

Evolution of the Organizer and the chordate body plan

JOHN GERHART*

Department of Molecular and Cell Biology, University of California, USA

ABSTRACT The discovery of the organizer by Spemann and Mangold in 1924 raised two kinds of questions: those about the means of patterning the chordate body axis and those about the mechanisms of cell determination by induction. Some researchers, stressing the second, have suggested over the years that the organizer is poorly named and doesn't really organize because inducers act permissively, because they are not unique to the organizer, and because multipotent responsive cells develop complex local differentiations under artificial conditions. Furthermore, with the discovery of meso-endoderm induction in 1969, the possibility arose that this earlier induction generates as much organization as, or more than, does the organizer itself. Evidence is summarized in this article that the organizer does fulfill its title with regard to pattern formation: it adds greatly to embryonic organization by providing information about time, place, scale, and orientation for development by nearby members of the large multipotent competence groups surrounding the organizer. Embryos having smaller or larger organizers due to experimental intervention develop defective axial organization. Without an organizer the embryo develops no body axis and none of the four chordate characters: the notochord, gill slits, dorsal hollow nerve chord, and post-anal tail. For normal axis formation, the organizer's tripartite organization is needed. Each part differs in inducers, morphogenesis, and self-differentiation. The organizer is a trait of development of all members of the chordate phylum. In comparison to hemichordates, which constitute a phylum with some similarities to chordates, the chordamesoderm part is unique to the chordate organizer (the trunk-tail organizer). Its convergent extension displaces the gastrula posterior pole from alignment with the animal-vegetal axis and generates a new anteroposterior axis orthogonal to this old one. Once it has extended to full length, its signaling modifies the dorso-ventral dimension. This addition to the organizer is seen as a major event in chordate evolution, bringing body organization beyond that achieved by oocyte organization and meso-endoderm induction in other groups.

KEY WORDS: *Spemann-Mangold organizer, Nieuwkoop center, hemichordates, induction, cortical rotation.*

Introduction

Interest in the Spemann-Mangold experiment has endured for 75 years because the organizer is crucial for patterning the chordate body axis and because induction is a ubiquitous element of mechanisms of cellular determination in metazoa. The organizer, which includes approximately 10% of the cells of the early gastrula, stands out from other signaling centers in embryos by virtue of its very widespread patterning effect. It signals set off responses in at least half the cells of the gastrula embryo, and thanks to its activity as an elongating signaling source, egg organization is transformed into embryonic organization during gastrulation. In this article we will address four subjects about the organizer:

- The Spemann-Mangold experiment itself, to recall on this 75th anniversary the critical and illuminating points.
- The functions of the three parts of the organizer in terms of their morphogenesis, inductions, and self-differentiations. Then we discuss briefly the changing ideas about mechanisms of induction and how insights about mechanism do and don't bear on questions of axial patterning. We ask whether the organizer really "organizes", answering affirmatively with regard to the information it provides on the time, place, scale, orientation, and completeness of development by surrounding cells of the large competence groups of the gastrula, to generate the chordate body axis.
- The four steps in the formation of the organizer, tracing these back through the induction of the organizer by the Nieuwkoop center. Nieuwkoop's experiments on meso-endoderm induction are

*Address correspondence to: John Gerhart, Department of Molecular and Cell Biology, 142 LSA, 165 Crosscampus Road, University of California, Berkeley CA 94720 USA. FAX: +1-510-643-6791. e-mail: gerhart@socrates.berkeley.edu

reviewed. Then we go back to the establishment of the Nieuwkoop center itself by cortical rotation in the first cell cycle and by localizations of maternal materials in oogenesis. And

- The evolution of the organizer, which is involved centrally in the development of the chordate body axis and the four phylotypic characters of chordates, namely, the notochord, dorsal nerve cord, gill slits, and post-anal tail, either by the organizer's induction or self-differentiation. We suggest that the organizer has been a novel patterning agency in chordate evolution, adding significantly to what had already been achieved by meso-endoderm induction and oocyte localization in chordate precursors. We suggest that it generates a new body axis orthogonal to the axis of ancestral deuterostomes, which may have been aligned with the egg's animal-vegetal axis.

The Spemann-Mangold experiment

Throughout his career, Spemann worked with newt embryos (urodela, amphibia), a favored experimental material at the turn of the 20th century. In 1918 he published his first successes in grafting the upper lip ("obere Urmundlippe") of the blastopore from one gastrula to the opposite side of another gastrula. He did not call it the "dorsal" lip. That terminology came later when consensus was reached about dorsal and ventral locations on the fate map. Grafted embryos developed to the neurula stage with a secondary body axis near the graft site, and this axis contained a notochord and neural plate in nearly normal alignment and proportions. Since the donor and host embryos were from the same newt species, Spemann couldn't distinguish which parts of the secondary axis came from the graft or host. The result seemed not entirely new at the time, since ten years earlier W. Lewis (1908), whom Spemann cites, had grafted an upper lip of the blastopore of *Rana palustris* into a host embryo at the neurula stage. When Lewis found a secondary notochord and neural tube, he correctly concluded that the graft had self-differentiated. However, Spemann used a gastrula host, and so his experimental conditions were appropriate for his subsequent discovery of the organizer's inductive activity, whereas those of Lewis were not.

Spemann did not call much attention to the upper lip graft in the 1918 paper. He had done the graft as part of a large series of experiments of systematic exchanges of cell groups between different locations of different gastrulae, and letting them develop to see if the exchanged pieces pursued old or new developmental paths. In most cases the grafts developed in concert with cells of the new location, with no memory of development at the site of origin. For example, when two pieces of early gastrula ectoderm were exchanged between sites in the prospective neural plate and epidermis regions, the pieces healed seamlessly into their new sites and developed according to the new location as epidermis or neural plate. At the time of exchange, they were "indifferent" as to their developmental outcome. However, similar grafts exchanged at the end of gastrulation, retained the development of the site of origin. They were no longer indifferent. Thus, if determination is defined as the capacity of a cell group for autonomous development at a new location in the embryo, then determination had occurred during the gastrula period. Most ectodermal and mesodermal parts of the early gastrula were composed of "indifferent" cells, which could pursue any of a variety of developmental paths depending on their location during gastrulation. Yet not all cells of the early gastrula could be indifferent. Which few cells of the embryo might provide the determinative influence? In the context

of inquiry of the 1918 grafting experiments, Spemann could conclude that the upper lip behaved differently from the other grafts in that it possessed an advanced state of determination, revealed by its self-differentiation. He furthermore suggested in 1918 that the upper lip might be the source of determinative influences, although he could not support the suggestion as yet.

The objective of the famous 1924 experiment was to distinguish which tissues of the secondary axis derived from the graft donor and which from the host, by using donor and host embryos from newt species with eggs of differing pigmentation. This was the critical means to identify which tissues of the secondary axis were self-differentiated by the graft and to recognize induction, if host cells should happen to be contained in the secondary axis and if those cells could be shown to have changed fate. Spemann was alert to the possibility of induction from his extensive work on the interactions of the optic cup with head ectoderm in lens formation (1901-1912). He has used the term "induction" as early as 1901, and the concept was generally known for interaction effects in other organisms (Oppenheimer 1991).

But if Spemann realized the importance of induction by the upper lip, why did he wait six years after 1918 to complete and publish the upper lip grafts in marked donors and host? This is all the more puzzling when it is realized that he had already worked out the methods of interspecies grafting in the 1918-21 period and had conducted a large set of other grafts. In 1921 he published extensive results, supported by pigmentation markers, to document that prospective epidermis of one species could indeed take on neural development in an embryo of another species if grafted to the prospective neural plate region. Hamburger (1988) has summarized evidence that Spemann delayed the crucial graft because he didn't think it would be informative. He held prior conceptions about how the upper lip must exert its determinative influence. He thought it remained on the surface of the embryo during gastrulation and exerted a spreading influence on nearby surface ectoderm (a planar induction?), directing it into neural development (see also Fässler, 1996; Steinbeisser, 1996). Then the lip material would just become part of the neural plate, he seemed to think. From this viewpoint, grafting didn't seem informative, for Spemann already knew that neural plates could be of mixed donor and host tissue. He apparently didn't consider the unlikely possibility that the upper lip would involute during gastrulation, that it could induce neural tissue from an internal position, and that it then differentiates to notochord, a tissue entirely different from neural tube. According to Hamburger, Spemann realized the importance of the interspecies grafting of the upper lip as he proofed the 1921 article. He then added a postscript to the effect that Hilde Mangold (nee Pröschooldt), in a single experimental case, had found a graft-derived notochord and a host-derived neural tube in the second axis. In this postscript, Spemann introduced the term organizer ("Organisator") as the part of the embryo which "...is able to set up an organization field of a certain orientation and extent when introduced in the midst of indifferent tissue." (translated by V. Hamburger)

As a graduate student, Mangold performed hundreds of upper lip transplants to the ventral margin of gastrula embryos in the 1921-24 period, but only 5 lived to post neurula stages to give useful results (Fig. 1). Infection was the problem. It was only in 1931 that Holtfreter introduced the use of sterile technique and a balanced salt solution instead of undefined pond water. Cross sections of two of the most complete secondary axes are shown in

Fig. 1. The Spemann-Mangold experiment by which they discovered the organizer and its inductive effects.

(A) The grafting plan. The donor embryo on the left is the early gastrula of a newt species with lightly pigmented eggs (*Triton cristatus*). The host embryo is an early gastrula of a newt species with darkly pigmented eggs (*Triton taeniatus* or *alpestris*). The upper lip of the blastopore (shown on the right side of the donor gastrula), which is the organizer, is surgically removed and inserted into an incision in the ventro-posterior blastocoel wall of the host, at the farthest location from the host's upper lip. Host tissue from the incision is discarded or put into the donor embryo in place of the upper lip. **(B) Five surviving embryos**, external views, from several hundred operations done in the 1921-24 period. These were the only ones to develop to post-neurula stages with differentiated axial tissues. Primary and secondary body axes are visible as dorsal midline grooves at the sites of closure of the neural tubes. Head parts are generally missing from the secondary axes. Embryo Um 8 is shown from two sides to view its two neural plates. The embryos are scanned and recomposed from Spemann and Mangold (1924). **(C) Trunk-level cross sections of two embryos with well-differentiated secondary axes.** Primary and secondary axes are designated by 1° and 2°, respectively. Tissues of the primary axis are entirely of host origin. Tissues of the secondary axis are marked g, or h, or g+h to indicate graft or host origin. Tissues of graft origin can be identified by their light pigmentation. Note that the host contributes mostly neural tube, somites, pronephros, and gut to the secondary axis, whereas the graft contributes mostly notochord and floor plate. Cross sections are scanned, modified, and relabeled from Spemann and Mangold (1924).

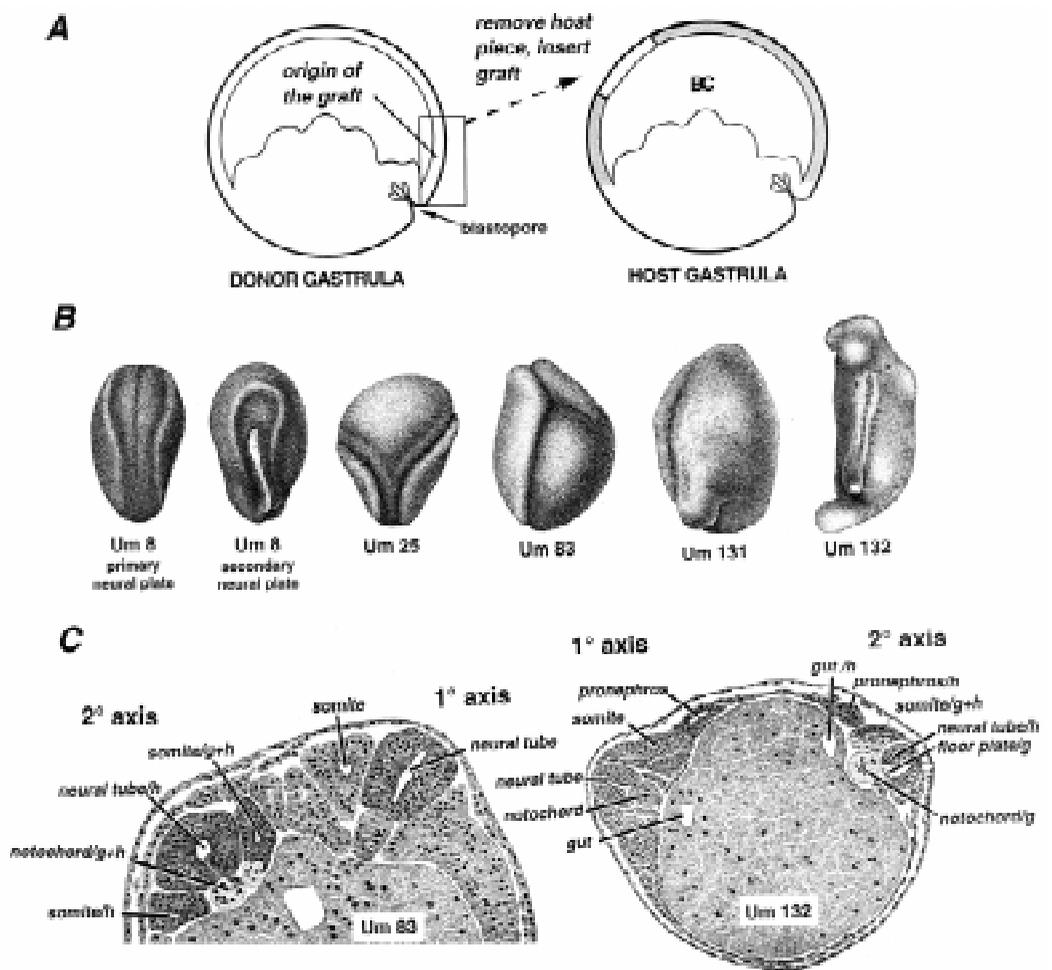


Fig. 1. The secondary axes uniformly lacked head structures anterior to the ear vesicle, a point to be discussed later. The axis had a neural tube, notochord, bilateral rows of somites, kidney tubules, and a gut lumen, well-proportioned and arranged in near normal organization.

