoplasmic components and changes the axis accordingly (Ancel and Vintem-

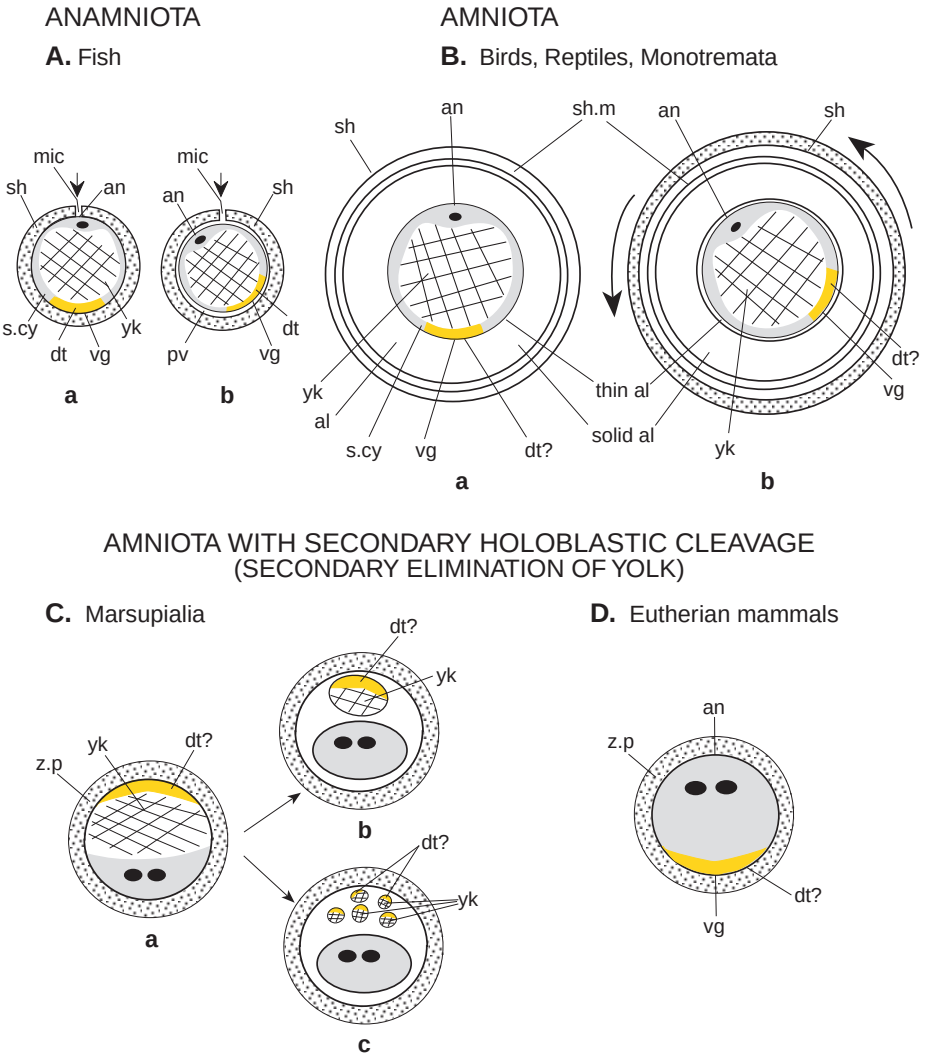

Fig. 2. Vertebrates with meroblastic cleavage or a secondary holoblastic cleavage. (A) Fish; (a) before and (b) after fertilization. (B) Birds. Interpretation of Kochav and Eyal-Giladi (1971). (a) After fertilization the still radially symmetric egg is being enveloped in the oviduct by albumin and shell membranes. (b) On entering into the uterus the egg is starting to rotate on its long axis. The egg shell is being secreted and at the same time the egg acquires a tilted position, which gradually determines its posterioanterior axis, which is perpendicular to the uterus. Axialization in Reptiles and Monotremata is probably acquired on a similar basis. (C) Marsupials. Interpretation of Selwood 1992. As it is difficult to define the animal and vegetal poles, the yolk mass is shown uppermost because this is where the blastomeres flatten. This representation makes it easier for comparison with other blastoderms (Selwood, personal communication). (a) The radially symmetric egg prior to fertilization. (b,c) two different types of yolk emission following fertilization. (D) Eutherian mammals. The radially symmetric egg prior to fertilization. The symmetry of A, B and D is probably determined by their spatial position (gravity). The bilateral symmetry of C might be determined by determinants which are separated together with the yolk from the bulk of the cytoplasm and occupy an eccentric position near the zona pellucida.

berger, 1948; Gerhart et al., 1981). SEP was shown to be sufficient for achieving axialization in microgravity experiments with *Xenopus* eggs that have been fertilized in outer space (Souza et al., 1995).

In telolecithal eggs with big amounts of yolk, most of the cytoplasm containing the female pronucleus is confined to the animal pole as a germinal disc, which is continuous with a very thin layer of peripheral subcortical cytoplasm covering the entire surface of the egg. The fertilizing sperm therefore has to penetrate near the animal pole where cleavage will later begin. The 'maternal determinants' involved in axis formation, presumably confined to the thin vegetal cytoplasm, are at that point still far away from where the 'action' is. This is probably why the blastomeres, formed from the germinal disc during the initial steps of cleavage, are equipotential. In such eggs, the aster seems not to be involved in the translocation of the distant vegetal determinants and the alternative mechanism, gravitational influence, is implemented.

The difference in the pace of symmetrization between the holoblastic and telolecithal egg types is not clear cut and there are eggs that are in between. In the holoblastic sturgeon (Acipenser), the relatively large amount of yolk causes cleavage to be slower in the vegetal than in the animal half. As a result, the more vegetally situated cells remain 'open' for a longer time, which may allow more time for the vegetal determinants to continue their gravity-directed movement towards the future dorsoposterior side.

