

FORMATION AND FUNCTION OF SPEMANN'S ORGANIZER

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ABSTRACT

The organizer is formed in an equatorial sector of the blastula stage amphibian embryo by cells that have responded to two maternal agents: a general meso-endoderm inducer (involving the TFG- β signaling pathway) and a dorsal modifier (probably involving the Wnt signaling pathway). The meso-endoderm inducer is secreted by most vegetal cells, those containing maternal materials that had been localized in the vegetal hemisphere of the oocyte during oogenesis. As a consequence of the inducer's distribution and action, the competence domains of prospective ectoderm, mesoderm, and endoderm are established in an animal-to-vegetal order in the blastula. The dorsal modifier signal is secreted by a sector of cells of the animal and vegetal hemispheres on one side of the blastula. These cells contain maternal materials transported there in the first cell cycle from the vegetal pole of the egg along microtubules aligned by cortical rotation. The Nieuwkoop center is the region of blastula cells secreting both maternal signals, and hence specifying the organizer in an equatorial sector. Final steps of organizer formation at the late blastula or early gastrula stage may involve locally secreted zygotic signals as well. At the gastrula stage, the organizer secretes a variety of zygotic proteins that act as antagonists to various members of the BMP and Wnt families of ligands, which are secreted by cells of the competence domains surrounding the organizer. BMPs and Wnts favor ventral development, and cells near the organizer are protected from these agents by the organizer's inducers. The nearby cells are derepressed in their inherent capacity for dorsal development, which is apparent in the neural induction of the ectoderm, dorsalization of the mesoderm, and anteriorization of the endoderm. The organizer also engages in extensive specialized morphogenesis, which brings it within range of responsive cell groups. It also self-differentiates to a variety of axial tissues of the body.

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WHAT IS THE ORGANIZER?

The Spemann organizer is formed at the blastula stage of amphibian development and acts at the gastrula stage as a cell population capable of (a) releasing inducers to adjacent cells, (b) engaging in distinctive morphogenesis, and (c) differentiating several tissues of the embryonic body axis. As its name implies, the Spemann organizer is able to organize embryonic development, not only by instructing surrounding cells by way of its secreted signals to differentiate as certain types of tissues, but also by organizing the scale, placement, and orientation of the tissues. It also has a role in left-right asymmetry, as reviewed by WB Wood (this volume). At least half the cells of the gastrula require signals from the organizer for their normal development, whereas the organizer

itself consists of only 5% of the cells, located in the dorsal marginal zone. Some affected cells are located far from the organizer, even on the other side of the embryo at the start of gastrulation. The organizer's morphogenesis and inductive signaling are coordinated in its far-reaching effects on development.

The organizer is not the sole source of information at the gastrula stage. It activates or derepresses surrounding cells to initiate the specific differentiation and morphogenesis of which these cells are capable, as specified by their particular competence to respond to its signals. Thus ectoderm responds to the organizer's signals to become neural plate (by neural induction), mesoderm responds to become dorsal mesoderm (by dorsalization of the mesoderm), and the endoderm responds to become anterior gut (by anteriorization of the endoderm), all perhaps responding to the same signals. The inducing properties of the organizer, as well as the competence of surrounding tissues to respond, change over time. These changes in induction and competence, which are poorly understood, contribute much of the specificity of tissue induction, as well as the spatial organization of the embryo.

The instructive properties of the organizer were discovered by Hans Spemann and his colleagues, with the initial experiments done by Hilde Mangold (reviewed by Spemann 1938, Hamburger 1988). In their classic experiments, a tissue fragment was taken from the dorsal lip of a gastrulating embryo of a light-colored newt, and this was grafted into the ventral side of a darkly pigmented host of the same age. The resulting embryo developed a secondary neural plate with good anterior to posterior organization, and this was underlain by a secondary notochord, somites and, in some cases, a gut. Pigmentation differences allowed a clear discrimination between host and donor cells, so that although the graft differentiated into notochord, and variably into floor plate and some somites, the bulk of the second axis, including most of the neural plate and somites, was induced from host tissues that otherwise would not have formed axial embryonic structures. These experiments not only demonstrated embryonic induction, but also laid the groundwork for subsequent work that demonstrated the ability of the organizer to induce a complete second axis (from head to tail), the ability of partial organizers to induce partial axes, and the ability of specific surrounding cells to give specific responses to the organizer's signals, i.e. their competence. The Spemann-Mangold experiment has been repeated in recent years with cells marked with lineage tracers to document more precisely the contributions of graft and host to the secondary axis (Gimlich & Cooke 1983, Smith & Slack 1983).

All chordates are thought to have an organizer that releases inductive signals. It is located in the dorsal lip of the blastopore of amphibia, cephalochordates (amphioxus), cyclostomes (hagfish, lampreys), and non-teleost fish (such as shark and sturgeon). Urochordates have an organizer in the dorsal lip that acts

in neural induction but probably not in dorsalization. The embryonic shield of teleost fish is equivalent to the amphibian organizer, as is the anterior end of the early primitive streak of amniotes (reptiles, birds, mammals), in which are located Hensen's node and the prospective pre-chordal plate mesoderm. Organizer function is a distinguishing characteristic of chordate development.

In this review, we first discuss how the amphibian organizer is induced, drawing on both embryological and molecular literature. In the second part, we discuss the activities of the organizer in induction, self-differentiation, and morphogenesis. Recent reviews of aspects of these subjects include Gerhart et al 1991, Kessler & Melton 1994, Slack 1994, Harland 1994, 1996, Sasai & De Robertis 1997).

INDUCTION OF THE ORGANIZER: MESO-ENDODERM INDUCTION

Induction of the organizer is a part of meso-endoderm induction, the process by which the large germ layer-specific competence domains of the embryo—the ectoderm, mesoderm, and endoderm groups—are established that will figure prominently in gastrulation in their responses to signals from the organizer. As discussed below, meso-endoderm induction must be supplemented by a local dorsal modifier signal for the organizer to form. During cleavage stages, the mesoderm is specified, such that at the midblastula transition (4000 cells), a number of mesoderm-specific genes are activated in the marginal zone, the belt of tissue around the equator of the embryo. The organizer is the most dorsal mesoderm, as diagrammed in Figure 1. The remaining mesoderm of the marginal zone is ventral mesoderm. Endoderm is also specified, although less is known about its activated genes. The dorsal sector contains prospective pharyngeal endoderm, a part of the organizer. In addition to genes that are activated throughout the mesoderm and endoderm, several genes are activated in only the dorsal or ventral domains. Below we discuss the two extreme possibilities that the initial specification of mesoderm and endoderm includes a binary decision to be either dorsal or ventral, or includes a more graded organization, with dorsal, dorso-lateral, lateral, and ventral states of specification from the outset.

Also reviewed are four types of signal transduction pathways that have been implicated in the formation of meso-endoderm and dorsal ventral patterning (Figure 2). The first is a pathway activated by activin and its relatives within the transforming growth factor-beta (TGF- β) superfamily. This pathway can induce various mesodermal and endodermal tissues in the embryo and, at high doses in vitro, can induce the organizer and various dorsal structures. The activin pathway appears to act during cleavage and blastula stages. Second, fibroblast growth factor (FGF) signals are important for mesoderm induction or maintenance but do not appear to impose dorso-ventral information. Third, the

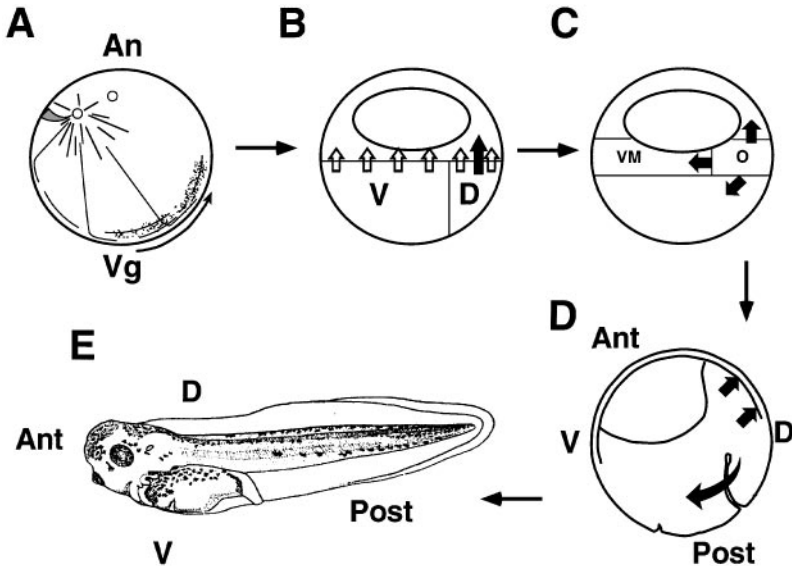


Figure 1 Events of amphibian early development, schematic view. *Xenopus laevis* is exemplified. (A) A cross-section view of the egg in the middle of the first cell cycle after fertilization, as cortical rotation occurs. The sperm has entered on the left and the sperm aster is shown in the animal hemisphere. The arrow indicates the 30° rotation of the cortex of the egg cell. This movement aligns microtubules in a parallel array close to the cortex, and cytoplasmic materials (dotted) at the vegetal pole are transported along the array to a site at the equatorial level on the right side. The cylindrical symmetry of the unfertilized egg has now changed to bilateral symmetry. At this equatorial site the organizer will later arise. (B) Cross-section of the midblastula stage (4000 cells), as meso-endoderm induction occurs. A blastocoel space has formed in the animal hemisphere. Gene expression has just begun. Open arrows indicate general meso-endoderm inducers released from the vegetal cells (V), impinging on animal hemisphere cells. The zygotic response of adjacent animal hemisphere cells establishes the marginal zone of prospective ventral-posterior mesoderm and endoderm, encompassing the blastula. The large closed arrow indicates the dorsal competence modifier released by dorsal (D) vegetal cells (of the Nieuwkoop center). When adjacent animal hemisphere cells respond to the combination of general meso-endoderm inducers and the competence modifier, they form the organizer in the dorsal marginal zone. (C) The early-gastrula stage (10,000 cells), cross section. The organizer (O) has begun to release signals (arrows) to the ectoderm above (neural induction), the ventral mesoderm (VM) lateral to it (dorsalization of the mesoderm), and the endoderm below it (anteriorization of the endoderm). These signals reach a limited distance. (D) The midgastrula, cross-section view. Gastrulation has proceeded with the internalization of endoderm and involution of mesoderm. The organizer has moved under the ectoderm, and vertical neural induction occurs in regions of contact (blunt arrows). Dorsalization of the mesoderm continues (curved arrow). Ant, anterior; Post, posterior. (E) Hatching tadpole, stage 40. External view. Note the orientation of the dorso-ventral axis (D and V) and the antero-posterior axis (Ant-Post).