In the 1924 paper, Spemann and Mangold could conclude that the graft always self-differentiated as notochord or as centrally located mesenchyme, and sometimes also as the floor plate of the neural tube and as minor amounts of somite and gut roof. The host tissue, though, gave rise to most of the neural tube tissue (sometimes even the floorplate), the kidneys, most of the somites, and the gut. Host derivatives of all three germ layers were present, and the majority of tissue of the secondary axis was derived from the host. The authors could therefore suggest that the graft had induced indifferent neighboring tissues of the host to form a variety of tissues that they otherwise would not, and had organized them into an axis of near-normal proportions and arrangement. Cells near the resident upper lip on the other side, or near the lip of a normal embryo, were deduced to gain their determination in the same way, depending on their distance from the resident lip.

When Vogt published his fate maps of urodeles in 1929, the interpretation could be strengthened regarding "changes of fate"

of host cells near the graft. In addition to the induced neural tube of the secondary axis deriving from prospective epidermis tissue near the graft, the induced somites could be recognized as derived from adjacent tissue that would have developed into lateral plate. Thus it was concluded that induction by the organizer did indeed lead to a change of fate, where "fate" is defined merely as developmental outcome, not as developmental predestination. Indifferent cells of the early gastrula didn't as yet have a determined state from which to change, but only a broad competence from which to take various alternatives, depending on conditions.

The conclusion about change of fate was questioned as recently as 1983 by M. Jacobson (1984) who pointed out that the Spemann-Mangold experiment with differently pigmented donor and recipient embryos did not eliminate the possibility that the secondary axis was formed by host cells that migrated over to the graft from their fate map location near the resident upper lip and then constituted the secondary axis without changing fate. (The organizer would still be exerting an organizing though not determinative influence, even in this scheme.) However, when lineage tracing experiments with macromolecular fluorescent dyes were done to distinguish parts of the host nearby or far from the graft, it

was found that the secondary axis indeed derived from host cells near the graft, as Spemann and Mangold assumed (Gimlich and Cooke, 1983; Jacobson, 1984). This was expected from a variety of old results, such as Holtfreter's experiments of combining excised prospective epidermis (no prospective neural cells present) with the upper lip explant in a "sandwich" induction situation, and finding that a portion of neural tube was induced in the ectoderm (Holtfreter, 1933). The conclusions about determinative influences and changes of fate have been affirmed from many experimental directions. As Spemann and Mangold (1924) wrote:

"A piece taken from the upper blastopore lip of a gastrulating amphibian embryo exerts an organizing effect on its environment in such a way that, following its transplantation to an indifferent region of another embryo, it there causes the formation of a secondary embryo. Such a piece can therefore be designated an organizer."

The discovery of the organizer marked a great advance in the understanding of chordate axis specification. During the gastrula period, the upper lip of the blastopore, the organizer, serves as a coherent source of inductive signals, reaching indifferent but competent cells of the three prospective germ layers, providing information used in determinative decisions regarding subsequent paths of development. It affects the development of at least half the cells of the gastrula and mediates the formation of the body axis with all its identifying chordate characters. "Induction" was found in 1924 to be the organizer's mode of action, just as it had previously been established for the lens (Spemann's previous work). The generality and importance of induction in cell determination was thereby established (see Oppenheimer, 1991), and searches for inductive interactions in many kinds of organogenesis soon followed. However, as discussed below, advances in the understanding of pattern formation and axis specification were to be eclipsed by interest in the actual mechanism of cell determination by induction.

Embryos without organizers

As a logical criticism of the Spemann-Mangold experiment, one could say that although their results showed clearly that a second axis can be induced when the artificial conditions are created of grafting an upper lip into the ventral side of a gastrula, how does one know that normal development depends on such inductive effects? Organizer removal is such a test. Spemann explored the development of organizer-less embryos by removing the two dorsal blastomeres of the four cell cleaved egg (see Spemann, 1938). These half embryos cellularized, gastrulated weakly, and formed a belly piece (bauchstück) with a limited variety of cell types. But since an entire half of the embryo had been removed, maybe more than the organizer was missing. Organizer removal is difficult at later stages in urodeles because the organizer seems to be reestablished from surrounding tissues. Again, almost half of the embryo must be ablated to eliminate organizer-related development.

Xenopus laevis, an anuran amphibian, is suitable for such experiments (Gerhart *et al.*, 1989). Table 1 shows some of the many methods to produce organizer-less embryos. These methods not only reveal the dependence of axis formation on the organizer but also allow insights into the steps of organizer formation. A block of any of the steps leads to a gastrula with a reduced or no organizer. For example, UV irradiation of the newly fertilized egg on the vegetal pole is effective because cortical rotation fails (as discussed later). The egg forms a full sized bauchstück, like Spemann's ventral half embryo. It cleaves on schedule and internalizes the marginal zone mesoderm and vegetal hemisphere endoderm by a ventral type of gastrulation. The blastopore closes to a point. The embryo does not elongate by convergent extension. It forms no neural plate, notochord, gill slits or tail, which are the chordate typifying traits. The embryo develops cell types of the kind normally found ventrally and posteriorly, namely, ciliated epidermis, coelomic mesoderm, blood cells, and posterior gut. It lives for several weeks digesting yolk and

TABLE 1

METHODS TO MODIFY ORGANIZER FORMATION AND FUNCTION IN *XENOPUS LAEVIS*

Developmental Outcome and Treatment Stage	Treatment
Ventro-posteriorized embryos (reduced or no organizer and Nieuwkoop center)	
Stage 6 oocyte	Deplete β -catenin mRNA. Deplete Frizzled mRNA.
Egg, first cell cycle	UV irradiate vegetal surface, or expose to cold, or pressure, or nocodazole to block microtubule formation needed for cortical rotation
2, 4, or 8- cell stage	Remove dorsal half, make double ventral embryo. Inject with mRNA for dnTcf, GSK3, axin, APC. Inject with mRNA for BMP2,4, BMPR, Smads.
Blastula	Excise Nieuwkoop center.
Gastrula	Excise Spemann's organizer. Inject sulfonated polycyclics (e.g. suramin) into blastocoel.
Dorso-anteriorized or twinned embryos (expanded organizer and Nieuwkoop center)	
Egg, first cell cycle	Treat with D ₂ O before cortical rotation to polymerize MT randomly. Centrifuge or tip eggs to displace cytoplasmic core in two directions. Transplant vegetal cortex to ventral side.
2, 4, or 8-cell stage	Surgically prepare double dorsal embryos. Inject with mRNA for Wnt1 or 8, Dsh, dnGSK3, β -catenin (stable form), GBP, CKII, Tcf. Inject ventrally with mRNAs for Noggin, Chordin, Cerberus, Dickkopf, inhibitory Smads.
16-cell to midblastula	Treat briefly with Li ⁺ before MBT. Graft in additional Nieuwkoop center.
Early gastrula	Graft in additional organizer.
Normal or rescued embryos (Nieuwkoop center and organizer: 60° of circumference)	
Stage 6 oocyte	Normal localization of VegT and β -catenin mRNAs to vegetal hemisphere.
Egg, first cell cycle	Normal cortical rotation. Centrifuge or tip rotation-defective eggs in one direction to displace cytoplasmic core. Graft in vegetal cortex.
2, 4, or 8- cell stage	Inject mRNA for β -catenin or its stabilizing agents, or for high levels of Nodals, into one side of rotation-defective or mRNA depleted embryos.
Blastula	Normal endo-mesoderm induction.
Early gastrula	Graft in Nieuwkoop center or organizer.

Abbreviations: APC, adenopolyposis coli protein; CKII, casein kinase II; dn, dominant negative form of a protein; GBP, GSK3 binding protein; BMP, bone morphogenetic protein; BMPR, BMP receptor; Smad, transcription factor of BMP pathway; Tcf, T cell factor.

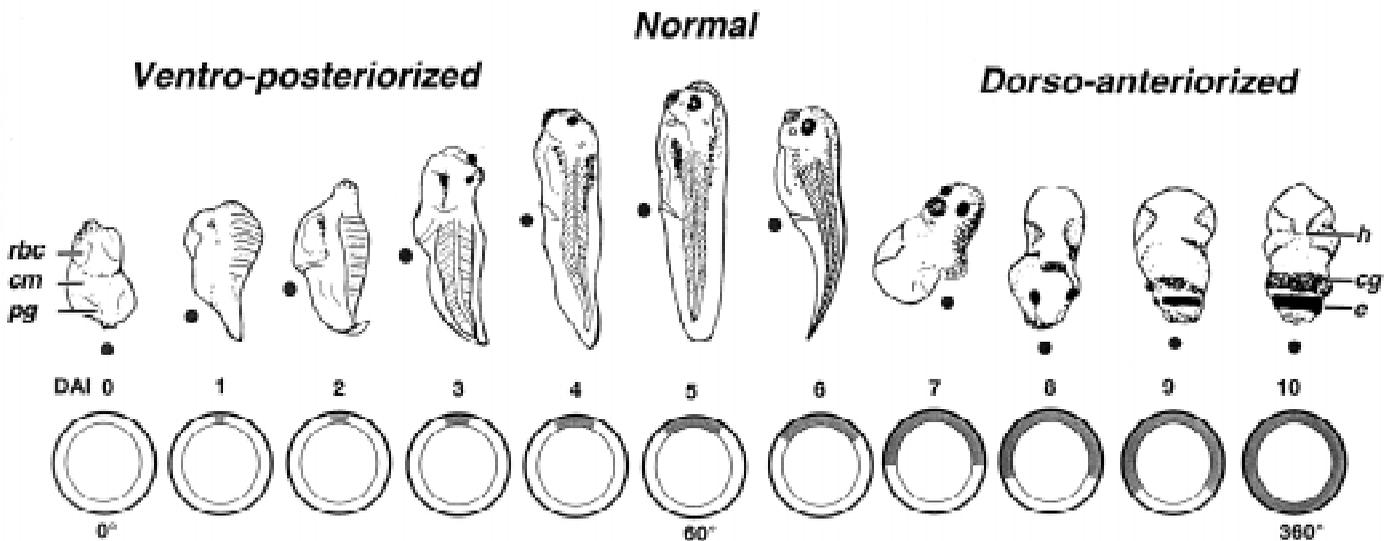


Fig. 2. Chordate axial organization depends on the size and composition of the organizer. (Upper row) The anatomies that develop from *Xenopus* gastrulae containing different amounts of Spemann's organizer in the marginal zone. Amounts can be experimentally controlled in a variety of ways listed in Table 1. DAI ("dorso-anterior index") grades from 1 to 10 are assigned to the anatomies. The drawings are modified from those of Kao and Elinson (1989), who defined the DAI scale. DAI 0 is a ventro-posteriorized limit form embryo with no axial mesoderm (notochord, somites), no gill slits, no neural tube, and no tail. It is cylindrically symmetric, and the old animal-vegetal axis remains the axis of the post-gastrula embryoid. DAI 5 is a normal embryo. Its anteroposterior axis is orthogonal to the animal-vegetal axis (see Fig. 6 for further detail). DAI 10 is a dorso-anteriorized limit form embryo with excessive neural tissue, notochord, and heart. It is cylindrically symmetric and the old animal-vegetal axis remains the axis of this post-gastrula embryoid. Intermediate DAI grades have intermediate anatomies. Dark circles indicate the position at which the blastopore closed. **(Lower row) The estimated amount of organizer in the marginal zone of the early gastrula** is shown for each DAI grade. The DAI 0 gastrula has no organizer. It has 360° of marginal zone mesoderm that would be inducible by dorsalizing signals from the organizer, if it had an organizer. DAI 5, the normal embryo, has a 60° of organizer and 300° of marginal zone mesoderm available for dorsalization by the organizer's signals. The DAI 10 embryo has 360° of organizer in its marginal zone but no responsive mesoderm for dorsalization. Hence it forms no somites. Estimates for the amount of organizer in DAI 1-5 have an experimental basis (Stewart and Gerhart, 1990). Estimates for grades 6-9 are approximate. Grade 10 is experimentally estimated (Kao and Elinson, 1989). cg, cement gland; cm, coelomic mesoderm; e, eye; h, heart; pg, posterior gut; rbc, red blood cells.

moving by cilia. At the gastrula stage, this embryo can be rescued by an organizer graft, or by combining half of it with a lateral half of a normal embryo, in which case it gains at least half an organizer (Stewart and Gerhart, 1990). A bilateral chordate body axis develops and the initially ventralized parts contribute extensively to it. At the early gastrula stage, a ventro-posteriorized embryo resembles 300° of the 360° circumference of the normal embryo, that is, everything except the organizer. The requirement for the organizer's function is revealed at and after gastrulation.

Furthermore, the quantity of organizer is related to the size and completeness of the body axis (Stewart and Gerhart, 1990). Normally the organizer is about 60° wide in the marginal zone of the *Xenopus* early gastrula. As the amount of organizer is reduced (cut vertically to reduce amounts of both the head and trunk-tail organizer regions), the anterior parts of the body axis fail to form. As shown in Fig. 2 (DAI 0-5) the brain and nasal pits are lost first, eyes next, the heart, otic vesicles, and midbrain next, then hindbrain, trunk somites, spinal cord, and the tail last. These incomplete axes serve as evidence that the size and composition of the organizer are essential for normal axis specification.

The organizer can also be made to develop excessively (see Table 1), by exaggerating or duplicating the steps of its formation. Dorso-anteriorized embryos are then formed. The DAI 10 embryo gastrulates early and vigorously, often with excessive convergent extension (but not always). It forms excess neural tissue (via a

highly modified neurulation), circumferential bands of cement gland and eye pigment close to the blastopore, a large central heart far from the blastopore, and a short gut. These are the tissues that are normally self-differentiated by the organizer or are induced by the organizer from ectoderm. Posterior neural tissue is missing. In the DAI 10 case, the organizer occupies the entire marginal zone, since as shown by Kao and Elinson (1988), several equi-spaced organizer grafts can be taken from a single dorso-anteriorized embryo (DAI>8), and all of these organize secondary axes in the ventral sides of normal gastrulae. In the absence of latero-ventral mesoderm, the organizer has nothing to dorsalize, and so the DAI 10 embryo differentiates no somites. The embryo also may not have gill slits, perhaps due to a lack of non-neuralized ectoderm. Intermediate forms (DAI 6-9) have enlarged heads, reduced trunks, and less or no tail.