In anamniotic telolecithal eggs, there are two different modes of fertilization. While, in most teleosts, fertilization is external via a micropyle (Fig. 2Aa), in elasmobranchs, it is internal and usually polyspermic. Nevertheless only the sperm closest to the female pronucleus contributes to the zygotic nucleus (Ginsburg, 1968). This is why, in both types, the axis seems unrelated to the SEP, as the aster of the fertilizing sperm, which is very distant from the 'determinants', is unable to shift them into an effective asymmetric position. Instead, gravity is utilized as the vectorial force to gradually translocate the determinants towards the future dorsal position of the already cleaving tilted blastoderm (Devillers, 1951; Wintrebert, 1922; Clavert, 1962; Clavert and Filogamo, 1966) (Fig. 2Ab). We do not know at present how the slow, gravity-mediated translocation functions. The above scenario contradicts the ideas of Strehlow and Gilbert (1993) who believe that, in zebrafish, the three first cleavages indicate the adult body axes. Nevertheless the majority of fish investigators (see Helde et al., 1994; Abdelilah et al., 1994) think that, in zebrafish as in *Xenopus*, the dorsoventral axis and the first cleavage planes are determined by separate mechanisms and cell fate is determined only shortly before the onset of gastrulation (Ho and Kimmel, 1993; Helde et al., 1994; Vivien and Hau, 1954; Clavert, 1962; Ho, 1992). Helde and Grunwald (1993) mention the idea that maternal information is sequestered in the yolk cell and becomes active during gastrulation when prospective cell fates become predictable. The yolk component is also stressed by Oppenheimer (1936), Tung et al., (1944, 1945) and Devillers (1949) who found that, in different teleosts, there is a critical stage for the axialization of the blastoderm, which depends on the relative amount of yolk in the egg; namely the bigger the yolk volume, the later in development the critical stage is. If a blastoderm is separated from the underlying yolk and cultured in vitro before the critical stage, it will turn into a radial symmetric hyperblastula, while separation from yolk after the critical stage enables normal differentiation. In a complementary experiment performed by Oppenheimer (1936) on *Fundulus* and by Tung et al. (1945) on *Carassius*, young cleaving blastoderms were cut off the egg with different amounts of yolk adhering to them. The younger the blastoderm, the bigger the portion of the original yolk ball that was needed to enable normal development. Long (1983) marked the dorsal side of trout eggs by chalk particles inserted onto the yolk underneath the embryonic shield and then removed the axial blastoderm and replaced it with a younger blastoderm without a visible bilateral symmetry. The transplanted blastoderms formed an axis that complied with the mark on the yolk and the syncytial layer attached to it.

This indicates that, in fish, determinants with a migrating capacity are localized either in the yolk or syncytial layer. The stage at which the highest concentration of determinants reaches the posterior marginal area is critical for axialization, after which differentiation can go on without further involvement of the yolk ball. Due to the gradual process of axialization, a distinction should be made between the axialization of the egg as an entity and the axialization of the cellular blastoderm. The first starts immediately after fertilization when the determinants start their migration, while the second is materialized at the critical stage, after the arrival of determinants at the future posterior side of the blastoderm.

Amniota

Axialization in lower amniotes such as reptiles and birds proceeds according to the same principles as in the telolecithal Anamniota. They also have a relatively enormous amount of yolk, which separates the germinal disc from the vegetal subcortical area and therefore probably excludes the possibility that the sperm aster might be involved in the translocation of the 'vegetal determinants'. Thus, from the two previously described alternative mechanisms that are presently known to be involved in axialization, only gravity remains an option for the amniotic meroblastic eggs.

In both reptilian (Clavert and Zahnd, 1955; Pasteels, 1955; Raynaud, 1960; Clavert, 1960, 1962) and avian eggs (Vintenberger and Clavert, 1954, 1960), axialization takes place during the uterine period, while the egg is rotating on its long axis. Kochav and Eyal-Giladi (1971) realized that the rotation forces the blastoderm into an oblique position in the direction of the rotation and that the upper edge of the blastoderm is gradually committed to become the posterior-dorsal side (also supported by Callebout, 1993a,b). In addition, according to the model presented in Fig. 2Ab and Bb, the maternal determinants on the other side of the yolk are also forced into an oblique position, which is a mirror image of the blastodisc's position. The oblique position of the determinant-rich cytoplasm might be the factor influencing directional migration upwards towards the future posterior side.

Kochav and Eyal-Giladi (1971) showed that there is a labile period when the blastodisc that has already cleaved into

thousands of cells can be forced, after changing the egg's spatial position by 180°, to form an axis according to the new position. At a slightly later stage, a similar change of position can only cause the formation of an axis that is a compromise between the two positions. Somewhat later (similar to the critical stage of fish), the turning of the egg by 180° no longer affects axialization (Eyal-Giladi and Fabian, 1980). The somewhat unstable axis, determined in birds during the uterine period, is already manifested in cultured blastoderms of stages X E.G. & K. (Eyal-Giladi and Kochav, 1976) which have not been subjected to experimental interventions. Manipulations such as cutting or folding of the blastoderm may cause a lateral deviation of the axis (Eyal-Giladi, 1969, 1970, 1991). This topic is however beyond the scope of the present paper.