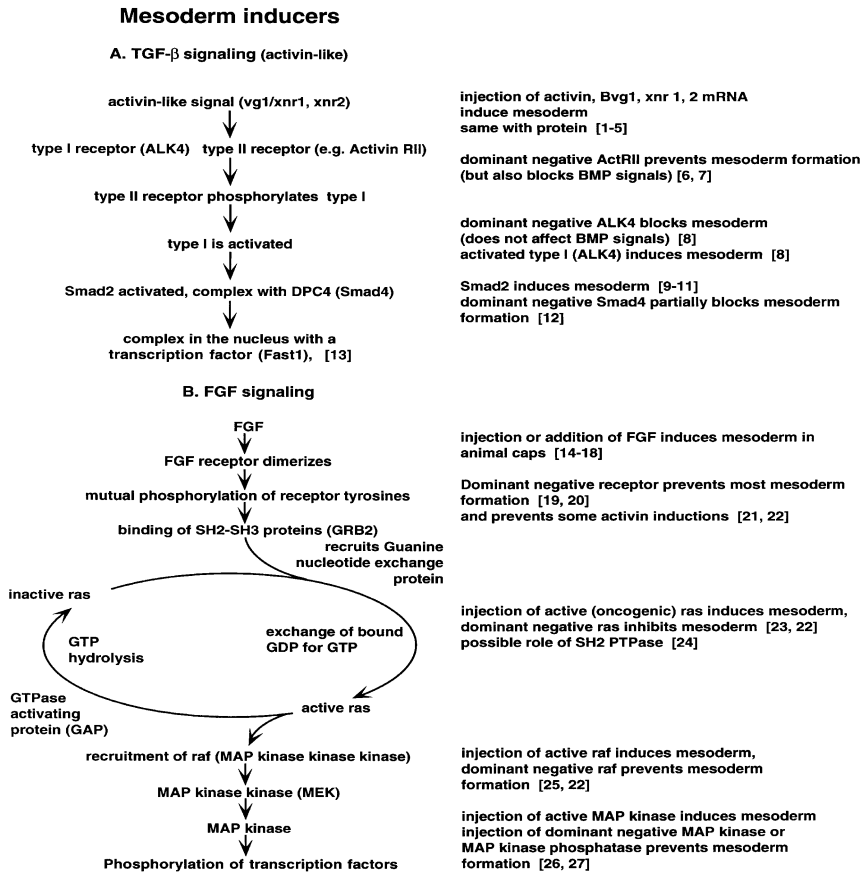
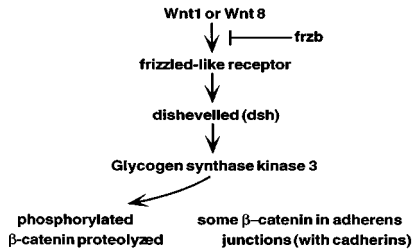


Figure 2 Summary of signal transduction pathways that contribute to early embryonic patterning. An outline of the pathway is given on the left, and experiments that activate, or interfere with the pathway are adjacent on the right. Numbers in parentheses refer to references. Mesoderm inducers: (A) TGF- β signaling: (1, Asashima et al 1990; 2, Smith et al 1990; 3, Thomsen et al 1990; 4, Thomsen & Melton 1993; 5, Jones et al 1995; 6, Hemmati-Brivanlou & Melton 1992; 7, Hemmati-Brivanlou & Thomsen 1995; 8, Chang et al 1997; 9, Baker & Harland 1996; 10, Eppert et al 1996; 11, Graff et al 1996; 12, Lagna et al 1996; 13, Chen et al 1996). (B) FGF signaling: (14, Kimelman & Kirschner 1987; 15, Slack et al 1987; 16, Green et al 1990; 17, Isaacs et al 1992; 18, Thompson & Slack 1992; 19, Amaya et al 1991; 20, Amaya et al 1993; 21, Cornell & Kimelman 1994; 22, LaBonne & Whitman 1994; 23, Whitman & Melton 1992; 24, Tang et al 1995; 25, MacNicol et al 1993; 26, LaBonne et al 1995; 27, Umbhauer et al 1995).

Modifiers of mesoderm**C. wnt signaling**

injection of wnt8 or wnt1 mRNA mimics a Nieuwkoop center [28, 29] providing a dorsal competence modifier [30, 31]. Late expression of wnt8 ventralizes [32] dominant negative wnt [33] or the antagonist frzb acts (during gastrulation) to block muscle formation [34, 35]

overexpression of receptor mimics wnt signal [36]

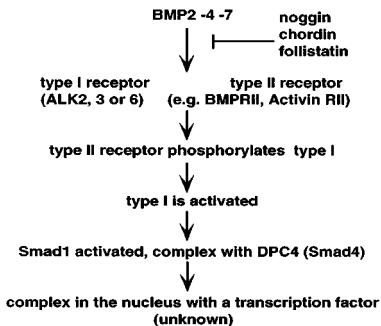
overexpression of dsh mimics, dominant negative dsh acts (during gastrulation) to block dorsal signals [37]

Overexpressed GSK3 mildly ventralizes, dominant negative GSK3 dorsalizes [38-40]

If GSK3 inactive free β -catenin stable
 ↓
 translocation of β -catenin into nucleus
 ↓
 complex with tcf3/LEF1 in the nucleus

overexpressed β -catenin dorsalizes [41-43]
 removal of β -catenin mRNA ventralizes [41]

removal of tcf-3 β -catenin interaction domain ventralizes
 Injection of LEF1 dorsalizes [44]

D. BMP signaling

injection of BMP mRNA or addition of protein ventralizes [45-47]
 BMP4 induces epidermis [48]
 BMP antagonists dorsalize mesoderm [49-51]

dominant negative ALK3 prevents ventral mesoderm formation [52, 53]

activated ALK3 (tkv) reverses noggin action [54]

Smad 1 expression ventralizes [11, 55-57]

Figure 2 Modifiers of mesoderm: (C) Wnt signaling: (28, Smith & Harland 1991; 29, Sokol et al 1991; 30, Christian et al 1992; 31, Sokol & Melton 1992; 32, Christian & Moon 1993; 33, Hoppler et al 1996; 34, Leyns et al 1997; 35, Wang et al 1997; 36, Yang-Snyder et al 1996; 37, Sokol 1996; 38, Dominguez et al 1995a; 39, He et al 1995; 40, Pierce & Kimelman 1995; 41, Heasman et al 1994; 42, Guger & Gumbiner 1995; 43, Karnovsky & Klymkowsky 1995; 44, Molenaar et al 1996). (D) BMP signaling: (45, Koster et al 1991; 46, Dale et al 1992; 47, Jones et al 1992; 48, Wilson & Hemmati-Brivanlou 1995; 49, Smith et al 1993; 50, Sasai et al 1994; 51, Sasai et al 1995; 52, Graff et al 1994; 53, Suzuki et al 1994; 54, Holley et al 1996; 55, Hoodless et al 1996; 56, Liu et al 1996; 57, Thomsen 1996).

pathway activated by Wnt signals is likely to be the pathway that specifies dorsal information in the blastula. And fourth, signaling by bone morphogenetic proteins (BMPs) is thought to be important in providing ventral specification during gastrulation, and the prevention of their signaling results in dorsal development. Finally, the Wnt signaling pathway is reused during gastrulation, but oddly enough in the opposite direction from its early activity, by inducing ventral and posterior types of tissues, and prevention of these signals facilitated dorsal development.

What Is the Source of Signals?

The prevailing view holds that signals from the vegetal hemisphere induce mesoderm and endoderm around the equator. This view derives from the experiments of Pieter Nieuwkoop, who first demonstrated the phenomenon of meso-endoderm induction. He included both the mesoderm and endoderm in his considerations; later authors emphasized the mesoderm alone and gradually came to call the interaction mesoderm induction, although in the past two years, endoderm has regained attention. These experiments of Nieuwkoop exploited the ability of explants of amphibian embryos to develop in isolation in a simple buffered saline. Animal caps develop into a kind of epidermis, and vegetal explants either do not develop into recognizable tissues, or develop some posterior endodermal characters. However, when animal caps are grafted onto vegetal explants, mesoderm and pharyngeal endoderm are the result (Nieuwkoop 1969a,b). Using pigmentation and ^3H -thymidine-labeled cells as markers, Nieuwkoop concluded that the mesoderm and head endoderm developed exclusively from the animal cap tissue and were therefore induced by the vegetal cells (Nieuwkoop & Ubbels 1972). Explants of dorsal vegetal cells induce dorsal mesoderm such as notochord and muscle, and induce head endoderm (and hence an organizer had been induced, from which these tissues derive). In contrast, ventral vegetal cells induce blood and mesenchyme, and intermediate vegetal cells induce intermediate types of mesoderm (Boterenbrood & Nieuwkoop 1973). Nieuwkoop and coworkers therefore favored the view that the mesoderm and head endoderm were induced by an interaction of animal hemisphere cells with vegetal hemisphere cells and that the signal produced by the vegetal cells was graded from the dorsal to the ventral side. A somewhat different view was advanced by Smith & Slack (1983); a three signal model for mesoderm patterning, consisting of an early pair of signals differing qualitatively between dorsal and ventral vegetal blastomeres (Dale et al 1985), that acted in the blastula stage to divide the early marginal zone of mesoderm into only two distinct territories: the dorsal (axial) and ventral mesoderm, the former being the organizer. The third signal, a dorsalizing inductive signal from the organizer, would then impose more dorsal (paraxial) and intermediate fates on

neighboring ventral mesoderm in the gastrula stage. This model has continued to be a useful interpretation of the embryological results, although it has been modified in molecular detail (see below). Here we support the argument for two distinct signals from the vegetal hemisphere: a generic meso-endoderm-inducing signal and a dorsal-modifying signal superimposed on this, as diagrammed in Figure 1.