Thus, the amphibian egg has the potential to form any amount of organizer from 0° to 360° of the marginal zone, depending on the orientation and intensity of the steps of organizer formation (Elinson, 1995). A remarkable range of anatomies is generated depending on the size of the organizer. The normal embryo has a balanced proportion of both organizer (60°) and non-organizer tissue (300°) in its equatorial marginal zone. The DAI series shows the importance of the organizer's normal constitution for normal axis formation. Deficiencies in the organizer lead to specific deficiencies in the body axis, in both the anteroposterior and dorsoventral dimensions.

Induction as a mechanism of determination

After the 1924 discovery of the organizer, interpretations soon varied widely about the information that it might transfer during its inductions to effect cell determination. Questions about the mechanism of determination by induction became equated with questions

TABLE 2

THREE PARTS OF THE ORGANIZER: DIFFERENCES OF MORPHOGENESIS, INDUCTIONS, AND SELF-DIFFERENTIATION

Subregion of organizer and Functions

Head organizer

Subregion of prospective prechordal mesoderm

Morphogenesis

- Spreading, migrating cell population

Induction

- Induces anterior neural plate by neuralizing induction (Chordin, Noggin, Follistatin, Frzb). Also induces *emx*, *otx* expression.
- Splits eye field (by *Shh* repression) and induces ventral brain region.
- Counteracts posteriorization? (Frzb?)
- Mesoderm: heart induction?

Differentiation

- to prechordal mesoderm (cranium floor, some jaw parts)

Subregion of pharyngeal endoderm

Morphogenesis

- Bottle cell apical contraction, then spreading of epithelial population

Induction

- Induces prechordal mesoderm beneath it?
- Induces gill slits in ectoderm and branchial arches in mesoderm.
- Induces epibranchial placodes in ectoderm, provides sites for neural crest immigration in branchial arches.

Differentiation

- to pharyngeal lining, gill pouches, and endostyle/thyroid

Trunk-tail organizer

Subregion of prospective chorda-mesoderm

Morphogenesis

- Convergent extending cell population

Induction

- Neuralizing induction of neural plate (Chordin, Noggin, Follistatin)
- Posteriorization of neural tissue (anterior) to hindbrain-spinal cord and prospective trunk neural crest with convergent extension and *Hox* expression (Wnts?).
- Dorsalization of mesoderm; induce somite formation in marginal mesoderm (Chordin, Noggin, Follistatin).
- Inductive prevention of head organizer (ADMP)
- Induce sclerotome from somites (*Shh*) and floorplate of neural tube (*Shh*)
- Induce pancreas/liver sites in endoderm (*Shh*?)
- Induce tail primordium at juncture of neural ectoderm, chordamesoderm, and marginal mesoderm (eFGF, *Shh*?)
- Induce hypochord from gut (*Shh*).

Differentiation

- to notochord

Subregion of prospective gut roof endoderm

Morphogenesis

??

Induction

- induces underlying chordamesoderm

Differentiation

- to gut roof and hypochord

Deep yolk endoderm

Morphogenesis

- Inrolling of yolk mass in gastrulation, cleft of Brachet formation

Induction

- Induce very anterior neural structures and cement gland (Dickkopf [anti-BMP]; Cerberus [anti-BMP and anti-Wnt])
- Inductive prevention of ectoderm conversion to mesoderm (Cerberus = anti-Nodal)
- Inductive prevention of posteriorization (Cerberus = anti-Wnt)
- Mesoderm: heart induction
- Endoderm: anteriorization of gut

Differentiation

- to liver and anterior gut

about whether the organizer really “organizes” the chordate body axis. In retrospect, this equation seems to have created some confusion. Insights into the scheme of patterning of the body axis are somewhat independent of insights into the actual mechanisms by which patterning is achieved. Spemann and Mangold (1924), who seemed to hold a deeper interest in patterning, wrote:

“The term ‘organizer’ (rather than, perhaps, determiner) is supposed to express the idea that the effect emanating from these preferential regions is not only determinative in a definite restrictive direction, but that it possesses all those enigmatic peculiarities which are known to us only from living organisms.”

The “enigmatic peculiarities” presumably included the organizer’s capacity for large scale organization of the body axis and for assimilating neighboring tissue seamlessly into normally proportioned structures integrated with the structures from its own self-differentiation. Spemann thought that the organizer’s architecture was important for its organizing function (although he did not imply that it contains a miniature representation of the body axis, to be expanded onto nearby cells by induction). Evidence for this view came not only from Spemann’s own experiments (1931) but also from O. Mangold’s (1933) analysis of inductions by fragments of archenteron roof (the transient location of the organizer’s chordamesoderm). From this analysis, the organizer seemed to contain a rich pattern of numerous different inducers arranged sequentially along its length, acting to induce the rich pattern of differences along the body axis. Spemann’s emphasis on architecture has indeed been borne out for understanding the patterning of the body axis, as discussed below, but was not essential for understanding individual determination events.

Instructive induction was one possibility for information transfer. It connoted a situation in which responding cells are largely unable to pursue a particular developmental path on their own (its determination), and in which the inducer introduces essential information for that path. An indifferent cell is inept but programmable. Permissive induction connoted a situation in which responding cells would carry extensive readiness for a path of development but are blocked (by themselves or others) from pursuing it. The inducer would simply lift the block, acting as a “releaser” (summarized by Oppenheimer, 1974). “Indifferent” here might mean the cell has many responses open to it but favors one until released from this weak bias by an external signal. The level of information transfer differs greatly in the two possibilities.

In the two decades after 1924, experimental evidence for instructive induction often seemed tantalizing but weak, while that for permissive induction seemed unattractive but strong. Holtfreter (1991), who was a major contributor to studies of the organizer in the 1930’s-1950’s, took a position distant from that of Spemann and built up evidence that the organizer did not have “...enigmatic peculiarities known to us only from living organisms”. He wrote:

“It would be entirely misleading to conceive of the organizer material as a kind of general manager which determines the destiny of the entire remainder of the embryo.” (Holtfreter and Hamburger, 1955).

He introduced many experimental advances (see Gerhart, 1997), and one of the first was sterile technique in the operations on amphibian embryos and a balanced salt solution (“Holtfreter’s solu-

tion") to replace undefined pond water. These modifications greatly increased the frequency of success in transplantations and widened the range of operations that could be done. He invented the sandwich technique in which designated small pieces of inductive tissue and responsive tissue are combined *in vitro* and scored for subsequent induction and development (Holtfreter, 1933). He incubated gastrula pieces in isolation in salt solution to assess their capacity for autonomous differentiation, nowadays called a "specification test".

On the issue of the organizer's indispensable architecture, he showed that dead and disorganized organizer tissue could still induce parts of the nervous system such as forebrain vesicles and eyes or nasal pits. The organizer's architecture wasn't needed for the ectoderm to generate this substantial organization. Next he showed that extracts from a wide variety of animals, tissues, and stages, could lead to inductions in amphibia (mostly neural inductions were scored), and hence the inductive materials are not exclusive agents of the upper lip of the blastopore of the embryo. These latter experiments led to attempts by several groups of researchers to purify inducers, and soon it was found that a variety of chemicals and extracts would work. From all this work, proposals about the importance of the organizer's architecture and about the uniqueness of its inducers were losing plausibility.

Waddington and Needham visited O. Mangold and Holtfreter in 1933 in Berlin to learn the bioassay methods and to try to purify inducers. Waddington (1932) had already proposed the term "competence" to connote the manifold responsiveness of embryonic cells to various inductors (although C. Stern [1999] has noted that Waddington later redefined his own term several times). This term gave emphasize to the poised multipotent state of responding tissue in the early gastrula. The indifference of cells reflected their multipotency, not their inadequacy. Holtfreter extended Spemann's tests of indifference and showed that the competence groups of the ectoderm, mesoderm, and endoderm of the embryo are very large, with only half the members eventually taking organizer-induced paths of development. Waddington (1954) later sought to remove the remaining vestiges of connoted instructiveness from inducers by introducing the terms "evocation" and "evocator" to replace induction and inducer, to make clear the readiness of responding cells to develop, and the inducer's mere calling forth or unleashing of this readiness.

The final blow to organizer structure and instructiveness perhaps came in 1941 when L. Barth found that gastrula ectoderm would develop along any of a wide variety of paths, when treated with pH extremes or high salt shocks of various kinds. He introduced a three step regimen of shock, recovery, and differentiation conditions, and produced brain parts, eyes, kidney, or muscle, to mention a few outcomes (Barth and Barth, 1974). Here the inducer was undeniably non-biological and unstructured (H^+ , OH^- , and ions), yet eventual tissues were locally structured, for example, a brain vesicle with an attached eye. Holtfreter repeated and extended Barth's results and devised an "autoneuralization model" in which the ectoderm possessed internal instructive agents for neural development, but was self-inhibited from using these for this development. Shock conditions, but also normal inductions, were seen as ones disrupting self-inhibition and releasing neural development (Holtfreter, 1948). Autoneuralization is close to the modern default model of neural induction (Weinstein and Hemmati-Brivanlou, 1999), except that current evidence favors an intercellular inhibition (via BMP2,4) rather than an intracellular self-inhibition. The intercellular inhibition can be upset by disaggregating cells with pH and ion shocks, whereas

Holtfreter thought the shock upset internal ionic balance (a "sublethal cytolysis" of the cell) and thereby disrupted intracellular inhibition.

As discussed elsewhere in this issue, inducers released by the organizer indeed block an anti-neural repression and pro-epidermal activation mediated by BMP signaling among competent responding cells. Inducers include Chordin, Noggin, Follistatin, Frzb, Xnr3, Cerberus, and Dickkopf. These insights are a great advance in the understanding of the mechanism of induction and also have import for understanding the organizer's role in patterning the chordate body axis.

In the 1924-1950 period, the pH and ion effects perhaps represented the greatest deviation of experimental results from Spemann's initial views of the importance of organizer structure and the "enigmatic properties of living organisms". However, when gastrula ectoderm fragments were treated with purified inducer proteins or shocked by pH extremes, global axial structure was not achieved. At the end of the long line of experimental analysis of the mechanism of determination via induction, the rather different question still remained as to the information the organizer provides as "an organizer" as opposed to as an inducer. This will be discussed later.

Organization and function of the organizer

Current evidence generally favors the view that the organizer has at least three parts differing in their inducers, their morphogenesis, and their self-differentiations. These are the head organizer, the trunk-tail organizer, and the deep yolk endoderm, all located in a 60-90° sector bridging the midline on the dorso-anterior side of the early gastrula. They have a particular arrangement relative to each other and to the competence groups surrounding them. Together they constitute approximately 10% of the cells of the early gastrula. As far as can be presently discerned, they jointly provide all functions of the organizer. O. Mangold's results (1933) from transplantations of small fragments of archenteron roof of the late gastrula in which he obtained many different locally specific inductions, seemed to suggest that the organizer had many more than three inductive subregions, but these results have been interpreted as the outcome of the responses of competent cells to different amounts and ratios of a few inducers distributed from a few organizer regions (Nieuwkoop *et al.*, 1952; Saxen and Toivonen, 1962).

Table 2 lists the differences of the three regions, to be discussed in turn. Spemann (1931) discovered the head and trunk-tail organizers. When taken from the early gastrula, the upper lip of the blastopore induces mostly head structures in ectoderm at the responding ventral site of grafting, whereas taken from the late gastrula, it induces trunk and tail structures in the same surrounding cells. During gastrulation the cellular composition of the lip changes with time as mesodermal and endodermal cells of the marginal zone invaginate, involute, and ingress at the lip and move inside. The early lip contains cells derived from the vegetal edge (ventral band) of the marginal zone, and the late lip contains cells derived from the animal edge (dorsal band), which is the limit of involution. The deep yolk endoderm part lies internal to the head organizer in the early gastrula (Sasai *et al.*, 1995).

The head organizer originates in the ventral band of the marginal zone and, after gastrulation, eventually differentiates into ventrally located head tissues such as the prechordal plate mesoderm (e.g., cartilaginous elements of the cranium floor, jaw, and gill slits) and pharyngeal endoderm (gill pouches and endostyle). Both its mesodermal and endodermal subregions seem important in morphogen-

esis and induction. The mesoderm subregion of the head organizer induces anterior head parts such as anterior neural plate (forebrain, midbrain), and gill slits. The neural induction is one of neuralization, currently thought to rely on the secretion of proteins antagonizing BMP and Wnt signaling, such as Chordin, Noggin, Follistatin, and Frzb. Neuralized tissue develops to forebrain and midbrain, and *emx* and *otx* genes are expressed there, but not the Hox genes. The same signals may operate in the head organizer's dorsalization of nearby mesoderm (to parachordal anterior mesoderm?), since the competence of mesoderm cells to respond differs from the competence of ectodermal cells. The endodermal portion of the head organizer induces gill slits in ectoderm, though little is known about this induction. *Xnr3* is secreted mostly by this subregion.

As shown in Table 2, the head organizer region is the site of expression of genes for particular transcription factors, namely, *gooseoid*, *HNF3b* (pintallavis), and *Lim1* genes, and of genes for secreted signaling proteins such as Chordin, Noggin, *Xnr3*, Follistatin, and Frzb (Harland and Gerhart, 1997; Lemaire, 1998). As noted above, *xnr3* expression is limited to the endodermal layer. The morphogenesis of the head organizer consists of two kinds. The mesoderm migrates as loose cells clumps along the blastocoel wall, with combined underlapping lamellapodia of several cells tracking along oriented fibronectin strands (Nagel and Winklbauer, 1998). The endoderm engages in bottle cell formation at the time of lip formation. This cell shape change entails a 50 fold apical contraction of the surface area facing the external medium. The cells, which comprise the leading part of the archenteron, later relax and spread in the pharyngeal region.

The trunk-tail organizer originates in the dorsal band of the marginal zone and, after gastrulation, eventually differentiates into dorsal mesoderm, particularly the notochord. The endodermal subregion differentiates to the gut roof. The morphogenesis of the trunk-tail organizer is particularly distinctive. The cell population engages in convergent extension, eventually narrowing from a rectangular array about 20 cells wide and long and several cell layers in thickness, eventually to a rod, one cell thick (Shih and Keller, 1992). In the course of this morphogenesis it pushes across the embryo to the other side of the marginal zone, bringing its secreted inducers to regions of the embryo initially too far away. In axial patterning, it acts as an elongating signal source.