Eyal-Giladi et al. (1994) recently developed a system that enables the culture of very early aborted avian eggs (quail) in emptied foster shells. Uterine eggs surrounded only by soft egg membranes were aborted immediately after arriving in the mother's uterus where they were about to start their rotations. Three groups of shell-less eggs were released to different extents from the attached egg membranes and put into foster shells to enable proper incubation conditions. It was shown that the more the yolk ball (with the germinal disc) was freed from the attached envelopes, and the germinal disc was made free to float and acquire an horizontal position, the more the blastoderms tended to develop into extraembryonic tissues and failed to form an axis. We can therefore assume that, in avian eggs (and probably in reptiles), similar to fish, the subcortical vegetal determinants essential for axialization have to migrate a long distance to reach the uppermost margin of the blastodisc, which will become the posterior side (Fig. 2Ba, b). However, in those cases in which the blastodisc is horizontal and there is no uppermost margin, the determinants on the opposite side of the yolk either do not migrate, or more probably migrate equally towards the entire periphery of the blastodisc, thus creating a radial symmetric condition. Another observation might also support the idea of a gradual migration of the vegetal determinants towards the uppermost margin of the bastodisc in birds. Eyal-Giladi (unpublished data) never succeeded in getting axis formation in cultured blastodiscs isolated from the yolk at early uterine stages. Very small axes did develop in such blastoderms, only when cultured after stage VII (Eyal-Giladi and Kochav, 1976). Stage VI-VII (Eyal-Giladi and Kochav, 1976) in avians might therefore be the critical stage at which the determinants reach the posterior margin of the blastodisc. It can therefore be forseen that, in microgravity experiments with avian eggs ovulated and fertilized in space, there would be no axis formation. This prediction contradicts the findings in amphibian embryos (Souza et al., 1995) in which an axis developed after fertilization in outer space, probably because axialization was determined by the SEP which is the conventional option for holoblastic eggs.

The situation in early mammalian embryos is more obscure. The most primitive mammals are the egg laying Monotremata (Prototheria) the eggs of which, as well as their genital tracts, resemble those of reptiles and no information is available concerning their axialization. More information exists about marsupials (Metatheria), which includes the description, in different genera, of cleavage patterns, the relation of the blastomeres to the yolk and the formation of the blastocyst (Selwood, 1994). The marsupials have a holoblastic cleavage despite the fact that some have a large amount of yolk. During the first cleavage division, the yolky cytoplasm is eliminated into the cleavage cavity either as separate vesicles or as a single large membrane-bound yolk mass (Selwood, 1994) (Fig. 2C). Additional materials may be eliminiated from the blastomeres into the perivitelline space at the 2- and 4-cell stages (Selwood, 1992). The first blastomeres do not adhere to one another, but rather adhere to the zona pellucida and there are some hints that axialization might be related to the way they attach to fixed locations of the zona. Selwood (1992) mentions that, in embryos with a polarized emission of yolk, there might be an attachment of blastomeres to particular sites on the zona, which is followed by the stretching of blastomeres between those sites and influences the cleavage pattern. Selwood suggests that 'determination of bilateral symmetry might be due to the accumulation of positional signals combined with autosuppressive effects and/or uneven distribution of maternal determinants'.

Eutherian cleaving embryos were regarded not to be polarized, as single blastomeres from the 2- to 8-cell stage, on the one hand, and chimeric embryos, on the other, can develop into normal blastocysts (Gardner and Rossant, 1976; McLaren, 1976). It was also shown that one can scramble the cytoplasm of a mouse zygote (Evsikov et al., 1994) and get normal embryos. The interpretation of the above data went as far as to abolish of the terms animal-vegetal for eutherians, which means that the egg does not even possess radial symmetry. Gardner (1996) in a recent review challenges the above approach and rightly claims that the issue of animal-vegetal polarity should be reinvestigated. He supports his doubts with the experimental results of several, mainly earlier investigators such Denker (1976). The latter raised the possibility that the eutherian egg has an initial polarity, which depends on the eccentric localization of 'determining factors', so that the blastomeres of the early embryo are basically unequal. Some of the blastomeres tend to develop into trophoblast, while the others will become ICM. The above initial segregation (preformation) is however quite labile and does not exclude, especially under experimental conditions, an impact of the inside-outside positional effect, which is believed to cause the segregation between trophoblast and ICM. However, the cells of the ICM at the 32-cell stage are pluripotent (Pedersen, 1986) and the ICM as a whole is probably radially symmetric and does not yet have a posterioanterior axis. It has been frequently suggested that the blastocyst is also radially symmetrical prior to implantation and that the above condition persists through the implantation period until the onset of gastrulation. However, Smith (1980) realised that, prior to implantation, all of the 3.5 day mouse blastocysts that she studied had a distinctive orientation within the uterus, the axis connecting the ICM with the abembryonic pole of the blastocyst being parallel to the uterine floor. Those blastocysts normally pass down the uterus with the above axis almost horizontal, even though they are still surrounded by the zona pellucida (Fig. 3D). In the

above blastocysts, there are also consistent morphological expressions of bilateral symmetry in the shapes of both the ICM and the blastocystic cavity. With this characteristic horizontal position of the blastocyst in the uterus, implantation begins at the abembryonic side. Smith (1980) suggests that differences in the environmental influences to which the surfaces of the blastocyst are exposed by virtue of their horizontal position in the uterus, lead to the morphological asymmetries, including a flattening of the blastocyst (Enders, 1971; Huber, 1985). Such a flattening might ensure that, even when still surrounded by the zona pellucida, while being moved along the uterine horn to their implantation site, they are capable of keeping their original spatial orientation.

As to what later on concerns the orientation of the primitive streak and embryonic axis, Smith (1980, 1985) and Gardner et al. (1992) agree that there is a clear relationship between the posterioanterior (PA) axis of the embryo and the orientation of the uterine horn, the PA axis being roughly perpendicular to the long axis of the uterine horn. They also find a clear correlation between the embryonic axis and the tilt of the ectoplacental cone which develops on the ICM side, opposite to the abembryonic pole. During the implantation process, it is the ectoplacental cone that anchors the polar trophoblastic side to the uterine wall. While Smith (1980) claims that the posterior side of the embryo is always at the direction of the tilt, Gardner et al. (1992) claim that the two are either parallel or antiparallel. This dispute does not change the fact that the PA axis, which must be determined prior to PS formation, is probably determined either prior to, or shortly after implantation. The importance of the positioning of the conceptus in the uterine wall implies the involvement of spatial information, or in other words – gravitational information. We can only speculate at present on how this takes place as there is no experimental evidence on the above stages. However, if the ideas of Gardner and Denker about a cytoplasmic segregation in the egg are correct, then some determinants localized eccentrically at the future abembryonic region could be responsible for axis formation. In that case, the migration of maternal determinants to a dorsoposterior position at the margin of the ICM would be required. A possible route for the migration of the determinants might be intercellular bridges preserved between blastomeres at least until the 8-cell stage. These connections, probably unique for the eutherian embryo, are actually persistent midbodies, which allow the passage of molecules as big as horseradish peroxidase (Goodall and Johnson, 1984). If the above scenario is correct then axialization in eutherian mammals, despite the lack of yolk in the egg, fits into the general picture of axialization in amniotes and seems to be gravity dependent.