Although recombinates of vegetal and animal cells can be used successfully to document the phenomenon of meso-endoderm induction, the mesoderm normally differentiates from the equator, and it is difficult to use cut-and-paste embryological experiments to demonstrate that normal meso-endoderm formation is a direct result of cell interaction. As soon as it is feasible to take explants from the marginal zone, those cells can develop autonomously as mesoderm. Thus it is possible that the phenomenon of meso-endoderm induction is a synthetic, experimental construct and that the embryo normally uses cell-autonomous determinants for activating mesoderm and endoderm. However, mesoderm formation can be blocked by reagents that block signaling by secreted polypeptide growth factors such as activin and FGF, which lends support to the idea that meso-endoderm formation is a result of the activation of cell surface receptors and, therefore, is a result of induction, broadly defined to include paracrine and autocrine signaling (Figure 2). The best evidence that mesoderm formation may be at least in part a result of the segregation of cell-autonomous determinants comes from experiments with dissociated embryos. The most dorsal and most ventral equatorial blastomeres still initiate expression of dorsal- and ventral-specific genes, even though they are kept from cell contact (Lemaire & Gurdon 1994). However, the same genes do not turn on in embryos where signaling by TGF- β superfamily members is blocked with a truncated activin receptor (Hemmati-Brivanlou & Melton 1992). The results could be reconciled if the activity of localized determinants requires autocrine signaling by members of the TGF- β superfamily. Certain natural (*xnr2*) or synthetic (*Bvg1*) TGF- β relatives are not secreted efficiently by cells, but still have biological activity as meso-endoderm inducers (Dohrmann et al 1996, Jones et al 1996b). Perhaps they act within the secretory pathway on receptors that have not yet traversed to the cell surface, as is thought to occur for other growth factors (Bejcek et al 1989).

The graded action of vegetal cells in inducing dorsal to ventral types of mesoderm suggests graded signals (Boterenbrood & Nieuwkoop 1973). The first candidate mesoderm inducers appeared to fit this model. FGF induced ventral types of mesoderm in animal caps, whereas activin induced dorsal types of mesoderm. Further work has modified this view extensively. First, expression of a large excess of dominant-negative receptors can be used to prevent signaling in the embryo. A truncated, dominant-negative FGF receptor resulted in blockage of most kinds of mesoderm induction, not just of ventral mesoderm but

also of muscle and notochord. Only parts of the head mesoderm differentiate in the absence of FGF signaling (Amaya et al 1991, 1993). A dominant-negative activin receptor, which we now know blocks most TGF- β family signals, prevents the formation of any mesoderm (Hemmati-Brivanlou & Melton 1992). Such experiments support the possibility that the induction of dorsal versus ventral mesoderm may not depend on the different exposure of cells to activin- or FGF-like signals. Instead, activin and FGF signals may induce the development of a generic kind of ventral mesoderm, uniformly arrayed around the equator, upon which the organizer sub-region would be patterned by a separate signal, a dorsal modifier of meso-endoderm induction.

When Are the Meso-Endoderm-Inducing Signals Active?

To define when a signal ceases to be sent, progressively older vegetal blastomeres are recombined with tissue that is competent to respond, usually mid-blastula animal cap. Boterenbrood & Nieuwkoop (1973) and then Jones & Woodland (1987) carried out the first extensive analysis and concluded that the signaling center loses activity at early to midgastrula stages. By grafting progressively older animal caps onto early, strong-inducing cells, it was concluded that animal caps lose their competence to respond to the vegetal signals at the late blastula (urodeles; Boterenbrood & Nieuwkoop 1973) or early gastrula (*Xenopus*; Gurdon et al 1985, Jones & Woodland 1987) stages. This conclusion is supported by measurements of the period of competence of animal cap cells to respond to soluble mesoderm inducers (activin or FGF). Competence declines gradually in late-blastula and early-gastrula stages, but with larger doses of inducer and sensitive measurements, mesoderm induction is still detectable in caps cut from midgastrula stages (Green et al 1990, Lamb & Harland 1995).

In addition, the start of signaling was measured by combining young vegetal plugs with old caps cut from very early gastrulae, which have only a brief period of competence left. If the caps respond at all, the vegetal cells already must be signaling at the time of first contact. The conclusion was that vegetal cells are signaling as soon as it is feasible to isolate them and measure their activity, at the 16- to 32-cell stage (Jones & Woodland 1987). The strong implication of this work is that the start of meso-endoderm induction must rely on maternal mRNA or protein since transcription does not start in earnest until the midblastula stage (4000 cells).

Although meso-endoderm induction may initially rely on maternal signals, it does not mean that all meso-endoderm induction relies only on maternal information. In particular, a small early maternal signal may initiate zygotic inductions or sensitize cells to further zygotic inductions. Such changes in competence have not been explicitly measured, although mesoderm inducers are known to prolong the lifetime of FGF receptor and may thereby prolong the competence to respond to FGF (Musci et al 1990, Friesel & Dawid 1991).

THE IDENTITY OF MESO-ENDODERM INDUCERS

There are several candidates for a meso-endoderm inducer, but to date none has been proven essential in the embryo. The proposed signals include various TGF- β family members such as activin (Asashima et al 1990, Smith et al 1990, Thomsen et al 1990), Vg1 (Thomsen & Melton 1993), TGF- β 2 (Rosa et al 1988), and *Xenopus* nodal-related 1 and 2 (Xnr1, Xnr2) (Jones et al 1995). All are present in the egg either as mRNA or protein, or are transcribed in the late blastula. Activin protein, for example, is present, and the current data indicate its location in yolk platelets as a complex with lipovitellin, taken up by the oocyte during vitellogenesis (Dohrmann et al 1993, Uchiyama et al 1994). Although BMPs are now considered epidermal inducers (see below), they also have ventral mesoderm-inducing activity at high doses (Wilson & Hemmati-Brivanlou 1995) and may be a part of the mesoderm-inducing signal. From the FGF family, basic FGF protein (not secretable by the usual secretory pathway) has been found in the egg and embryo, and eFGF (embryonic FGF, a secreted form) has been found in the embryo, as have novel ligands that also can activate the FGF receptor (Kimelman et al 1988, Slack & Isaacs 1989, Isaacs et al 1995, Kinoshita et al 1995).

The TGF- β Family

Although all of the activities listed can induce meso-endoderm when presented to blastula-stage animal caps as mature active protein, we do not know how much protein is present in the embryo, and it has been particularly difficult to block the activity of specific molecules to test their essentiality. The use of dominant-negative receptors has provided convincing evidence that the TGF- β superfamily and FGF family are involved in induction, but the approach suffers from the limitation that the dominant-negative forms may be promiscuous in their inactivation of receptors and that the receptors themselves may have broad specificity in their binding of ligands. A more detailed discussion of dominant-negative receptors is presented by Harland (1996), and a recent review of TGF- β signaling is presented by Massagué (1996).

A truncated activin type II receptor (truncated ActRII), which retains a transmembrane domain, blocks signaling by activin (Hemmati-Brivanlou & Melton 1992), Vg1 (Schulte-Merker et al 1994), and BMPs (Hemmati-Brivanlou & Thomsen 1995). It is surprising that this truncated receptor blocks Vg1 signaling because it does not bind Vg1 (Kessler & Melton 1995). Presumably it inhibits by interfering with formation of the ternary complex between Vg1, its type II receptor, and the transducing type I receptor. Even though the truncated type II receptor lacks specificity within the TGF- β family, it does not block signaling by the FGF pathway; thus its specificity may be limited to the TGF- β superfamily (Hemmati-Brivanlou & Melton 1992). Embryos containing

a large excess of truncated ActRII form no mesoderm, suggesting that one or more members of the family are crucial in mesoderm induction. Additional evidence that the dominant-negative receptor has some specificity and is not just gumming up the works comes from rescue experiments where wild-type receptor mRNA is mixed with the mRNA for the dominant-negative receptor, and normal development is restored. However, this approach may test only whether gumming is irreversible. A similar conclusion that signaling by TGF- β family members is essential for mesoderm induction comes from experiments with a truncated activin type I receptor, ALK4 (or ActRIb) (Chang et al 1997).

As an alternative approach, a truncated activin type II receptor has been used, consisting of the extracellular domain without the transmembrane domain. This modified receptor, which presumably is soluble and still binds its ligand, should be more specific in its inhibition than a truncated receptor that retains a transmembrane domain. This dominant-negative form does not affect signaling by Vg1 or Xnr1 and has only a small effect on signaling by BMP4 (Dyson & Gurdon 1997). Embryos containing this dominant-negative receptor develop abnormally and seem delayed in mesoderm formation, although mesoderm eventually does form. However, the activin type II receptor is known to bind other family members such as BMP7 (Yamashita et al 1995), so while the specificity of this form is improved, it is still not narrow enough to conclude that activin is the required ligand. The best evidence against activin being a required ligand is from follistatin mRNA injection. Although follistatin blocks the activity of activin added to animal caps, it does not prevent mesoderm induction in the embryo (Schulte-Merker et al 1994, Kessler & Melton 1995).