The trunk-tail organizer induces several kinds of development in nearby ectodermal, mesodermal, and endodermal competence groups. In the ectoderm, it induces the middle and posterior part of the neural plate, the parts differentiating to hindbrain, spinal cord, and trunk neural crest derivatives. These are the regions of Hox gene expression. At least two signals are thought to be involved in the two-step neural induction (Nieuwkoop *et al.*, 1952). One is a neuralization signal, probably a BMP antagonist as in the head organizer. The second is a posteriorizing agent, yet to be identified but suspected to be a Wnt or FGF member, that transforms neuralized tissue to posterior neural development, namely, hindbrain and spinal cord (McGrew *et al.*, 1995; Lamb and Harland, 1995). Additionally the trunk-tail organizer, as it elongates to the notochord, induces the neural tube to form the floor plate in the sector closest to it. Nodal is the signal.

In the mesoderm competence group (the dorsal band of the marginal zone) the trunk-tail organizer induces trunk somite formation. The BMP antagonist signal of neuralization may also mediate this dorsalization of mesoderm. Additionally the trunk-tail organizer, as it elongates to the notochord, releases Shh which induces the

spherical somites to form the sclerotome portion on the side nearest the notochord. In the endoderm competence group, the trunk-tail organizer induces the hypocoel from the gut roof (Cleaver *et al.*, 2000). Finally in a combination of ectoderm and mesoderm competence groups, it induces the proliferative tail bud, in which growth and inductions continue for many weeks. eFGF and Shh are thought to act among the inducers of the tail bud. The tail eventually contains 30 or so somites, many more than in the trunk, as well as a longer section of notochord and neural tube.

The trunk-tail organizer is the site of expression of genes for the transcription factors Not2 (Xnot2), Lim1, and Xbra and for the secreted proteins Noggin, Chordin, Follistatin, ADMP, Shh, Nodal and eFGF. The *Xbra* gene is initially expressed at the border of the head and trunk-tail organizer regions at early times, perhaps within the trunk-tail region (Zoltewicz and Gerhart, 1997). Some of the locally expressed gene products are known to have roles in suppressing other organizer regions. For example, Gsc of the head organizer represses *Xbra* of the trunk-tail organizer, whereas Xbra and Lim1 of the tail organizer together repress *gsc*. The secreted ADMP protein of the trunk-tail organizer inhibits head organizer formation (Dosch and Niehrs 2000). Thus, the organizer subregions have means to pattern themselves as adjacent but different.

The third part, the deep yolky endoderm, is the most recently found (Bouwmeester *et al.*, 1996) and least characterized. It was probably not included in the original Spemann-Mangold grafts or in those of most later researchers. It has been difficult to demonstrate its inductions by grafting it alone (Bouwmeester *et al.*, 1996). Its effects are subtle. It probably induces heart from the ventral band of marginal zone mesoderm (Schneider and Mercola, 1999), and anterior neural tissue and cement gland from ectoderm, perhaps by working in concert with the head organizer. It seems to block posteriorization of neural tissue, and this is an important contribution to patterning (Brickman *et al.*, 2000). It anteriorizes endoderm. It eventually differentiates as liver and anterior gut. It locally expresses the *HEX* gene, and those for the secreted proteins Cerberus and Dickkopf (Bouwmeester *et al.*, 1996; Glinka *et al.*, 1998; Jones *et al.*, 1999; Schneider and Mercola, 1999). The region has been identified early as a HEX expressing column which forms centrally in the vegetal yolky endoderm of the blastula, even in ventralized embryos, and then moves to the side of the organizer in normal embryos, while staying central in ventralized cases. This part has a morphogenetic role. It engages in changes of cell affinity that mediate the formation of a cleft ("cleft of Brachet") between the marginal zone and deep cells at the start of gastrulation, and probably exert an inrolling action important for internalizing the vegetal yolk mass (Winklbauer and Shurfeld, 1999)

In conclusion, the organizer has at least three qualitatively different parts distinguishable by their local gene expression, secreted inducers, kinds of morphogenesis, and eventual self-differentiations. All three are important in concert for the specification of a complete body axis. The head and trunk-tail organizer parts share some properties of gene expression and secreted inducers, but the trunk-tail part clearly has additional properties of its own. The deep yolky endoderm region may serve an enhancing role to the head organizer, and may add some inductions of its own, such as heart induction and anti-posteriorization. All three germ layer competence groups are affected by the inductions. Spemann's expectation that the organizer has structure, and that the structure is important to its function is in part fulfilled, as shown by the organizer ablation and grafting experiments.

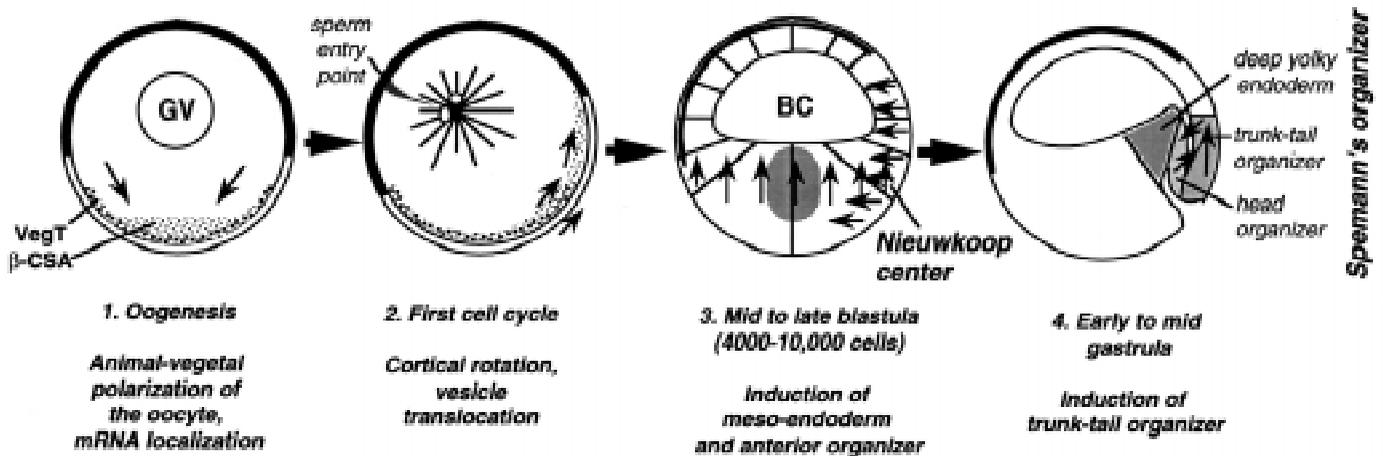


Fig. 3. Four steps in the formation of the organizer. (Step 1) Oogenesis. A β -catenin stabilizing agent (β -CSA, fine dots) is translocated to a small spot on the vegetal pole cortex. *VegT* mRNA (*VegT*, coarse dots) is later translocated to the vegetal cortex over a broad area. **(Step 2)** The first cell cycle after fertilization. The β -catenin stabilizing agent, which is associated with vesicles, moves along microtubules that are arranged as parallel tracks close to the cortex, with their plus ends pointed to the right as shown. The agent moves from the vegetal pole to the equator on one side. The Nieuwkoop center and organizer will later form there. Where the stabilizing agent is deposited, high β -catenin levels persist until the mid blastula stage. Maternal *VegT* mRNA remains broadly distributed throughout vegetal cells and symmetric about the animal-vegetal axis. **(Step 3)** Mid and late blastula period: gene expression starts and meso-endoderm induction begins, mediated by secreted Nodal signals from cells containing maternal *VegT*. Cells containing high β -catenin secrete *Xnr3* and perhaps other proteins. The head organizer is induced in ventral band cells receiving both Nodal and *Xnr3*, and perhaps also in cells receiving high Nodal doses from the Nieuwkoop center, the organizer of the organizer. The deep yolkly endoderm portion of the organizer arises in the central vegetal yolk mass (hatched region) and moves toward the right side where the head organizer is begin induced. It may assist head organizer formation. **(Step 4)** Cells of the dorsal band of the marginal zone mesoderm are recruited into the trunk-tail organizer by a spreading induction about which little is known. The head organizer may initiate the recruitment, and the trunk-tail organizer may continue the recruitment once a part of it is formed. The three parts of Spemann's organizer are shown. GV, germinal vesicle of the oocyte (the nucleus); BC, blastocoel.

The Nieuwkoop center as “the organizer of the organizer”

How does the three part organizer gain its organization in the pre-gastrula stages of development? There are at least 4 steps in the formation of the amphibian organizer in the period from oogenesis to gastrulation, as shown in Fig. 3. In amphibia, the steps involve both induction and cytoplasmic localization, as summarized in the next two sections.

As Nieuwkoop has pointed out, embryologists assumed for many years that mesoderm, like the ectoderm and endoderm, must arise from a lineage of cells specified by special cytoplasmic materials localized in the egg (Nieuwkoop, 1997), as if no germ layer could arise by induction. Nieuwkoop examined the assumption of autonomous mesoderm formation by a series of ablation and recombination experiments, which led to the discovery of “meso-endoderm induction”. The organizer is formed as an aspect of this induction. Nakamura in Japan also explored the question of the “organizer of the organizer” and did similar experiments, which he interpreted according to a double gradient model rather than as induction (Nakamura, 1978).

As shown in Fig. 4, Nieuwkoop removed equatorial cells from a mid blastula newt embryo, those which autonomously develop mesoderm (a point discussed later), and showed that neither the animal cap piece nor the vegetal base piece could develop mesodermal derivatives on its own. Then he combined the cap and base pieces, and they developed substantial endoderm and mesoderm including tissues derived from and induced by the organizer, such as the notochord, prechordal plate, neural tube, somites, pharyngeal endoderm, and tail parts (Nieuwkoop, 1969a,b). By lineage tracing and ^3H -thymidine labeling, he and his colleagues showed that the

mesoderm and pharyngeal endoderm derive exclusively from the animal cap portion of the recombinant (Nieuwkoop and Ubbels, 1972), the portion which is composed of cells descended from blastomeres of the upper quartet of the 8-cell embryo. The vegetal part of the recombinant was the source of the inductive signals.

This was the first evidence that all mesoderm and some endoderm could be formed by an inductive interaction between cells of the two polar parts. He called this induction “meso-endoderm induction” (Nieuwkoop *et al.*, 1985). Based on the variety of neural tissues in the differentiated recombinants, both the head and trunk-tail parts of the organizer had been formed by induction. Dorsalization of the mesoderm (to give somites) by the organizer also occurred, as did anteriorization of the gut.

Boterenbrood and Nieuwkoop (1973) then explored the inductive capabilities of parts of the vegetal hemisphere cells by cutting the hemisphere into dorsal, lateral, and ventral quarters, as shown in Fig. 4. Lateral and ventral quarters, when combined with animal cap ectoderm, induced lateral and ventral mesoderm (red blood cells, coelomic mesoderm, germ cells [only in urodeles, not anurans]), but did not induce the organizer, as deduced from the absence of differentiated notochord and neural tube. At best, minor amounts of somite tissue were formed, perhaps reflecting a weak dorsalization. The dorsal quadrant, however, induced animal cap cells to form not only blood and coelomic mesoderm, but also the organizer, as judged by its self-differentiated and induced derivatives (Fig. 4C). A notochord did form, and neural induction of brain and spinal cord occurred. Thus the dorsal quadrant demonstrated its special properties as the inducer of the organizer. This region has been called, the “organizer of the organizer” (Nakamura, 1978), or the “Nieuwkoop center” (Gerhart *et al.*, 1991).

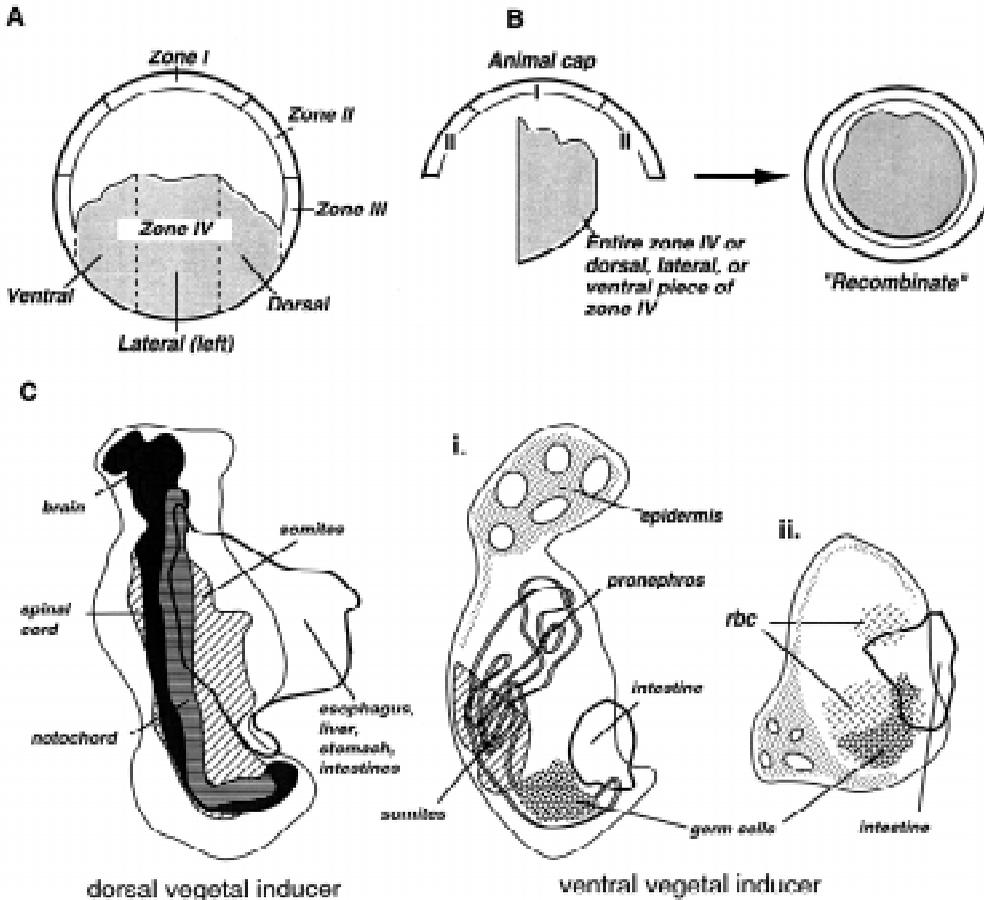


Fig. 4. The Nieuwkoop experiment.