COMPARING THE EARLY DEVELOPMENTAL STAGES IN CHORDATES

The *Xenopus* system is the most extensively studied from the cytologic, morphogenetic and molecular points of view. We can therefore use *Xenopus* as a model with which the developmental events in other groups can be compared. In the vegetal half of a *Xenopus* oocyte, there are radially distributed 'maternal determinants' (Gerhart et al., 1989; Fukui and Asashima, 1994). After fertilization, a microtubule-mediated cortical rotation takes place and the determinants become localized asymmetrically in a vegetal-dorsal position (Fig. 1C). As a consequence, the determinants are specifically included during cleavage in a limited region of dorsoposterior cells. The above region, after being activated (Thomsen and Melton, 1993), will form the organizer-inducing center – 'Nieuwkoop's center' – in the early blastula. Nieuwkoop's center, around the midblastula stage, induces the formation of the dorsal marginal zone or 'Spemann's organizer' in the competent neighboring cells of the animal hemisphere. Spemann's organizer cells then, at the end of the induction process, start to gastrulate via the blastopore and form both axial mesoderm and definitive endoderm. Trying to correlate the above processes with the

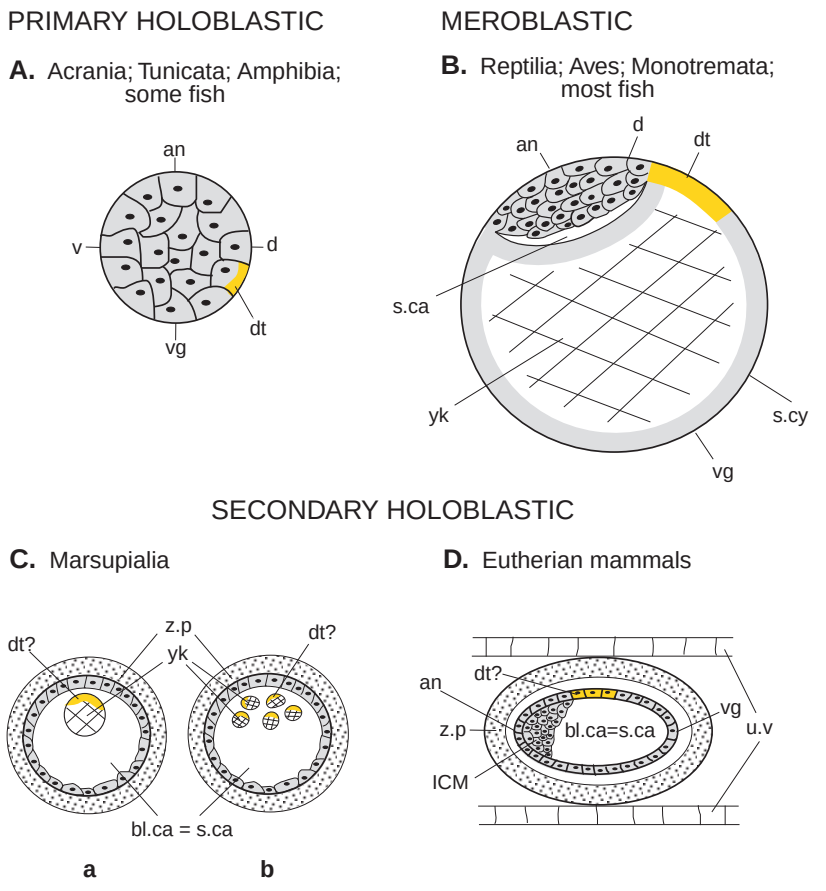

Fig. 3. Nieuwkoop's center and its proposed parallels in typical morulae of other chordates. (A) In amphibians and other holoblastic chordates.(B) In morulae of vertebrates with meroblastic cleavage.(C,D) A meroblastic pattern in the secondary holoblastic types. (C) Marsupials (interpretation of Selwood, 1992), (D) eutherian mammals (interpretation of Smith, 1980).

developmental stages of *Xenopus* we can generalize that, during the early blastula stage, the mesoderm-inducing center has to be formed in the right place. During the more advanced blastula, the induction of the endomesoderm (Spemann's organizer) will take place and the result will be manifested at the gastrula stage by the latter's involution via the blastopore (Fig. 5).

In all chordates, similar developmental steps probably have to take place. One may therefore define in the different chordates the homologous developmental stages, as well as the homology of germ layers and cell clones at the above stages. Eyal-Giladi (1995) has started in this direction and compared the morulae and blastulae of amphibian, avian and mammalian (Eutheria) embryos.

In the present paper, the discussion is extended by including all chordate types and by considering also criteria related to the gastrula stage (Fig. 5).

Two homologous developmental stages can be easily determined in all chordates, namely the zygote and the gastrula stages. The stages in between are much more difficult to define in embryos with a meroblastic cleavage and hard to compare with holoblastic embryos. We have therefore to look for criteria that would be generally applicable to all chordate embryos. Eyal-Giladi (1995) stresses that, in order to be able to compare meroblastic embryos with holoblastic ones, one should refer only to the formative part of the embryo and exclude the trophoblastic part, which particularly concerns the marsupial and eutherian mammals. In all chordates, the period between fertilization and gastrulation can be described as: cleavage, leading to the formation of a compact morula (Fig. 3). The next stage, blastula, is characterized by formation of a blastocoelic cavity, which separates the ectoderm (epiblast) of the animal half from the endoderm (hypoblast) of the vegetal half (Fig. 4). 'Maternal vegetal determinants' involved in axialization must be shifted to the future posterior-dorsal side of the embryo either in the zygote (holoblastic) or during cleavage and morula stage (meroblastic) (Fig. 2). At the end of this period, an induction center is formed (homologous to Nieuwkoop's center) in a region comparable to the dorsal side of the vegetal half (Fig. 3). This center induces Spemann's organizer (or its homologue), in the ectoderm or epiblast (Fig. 4). The endomesoderm, which is the morphogenetic manifestation of Spemann's organizer, will invaginate either via the blastopore or via its homologue – the primitive streak (Fig. 5).