Despite the large body of work on meso-endoderm induction, and prevention of this induction by manipulating signaling by the TGF- β family, we still do not know definitively whether the inducer used in the embryo is one of those already identified or is yet to be identified. However, it is fairly safe to conclude that signaling by TGF- β family members plays an essential role in meso-endoderm induction.

Signaling by FGF

bFGF was the first purified (and known) molecule able to induce mesoderm (Kimelman & Kirschner 1987, Slack et al 1987). It is not a potent inducer of dorsal mesoderm, and it was initially suggested that it is the ventral mesoderm inducer. Models for patterning the marginal zone based on an activin-like dorsal signal and an FGF ventral signal were developed in considerable detail (Green et al 1992). However, the hypothesis rests on the assumption that extensive patterning of the marginal zone is carried out by meso-endoderm inducers

during cleavage and blastula stages, whereas there is good evidence that most patterning of the marginal zone occurs during the gastrula stages, when cells are losing their responsiveness to mesoderm inducers and when mesoderm dorsalizing activities from the organizer become important (the third signal in the three signal model) (Green et al 1992; see below). The main evidence against FGF as a predominantly ventral signal came from interference with signaling. Dominant-negative FGF receptors (Amaya et al 1991, 1993), as well as interference with FGF signaling downstream of the receptor (Whitman & Melton 1992, LaBonne et al 1995), showed that the FGF signal transduction pathway is required for production or maintenance of most of the mesoderm, including dorsal mesodermal tissues like the notochord and somites (Amaya et al 1993, Kroll & Amaya 1996). In addition, dorsal injections of the dominant-negative receptor have more severe consequences than ventral injections, suggesting FGF has important roles on the dorsal side (Isaacs et al 1994). The only mesoderm that appears to form without the involvement of FGF is the head (prechordal) mesoderm (Amaya et al 1993, LaBonne & Whitman 1994, Cornell et al 1995), because embryos expressing the dominant-negative FGF receptor still develop heads and express *gooseoid*, a marker gene of prechordal mesoderm in the late gastrula. Furthermore, when animal cap explants expressing the truncated receptor are treated with activin, they too express *gooseoid*. Interestingly, inhibition of FGF signaling prevents continued expression of most other mesodermal properties in response to activin, including expression of most markers of mesoderm, such as *brachyury* (*Xbra*). Therefore FGF signaling appears to provide a permissive environment in which activin can signal with full effect (LaBonne & Whitman 1994). The molecular basis for this partial dependence of activin signaling on an intact FGF pathway is not understood.

WHEN DOES FGF SIGNALING ACT? Although several observations have shown that FGF signaling is crucial for mesoderm maintenance at the early-gastrula stage, they have called into question any role for FGF signals in the induction itself in the blastula stage (Isaacs et al 1994, Schulte-Merker & Smith 1995). These experiments showed that the truncated FGF receptor prevents continued expression of *Xbra*, which is considered to be initially a generic mesoderm marker gene, expressed throughout the marginal zone, although subsequently it narrows to expression in the notochord and posterior mesoderm. Expression of *Xbra* is not maintained when cells are dissociated, but expression is restored by application of recombinant FGF protein. Increased FGF expression in the embryo causes excess posterior development and elevated expression of genes that are normally expressed in the posterior part of the neurula (Isaacs et al 1994, Pownall et al 1996).

DOES FGF HAVE A ROLE IN MESO-ENDODERM INDUCTION? If FGF signaling is required for mesoderm maintenance, then it becomes more difficult to ask whether it is also required for the first steps of induction. One might expect that in the presence of a truncated FGF receptor, mesoderm-specific marker genes would be transiently activated if FGF has no role in mesoderm induction but does have a role in maintenance, or would never be activated if FGF has also a role in initial induction. The data are incomplete but indicate a transient expression (Isaacs et al 1994, Schulte-Merker & Smith 1995). A suggested role for FGF is in distinguishing between mesodermal and endodermal germ layers. While mesodermal fates require FGF signaling, endodermal fates are suppressed by FGF (Cornell et al 1995, Gamer & Wright 1995). It is attractive to think that activin-like signals are restricted to the equatorial and vegetal regions, whereas FGF signals are restricted to the animal and equatorial regions. Thus an overlap of activin-like and FGF signals would specify mesoderm at the equator (the marginal zone), leaving endoderm in the vegetal region. Ectoderm would be left as the animal cap region receiving FGF but not activin signals. Hence the germ layers would be established from a common cell population, and the relatedness of layers would be based on their having received FGF, activin, or both. The main problem with this attractive conclusion is that FGF signaling does not appear to be restricted to the ectoderm and mesoderm. From measurements of the activation of MAP kinase, an intracellular mediator of FGF signaling, there is good evidence that FGF receptor activation occurs throughout the entire embryo at the late blastula and gastrula stages (LaBonne & Whitman 1997). From experiments like these, it is hard to conclude that FGF has any instructive role in meso-endoderm induction, and we are left with an incomplete picture of how the tissue layers are formed.

A Role for Localized Transcription Factors?

Although dominant-negative receptor experiments and cell dissociation experiments have shown convincingly that signaling is required for meso-endoderm induction and/or maintenance, localized transcription factors may also contribute to germ layer formation. A T-box factor (related to the T gene *brachyury*), whose mRNA is localized in the vegetal hemisphere of eggs, has been identified by several independent approaches (Lustig et al 1996, Stennard et al 1996, Zhang & King 1996). Synthetic mRNA transcribed from this gene (*Xombi*, *VegT*, or *antipodean*) can specify mesodermal fates in animal cap cells explanted from injected embryos. This localized transcript is therefore a likely contributor to mesoderm specification. As yet, the RNAs have not been eliminated in the oocyte to determine the essentiality of their encoded proteins in early development.

DORSAL-VENTRAL PATTERN AS SEPARATE FROM MESO-ENDODERM INDUCTION

A Competence Modifier Is Moved to the Dorsal Side

Although graded activity of mesoderm inducers such as activin, Vg1, or Xnr1 and 2 could in principle account for dorso-ventral differences in the mesoderm, there is currently no direct evidence for this view because none of the inducers is found in a clear dorso-ventral gradient, and because inhibiting TGF- β signaling eliminates all mesoderm rather than leading to defects in the dorso-ventral patterning of mesoderm of the late blastula marginal zone. Instead, we favor the proposal that a competence modifier, with no inherent meso-endoderm-inducing activity of its own, acts on the dorsal side of the embryo and synergizes with meso-endoderm inducers to produce the organizer, as proposed by Christian et al (1992) and Kimelman et al (1992).

Dorsal signals are specifically localized and activated after fertilization. A number of experiments have suggested that most of the dorsal side of the embryo is modified as a result of cytoplasmic rearrangements that occur after fertilization. The cytoplasmic rearrangement (called cortical rotation) is manifest as a displacement of the cortex of the egg by 30° relative to the central core of cytoplasm of the egg. Between the cortex (4 μ m thick) and the core is a shear zone (also 4 μ m thick) faced with parallel microtubules that originate from the core and lie on its surface, bordering the shear zone, oriented with their plus ends toward the prospective dorsal side. These are the likely tracks upon which molecular motors, attached to the cortex, drive rotation (Elinson & Rowning 1988, Houlston & Elinson 1991, Rowning et al 1997). Although the cortex rotates by only 30° relative to the core, small particles, membrane-bound organelles, pigment granules, and possibly informational molecules are transported in saltatory bursts for much greater distances, as much as 60-100°, along the microtubules. Since particles are moved toward the plus ends of microtubules, a kinesin may be involved. The shear zone is also a transport zone. Rotation of the cortex is now thought to be a device to align microtubules into a single parallel array used for efficient transport (Rowning et al 1997). When eggs are exposed to UV irradiation on the vegetal pole or to low temperature or high pressure, the microtubules depolymerize and cortical rotation stops, as does vesicle transport. Such eggs develop as ventralized embryos (Gerhart et al 1989). They fail to form a Nieuwkoop center and an organizer (discussed below).

The result of transport along microtubules during cortical rotation is that particles starting near the vegetal pole may reach the equator or even enter the animal hemisphere. Considerable evidence supports the idea of transport. These experiments include ablations of vegetal regions of the egg, which abolish

dorsal-ventral polarity if vegetal pole cortex is removed before, but not after, cortical rotation (Sakai 1996). Not only is the vegetal region required for dorsal inductions, but the cytoplasm near the vegetal cortex contains transplantable dorsalizing material (Yuge et al 1990, Fujisue et al 1993). This material is probably attached to the cortex at various times because transplants of cortex can initiate the development of a secondary axis (Kageura 1997). Interestingly, although this material has the ability to act in the marginal zone to produce dorsal mesoderm, in the animal cap it has no mesoderm-inducing activity, although it occasionally induces some neural tissue (Holowacz & Elinson 1995). As discussed below, this behavior is also found for injected protein intermediates of the Wnt signaling pathway.

The Nieuwkoop Center

At early cleavage stages, the highest concentration of dorsal meso-endoderm-inducing activity resides in the dorsal vegetal blastomeres (tiers 3 and 4 in a regularly cleaving 32-cell embryo; see Figure 3). Transplantation of pairs of dorsal vegetal blastomeres, either into a ventralized embryo or into the ventral side of a normal embryo, leads to axis induction (Gimlich & Gerhart 1984, Gimlich 1986). The most striking demonstration that the signal is an inductive signal, and not limited to autocrine effects, is from experiments with the tier 4 dorsal blastomeres. In normal development, lineage tracing shows that these cells do not differentiate into mesoderm, but form part of the anterior gut endoderm. When transplanted into the ventral side of tier 4, they induce an axis (hence an organizer is formed), but themselves still form part of the endoderm. This procedure is a clear experimental demonstration of induction, where the signaling cell instructs neighboring cells to change their fate, but does not itself participate in the differentiation of dorsal mesoderm. Tier 3 dorsal blastomeres are also active (Gimlich 1986), but since their progeny cells are normally fated to be part of the organizer and to form some dorsal mesoderm, the experimental demonstration that the signaling is inductive is not quite as obvious as with the tier 4 blastomeres. Nonetheless, the tier 3 dorsal blastomeres do recruit additional tissues to the new axis, and thus show inductive activity. In fact, they are more active in inducing an axis than are dorsal blastomeres of the fourth tier, and in the context of normal development are likely to be sufficient to induce a complete axis because all tier 4 blastomeres can be removed entirely without affecting dorsal development (Gimlich 1986).