The results allowed Nieuwkoop (1969a) and Boterenbrood and Nieuwkoop (1973) to conclude that mesoderm and pharyngeal endoderm are formed from animal hemisphere cells by meso-endoderm induction from vegetal cells in the blastula period, and that the organizer is induced by the dorsal vegetal quadrant. (A) shows the origin of the inductive and responsive cells in a newt or axolotl (urodele) mid-blastula embryo, in cross section. The embryo is divided into 4 zones, the polar animal cap (zone I), the rest of the animal cap (zone II), the sub-equatorial margin (zone III), and the vegetal base (IV). Zone IV is further divided into dorsal, lateral, and ventral quadrants. When the individual zones are allowed to develop in isolation for 10 days, zones I and II develop ciliated epidermis, zone III develops some mesoderm, both dorsal and ventral, though less than expected from the fate map, and zone IV remains as yolk cells ("endoderm"), with little identifiable differentiation. (B) shows the surgical preparation of various recombinates of zones. Zones I and II are combined with the entire zone IV or with quarters of IV. They heal into a ball with the yolk mass inside. (C) shows the meso-endoderm in 3 embryoids developed from the recombinates after 10 days, in cross section. The embryoid on

the left developed from a recombinant containing the zone IV dorsal quadrant. It contains abundant dorsal and ventral mesoderm and pharyngeal endoderm, none of which was developed by zone I, II, or IV alone. The embryoid contains a notochord, a neural tube, and abundant somites, evidence that it formed an organizer and that the organizer functioned in induction. Lineage labeling showed that the mesoderm and pharyngeal endoderm derived from zones I and II. Zone IV was the source of inductive signals. The center and right embryoids (i and ii) developed from recombinates containing the zone IV ventral quadrant. The right form is more typical. Both developed germ cells and posterior intestine. The center case (i) also developed pronephros and a small amount of somite muscle. The right case developed abundant red blood cells. Note the absence of notochord and neural tube, indicating little or no organizer. Modified from Boterenbrood and Nieuwkoop (1973). rbc, red blood cells.

Initially Weijer *et al* (1977) suggested that the dorsal quadrant exerted its patterning effect quantitatively as a high point of an inducer gradient. The lateral and ventral quarters were considered to release the same inducers, just less in amount. Slack and his colleagues (Smith and Slack, 1983; Dale and Slack, 1987), on the other hand, proposed that the dorsal quadrant differs qualitatively from the others, providing a unique second signal in a three signal model of mesoderm patterning, in which the lateral and ventral quadrants provided a different "first" signal. (The subsequent dorsalization of mesoderm by the organizer was seen as the third of three signals). Later Kimmel *et al* (1992) suggested that the dorsal quadrant was both alike and different: it shared a general mesoderm inducer with other quadrants but possessed a unique enhancer or "competence modifier" that locally acted in combination with the general inducer for organizer formation. These interpretations are not mutually exclusive, as discussed later.

The Nieuwkoop center was also demonstrable in experiments to rescue ventro-posteriorized embryos by grafts of certain blastomeres. Gimlich and Gerhart (1984) blocked cortical rotation with UV irradiation, thereby preventing formation of the Nieuwkoop center and the organizer. Then at the 64 cell stage they grafted into

the compromised embryo a pair of dorsal vegetal blastomeres from a normal embryo, replacing two of the embryo's own vegetal blastomeres. The operated embryo was extensively rescued. It developed almost normally. Based on the completeness of its body plan, it must have formed an organizer which induced neural tube and somites, and differentiated dorsal mesoderm. When graft blastomeres were filled with a high molecular weight lineage tracer (too large to pass through gap junctions of the rescued embryo) before grafting, the rescued tadpole was found to contain fluorescent descendent cells only in its yolky gut endoderm. None was in the dorsal axial tissues. Therefore, these axial tissues, which included the organizer's self-differentiations, must have been induced in one or more steps by the grafted vegetal cells. The graft blastomeres had served as a Nieuwkoop center inducing an organizer. Gimlich (1986) later showed that certain equatorial cells could also rescue axis development when grafted into a 64 cell ventralized host. Many induced axial parts were detectable. However, since descendants of these cells actually form part of the organizer and axis, it was more difficult to distinguish induction from self-differentiation. Kageura (1997) further showed that even certain dorsal animal hemisphere blastomeres, when transplanted

to the ventral side at the 32 cell stage, can have later organizer-inducing effects, a result that would seem to contradict the vegetal location of the Nieuwkoop center. The questions remained, then, how extensive is the Nieuwkoop center and how does it act to induce the organizer? These questions will be discussed after a summary of steps by which the Nieuwkoop center itself is formed.

As part of meso-endoderm induction of the organizer's complexity, it must be noted that marginal zone mesoderm arising from this induction is not uniform in the animal-vegetal dimension. The zone has two circumferential bands. The ventral band (the more vegetal of the two) develops mostly to coelomic mesoderm (lateral plate), heart and blood, ventral tissues in the tadpole. The dorsal band develops mostly to somites, dorso-lateral tissues. This difference has relevance to the organizer because the bands divide it. The head organizer comes from the ventral band (and its differentiations are mostly ventral) and the trunk-tail organizer from the dorsal band. What makes the two bands different? Kimelman *et al.* (1992) and Lane and Smith (1999) have proposed that FGF signaling along the animal-vegetal axis of the egg (maternal and then zygotic) is needed to establish the dorsal band of the marginal zone. The expression of *bra* is part of the response and differentiation of this band. When FGF signaling is blocked by a dominant negative FGF receptor, the dorsal band fails to become different from the ventral band. The ventral band may depend only on Nodal signaling for its formation and may be repressed by FGF.

Although Nieuwkoop showed that the induction occurred in the mid and late blastula stage and was gone by the early gastrula stage, he did not test or suggest how early it occurred. Because recombinates could be made early, it was assumed that the induction occurred before the mid blastula stage (4000 cells) when zygotic gene expression and therefore that it exclusively involved maternal secreted proteins. Jones and Woodland (1987) obtained some evidence that induction might begin as early as the 32 cell stage. However, this assumption has been called into question recently, as discussed below.

Although meso-endoderm induction can be shown experimentally in recombinates, is it normally needed? The answer is substantially yes. Fate maps show that most mesoderm, including all the prospective notochord (that is, the trunk-tail organizer), derives from progeny cells of the upper quartet of the 8 cell stage cleaved egg (Vodicka and Gerhart, 1996). Since these blastomeres and their descendents are incapable of mesoderm development on their own, they seem entirely dependent on meso-endoderm induction. However, blastomeres of the vegetal quartet, which give rise to the lower part of the marginal zone (the ventral band) including the head organizer, are autonomous for mesoderm formation. The amount of mesoderm, though, is less than expected from the fate map (Nieuwkoop, 1969a, Gurdon *et al.*, 1984). These cells probably rely on meso-endoderm induction. This is argued from the fact that if TGF- β signaling is blocked by a dominant negative activin receptor, mesoderm formation fails here as well as elsewhere in the embryo, as if they must secrete and receive inductive signals (Hemmati-Brivanlou and Melton, 1992). As discussed below and elsewhere in this Volume, Nodal signals (*Xnr1*, *2,4* and *Derriere*) are probably the secreted agents of the induction, and they probably signal through a receptor similar to the activin receptor. The vegetal cells behave autonomously because they contain all the components needed to induce meso-endoderm among themselves.

In conclusion of this section, meso-endoderm induction clearly generates considerable organization in the blastula, mostly along

the egg's animal-vegetal axis but also on one side of the embryo where the organizer resides. Some authors have suggested that most patterning occurs by this induction, and that morphogenesis at gastrulation just puts the different regions in place (Weijer *et al.*, 1977). Since the organization of the late blastula seems already so extensive, we will later ask what the organizer's inductions add to blastula organization.

Formation of the Nieuwkoop center

How does the Nieuwkoop center become established in just one quadrant of the blastula? In the first cell cycle the egg undergoes a cortical rotation which is intrinsically asymmetric and transforms animal-vegetal cylindrical symmetry into bilateral symmetry of a first kind (Gerhart *et al.*, 1989; Elinson, 1995). After rotation, the egg's cytoplasmic contents are no longer equivalent around the equator. A new axis has been established orthogonal to the animal-vegetal axis. This axis is usually called a dorsoventral axis, but Lane and Smith (1999) point out that it might more accurately be called an anteroposterior axis of the mesoderm, with the anterior pole at the side where the organizer will form. The identity of this axis is difficult to decide, and the inquiry raises questions, which we think leads into the subject of the evolution of the chordate body axis. As discussed later, the chordate anteroposterior axis may have been a dorsoventral axis in a pre-chordate ancestor. In the meantime we will use a compromise terminology of dorso-anterior and ventro-posterior to refer to the poles of this new axis emplaced by cortical rotation.

Cortical rotation entails the translocation of the cortical layer of egg cytoplasm relative to the deep cytoplasmic core. It orients elongating microtubules into a parallel array in a subcortical layer, and the plus ends of the microtubules point in the direction of rotation (Elinson and Rowling, 1988). The parallel array then serves as a set of tracks on which maternal materials move from the vegetal pole to the equator of the egg on one side. The orientation of the array is unique, one of the innumerable possibilities in the 360° of circumference, any one of which the egg could take. Array formation is thought to succeed because of reciprocal positive feedbacks between cortical movement and microtubule growth. The direction of movement of the cortex depends on the vector summed directions of the growing microtubules, and the direction of elongation of microtubules depends on the single direction of movement of the cortex (Gerhart *et al.*, 1989). These feedbacks give a self-organizing quality to array formation. Normally the formation is biased in a direction aligned with the point of sperm entry, but even artificially activated eggs form a functional array, presumably aligned with some small random departure from cylindrical symmetry.

When completed, the array is a thin mat (4-8 μm thick) at the interface of the core and cortex, approximately 4 μm internal to the plasma membrane. The minus ends of the microtubules are embedded in the core, and the microtubules bend over at the cortex. The array appears only once in embryogenesis, at 40 min after fertilization, and disappears at 90 min, within the 100 min first cell cycle. Its function seems to be entirely that of tracks for the translocation of cytoplasmic components in the uncleaved egg. Agents that disrupt or prevent microtubule formation, such as UV irradiation of the vegetal pole, or cold, or pressure, or nocodazole applied in the first cell cycle, prevent the array and hence prevent the translocation of maternal materials. Such an egg remains cylindrically symmetric and develops as a ventro-posteriorized embryo, unable to establish a Nieuwkoop center and an organizer.

Whereas the cortex moves slowly (7 $\mu\text{m}/\text{min}$, kinesin dependent) over the array toward the plus ends of the microtubules, maternal materials such as vesicles move rapidly in the same direction (25 $\mu\text{m}/\text{min}$, kinesin dependent). While the cortex moves about 30° of arc, the vesicles move 90-120°, reaching even into the animal hemisphere on one side of the egg (Rowning *et al.*, 1997). Among the observed moving vesicles are presumably the maternal dorso-anteriorizing materials that will persist at their new unilateral location for 6 hrs until zygotic transcription begins at the 4000 cell stage. Evidence that these materials are initially located at the vegetal pole includes:

- 1) Irradiation of the vegetal pole of the full grown oocyte, but not elsewhere, inactivates a material such that a fertilized egg derived from the oocyte is ventro-posteriorized even though it still undergoes cortical rotation (Holwill *et al.*, 1987; Elinson, 1995).
- 2) A fragment of cortex cut from the vegetal pole of a newly fertilized egg, but not from other regions of the surface, can be inserted into the equatorial level of normal eggs on the ventral side and will cause secondary axis formation. The same effect can be obtained with cytoplasm micropipetted from the vicinity of the vegetal cortex (Yuge *et al.*, 1990; Kageura, 1997).

Most illuminating in the search to identify the critical maternal materials have been experiments with injected mRNAs encoding various ligands and intermediates of the Wnt signal transduction pathway. β -catenin, which is a key intermediate of this pathway, is continuously degraded by proteolysis after phosphorylation and ubiquitination, and is continuously formed by translation of uniformly distributed maternal mRNA. Its level in an unsignaled cell is very low. Signaling leads to the inhibition of phosphorylation and breakdown, and so β -catenin accumulates in the cytoplasm. It complexes with the Tcf transcription factor (Molinaar *et al.*, 1996), and the complex activates transcription of genes not affected by Tcf alone (which acts as a repressor in combination with groucho protein).

As McMahon and Moon (1988) discovered, Wnt1 mRNA injected on the ventro-posterior side of normal eggs shortly after fertilization suffices for subsequent formation of a secondary axis which is as complete as the primary axis and as complete as any developed from a Spemann-Mangold organizer transplant! Clearly the injected embryo has developed a second Nieuwkoop center and second organizer on the injected side. Various pathway intermediates have axis-promoting or suppressing effects (see Table 1) consistent with their positive or negative action in Wnt signal transduction (reviewed by Moon and Kimelman, 1998). Thus, any component that increases β -catenin levels on the ventro-posterior side leads to twinning (or to rescue of a ventro-posteriorized embryo). These components include excess normal β -catenin or a mutationally stabilized form of it, GBP (the GSK3 binding protein), disheveled (Dsh), dominant negative GSK3, and Wnt ligands. In the opposite direction, any component that reduces β -catenin levels on the dorso-anterior side leads to ventro-posteriorization. These agents include excess GSK3, CamKII, depletion of β -catenin mRNA from oocytes by complementary oligonucleotide/RnaseH treatment (Wylie *et al.*, 1996), and a dominant negative Tcf which binds β -catenin but cannot interact with DNA to enhance transcription. Embryos ventro-posteriorized by such injections seem to lack an organizer, just as do those produced by UV-disruption of microtubules in the first cell cycle or by organizer ablation from the late blastula. These results suggest that β -catenin-stabilizing agents are the maternal materials normally stored at the vegetal pole in association with vesicles and then transported to one side on microtubules. Consistent with these effects, it has been found

that β -catenin indeed accumulates on the prospective dorso-anterior side of normal eggs by the end of the first cell cycle (Rowning *et al.*, 1997).