Embryos with holoblastic cleavage, Urochordata, Acrania, Cyclostomata, Chondrostei, Dipnoi and Amphibia, all have a similar morphology of the morula, blastula and gastrula stages, as well as similar morphogenetic movements. The maternal determinants, which have been demonstrated to exist in Urochordata, Acrania and Amphibia, both morphologically and experimentally, probably exist in the other holoblastic groups. They must be shifted after fertilization (mediated by microtubules) from the initial subcortical vegetal position into an eccentric, more dorsal position to form Nieuwkoop's center (Sawada and Schatten, 1985, 1988; Sardet et al., 1989; Gerhart et al., 1989). It is presently unclear how determinants involved in the formation of the center move to the posteriodorsal side of the zygote, when gravity is driving the migration. With the meroblastic yolky eggs, the situation is even more complex, but the same developmental principles seem to prevail here as well. In yolk-rich eggs, maternal determinants are localized in the vegetal subcortical cytoplasm and have to travel a long distance

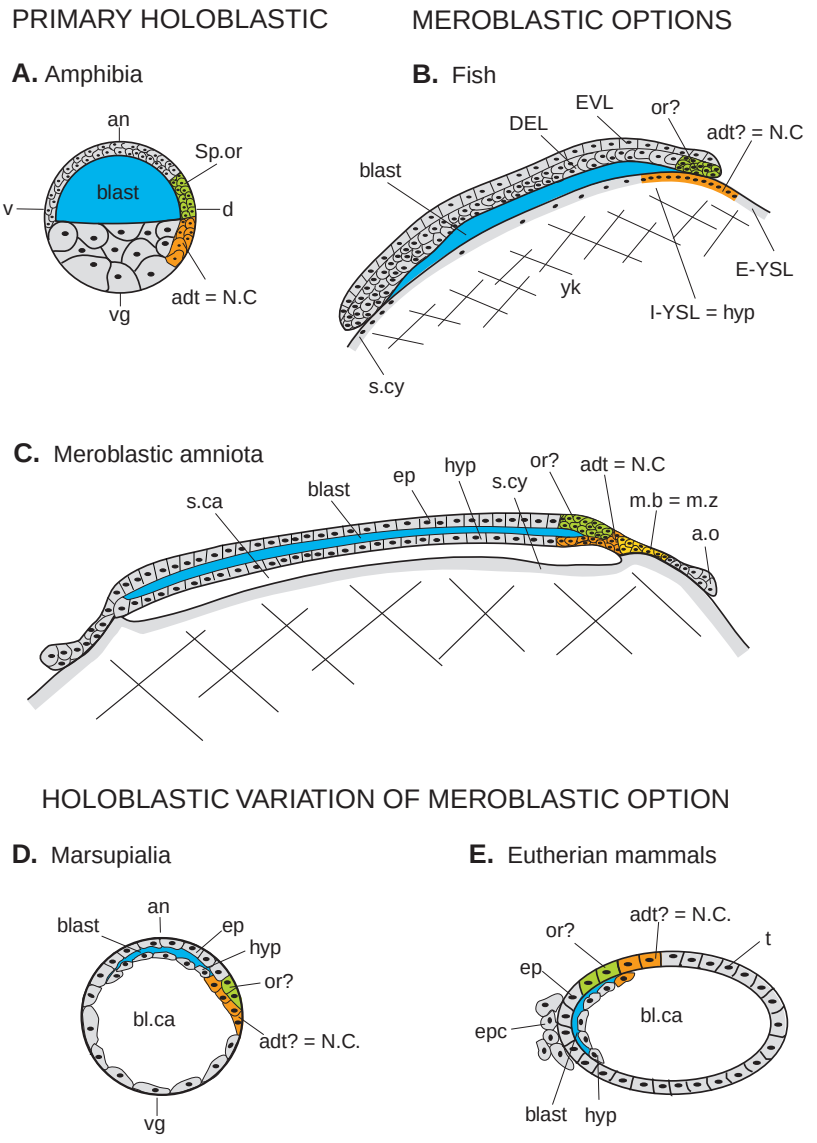

Fig. 4. Sagittal sections of blastulae. Proposition for the homology of blastulae and the gradual formation of a mesoderm-inducing center in them. (A) A holoblastic blastula in which Nieuwkoop's centre is the inducer of the organizer. (B-E) are four proposed types of meroblastic blastulae: B, fish; C, meroblastic amniota; D, marsupialia; E, eutherian mammal.

along the circumference of the egg to arrive in the right place, so as to form the induction center (Driever, 1995), at a stage that is comparable to the late morula and early blastula of the holoblastic embryos. Indeed, the experimental data indicate that axialization in such eggs is relatively late in development and can either be totally prevented, or its orientation changed even during cleavage (Oppenheimer, 1936; Tung et al., 1945; Kochav and Eyal-Giladi, 1971; Eyal-Giladi and Fabian, 1980). It is suggested that the definition of the various developmental stages should be based on the following criteria.