From these experiments we can conclude that cells of both tier 3 and 4 (and to a lesser extent tier 2 and 1 cells, as discussed below) transmit an inductive signal that can induce the organizer and dorsal meso-endodermal structures without themselves necessarily contributing progeny cells to these differentiations. An additional important characteristic of this vegetal dorsalizing center of cells is its time of activity. When progressively later blastomeres are taken for

transplantation, it is clear that the vegetal dorsalizing cells lose activity in the late blastula; even when they are transplanted into an early embryo that is competent to respond to the signals, no dorsal induction takes place (Boterenbrood & Nieuwkoop 1973, Jones & Woodland 1987). Therefore, an additional important operational criterion for the activity of the vegetal dorsalizing center is that it is present before, but lost at, the late blastula stage. This vegetal dorsalizing center has been called the Nieuwkoop center (to acknowledge Nieuwkoop's pioneering contributions) (Gerhart et al 1989). The center is defined as a group of cells able to induce the formation of the organizer from animal hemisphere cells. As discussed below, this probably requires the synergy of two kinds of signals, a meso-endoderm inducer and a dorsal competence modifier. Individual cells, able to release both signals, occupy the dorsal sector of the blastula at the vegetal and equatorial levels.

Although the strongest Nieuwkoop center activity is found in the dorsal quadrant of the vegetal hemisphere (Gimlich & Gerhart 1984, Gimlich 1986), the modification of signaling ability that results from cortical rotation extends nearly to the animal pole. Thus, tier 1 and 2 blastomeres have inducing ability when transplanted to the same tier on the ventral side (Kageura 1990), or even when placed in a vegetal tier (Gallagher et al 1991). Many progeny of dorsal tier 2 blastomeres enter the organizer and dorsal mesoderm, and some progeny of dorsal tier 1 blastomeres do this as well (Vodicka & Gerhart 1995). Cells of the dorsal sector of the animal hemisphere may secrete only the dorsal modifying signal, as discussed below. However, in regions where they are close to other cells releasing the meso-endoderm inducer, one would have to say a Nieuwkoop center is present, composed of individual cells secreting one or the other signal, but not both.

Finally, several experiments have shown that animal caps, which themselves do not form dorsal meso-endoderm autonomously, have a dorso-ventral polarity that leads to asymmetric expression of an epidermal antigen (London et al 1988), and to different responses of dorsal and ventral fragments of caps to inducers such as activin (Sokol & Melton 1991, Bolce et al 1992). These observations indicate that the dorsal side of the animal cap has been modified as the result of cortical rotation, even though the modification is not sufficient in itself for organizer formation. The necessary meso-endoderm inducer (the putative TGF- β signal) is not produced by animal cap cells.

What Is the Nature of the Dorsal Modifying Signal?

In principle Vg1 or activin-like signals could induce dorso-ventral polarity, and several in vitro results are consistent with this, such as the induction of ever more dorsal gene expression when disaggregated midblastula animal cap cells are exposed to ever higher concentrations of activin (Green et al 1994). However, as discussed above, direct evidence in the embryo is lacking for this

possibility. Signaling by BMPs has a clear role in dorso-ventral polarity at gastrula stages and is discussed below in the context of the activities of the organizer. That leaves the Wnt signaling pathway as the most attractive candidate for the early competence-modifying activity, and the evidence for the involvement of this pathway in dorsal-modifying activity is now strong. Several experiments have shown that Wnts can mimic the activity of the Nieuwkoop center when injected into vegetal blastomeres (Smith & Harland 1991). Although Wnt-injected animal caps do not form mesoderm, their response to FGF and activin is greatly enhanced such that they form even dorsal mesoderm (Christian et al 1992, Sokol & Melton 1992). Thus the injected caps produce a competence modifier.

An endogenous component of the Wnt signaling pathway in *Xenopus* eggs, glycogen synthase kinase (GSK3), has been implicated in the normal suppression of dorsal fates; in these experiments, a dominant-negative kinase expressed on the ventral side mimics Wnt signaling and induces a secondary axis (Dominguez et al 1995b, He et al 1995, Pierce & Kimelman 1995). Expression of excess active GSK3 on the dorsal side suppresses dorsal development. The wild-type, constitutively active kinase is thought to phosphorylate β -catenin, leading to proteolysis of the β -catenin. Activation of Wnt signaling, or overexpression of the dominant-negative kinase, prevents phosphorylation and stabilizes β -catenin (Yost et al 1996). Overexpressed β -catenin, or its close relative plakoglobin, also mimics Wnt signals in the embryo, (Guger & Gumbiner 1995, Karnovsky & Klymkowsky 1995), and stabilization of the β -catenin by amino acid substitutions increases its activity (Yost et al 1996).

Although the traditional role of β -catenin was thought to be part of the adherens junction, stabilization of β -catenin leads to accumulation of free cytoplasmic protein that can then accumulate in the nucleus (Funayama et al 1995, Yost et al 1996). β -catenin forms a complex with the transcription factors LEF1 or tcf3, enters the nucleus with them, and modifies the activity of these DNA-binding proteins (Behrens et al 1996, Molenaar et al 1996). Thus at the midblastula transition when transcription begins, Wnt signals appear to lead directly to transcriptional activation of genes such as *xnr3* and *siamois* (Smith et al 1995, Carnac et al 1996), which are normally expressed in the organizer and dorsal ectoderm; the signals may also synergize with mesoderm-inducing signals to activate genes like *gooseoid* (Steinbeisser et al 1993, Watabe et al 1995), which is expressed in the anterior half of the organizer (discussed below). The precise mechanism by which β -catenin modifies transcription factors has yet to be elucidated, and although it may act as a coactivator of transcription, as suggested by Behrens et al (1996) and Molenaar et al (1996), it is also possible that it blocks constitutive activity of the transcription factors because a membrane-tethered form of plakoglobin has axis-inducing activity (Merriam et al 1997). In this case, the activity of LEF1 or tcf3 might be viewed as promoting ventral fates,

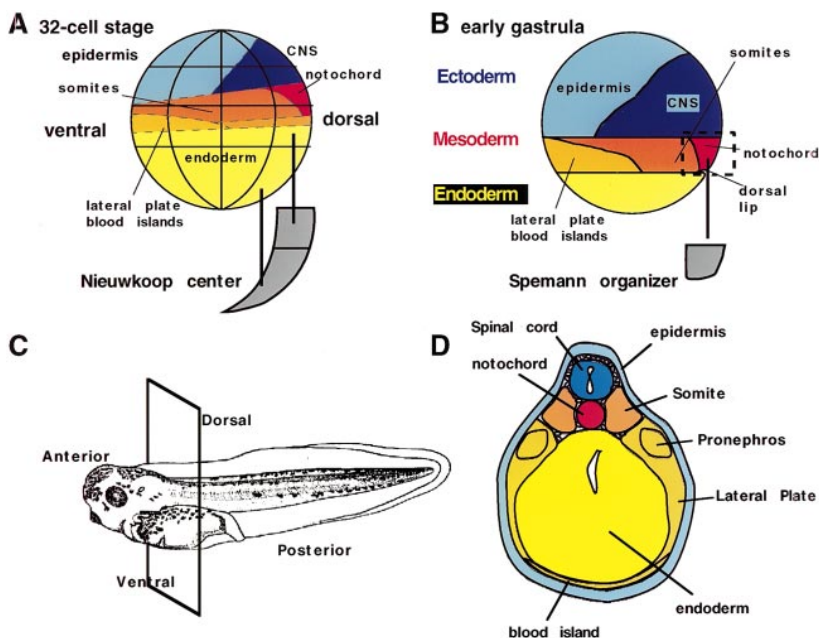


Figure 3 Summary of fate maps and organizing centers in the *Xenopus* embryo. (A) The four tiers of the 32-cell embryo, and the location of the Nieuwkoop center. Lines indicate cleavage planes in a regularly cleaving embryo. Tier 1 is the top tier and tier 4 the bottom tier. The colored fate map, drawn from data of Dale & Slack (1987a), is only approximate because extensive cell mixing will occur, and boundaries of different territories are not sharp. The Nieuwkoop center activity is strongest in tiers 3 and 4 on the dorsal side. CNS, central nervous system. (B) By early gastrulation, epiboly has spread the ectoderm, and internal gastrulation movements have begun. By this time, Spemann's organizer, located in the dorsal lip, has become active, and the Nieuwkoop center is inactive. The head and heart mesoderm have already moved inside the gastrula. The fate map is drawn from Keller (1975, 1976). The superficial layer of the marginal zone, which overlies the deep mesoderm and will form the lining of the archenteron and afterward the definitive gut, is not shown here (see Figure 5). (C) A section through the trunk of a tadpole is represented in (D), with the same colors used in the fate maps.