Disheveled (Dsh), which normally acts in the Wnt signaling pathway to stabilize β -catenin, also accumulates on the dorsal side in the first cell cycle (Miller *et al.*, 1999) and associates with vesicles that move along microtubules of the parallel array. It remains uncertain whether Dsh is itself initially localized at the vegetal pole or whether some agent upstream of Dsh is there. Sumanas *et al.* (2000) recently reported that eggs, obtained from oocytes in which Frizzled 11 mRNA has been depleted, develop to ventro-posteriorized embryos. This finding suggests that receptor activity is needed for β -catenin stabilization and axis formation. In light of this result, it seems plausible that a vesicle-associated Wnt ligand of unknown identity is localized at the vegetal pole. However, Dominguez and Green (2000) have found that GSK3 protein is destroyed on the dorso-anterior side of normal embryos, an effect not consistent with the known steps of Wnt signaling but simulated by injections of GBP protein. Therefore, they suggest that β -catenin is normally stabilized by agents which may not be Wnt pathway intermediates, even though such intermediates can affect β -catenin levels.

After β -catenin accumulates on the dorso-anterior side during the first cell cycle, it persists there until the midblastula transition when gene expression starts (Schneider *et al.*, 1996; Larabell *et al.*, 1997). Thereafter, the β -catenin/Tcf complex activates transcription of the *xnr3*, *siamois*, and *gsc* genes, and perhaps others. The complex also acts in concert with other transcription factors, such as VegT and Smads, as discussed later. Among the zygotic proteins, Xnr3 is secreted by β -catenin containing cells, many of which are members of the Nieuwkoop center. As a concordance test, Marikawa *et al.* (1997) found that genes activated in the cellularized animal pole region by β -catenin injection are also activated there by injections of vegetal pole cytoplasm, namely, *siamois* and *xnr3*. Also, the *bmp4* gene is repressed in the animal pole region by both kinds of injections. Thus, it seems likely from several lines of experimental inquiry that the dorso-anterior side of the egg is differentiated from other regions of the blastula by persistent high levels of β -catenin protein.

As currently understood, the formation of the Nieuwkoop center requires two inputs, of which β -catenin is only one. Meso-endoderm induction is also involved, and this induction requires a separate localized maternal material. Zhang *et al.* (1998) and Kofron *et al.* (1999) have recently found that oligonucleotide-mediated depletion of the *Xenopus* oocyte for a maternal mRNA encoding the VegT transcription factor (a T box family member), yields eggs that are severely deficient in meso-endoderm induction. VegT mRNA is normally localized to the entire vegetal cortex during oogenesis (Lustig *et al.*, 1996; Stennard *et al.*, 1996; Zhang and King, 1996). It partitions to cells cleaved from that region and is translated during cleavage. At the midblastula transition, VegT protein normally activates transcription of genes encoding several secreted signaling proteins, Nodal 1, 2, 4 and Derriere (all TGF β signals), which then induce meso-endoderm in animal hemisphere cells, as discussed elsewhere in this Volume. When VegT mRNA is depleted in the oocyte by oligonucleotide injection, the Nodal inducers are not produced later in the embryo. Then, organizer formation, which is an aspect of meso-endoderm induction, fails in these embryos (Kofron *et al.*, 1999).

The spatial pattern of meso-endoderm induction within the blastula depends first on the distribution in the egg of maternal transcrip-

tion factors and co-factors, namely, VegT and β -catenin, and second on the distribution in the late blastula of zygotic secreted inducer proteins, such as Nodals and Xnr3, encoded by genes activated by those factors. This is a new view of meso-endoderm induction, for just a few years ago it was thought that maternal (not zygotic) secreted proteins are the main inducers. Veg1, activin, and FGF had seemed strong candidates. Now their role in meso-endoderm induction is unclear. Veg1 may be a participant in endoderm formation and a co-contributor to meso-endoderm induction. FGF may participate in the patterning of the marginal zone, as noted above.

In light of this new molecular information, what and where is the Nieuwkoop center and how does it induce the 60°-wide, three part organizer in the marginal zone on one side? In devising tentative answers to these questions, we should recall that the center is defined as that part of the blastula stage embryo that induces the organizer (Fig. 5). Also, we should recall that all cells of the animal hemisphere and marginal zone are competent to be induced to membership in the organizer. This competence is shown by the fact that animal cap cells, that contain no VegT or β -catenin, can be grafted into the prospective organizer region at the blastula stage and do become part of the organizer (Gerhart *et al.*, 1991). The only non-competent cells seem to be those of the vegetal pole region (Fig. 5), where high levels of VegT protein activate the *Bix4* gene (Casey *et al.*, 1999).

It is not yet clear, though, what dose or variety of secreted inducer proteins a competent cell must receive in order to enter organizer formation. To give two possibilities, a mixed dose of Nodal proteins and Xnr3 (or some other secreted protein encoded by a β -catenin/Tcf activated gene) may suffice, or a high dose of Nodal proteins alone may suffice. At the same time, there are two impossibilities. If a competent cell receives only a moderate dose of Nodal inducers (from a cell containing only VegT), it becomes lateral ventral mesoderm, not the organizer. And if it receives only Xnr3 (from a cell containing only β -catenin), it remains ectoderm, though it perhaps differs from other ectoderm cells in its enhanced responsiveness to neural inducers.

With these assumptions, we suggest that the center contains two kinds of cells. It includes all blastula cells that contain both maternal VegT and maternal β -catenin. These would be capable of releasing both categories of secreted zygotic materials, and maybe they also release high Nodal amounts due to a synergism of VegT and β -catenin within those cells (Agius *et al.*, 2000). If synergistic enhancement of Nodal production dominates, then organizer formation would fit the original model of Weijer *et al.* (1977) who proposed that the vegetal hemisphere releases a gradient of meso-endoderm inducer with the dorsal quadrant the highest. If an inducing combination of Nodals and Xnr3 is more important, then the overlap model of Kimelman *et al.* (1992) would fit better. Maybe both suffice. Blastula cells containing VegT as well as β -catenin occupy the dorsal vegetal quadrant up to the equatorial level. Many dorsal equatorial cells contribute cellular descendents to the head organizer (an auto-induction).

But the Nieuwkoop center may also consist of cells that contain only VegT (releasing only Nodals) or only β -catenin (releasing only Xnr3) but not both (Fig. 5). The cells would have to be located near enough to each other that responding cells would receive both kinds of inducers, or that different secreting cells will synergize each other's Nodal production so that responsive cells receive high Nodal doses, or both. Some animal hemisphere cells do contain only β -catenin and will only secrete Xnr3, and most marginal zone cells do contain only

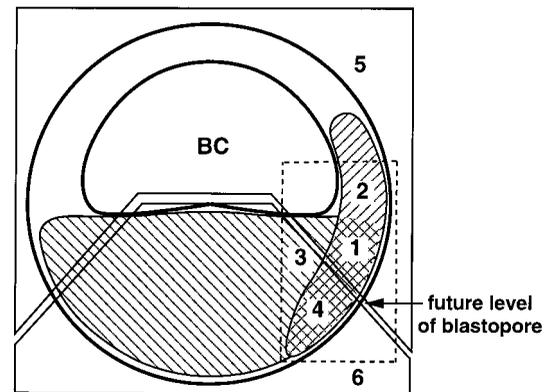


Fig. 5. The Nieuwkoop center. A *Xenopus* blastula in cross section is shown. The Nieuwkoop center, sometimes called the “organizer of the organizer” is defined as that part of the blastula that induces the organizer to form. This figure shows the center, which is enclosed by the dashed lines, to have several subregions, here identified by number. **(1)** The autonomous part of the Nieuwkoop center composed of cells that have received both maternal VegT and the maternal β -catenin stabilizing agent. These cells secrete high levels of Nodal proteins and also Xnr3 protein. The cells are close to, and include, responsive cells, and so organizer cells will be induced. **(2)** A non-autonomous part of the Nieuwkoop center. The cells have received the maternal β -catenin stabilizing agent but not maternal VegT mRNA. They secrete Xnr3 and perhaps other proteins but not VegT protein. If their secreted proteins reach responsive cells, including themselves, which are in a location to receive Nodal proteins from yet other cells, organizer cells will be induced. If their secreted proteins reach responsive cells, including themselves, which do not receive Nodal proteins, they won't induce organizer cells. The cells receiving only signals from these type 2 cells will remain ectoderm, perhaps with extra sensitivity to later neural induction. **(3)** A non-autonomous part of the Nieuwkoop center. These cells have received maternal VegT mRNA but not the maternal β -catenin stabilizing agent. They secrete Nodal proteins. If their secreted proteins reach responsive cells, including themselves, that are in a location to receive Xnr3 protein from yet other cells, organizer cells will be induced. If their secreted proteins reach responsive cells, including themselves, that do not receive Xnr3, they will induce non-organizer meso-endoderm cells. **(4)** An autonomous part of the Nieuwkoop center, like region 1, but located too far from responsive cells to actually induce organizer cells, and itself composed of cells that are not responsive (see region 6 description). They could induce an organizer upon appropriate transplantation. **(5)** Cells competent to form the head or trunk-tail parts of the organizer if they receive either very high levels of Nodal proteins alone or the combination of moderate levels of Nodals together with Xnr3 protein. **(6)** Cells of the vegetal hemisphere that are not competent to form the head or trunk-tail part of the organizer (due to excess VegT or yet another maternal factor?). They may be competent to form the deep yolkly endoderm part of the organizer. BC, blastocoele.

VegT and secrete only Nodals. Where these zones are close together, inducer overlap may occur. Such overlap may explain some of the results of Kageura (1995), who grafted dorsal animal cells (β -catenin containing) to the ventro-lateral side where VegT containing cells of the vegetal hemisphere are located. A secondary axis was produced, perhaps because a two part Nieuwkoop center was surgically constituted, capable of inducing an organizer.

Does the Nieuwkoop center induce the entire three part organizer in a single step of meso-endoderm induction in the late blastula period? Various results indicate not. Suzuki *et al.* (1984) have argued that the head organizer in urodeles doesn't yet have all its properties at the start of gastrulation and initially acts like a weak trunk-tail

organizer. Their evidence indicates that as gastrulation begins, the involuted part of the organizer interacts with the part still on the surface, enabling the head and trunk-tail organizers to differentiate from one another. This is at a time when the Nieuwkoop center has supposedly ceased activity (Boterenbrood and Nieuwkoop, 1973). Evidence in *Xenopus* also favors a secondary induction of some aspect of the trunk-tail organizer. As mentioned above, the *Xenopus* organizer, in contrast to that of urodeles, has distinct parts at the start of gastrulation, which is defined as the time of bottle cell formation. However, *Xenopus* differs from urodeles in relevant ways. It begins gastrulation internally at least an hour before bottle cells form, and its mesoderm is internal even before gastrulation (Nieuwkoop and Florschütz, 1950). The final patterning of the *Xenopus* organizer via intra-organizer interactions may occur before the blastopore lip appears. The separability of the final steps of organizer formation may be revealed by embryos deficient in FGF signaling because of a dominant negative FGF receptor (Amaya *et al.*, 1991). In these embryos the head organizer forms successfully but the trunk tail organizer does not (or does not persist). Stewart and Gerhart (1991; Gerhart *et al.*, 1991) showed that an animal cap from an organizer-less embryo, when grafted onto the vegetal base of a normal embryo at the late blastula stage, can still form chordamesoderm and a trunk-tail organizer in large amounts. This result suggests that the head organizer part of the organizer, and/or the deep endodermal part, can induce the trunk-tail organizer after the Nieuwkoop center has lost activity. Finally, Domingo and Keller (1995) have shown in normal embryos that non-organizer cells can be grafted into the trunk-tail organizer at the mid or late gastrula stage, and they are still induced to behaviors of trunk-tail organizer cells. A spreading trunk-tail induction seems to continue into these advanced stages. Thus, it may be that the Nieuwkoop center induces the head organizer region in the ventral band of the marginal zone, which later induces the trunk tail organizer in nearby mesoderm of the dorsal band. It remains to be clarified whether all properties of the trunk-tail organizer require this secondary interaction or just a subset, such as the posteriorization/convergent-extension activities of the trunk-tail organizer.

Finally, if we trace the steps of organizer formation to developmental stages, we come to oogenesis in the case of amphibia (Fig. 3). This is when the animal-vegetal axis is generated. The β -catenin stabilizing agent (Dsh, or Wnt, or Frizzled?) is localized to the vegetal pole cortex of the oocyte, and VegT mRNA is localized to the entire vegetal cortex. Two pathways are thought to operate in *Xenopus* oocytes in mRNA localization (Kloc and Etkin, 1995). The METRO pathway (oocyte stages 1-2) moves newly synthesized materials to a small patch of cortex at the vegetal pole, the region where germ plasm is formed. Later, the Vg1 pathway (stages 3-5) moves materials widely to the vegetal cortex. Presumably the β -catenin stabilizer, which resides directly at the pole, depends on the former, and VegT, which is spread over the entire vegetal cortex, depends on the latter. These localizations along the animal-vegetal axis constitute the first step of organizer formation. The second axis, which is established by cortical rotation in the fertilized egg, is then a partial unilateral distortion of the animal-vegetal axis.

Does the organizer really organize chordate development?

We will consider this question in a pattern formation context, namely, the specification of the chordate body plan, rather than in the context of mechanisms of cell determination via induction. In the

pattern formation context the organizer clearly lives up to its title. The question is then, what aspects of the body plan does it organize, in comparison to those achieved by other patterning events of chordate development?

Even before gastrulation the embryo has extensive organization based on the animal-vegetal organization of the oocyte, on modifications of this axis introduced by cortical rotation in the first cell cycle (a second axis), and on the elaborations of these axes due to meso-endoderm induction in the blastula period. Meso-endoderm induction is certainly a widespread patterning event of great significance for the body plan. Its inductions are generally aligned with the axes of the egg. The responses of competent cells to this induction event ensure that the three large competence groups are emplaced with mesoderm in the middle, that the mesoderm is divided into dorsal and ventral bands, and that the three part organizer is in the center of the groups.