(1) Morula

A morula is a compact aggregate of cells without a well-defined central cavity, from which the first germ layers are formed. In Fig. 3, four types of morulae are presented. The first represents all chordate anamniotic embryos with an holoblastic cleavage (Fig. 3A), all the cells of which are formative and in which the determinants forming Nieuwkoop's center are included in the dorsovegetal blastomeres. In embryos with a meroblastic cleavage, there are at least two different types of morulae. The first type is characteristic for both anamniotic (fish) and amniotic embryos that develop on top of a large yolk ball. The morula proper is composed of a thick disc or a lens-shaped group of blastomeres (Fig. 3B). The blastomeres at the margin of the disc are 'open' or, in other words, their cytoplasm is continuous with the thin layer of cytoplasm that covers the yolk's surface, on the bottom of the subblastodermic cavity and on the superficial face of the yolk underneath the plasma membrane. The second type is the eutherian variation of a meroblastic morula in which the ICM only, is identical to the blastomeres of the first type (Fig. 3D). The ICM is surrounded by the trophoblast, which is actually an empty yolk sac. According to the experimental data for fish, birds, reptiles and probably also mammals, the following prediction can be made. At the morula stage, the determinants that will form Nieuwkoop's center, are on their way from the vegetal subcortical area to the future posterior side of the blastodisc, so that the blastodisc itself is still radially symmetric despite the fact that the whole egg is not. It is at the late morula stage that the determinants reach the posterior side of the blastodisc and are incorporated into its posterior margin, where they are probably activated (Thomsen and Melton, 1993) and start to drive the morphogenetic processes involved in blastula formation as well as in the induction of Spemann's organizer. The situation in marsupials is quite strange and it is possible that the individual non-committed blastomeres arrange themselves around a polarizing information source attached to the inside of the zona pellucida (Fig. 3C).

(2) Blastula

A blastula in holoblastic embryos has a wide blastocoelic cavity, which separates most animal blastomeres from vegetal ones, and cellular continuity, as well as cell-to-cell connection, between the animal and vegetal halves is confined to the periphery of the ball-shaped embryo. It is there that induction of Spemann's organizer by Nieuwkoop's center takes place (Fig. 4A). The transition from morula to blastula stage in the meroblastic embryos is gradual. The most systematically studied embryo is the chick, in which the transition from the young morula to blastula starts during the uterine period 10-8 hours prior to laying, with a gradual and orderly loss of cells (cell shedding) into the sub-blastodermic cavity, progressing from the future posterior towards the future anterior side (Eyal-Giladi and Kochav, 1976; Kochav et al., 1980; Fabian and Eyal-Giladi, 1981; Eyal-Giladi, 1995). This is an indication for the gradual axialization of the radial symmetric young morula, a process that remains reversible, until cell shedding has roughly passed the middle of the blastodisc (Eyal-Giladi and Fabian, 1980). The above morphogenetic process is accompanied by directional physiologic and probably molecular changes, such as glycogenolysis (Eyal-Giladi et al., 1976, 1979), nucleolar activation (Raveh et al., 1976) and oxygen flux (Raddatz et al., 1987). At the end, a 1-cell-thick blastoderm is formed, which despite its thinness can be compared to a late holoblastic morula (Eyal-Giladi, 1995). In such a blastoderm the active homologue of Nieuwkoop's center is the posterior section of the marginal belt (previously marginal zone),* the anterior limit of which is marked by Koller's sickle. From that stage on, the active morphogenetic process of blastula formation is manifested by the growth of a lower layer – the hypoblast, which by growing anteriorly from the sickle, gradually cuts off the upper part of the subblastodermic cavity from the rest of it. Only the very narrow slit confined between the epiblast and hypoblast can thus be compared with the blastocoele of holoblastic embryos (Fig. 4C). As is widely accepted, the epiblast is comparable to the animal half while the hypoblast is homologous to the vegetal half of a holoblastic blastula (Waddington, 1956). However, sections of the embryo, crucial for further development, namely the marginal belt + area opaca, constitute a flat ring protruding from and surrounding 'the waist' of the blastula, at the connection line between epiblast and hypoblast. The maternal vegetal 'determinants' that have been incorporated into the cells of the

*Attention should be drawn to the evolution of the confusing term 'marginal zone', which until now refers to two different structures in amphibians and birds. In amphibian literature, the dorsal marginal zone is Spemann's organizer, induced during the blastula stage by Nieuwkoop's center. In birds, the term 'marginal zone' has been applied by Spratt and Haas (1960) to the peripheral belt of the area pellucida which, according to them, was previously named by Lillie 'the inner germ wall'. Spratt and Haas who have realized the importance of that area for axis formation even explained that the change was justified, as the marginal zone of the bird blastoderm is functionally analogous with the amphibian 'Rand zone' and the teleost 'germ ring'. However, later studies showed that the above analogy is erroneous and that, in birds, the belt named the marginal zone and especially its posterior section are involved in an earlier step of axialization, which makes them homologous to the amphibian Nieuwkoop's center. It is posterior marginal cells that are actviated while moving into the hypoblast (Eyal-Giladi et al., 1994) and induce the primitive streak in the epiblast (Azar and Eyal-Giladi, 1979; Khaner and Eyal-Giladi, 1989; Eyal-Giladi et al.. 1992; Eyal-Giladi, 1995). In order to put an end to the above confusion, it is suggested that the term 'marginal zone' should be limited to the amphibians while the until now 'marginal zone' of birds should be renamed and called the 'marginal belt'. The proposed term 'marginal belt' by its being reminiscent of the until now used term 'marginal zone,' might help to preserve the link between the former publications and new ones in which the new term will be used.

marginal belt, with the highest concentration at the posterior section, are transported by the cells that move via Koller's sickle into the central strip of the hypoblast (Eyal-Giladi et al., 1994) while being activated. From that new location, they induce a primitive streak (the anterior part of which is homologous to Spemann's organizer) in the epiblast (Azar and Eyal-Giladi, 1979; Khaner and Eyal-Giladi, 1986, 1989; Eyal-Giladi and Khaner, 1989; Eyal-Giladi et al., 1992, 1994; Eyal-Giladi, 1992). Due to the intimate contact of the hypoblast and the epiblast, this induction is probably mostly vertical and not planar as in an holoblastic blastula. The homology that we made between the vegetal half of an holoblastic blastula and the meroblastic hypoblast is mainly based on the fact that they are the first two germ layers separated by a blastocoelic cavity and that the posterior section of both induces the 'organizer' in the animal-epiblastic layer (Nieuwkoop, 1969; Boterenbrood and Nieuwkoop, 1973; Eyal-Giladi and Wolk, 1970; Azar and Eyal-Giladi, 1981).