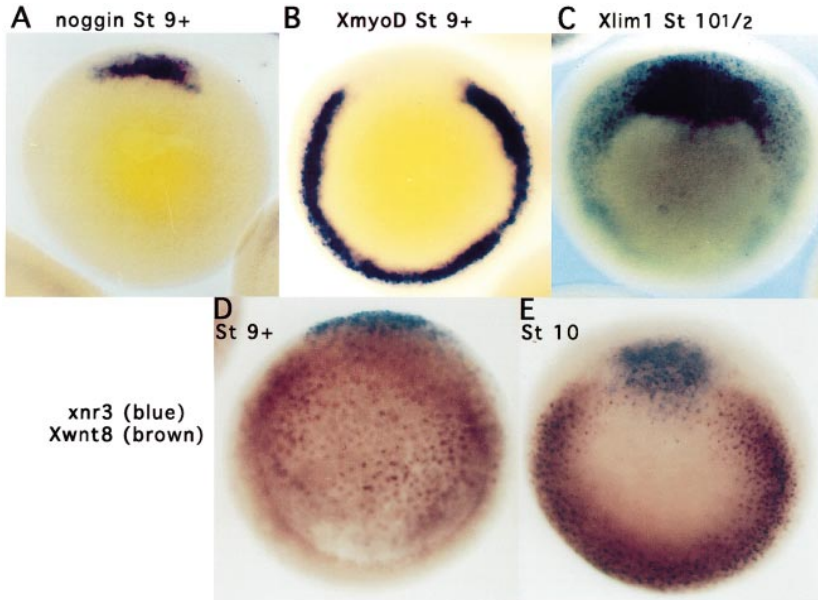


Figure 4 In situ hybridizations of the *Xenopus* late blastula and early gastrula, illustrating the binary decision of cells of the early marginal zone to become dorsal or ventrolateral mesoderm, and the rapid changes that occur by the early-gastrula stage as a result of dorsalizing signals from the organizer. These are all viewed from the vegetal pole, so that the mesoderm appears as a ring of tissue at the equator. (A) Late blastula stained for *noggin* mRNA (Smith & Harland 1992), showing expression restricted to the organizer. (B) Late blastula stained for *XmyoD* mRNA (Hopwood et al 1989, Frank & Harland 1991), showing a complementary pattern to A. (C) Early gastrula stained for *Xlim-1* mRNA (Taira et al 1992). While the onset of expression of this gene is more restricted in the blastula, by the early-gastrula stage there is an extensive dorsal-to-ventral gradient of expression in the marginal zone. (D) A late blastula stained for *xnr3* mRNA (Smith et al 1995) (blue) and *Xwnt8* mRNA (Christian et al 1991) (brown) mRNAs. The *Xwnt8* gene is expressed in the entire marginal zone except for the organizer region, in which the *xnr3* gene is exclusively expressed. (E) Late blastula stage (as in D), 60 min later. Signals from the organizer have spread laterally, and *Xwnt8* mRNA is now absent from cells close to the organizer, but still present in more distant cells of the marginal zone. The region stained for *xnr3* mRNA is slightly narrower than in D, probably because of convergence movements of cells to the dorsal midline.

whereas blockage of their activity allows derepression of dorsal gene activity. Whichever scenario is more accurate, the conclusion that excess β -catenin can act as a dorsalizing activity is robust.

Destruction of β -Catenin mRNA Blocks Axis Formation

As discussed in regard to the activin receptor, dominant-negative experiments have been informative but have the possible weakness that the dominant-negative protein binds promiscuously to other proteins. Hence it must be demonstrated that only the targeted activity is being blocked, and this is often difficult to do. The control of achieving rescue by the injection of excess wild-type protein may simply demonstrate mass action effects rather than specificity.

A more rigorous analysis would use mutational inactivation of components, but that is not applicable to *Xenopus*. However, experiments that analyzed the requirement for β -catenin have come close to the rigor of a genetic mutation. In these experiments, injected antisense oligonucleotides, in concert with endogenous RNase H, were used to deplete the maternal pool of β -catenin mRNA (Heasman et al 1994). After maturation and fertilization, these embryos developed no dorsal structures but developed ventral structures resembling those of ventralized embryos produced by blocking cortical rotation or removing the organizer (Gerhart et al 1989). Restoration of β -catenin mRNA by injection of a non-degradable form led to rescue of the phenotype, demonstrating the specificity of the effect. Such deficient eggs were not rescued by injection of mRNAs for Wnt8 or the GSK3 dominant-negative protein, showing that the encoded proteins must act through β -catenin. This powerful experiment indicates that blocking the Wnt signaling pathway is sufficient to prevent dorsal development and strongly suggests that only the Wnt signaling pathway leads to dorso-ventral polarity in the early embryo. The results suggest that even if dorsal-to-ventral gradients of TGF- β proteins, such as Vg1, are present in the embryo, they are not sufficient to impose any dorso-ventral polarity. This experiment, more than any other, leads us to favor the view that whereas the TGF- β pathway is likely to be involved in general meso-endoderm induction, it does not provide dorso-ventral information (Slack 1994). Also included in this view, the dorsalizing effect of lithium ion may result from its inhibition of GSK3 and hence its stabilization of β -catenin throughout the embryo (Klein et al 1996).

What Activates the Wnt Signaling Pathway?

The most obvious way that Wnt signaling might occur on the dorsal side is through activated expression of localized maternal *wnt* mRNAs. Although maternal *wnt* mRNAs have been identified (Ku & Melton 1993, Cui et al 1995), there is growing evidence that the Wnt pathway may be activated inside the eggs, circumventing a Wnt ligand. Reagents that are expected to block endogenous Wnt or disheveled activity in the egg do not prevent axis formation, even

though they block the axis-inducing activity of injected *wnt* mRNA (Hoppler et al 1996, Sokol 1996, Leyns et al 1997, Wang et al 1997); whereas overexpression of GSK3 and depletion of β -catenin does block axis formation. It is attractive to speculate that cortical rotation activates the Wnt pathway downstream of the Wnt receptor, bypassing the need for an extracellular signal. In recent antibody-staining experiments, β -catenin protein is seen to accumulate on the dorsal side of the egg after cortical rotation and to be maintained at least until the midblastula transition (Schneider et al 1996, Larabell et al 1997, Rowning et al 1997). Because β -catenin mRNA and GSK3 mRNA are uniformly distributed in the egg, it is speculated that cortical rotation transports some locally acting inhibitor of GSK3 activity to the dorsal side (Rowning et al 1997).

What Is Activated by Wnt Signaling?

Gene transcription is different between the dorsal and ventral sides of the normal embryo even at the onset of gene activation at the midblastula transition. Thus the dorsal modifier signal, such as maternal β -catenin, must either affect transcription directly in the dorsal mesoderm or cause the synthesis and release of signals that can soon act on transcription in the dorsal mesoderm. The transported Wnt intermediate may stabilize β -catenin, which then complexes with transcription factors to directly activate organizer-specific genes, such as *xnr3* (Smith et al 1995) and *siamois* (Lemaire et al 1995) (indeed, these genes are expressed in animal cap cells injected with β -catenin mRNA). The transported intermediate may synergize with other transcription factors activated by the TGF- β pathway to enhance expression of genes such as *gooseoid* (Steinbeisser et al 1993).

However, the effects of overexpressed β -catenin are not entirely cell autonomous, and animal caps overexpressing β -catenin can dorsalize adjacent mesoderm without also having meso-endoderm-inducing properties (Wylie et al 1996). Such experiments strongly support the idea that activation of the Wnt pathway causes release of a dorsal signal that can act on other cells and can synergize with the TGF- β signaling pathway. Therefore, activation of the Wnt pathway (i.e. an increase of β -catenin levels) could have at least two effects on the secretion of protein signals to neighboring cells. The first would be a direct effect on the transcription of genes that encode secreted products (such as *Xnr3*). This effect would require a zygotic step, and hence the induction would occur only after the midblastula transition. The second would involve activation of maternal mRNA translation or secretion of stored maternal proteins. This effect could occur prior to zygotic transcription. A recent report (Wylie et al 1996) supports the idea that significant effects of the Wnt pathway activation follow the midblastula transition. To date, no compelling mechanism has been proposed for the expected Wnt effects on maternal inductive events. Wnt signaling has been shown to enhance gap junctional communication within minutes at

early blastula stages (Olson et al 1991). Other effects of β -catenin on secretion or translation remain to be discovered.

GRADIENTS AND RELAYS OF SIGNALING PROTEINS

Developmental biologists have been almost obsessed by the idea of morphogens, substances that when presented at different concentrations elicit different fates in a responding tissue. In *Xenopus*, TGF- β family members have been particularly attractive candidates for endogenous morphogens. Both activin and mature Vg1 protein can induce progressively more dorsal fates at higher concentrations, as discussed above. But how would gradients of these proteins be produced? One method is by diffusion from a localized source. Activin appears in some experiments to be able to diffuse over several cell diameters, and when released from a local source such as a bead, it induces rings of expression of various genes that likely activate at various decreasing doses of protein at increasing distances from the source (Gurdon et al 1994, 1995, 1996, Harger & Gurdon 1996). An alternative view is that TGF- β proteins do not diffuse but act on neighboring cells, inducing the expression of a secreted inducer (the same or a different protein) in that neighbor (Reilly & Melton 1996). This inducer might then induce synthesis of an inducer in the next cell, propagating the signal. Different experiments have been used to argue either case, but a recent comparison of a single ligand that behaves differently when processed by different means, strongly argues that ligands can diffuse or not, depending on conditions. In these experiments, Xnr2 was expressed in wild-type form, or as an activin-Xnr2 chimera. The activin pro-region allowed the cleaved Xnr2 ligand to act over several cells, whereas the wild-type pro-region allowed the cleaved Xnr2 to act over only very short distances, perhaps on only the adjacent cell (Jones et al 1996a). It is difficult to argue that the different processing of Xnr2 would cause a different ability to activate a relay, but it seems reasonable to conclude that different processing may allow the active ligand to be secreted or not, as has been found for Vg1 chimeras (Kessler & Melton 1995, Dohrmann et al 1996). The activation of relays also has been controversial, with some experiments showing cell-autonomous activity of the activated activin receptor (Jones et al 1996a), while others argue that a relay must occur (Reilly & Melton 1996). Activation of a relay should not be surprising because signals such as activin are known to activate the expression of genes such as *xnr1* and *xnr2* encoding other mesoderm-inducing signals (Jones et al 1995).