What more can be added by the organizer's inductions, morphogenesis, and self-differentiation? Gastrulation and neurulation, which are when the organizer acts, are periods of extensive remodeling of egg organization into embryonic organization. Chordates are said to be unusual in the large extent of this late remodeling, which is reflected in their highly convoluted fate maps. The trunk-tail organizer (the chordamesoderm) is the agent of much of this morphogenetic remodeling. By its convergent extension, it generates a new antero-posterior dimension, reaching to distant locations of the embryo, and exerting inductions on competence groups at these locations. It is an elongating signal source. The entire trunk and tail portions of the axis are added, and the dorsoventral dimension is extensively patterned. While not providing instructive detail about local development, the organizer provides global information on the time, place, scale, and orientation of axis formation via a small set of spatially coherent signals delivered over a great expanse of the embryo. The organizer's inductive signaling, together with the multipotent responsiveness of the embryo's large competence groups, may be especially compatible with large scale morphogenesis. Patterning succeeds with whatever cells of a competence group are there to receive signals, with tolerance to variable cell numbers and cell positions inherent in the extensive morphogenetic remodeling of the egg's initial organization. The responses of the competence groups are robust and self-organizing (as shown by the near normal local differentiations of gastrula tissue exposed to artificial inducers). Thus, the organizer exerts an overall organizing role in axis specification, not one of micromanagement of local differentiations.

The organizer provides reliable conditions for the establishment of global spatial organization of the body plan, namely, the place, orientation, and scale of dorso-anterior development by approximately half of the cells of the large ectodermal, mesodermal, and endodermal competence groups. The subsequent autonomous development by members of these groups is thereby organized in the anteroposterior and dorsoventral dimensions of the body axis in relation to the notochord and to each other. The importance of the organizer for the place and orientation of development by these groups is shown by the Spemann-Mangold results. That is, if the organizer is grafted to the ventro-posterior marginal zone, a body axis forms at the new location with anteroposterior and dorsoventral axes placed and oriented in relation to the graft.

The importance of the organizer for the scale and completeness of development by these groups is shown by the experiments to reduce or increase the size of the organizer (and hence the quantity and placement of its signals). If the size of the organizer is reduced

systematically and progressively by surgery at the early gastrula stage, or by other means interfering with its formation at earlier stages, the completeness of the body axis of the tadpole is reduced progressively from anterior to posterior, as described above. If the organizer is removed entirely, the embryo develops no chordate body axis and none of the four phylotypic traits. In the reverse direction, too much organizer also changes the anatomical scale systematically and progressively. Dorso-anteriorized anatomies are deficient in ventral and posterior parts of the body axis. Clearly the organizer determines the anteroposterior and dorsoventral completeness of development by the competence groups, based on the relative amounts of signaling and responding tissues. Since the development of the competent gastrula cells surrounding the organizer is contingent on its signals for the time, place, orientation, and scale for their responses, the organizer can be said to organize the chordate body axis. In contrast to the organizer's effects, the manifold inductions by artificial agents, purified proteins, or by subregions of the organizer lead to locally well organized derivatives but not an axis with the full variety of components in normal proportions and placement.

To do all this, the organizer needs internal organization, namely, the three parts. In many conditions it is hard to recognize the effects of absent deep yolk endoderm, but there may be unscrutinized deficiencies of heart, anterior gut, and anterior neural plate. The organizer may have considerable capacity to restore its tripartite organization after damage. Holtfreter (1951) cut a rectangle of tissue from the dorsal marginal zone and prospective neural ectoderm of the early urodele gastrula, rotated it 180° and grafted it back, and got normal axial patterning of the embryo. He also removed head mesoderm (part of the head organizer) from the early gastrula and still obtained good axial development. As noted above, when the urodele organizer is ablated, nearby parts of the gastrula regenerate it. Lastly, when he mixed organizer cells randomly and implanted them in the blastocoel of a gastrula, a rather complete secondary axis was still induced. These results suggest that the organizer has self-organizing properties, and that regions around it can regenerate parts of the organizer and contribute to its organization.

The medio-lateral organization of the organizer is also self-regulating. When a left or right half lip is transplanted into the ventral marginal zone of the gastrula, a bilateral body axis is still formed, slightly truncated at the anterior end (Stewart and Gerhart, 1990). The axis is bilateral because, although the graft is unilateral, the cells responding to inducers are bilaterally distributed around the graft. Holtfreter showed that isolated pieces of urodele lateral marginal zone, which are initially not bilateral, have a great capacity to take on bilateral polarized organization and to release inducers in a non-homogeneous array (Holtfreter and Hamburger, 1955). We conclude that the organizer's architecture is important for its axis organizing function in development, and that this architecture is ensured by a variety of self-organizing and regulatory behaviors.

The organizer is a strategy of chordate axis formation, a means to provide a reliable spatial array of signals for orienting, placing, and scaling the development of neighboring groups of cells in relation to it. For the remarkably broad expanse of its effects, it acts rather late in development. Other groups of animals such as arthropods develop without a late-acting global organizer, at least as judged by *Drosophila*, and instead use follicle-imposed patterning of the oocyte and a form of meso-endoderm induction (via the trunk and spätzle signals via the torso-like and toll receptors, respectively) at early stages. Different phyla organize their organizing processes in different ways.

Evolution of the organizer

In the evolution of the chordates from a non-chordate ancestor, when and in what order did the organizer gain its several parts and its many inductive, morphogenetic, and differentiative capacities? Also, since meso-endoderm induction precedes the organizer's inductions in chordate development, and probably predated the organizer in the evolving chordate line, what do the organizer's inductions add to the organization of the chordate body axis that wasn't already achieved by meso-endoderm induction?

Chordates, which include urochordates, cephalochordates, and vertebrates, are distinguished by four characteristics: their notochord, dorsal hollow nerve cord, gill slits (the branchio-pharyngeal apparatus), and post anal tail. Some authors add somites and the endostyle (or thyroid) to the list. The organizer is associated closely with the development of all these characters either by self-differentiation (i.e., to the endodermal part of the gill slits and endostyle, and to the notochord) or by induction (the dorsal hollow nerve cord, somites, the ectodermal part of the gill slits, and parts of the post-anal tail). The organizer is a trait of all members of the phylum. The evolution of the organizer is plausibly linked with chordate evolution. The origin of chordates has been much speculated upon, though little is known (see Cameron *et al.*, 2000; Gerhart, 2000). The present set of speculations differs from others in emphasizing the organizer and in comparing the body axis of chordates and hemichordates.

The organizer has been identified in all classes of vertebrates, by the grafting to a ventral site of a gastrula, followed by development of a secondary axis. The less well known examples include organizer grafts in jawless fish (lamprey, Yamada, 1938) and in cephalochordates (amphioxus, Tung *et al.*, 1962). In amniotes (reptiles, birds, and mammals) the organizer is called Hensen's node. Descriptions of the node tend to emphasize its trunk-tail chordomesodermal element while underplaying the head organizer part. The AVE equivalent of the amphibian deep endodermal part has been described recently in mammals (Beddington and Robertson, 1999). Although the organizer's characteristics are mostly conserved across the phylum, the ascidian urochordates have the most differences. Induction is limited to neural induction and a minor amount of mesoderm dorsalization. Most tail somites of ascidians arise autonomously in a lineage of myoplasm-containing cells. The neural tube is formed by a lineage which is not also competent for epidermal development. Still, as in other chordates, the ascidian organizer undergoes dramatic convergent extension, induces the neural tube, and differentiates to a notochord (Miyamoto and Crowther, 1985).

All chordates probably have meso-endoderm induction, a generalization Nieuwkoop explored in detail (Nieuwkoop *et al.*, 1985). Recent experimentation has supported this generalization. For example, in ascidians (urochordates), the notochord lineage of cells is induced by an endodermal cell, and the interaction can be simulated by FGF addition (Nakatani *et al.*, 1996). As Nieuwkoop pointed out, mesoderm is as ancient as bilateral animals, and so meso-endoderm induction may substantially pre-date chordates, having been present already in the deuterostome ancestor of chordates.

The metazoan phyla closest to chordates are the echinoderms and hemichordates. Together they constitute the sister group of chordates (Turbeville *et al.*, 1994). Does their development give evidence of an organizer or of meso-endoderm induction? As recently found, sea urchins (members of the echinoderms) stabilize β -catenin at the vegetal pole of the fertilized egg, the site at which micromeres arise (Logan *et al.*, 1999; Angerer *et al.*, 2000). Micromeres

induce nearby cells to form secondary mesenchyme and gut endoderm. They themselves develop to primary mesenchyme of the larva and to the adult rudiments. Uninduced cells, which are farthest from the vegetal pole, express the *bmp4* gene in the gastrula and become epidermal ectoderm. Thus the vegetal pole region has inductive and organizing properties, although these seem rather different from those of the chordate organizer, just as the embryonic organization of echinoderms and chordates is quite different. The echinoderm inductions seem mostly ones of meso-endoderm induction patterning a single axis in alignment with the animal-vegetal axis of the egg. However, sea urchin embryos also have an oral-aboral asymmetry associated with BMP expression on the prospective oral side and repression on the aboral (ciliary band/neuron) side, as well as repression in the vegetal region. Angerer *et al.* (2000) have suggested that β -catenin accumulates in the vegetal hemisphere but tilted toward the side the prospective aboral side, the regions where BMP gene expression is blocked. The repression is similar to the dorso-anterior β -catenin repression of BMP gene expression in *Xenopus* blastulae.

The asymmetry of β -catenin in the echinoderm egg is strikingly similar to the amphibian egg's early asymmetry following cortical rotation. These data may indicate that the means to generate bilateral symmetry in eggs via β -catenin (and possibly VegT) predates chordates and that meso-endoderm induction predates the organizer. However, echinoderms and chordates differ in what they do with these asymmetries. In echinoderms, meso-endoderm induction seems to have a role in axis specification in alignment with the animal-vegetal axis. While a second axis (orthogonal to the animal-vegetal axis) exists in echinoderm larvae, it seems not as elaborate as in chordates.

Although the information on these inductions and fates in sea urchins is intriguing, the penta-radial body organization of the adult and the extreme metamorphosis of the larva make it difficult to compare the urchin embryo, larva, or adult with the chordate embryo and adult. Hemichordate adults, on the other hand, may be more comparable (although cross phylum extrapolations are notoriously difficult). They have but a single body axis, and although many hemichordates have a larval stage, their metamorphosis is relatively modest. Little larval tissue is lost in the transition while new tissues are added, and all are aligned to the same body axis. For example, the trimeric coelom and gut of the larva are retained by the adult.

Hemichordates are named because of their several similarities to chordates. After the work of Bateson (1884) over a century ago, they were briefly included in the chordate phylum as non-vertebrate chordates (until ca. 1930). The adult has a long proboscis in the anterior, followed by a short collar with a ventral mouth, a pharyngeal region with a series of complex dorsolateral gill slits (up to 70 pairs added successively), and then a long posterior gut region with an anus at the tip. The dorsal midline contains a dorsal nerve cord that forms by a neurulation-like involution of surface ectoderm in some species (discovered by T.H. Morgan). The ventral midline contains a ventral nerve cord. Its development, which proceeds without neurulation, is poorly known. Protruding from the collar into the proboscis is a stomacord or buccal diverticulum, a short rod of vacuolated cells that Bateson homologized to the notochord. It develops from the gut roof as do the notochord and prechordal plate mesoderm of many chordates. More recent authors have disputed the homology or have supported it (Balsler and Ruppert, 1990). The juvenile temporarily bears a ventral post-anal tail-like extension in some species, although its tail characteristics are limited. Thus,

hemichordates have some chordate similarities and some non-chordate characters. The ventral nerve cord, for example, is reminiscent of annelids and arthropods (protostomes).

Hemichordates have received little embryological study, and it is not known whether the embryo has an organizer or meso-endoderm induction, although they probably resemble echinoderms and chordates in the latter respect. For purposes of discussion, we will assume that a pre-chordate ancestor resembled a hemichordate in various respects and will suggest five steps in the evolution of the organizer and in the evolution of the chordate body axis from a hemichordate-like condition.

1. Origins of the organizer: Since gill slits, endostyle, and pharyngeal mesoderm (gill bars) are clearly present in hemichordates, and since the head organizer of chordates differentiates to these structures in chordates, we suggest that hemichordates may have a head organizer-like region. However, since their pharynx and gill slits are located in the posterior coelomic region (metacoel) and not in the "head" (anterior by convention), one might call it a pharyngeal organizer. Whether such an organizer would have neural inducing properties in addition to gill slit inducing properties is hard to anticipate. The dorsal nerve cord in hemichordates does not arise in ectoderm near the pharynx but in ectoderm directly over the stomacord territory of the archenteron. Although the stomacord has long been homologized to the chordate notochord, it may also resemble the prechordal mesoderm (prechordal plate, head mesoderm) of chordates. This territory is included in the chordate head organizer and derives from the archenteron roof, as does the notochord (e.g., in urodeles, not in *Xenopus*). Therefore it seems plausible that the pharyngeal organizer and stomacord together constitute a modest organizer in hemichordates, and that the stomacord territory induces the dorsal neural tube. One may also guess that this pharyngeal organizer is induced by meso-endoderm induction via an asymmetric β -catenin-VegT mechanism, the kind of asymmetry seen in the sea urchin egg. Thus, hemichordates would have a modest Nieuwkoop center. Meso-endoderm induction presumably extends around the vegetal hemisphere to the ventral side of the embryo as well, aligned with the animal-vegetal axis of the egg, as in chordates and sea urchins. Compared to chordates, the body axis of hemichordates seems patterned mostly along the animal-vegetal axis of the egg, with rather little additional patterning orthogonal to this axis. Perhaps hemichordates use the egg's animal-vegetal axis and the co-aligned meso-endoderm induction to accomplish their major axis specification before gastrulation, and their modest pharyngula organizer acts only locally on the dorsal side during and after gastrulation.