There is however an important difference between the above blastular types. While, in most holoblastic blastulae, vegetal blastomeres participate in the formation of the embryonic endoderm, in the meroblastic blastulae, the hypoblastic cells move out into the extraembryonic areas (yolk sac) after executing their inductive role. The entire endoderm in the latter embryo is formed, together with the mesoderm, from the invaginating cells of the PS in amniotes and of the dorsal lip of the blastopore (the dorsal margin of the blastoderm) in fish. The reptiles have not been studied as thoroughly as birds, but it can be assumed that the same processes apply also to them. As to mammals, there are hints that the morphogenetic processes in both monotremes (Flynn and Hill, 1947) and marsupials (Selwood, 1994) resemble the processes of axialization in lower amniotes. There seems to be a process that is similar to the polarized cell shedding in birds (Eyal-Giladi and Kochav, 1976) and hypoblast formation in a number of marsupial species proceeds by migration of cells from the epiblast, similar to its formation in monotremes and in birds (Eyal-Giladi and Kochav, 1976). This is an indication that also in marsupials bilateral symmetry is determined prior to, and expressed at, the stage of hypoblast formation.

The exact morphogenetic details of blastula formation in eutherian mammals are mainly unknown, as the process probably takes place during the early stages of implantation. We do not know yet how the compact ICM (morula) turns into a blastula composed of two germ layers. Is there a cell-shedding process and is the growth of the hypoblast directional along the posterioanterior axis? One thing that can be said without hesitation is that the homology of the germ layers and of the cavities of a mammalian embryo to all other types of amniotic embryos described above is quite clear. The epiblast and hypoblast are similar to those of the avian embryo and so is the appearance of the PS at the posterior side of the blastoderm, which must be a result of induction. Therefore, it is the narrow slit separating the epiblast from the hypoblast that should be identified as a blastocoele (Fig. 4E), while the big cavity underneath the hypoblast is the empty yolk sac or blastocystic cavity.

The formation of the anamniotic meroblastic blastula characteristic for the majority of fish, proceeds along a different path, which requires a new approach to the formation of fish germ layers. As the morula does not present new challenges, while the blastula and gastrula do, we should start with gastrulation and try to define what are the fish blastula and the first two germ layers. In the zebrafish, a clear distinction can be made between a surface enveloping monolayer (EVL), which does not participate in the formation of the germ layers, and the more loosely associated multilayer of deep cells (DEL) situated underneath the EVL, from which the germ layers develop. At the beginning of gastrulation, a thickening of the margin can be observed, the germ ring, which forms around the entire circumference of the blastoderm (Warga and Kimmel, 1990) and which probably can be compared with the amphibian blastopore. It is at the margin of the bastoderm that an involution/ingression of the DEL begins and a lower layer is gradually formed (Shih and Fraser, 1995). Schmitz and Campos-Ortega (1994) have observed that, in the zebrafish, 'the embryonic dorsoventral polarity axis is morphologically distinguishable prior to the onset of gastrulation and that the involution of the DEL starts on the prospective dorsal (posterior) side of the embryo', as is also the case in Salmo (Devillers, 1960). In zebrafish, the dorsal (posterior) side is thinner than the anterior one and it is there that the embryonic shield will form. The DEL cells that contribute to both endoderm and mesoderm move into the lower layer from all around the margin (blastopore), but mainly via its dorsal part, while the DEL cells that remain in the upper layer form ectodermal derivatives (Warga and Kimmel, 1990; Kimmel et al., 1990). This raises the question of the homology of the involuting/ingressing lower layer. According to the criteria of the present paper, the nomenclature applied by Thisse et al. (1993) should be adopted. They call the involuting layer mesendoderm (equal to endomesoderm) and not hypoblast, as it behaves like the middle layer of all the meroblastic amniotic gastrulae. The dorsal section of the mesendoderm is therefore the involuting organizer of fish (Figs 4, 5). The induced mesendoderm in fish gastrulates by involution/ingression of individual cells and not as a continuous layer like in amphibians, which is reminiscent of gastrulation in birds. The above homologies confront us with the problem of whether there is anything in fish that is homologous to the inductive part of the hypoblast and thus answers to the following criteria: (1) it should be a structure with an extraembryonic fate, (2) it should become inductive at the late morula, early blastula stage, (3) it should establish an intimate contact with the posterior side of the epiblast to allow for the induction of the organizer to take place and (4) the space between the epiblast and the candidate for a hypoblastic role should be eligible for the title of a blastocoele.

It is the internal yolk syncytial layer (I-YSL), which covers the yolk surface, facing the lower surface of the blastoderm, that answers to all the above criteria. The I-YSL replaces the thin anuclear yolk cytoplasmic layer (YCL) on top of the yolk underneath the blastoderm, by the penetration of nuclei into it. The above nuclei originate from open marginal blastomeres which, according to Trinkaus (1993), enter into the cytoplasm of the external yolk cell during the late cleavage stages and thus form the external yolk syncytial layer (E-YSL). After several