While these various experiments illustrate what principles might apply to intercellular signaling, none directly addresses the important question of whether a gradient of activity of any ligand exists in the embryo. Furthermore, as mentioned above, because depletion of β -catenin prevents the formation of dorsal mesoderm and because activation of Wnt signaling provides a dorsal-

modifying signal that is not a mesoderm inducer, it is likely that the doses of meso-endoderm-inducing signals in the embryo are well below those used in experimental constructions of diffusion gradients to achieve dorsal meso-endoderm formation. It is plausible that the dorsal modifier acts by enhancing the effects of the meso-endoderm inducer, for example by increasing the number of receptors to it or raising the activity of intermediates of the inducer's signal transduction pathway. Hence the *in vitro* results with artificially high doses of a meso-endoderm inducer may give some reflection of the cell's true range of responses, even though the embryo uses a synergy of two factors rather than very high levels of one to achieve dorsal meso-endoderm induction. With regard to gradients created experimentally, secreted growth factors bind to many low-affinity sites on cells, so that low concentrations may be severely limited in diffusion and hence look like relay signals when compared with high doses that exceed the saturation limit of the low-affinity sites and diffuse to more distant cells. Thus before we project gradients of growth factor activity onto the embryo, it is relevant to ask whether these mechanisms are likely to act *in vivo*.

THE DYNAMIC MARGINAL ZONE OF THE LATE BLASTULA AND EARLY GASTRULA

Two kinds of experiments address whether meso-endoderm induction leads to a graded organization of the early marginal zone, or whether the zone is split into two territories, a dorsal organizer and a ventrally specified mesoderm. Restricted patterns of gene transcription directly reflect the organization of the mesoderm, as discussed below, and embryological experiments that measure specification can also be used to assess differences of cells. Specification is an operational term describing the developmental capability of a piece of tissue explanted from the embryo: what it can differentiate on its own, what inductions it can mediate, or how it responds to inducers. Experimental results give a range of answers. Explants into neutral saline were used to determine what explants of the *Xenopus* marginal zone differentiate when taken at the early-gastrula stage (Dale & Slack 1987b). These experiments showed that the early gastrula is organized into a dorsal organizer region (which differentiates notochord and muscle), a dorso-lateral muscle-forming region, and a latero-ventral region that differentiates blood and mesenchyme. However, the late blastula marginal zone shows less organization, and although the dorso-lateral region differentiates differently from the ventral region, it is difficult to exclude the possibility that intermediate types of differentiation (such as muscle) result from errors in dissection or from activation of signaling factors by wounding, a problem that has recently been highlighted by the observation that cutting the embryo activates FGF signaling (LaBonne & Whitman 1997). The experiments were interpreted as supporting a model where the marginal zone is initially induced as separate dorsal and ventral

tissues, with little or no intermediate mesoderm, and that intermediate types of mesoderm are induced in the late blastula and gastrula stages by the organizer. More clear-cut results were obtained with embryo hemispheres, which were cut along longitudinal meridians at low temperature and recombined with a naive ventral half-embryo (Stewart & Gerhart 1990). While there may have been some residual activation of induction by wounding, the conclusion was that the organizer at the late blastula stage occupies a 60° sector of the marginal zone, and that outside this sector, the dorso-lateral marginal zone differentiates in the same way as the ventral half of the embryo. Thus, outside the organizer, the rest of the marginal zone is specified as ventral mesoderm. It is still possible that some fine degree of organization or responsiveness of dorso-lateral mesoderm is specified at an early stage, but the alternative view is that the departures from a simple two-part organization at the late blastula stage reflect, not meso-endoderm induction, but the beginning of dorsalization of the marginal zone by signals from the organizer.

Molecular markers have been used to argue for both extreme possibilities; either that there are only two states of mesoderm in the marginal zone or that there is a graded initial organization of mesoderm. Examples of early expression of genes such as *noggin* or *XmyoD* support the two-zone idea (see Figure 4A, B), whereas expression of other genes has been reported to be graded (e.g. *Xvent 1* and *2*; Gawantka et al 1995, Onichtchouk et al 1996). Unfortunately, most gene expression has been examined at the onset of *Xenopus* gastrulation when dorsalization by the organizer has already begun (*Xenopus* gastrulation begins internally 2 h before the external blastopore lip appears; Nieuwkoop & Florshutz 1950). Thus, although experiments have shown that graded expression of genes such as *goosecoid* could in principle reflect extensive organization of the marginal zone (Niehrs et al 1994), it is not clear whether this graded expression exists before dorsalizing signals have been released by the organizer. An example of graded expression of *Xlim-1* in the early gastrula is shown in Figure 4C. The expression of a few organizer-specific genes has been examined in the late blastula, and these already appear graded in their expression (FKH1; Dirksen & Jamrich 1992, Ruiz i Altaba & Jessell 1992) (*frzb*; Wang et al 1997). However, before it can be concluded that this reflects meso-endoderm induction, it should be recognized how quickly gene expression changes between the late blastula and early gastrula, as shown in Figure 4D, E. Regions of expression of *xnr-3* and *Xwnt-8* initially abut one another in the late blastula, but by the onset of gastrulation, dorsalization (Perhaps by *Xnr3* protein) has downregulated *Xwnt-8* expression close to the organizer. Opposite regulation occurs for genes such as *MyoD*, where organizer signals are required to maintain and upregulate expression (Frank & Harland 1991).

From both embryological experiments and molecular descriptive experiments, it is fair to conclude that the initial mesoderm, shortly after the midblastula

transition, contains two different zones, but that there may also be some graded organization at the boundary of the zones. However, the organization that is imposed on the mesoderm by organizer signals during gastrulation is far more extensive than any subtle organization of the marginal zone by meso-endoderm induction during the late blastula stage.

ORGANIZATION OF THE EARLY GASTRULA ORGANIZER

As the result of meso-endoderm induction at the blastula stage, the *Xenopus* early gastrula contains the organizer 60–90° wide and equally high, centered on the dorsal midline in the marginal zone (Gimlich & Cooke 1983, Stewart & Gerhart 1990, Zoltewicz & Gerhart 1997), surrounded by three large competence groups of responsive cells: the prospective ectoderm, mesoderm, and endoderm. Several lines of evidence, presented in this section, show that the organizer itself is a non-homogeneous population of cells in terms of the inductive signals released by cells, the morphogenetic activities of cells, and the specification of developmental fate. These regional differences raise the question of whether meso-endoderm induction suffices to generate the full pattern within the organizer, and in the section following this, evidence is given that further patterning must occur.

As noted above, the organizer's inductive effects can be assayed by grafting it to the ventral side of an early gastrula, but the organizer also can be assayed by inserting it in the blastocoel of an early gastrula (the "einsteck" method) or by wrapping it in responsive ectoderm or mesoderm. In such assays, a complete organizer induces most structures of the head, trunk, and tail. Pieces of the organizer do less. The lower (or anterior) half of the organizer induces only head structures, and the upper (or posterior) part induces only trunk-tail structures (Zoltewicz & Gerhart 1997). Hence, the subregions differ inductively and are sometimes called the head organizer and trunk-tail organizer, respectively. In *Xenopus*, the former coincides with the prospective pharyngeal endoderm and prechordal mesoderm, whereas the latter coincides with the notochord territory (Figure 5A). Underlying mesoderm cells have inducer activity, but so does the surface layer of cells of the organizer, which is prospective pharyngeal endoderm and gut roof (Shih & Keller 1992b). Furthermore, parts of the organizer already differ in their specification state. When the posterior part is explanted, it is already autonomous in its ability to differentiate the notochord (Zoltewicz & Gerhart 1997). The explanted anterior part, however, does not differentiate notochord. This is further evidence for regional differences in the organizer.

In urodeles, a similar analysis 50 years ago revealed that the early gastrula organizer also has a head-inducing region and a trunk-tail-inducing region. The

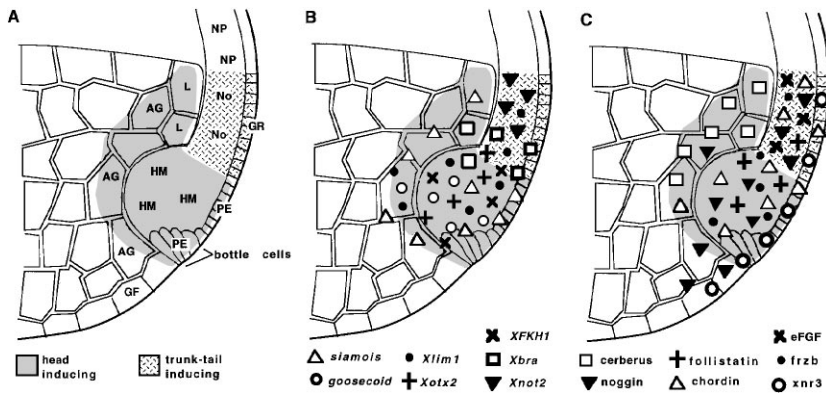


Figure 5 Organization of the organizer. Schematic sagittal cross section of the dorsal lip of the blastopore of an early *Xenopus* gastrula. In all three panels the head-inducing region (head organizer) is shown in gray; the trunk-tail-inducing region is indicated by cross-hatching. (A) Differentiative fates of organizer regions. AG, anterior gut; GF, gut floor; GR, gut roof; HM, head mesoderm; No, notochord; NP, neural plate; PE, pharyngeal endoderm; L, liver. (B) Localized expression of genes encoding transcription factors. Note different expressions in the head and trunk-tail inducing regions. *siamois* (Lemaire et al 1995), *goosecoid* (Cho et al 1991), *Xlim1* (Taira et al 1992), *XFKH1* (Dirksen & Jamrich 1992), and *Xotx2* (Blitz and Cho 1996) are expressed mostly in the organizer at this stage. *Xbr* (Smith et al 1991, Zoltewicz & Gerhart 1997) and *Xnot2* (von Dassow et al 1993) are expressed widely at first and then narrow to the organizer by the late gastrula stage. (C) Localized expression of genes encoding secreted proteins, the candidate organizer signals. *noggin* (Smith & Harland 1992), *chordin* (Sasai et al 1996), *folistatin* (Hemmati-Brivanlou et al 1994), *xnr3* (Smith et al 1995), *cerberus* (Boumeester et al 1996), *frzb* (Wang et al 1997, Leyns et al 1997), and *eFGF* (Isaaks et al 1995) are expressed in different regions, some more in the head-inducing part, or the trunk-tail-inducing part, the surface endoderm, or the deep endoderm.