2. Chordamesoderm, the trunk-tail organizer and the new anteroposterior axis of chordates: Compared to hemichordates, ancestors in the chordate line must have greatly increased the length and central prominence of the notochord, dorsal neural tube, and somites, creating a trunk and tail. We suggest that the ancestors, through a series of intermediates, greatly modified the body axis of the ancestral hemichordate-like deuterostome by generating a new posterior end while preserving the old anterior end, thereby creating a new anteroposterior axis orthogonal to the old one. Convergent extension is the novel morphogenetic activity of chordate gastrulation, performed mostly by the chordamesoderm, which is the trunk-tail organizer. The chordamesoderm is a

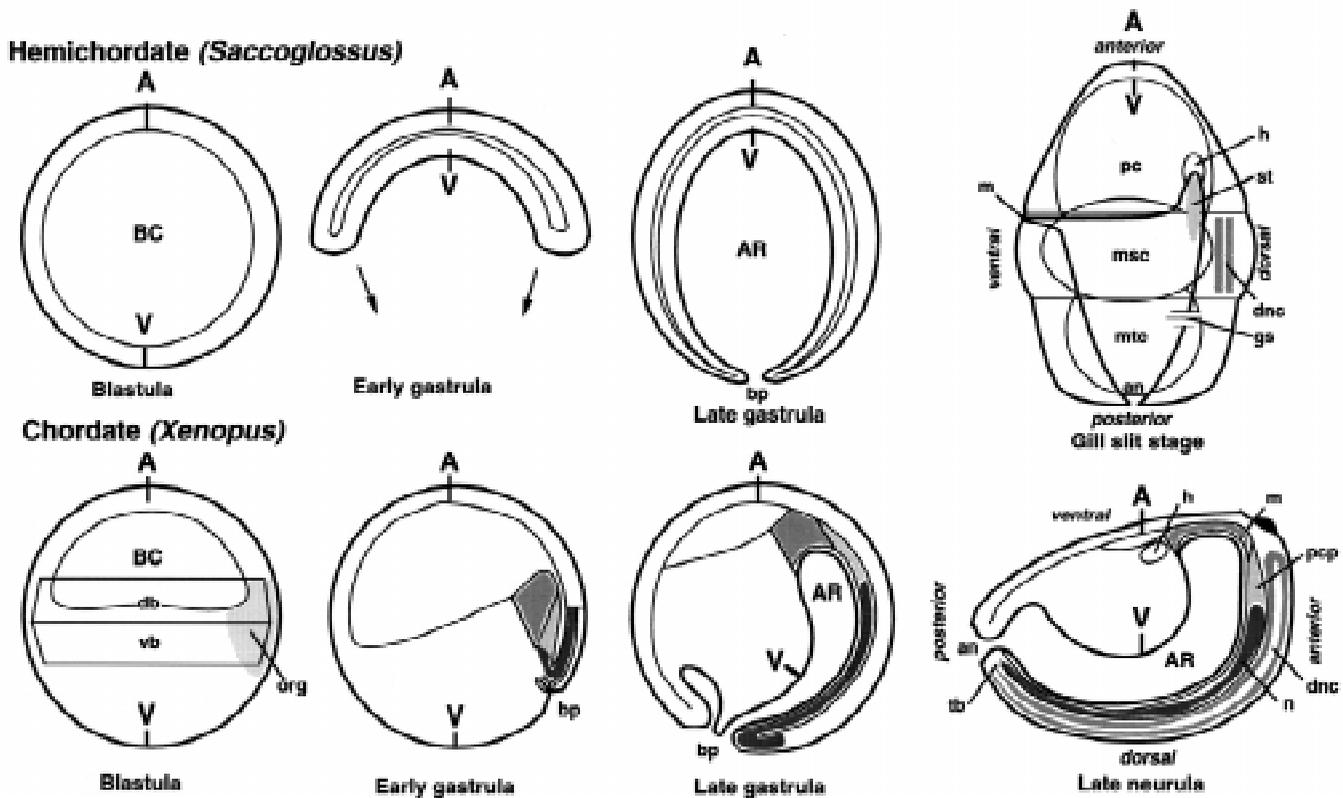


Fig. 6. The organizer and the new body axis of chordates. (Upper row) The anteroposterior axis of a hemichordate juvenile (*Saccoglossus kowalevskii*) develops in alignment with the animal-vegetal axis of the blastula. The vegetal hemisphere of the late blastula collapses inward beneath the animal hemisphere to give a double layered cup-shaped early gastrula. The edges of the cup close uniformly downward to give a prolate late gastrula with a blastopore aligned with the old animal and vegetal poles. In the upper right is shown the stage of the first gill slit (day 5). The dorsal nerve cord (dnc) has formed from ectoderm by neurulation, the stomach has protruded from the gut roof, and three coeloms have pouched from the archenteron. The anteroposterior axis is approximately aligned with the animal-vegetal axis. The dorsal and ventral sides are locally differentiated. Drawings are modified from Bateson, 1886. **(Lower row)** The anteroposterior axis of a chordate (*Xenopus laevis*, an amphibian) develops orthogonal to the animal-vegetal axis of the blastula. Invagination and involution begin earlier and more strongly on the organizer side, shown to the right. The head organizer involutes, followed by the trunk-tail organizer. The involuted head organizer moves upward but soon stops, setting the site of the future anterior pole. As the trunk-tail organizer (dotted region) involutes, it initiates a strong convergent extension morphogenesis. It pushes backwards (to the left in the figure) across the diameter of the blastopore, the closing point of which is displaced ever farther to the left, until the anus, the posterior pole, is opposite the anterior pole. The anteroposterior axis is now nearly orthogonal to the animal-vegetal axis. During convergent extension, the trunk-tail organizer passes through mesoderm initially opposite it in the blastula, inducing somites. A, animal pole; an, anus; AR, archenteron; bp, blastopore; BC, blastocoel; db, dorsal band of mesoderm; dnc, dorsal nerve cord; gs, gill slit; h, heart; m, mouth; msc, mesocoel (the collar region); mtc, metacoel (the pharynx-gut region); org, organizer; pc, protoceol (the proboscis region); pcp, prechordal plate mesoderm; st, stomach; tb, tailbud; V, vegetal pole; vb, ventral band of mesoderm.

large, asymmetrically placed cell population, initially located near the prospective anterior end. It lengthens in a direction orthogonal to the animal-vegetal axis and eventually differentiates to the notochord. The trunk-tail organizer presumably enlarged greatly in the ancestral chordate line, perhaps from the old prechordal plate-like stomach but perhaps from other gut roof sources posterior to the stomach territory. The addition may have been induced by the old pharyngula organizer rather than by the Nieuwkoop center. This is suggested because as noted above, the chordate head organizer seems to induce the trunk-tail organizer to gain some of its properties as gastrulation begins, for example, to add neural posteriorizing activity and convergent extension to its behaviors. Eventually the two parts settle into mutually exclusive states of differing activity within the chordate organizer, each expressing genes repressive to the other (Dosch and Niehrs 2000).

Chordamesoderm is also noteworthy as a new signaling center which extends the length of the embryo thanks to its convergent

extension. Cells that are initially far away from the organizer are later close to it, due to its movement toward them. Hemichordates do not have such an orthogonal extension during gastrulation (Fig. 6). Their blastopore closes symmetrically to a point, the anus, and the animal's anterior-posterior axis is aligned with the egg's animal-vegetal axis (though the vegetal pole is not the site of the anus). As the worm grows longer, the walls of the archenteron elongate circumferentially, and gill slits are added successively (anterior to posterior) in the pharyngeal region, as if there is a uniformly lengthening growth zone. The hemichordate dorsal side doesn't lengthen any more than the ventral side.

In contrast, the chordate blastopore closes very asymmetrically toward the ventral side as chordamesoderm pushes across the diameter of the blastopore by convergent extension (Fig. 6). The anus forms at a point on the ventral side of the perimeter, far offset from the animal-vegetal axis. (The anus continues moving ventrally even after gastrulation). Thus, the hemichordate posterior end is comparable to the chordate dorsal side, and the

hemichordate ventral side to the chordate posterior end. During its backward extension, the chordamesoderm passes through mesoderm of the entire marginal zone, creating a long domain of contacts and opportunities for signaling not present in hemichordates. This mesoderm of chordates corresponds to the mesoderm of the hemichordate metacoel. The metacoel may have become the somite mesoderm of chordates. Thus, the chordate anterior-posterior axis lies almost orthogonal to the egg's animal-vegetal axis and to the hemichordate anteroposterior axis. When a chordate embryo lacks an organizer and hence its capacity for convergent extension (DAI 0, Fig. 2), the blastopore closes on the animal-vegetal axis, and the embryo retains cylindrical symmetry around this axis, like the hemichordate embryo.

Hemichordates and chordates differ in many ways besides lengthening and signaling by the chordamesoderm. The tricoelomic organization of hemichordates and echinoderms, which is thought to be the basal body organization among deuterostomes, is greatly modified in chordates. Goodrich (1917) proposed that the large first and second coeloms of hemichordates have been greatly reduced in chordates, leaving only small cephalic and presomitic cavities in the head, whereas the large third coelomic cavity (the metacoel) is retained and expanded as the trunk coelom. However, if the new chordate body axis is really orthogonal to the old one of a hemichordate-like ancestor, the old proto-coel may have become the heart/blood cavities, the mesocoel the trunk coelom, and the metacoel the somite mesoderm (somites have cavities as they first form). In this realignment, the old anteroposterior axis of the ancestor would have become the new ventro-dorsal axis of chordates. The roles of chordamesoderm and prechordal (head) mesoderm in neural induction differ greatly in the chordate line. They have differentiated as the head and trunk-tail organizers, respectively. Whereas both are capable of neuralization, only the chordamesoderm has the posteriorizing activity that modifies neuralized tissue from the path of forebrain-midbrain development into the path of hind brain-spinal cord development. The short neural plate of hemichordates is presumed to be anterior-like (emx, otx expressing), that is, unposteriorized. It forms by a foreshortened neurulation. In the intermediates along the chordate line, the neural plate must have greatly increased in length. Posteriorization of neural tissue presumably arose as the plate elongated. The new posteriorized part of the neural plate itself engages in convergent extension, a new morphogenetic activity for neural tissue.

3. Anti-posteriorization: As posteriorization evolved as a chordamesoderm function in the proto-chordate lineage, and the posterior part of the nerve cord became increasingly different from the anterior, patterning problems may have arisen. Posteriorization is a dominant effect—it converts anterior neural tissue to posterior neural tissue, even the anterior neural tissue induced by the head organizer. Unchecked, it could result in a deficiency of forebrain and midbrain. Posteriorizing agents seem widely distributed in the chordate embryo. The best secondary heads are artificially produced on the ventral side of a *Xenopus* embryo when a combination of Wnt and BMP antagonists is injected (Glinka *et al* 1997). Each alone is much less effective. The failure of the delicate balance between the

head and trunk-tail organizers can be seen in frog and fish embryos that have impaired steps of organizer formation. They develop as if “over-posteriorized”, with microcephaly or acephaly. For example, they often have a reduced forebrain, no nose, and a cyclopic eye. The original Spemann-Mangold grafts gave axes without heads (Fig. 1).

The posteriorizing activity of the evolving trunk-tail organizer perhaps needed a regulatory counteraction to ensure normal anterior neural development, especially in the relatively large-brained chordates. Deep yolky endoderm is the third and least known part of the organizer. Its equivalent in mammals is the AVE (Beddington and Robertson, 1999). It secretes Cerberus and Dickkopf proteins, which may prevent over-posteriorization (Brickman *et al.*, 2000). Cerberus, as discussed elsewhere in this Volume, antagonizes BMP, Nodal, and Wnt signaling. Its anti-Wnt activity may counter posteriorization. Cerberus also counteracts meso-endoderm induction by its anti-Nodal activity, so ectoderm doesn't convert to mesoderm. Thus it preserves ectoderm for anterior neural development. It may also enhance the inductive effects of the head organizer and may contribute to neuralization by its anti-BMP activity. It may also contribute to heart induction (Schneider and Mercola, 1999). In amphibia, this region arises centrally in the vegetal yolk mass in a core that may gain β -catenin stabilizing activity from the vegetal pole. Then it moves to the organizer's side shortly before gastrulation and moves inward with the head organizer (Jones *et al.*, 1999). Thus, we propose that this region arose as the third and last part of the organizer, to offset the trunk-tail organizer and other posteriorizing tissues .

4. Post-gastrula notochord signaling: As the chordamesoderm extends, it becomes a signaling source running the length of the new anteroposterior axis of the animal. This signaling source (mostly Shh and FGF) is unique to chordates in being so extensive while arising so late in development, namely, during gastrulation. It has almost no relation to the egg's animal-vegetal axis. Non-chordate embryos only gain such global axis-specifying agencies as part of the egg's initial organization, for example, from the multicellular follicle's patterning of the oocyte (e.g., *Drosophila*), or the egg's animal-vegetal axis, or from meso-endoderm induction aligned with that axis, or from a proliferative growth zone. The notochord, though, generates its spatial orientation and placement by its own morphogenesis, not out of the egg's initial organization. Trunk-tail tissues are then organized around it during and after gastrulation, from cell populations spread around the blastopore periphery. Somites, for example, arise from marginal zone cells initially on the opposite side of blastopore from the organizer. The chordamesoderm extends over to them and induces them. Dorsalization of mesoderm may have evolved only after convergent extension was in place. This is consistent with the proposals of evolutionists who argue that somites were added anew within the chordate line and not carried over from a segmented pre-chordate ancestor. The extensive morphogenesis of the chordamesoderm sets the stage for other far reaching inductive patterning within all germ layers of the chordate trunk (the neural tube and floor plate, somites and sclerotome, hypocord and gut). From this evolutionary perspective, the organizer seems well named, as the agency of a new organization of the body plan in the chordate

phylum. This new organization seems superimposed on, yet derived from, the more modest organization of chordate ancestors (perhaps retained in modern hemichordates) that was achieved by meso-endoderm induction adding pattern along the dimension of the egg's animal-vegetal axis.

5. Ongoing changes of competence: Even before vertebrates arose within the chordates, the organizer probably reached its present status as a coherent and patterned inducer source of moderate complexity (head and trunk-tail parts) and morphogenetic capacity. Its organization and functions have been thereafter conserved. However, the body axis of vertebrates has added more constituents and has evolved a more complex arrangement than found in non-vertebrate chordates. Cells responding to the organizer's signals, namely, those of the large ectodermal, mesodermal, and endodermal competence groups, must have continued to evolve the variety of responses in their competence repertoires, still contingent on the organizer's conserved signals. For example, within the vertebrates the sclerotome and dermatome responses were added to somite mesoderm, the kidney has changed greatly, and the neural crest response was added to ectoderm. Furthermore, the repertoires of responses to secondary and tertiary inductions increased and changed as well. Conservation of the organizer's structure and function didn't limit the evolution of the competence of the surrounding cells.

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