divisions of the nuclei of the syncytial layer, the marginal blastomeres are cut off from the syncytial layer by the formation of plasma membranes at their marginal borders. The cellular blastoderm and the YSL then become independent (Trinkaus, 1993). After the cessation of mitosis, a contraction of the E-YSL also starts in an animal-vegetal orientation. The nuclei become tightly packed, towards the margin of the blastoderm until they are pushed underneath it, into the YCL which is thus turned into the I-YSL, shortly before gastrulation begins. Solnica-Krezel and Driever (1994) and Solnica-Krezel et al. (1995) describe an elaborate array of microtubules in the external yolk syncytial layer, reaching towards the vegetal pole, which were formed from centers belonging to the marginal cells. They believe that these microtubules are involved, among other things, in the crowding of the external-YSL nuclei towards the blastoderm's margin and their movement into the yolk cell cytoplasm underneath the margin. Trimble and Fluck (1995) claim that, following fertilization, a transient array of parallel microtubules forms at the vegetal pole of medaka zygotes and that a vector of saltatory motion is created along those microtubules, which points from the vegetal pole directly to the future dorsal surface of the embryo. If the idea about maternal determinants in the vegetal cytoplasm that have to be transported to the border of the blastoderm is correct, then the above microtubles could serve that purpose. The findings of Solnica-Krezel and Driever (1994) that, following a nocodazole treatment and the disruption of microtubules, the E-YSL nuclei do not perform the packing phenomenon, and also that neither a germ ring nor an embryonic shield can be detected, support the above hypothesis. It is therefore the combined translocation of the maternal determinants and E-YSL nuclei under the margin of the blastoderm that forms the syncytial I-YSL, which functions as the fish equivalent of Nieuwkoop's center and thus deserves the title of hypoblast in fish. Nieuwkoop et al. (1985) have already pointed in this direction by saying that: 'In the meroblastic teleost egg....the syncytial periblast may act as a mesodermal inductor, like the yolk mass in amphibians.'

A trial can thus be made to define a blastula and a morula in fish. We can use the now popular zebrafish and relate to the 'stages of embryonic development of the zebrafish' (Kimmel et al., 1995). Kimmel et al. (1995) divide early development into the cleavage period, blastula period and gastrula period, and do not mention a morula stage. However, according to the criteria suggested above, blastula period of Kimmel et al. (1995) should be divided in two. The blastula stage proper should be defined as the period during which the internal syncytial layer is formed underneath the blastoderm by the population of the I-YCL with nuclei pulled into it from the external syncytial layer (Fig. 4B). This process probably also includes the penetration of the 'vegetal determinants' into the I-YSL and the cooperation of both components in the formation of an 'organizer induction center'. The cavity between the I-YSL and the blastoderm can only then be termed a blastocoele. The blastula stage of zebrafish thus starts with the transition from the 'High stage' (3.3 h) to the 'oblong stage' (3.7 h) (Kimmel et al., 1995) while the morula stage is from the 128-cell stage, which is already ball-like ($2\frac{1}{4}$ h), to the 'high stage'. The cavity underneath the blastoderm at the morula stage is homologous to the subblastodermic cavity in birds before the formation of the hypoblast and to the blastocystic cavity in eutherians.

One can therefore conclude that, in all chordates, there is an obvious homology of the morphogenetic events. From the

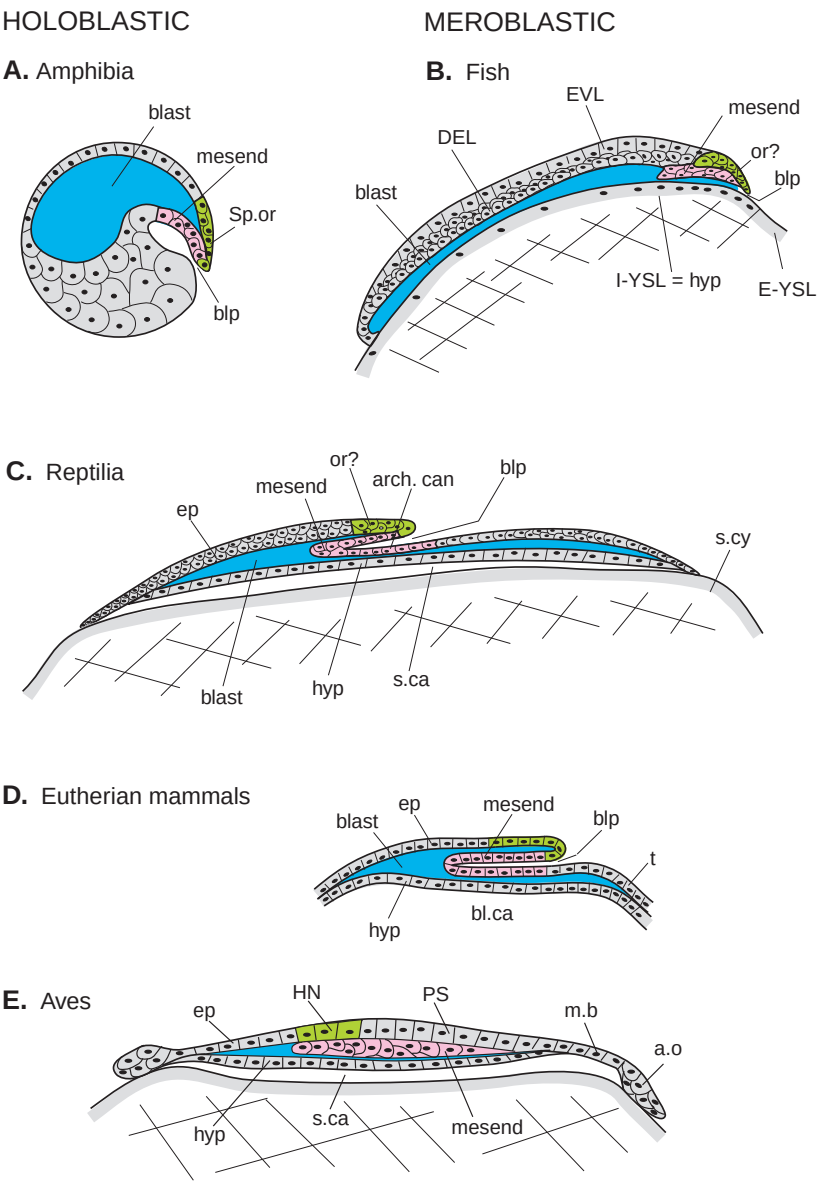

Fig. 5. Schematic gastrulae. (A) Holoblastic; (B) meroblastic fish; (C) reptiles; (D) eutherian mammals; (E) birds.

tunicates to the mammals, there is a similar pattern of cytoplasmic segregation, which is the first requirement for axialization and a subsequent chain of inductive events. On the basis of this functional homology, the morphological homology of the germ layers, and of cavities and spaces, in the different chordates was revised.

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