head organizer is small, about 60° wide and 30° high, centered on the dorsal midline right above the dorsal blastopore lip, and coincident with the prospective pharyngeal endoderm and prechordal mesoderm regions, as in *Xenopus*. The trunk-tail organizer, however, appears surprisingly large (at least 180° wide and 90° high). It too is centered on the dorsal midline above the blastopore lip, but does not include the head organizer region. It coincides not only with prospective notochord but also with prospective somites, heart, kidney, and even lateral plate (Holtfreter & Hamburger 1955). In fact it occupies most of the marginal zone. Most of these regions were not expected to exert such inductions in the embryo. Apparently, they had gained inductive capacity when explanted for the specification test, a point we return to below in connection with autodorsalization of mesoderm. Nonetheless, these experiments showed that the organizer has an early differentiation of parts. Recent results with mouse embryos raise the possibility that in this mammal the head organizer is quite

separate from the trunk-tail organizer and is recognizable as a group of primitive endoderm cells expressing *Hesx* and *nodal* genes (Thomas & Beddington 1996, Varlet et al 1997). Primitive endoderm is an extra-embryonic tissue precursor.

In the past few years, approximately 10 genes have been identified that are expressed within the organizer. Some encode transcription factors and some encode secreted proteins. Not only can the organizer now be located directly by staining methods (in situ hybridization for mRNAs), but also the organized heterogeneity of cells of the organizer can be seen. Among the transcription factors are *Siamois*, *Goosecoid*, *XFKH1* (Pintallavis), *Xotx2*, *Xlim1*, *Xbra*, and *Xnot2* proteins (all detected as localized mRNAs). Some such as *goosecoid* and *siamois* are exclusively expressed in the organizer, and expression appears cell autonomous. Others such as *Xnot2* and *Xbra* are initially expressed in the entire marginal zone, and their expression is maintained later only in the organizer, as the result of cell-cell signaling (Green et al 1994, Wilson & Melton 1994). As diagrammed in Figure 5B, several of these genes are not expressed uniformly in the organizer, but more in cells of the head organizer region or of the trunk-tail organizer region. *Xbra* expression may mark a boundary between the two regions, and Ban-Holtfreter (1965) noted the need for inclusion of cells of a mid-zone for maintenance of the differentiative fate of the anterior or posterior parts explanted in culture.

The secreted proteins are of particular interest as candidate inducers. These include *Noggin*, *Chordin*, *Cerberus*, *Xnr3*, *Follistatin*, *Frzb*, and *eFGF* (discussed below). The relevant mRNAs have been detected, although in most cases the protein has not. As shown in Figure 5C, several of the genes encoding these proteins are expressed more in the head organizer or trunk-tail organizer, a fact further implying that the early organizer is already a spatially differentiated cell population.

LATE STEPS OF ORGANIZER FORMATION

According to Hama, Kaneda & Suzuki, who studied the formation of the urodele organizer from 1945–1985, the head and trunk-tail-inducing subregions of the organizer have not yet been established when gastrulation begins (Kaneda 1981, Suzuki et al 1984) and meso-endoderm induction has ended (Boterenbrood & Nieuwkoop 1973). Early steps of morphogenesis and induction are needed to regionalize the organizer. The anterior part at first is not a head organizer, but is a trunk-tail-inducing region that differentiates notochord when explanted and cultured. When it involutes during gastrulation and contacts the still uninvolved prospective notochord region, it gains head-inducing capacity and become specified for prechordal plate differentiation. Simultaneously the prospective notochord region gains trunk-tail inducing properties and becomes specified for notochord differentiation as the result of receiving signals by a planar route from

the anterior part, whereas previously it had no inducing capacity and was only specified for latero-ventral mesoderm (Suzuki et al 1984). These interactions may involve zygotically expressed gene products, not maternal ones.

In *Xenopus*, the head- and trunk-tail-inducing parts of the organizer seem more differentiated at the start of gastrulation, which is defined for convenience as the time of formation of the external blastopore. However, *Xenopus* begins internal aspects of gastrulation 2 h before the external lip is formed, whereas urodeles initiate the external and internal events synchronously. Thus the *Xenopus* organizer may have engaged in late steps of organizer patterning at the late blastula stage. In fact, in the *Xenopus* late blastula, a large piece of ventral mesoderm can be grafted into the dorsal marginal zone, and it later forms the entire notochord of the normally developing tadpole (Gerhart et al 1991). The piece must have formed the entire posterior half of the organizer after it was grafted into place, induced to do so by neighboring cells, perhaps by the anterior half of the organizer. Most cells of the posterior organizer may be induced in this way because they derive from tier 2 blastomeres of the 32-cell stage (Vodicka & Gerhart 1995), which cannot form dorsal mesoderm in isolation. The inductive neighboring cells have been called the late blastula organizer to suggest that they have inductive activity even before overt gastrulation. They induce the posterior organizer, a gastrula organizer, active after the blastula stage (Gerhart et al 1991). In the chick embryo, this late blastula organizer may occupy Köller's sickle. Even at mid- and late-gastrula stages of *Xenopus*, individual cells or clumps of a few cells can be taken from the ventral mesoderm or animal cap ectoderm and transplanted into the posterior organizer region, and these cells successfully incorporate into this region and differentiate as notochord (Domingo & Keller 1995), presumably gaining inductive activity in the interim. In light of these results, it is possible that the notochord part of the organizer (the trunk-tail inducing part) is not formed directly by induction from the Nieuwkoop center but later by a zygotically based induction that lasts into the gastrula stage. Alternatively, the Nieuwkoop center initially does induce this part, but later zygotically based inductive interactions are needed for maintaining cells in their trunk-tail inducing state. Relevant to these results, it is interesting that large pieces of ventral mesoderm, when grafted into the organizer at the early-gastrula stage, are not incorporated and cause the organizer to behave as independent halves, which each eventually differentiates as notochord, while the intervening graft cells develop as muscle (Smith & Slack 1983). At the same time, the ventral graft cells did not subvert the neighboring organizer cells, as if any signals they might release have only short-range effect.

Embryos containing a dominant-negative form of the FGF receptor are able to establish and maintain the head-inducing part of the organizer but not the trunk-tail (posterior) part. This result suggests that FGF signaling is needed

to initiate and/or maintain that latter portion of the organizer (Amaya et al 1993, Kroll & Amaya 1996). As discussed above, current evidence favors the conclusion that FGF is needed for maintenance, not initiation.

The organizer remains a dynamic and replacable cell population at the late blastula and early-gastrula stages. It can be removed and is readily regenerated from neighboring cells of the urodele early gastrula, and the trunk-tail-inducing part is especially replaceable (Holtfreter & Hamburger 1955). The comparable part (the node) is readily regenerated in the chick early primitive streak embryo, from cells that would otherwise develop as somites (Grabowski 1956, Yuan et al 1995, Psychoyos & Stern 1996). Holtfreter showed that even the head mesoderm can be regenerated if removed at the urodele early-gastrula stage (Holtfreter & Hamburger 1955). In the *Xenopus* early gastrula, this regeneration is less effective, although perhaps partial (Cooke 1985). The *cerberus*-expressing zone of deep endoderm, which has been recently discovered (discussed below), may have a role in organizer regeneration at times after the Nieuwkoop center has lost activity.

In urodeles it is known that small explanted pieces of prospective somite and even lateral plate mesoderm can develop notochord and neural tube, although they would not do so in the embryo, as if they have an inherent but latent capacity to gain inductive activity and become dorsal, a point discussed below. The organizer is dynamic in the mid- and late-gastrula stages, as well, and changes its profile of secreted proteins. For example, new inducers such as Shh appear (Ekker et al 1995) and others such as Xnr3 disappear (Smith et al 1995).

DEVELOPMENT WITHOUT AN ORGANIZER

The importance of the organizer in normal embryonic patterning can be demonstrated by the development of ventralized embryos in which it is absent. A *Xenopus* gastrula can be made to lack an organizer if cortical rotation is blocked at the first cell cycle (Scharf & Gerhart 1983), if its maternal β -catenin mRNA is eliminated (Heasman et al 1994), or if the organizer is surgically removed at the late blastula stage (Stewart & Gerhart 1990). The gastrulating embryo retains cylindrical symmetry, internalizes the endoderm, closes the blastopore, and differentiates ectodermal, mesodermal, and endodermal tissues with ventral-posterior cell types, namely a ciliated epidermis (Scharf & Gerhart 1983), red blood cells at levels up to twenty times normal (Cooke & Smith 1987), coelomic mesoderm, and posterior gut in which the yolk mass is digested and in which *IFABP*, a midgut marker gene, is expressed. The foregut marker, *XlHbox8*, is not expressed (Henry et al 1996). The embryo has little or no body axis, head, trunk, tail, heart, kidney, somites, central or peripheral nervous systems, or placodes. The four chordate-distinguishing characteristics are absent: gill slits, notochord, dorsal hollow nerve cord, and post-anal tail.

Such a ventralized embryo can be fully rescued at the early-gastrula stage by grafting an organizer into its marginal zone (i.e. a dorsal blastopore lip from a normal early gastrula). A single-axis embryo is obtained (Stewart & Gerhart 1990). Rescue proceeds in much the same way as grafting a dorsal lip into the ventral marginal zone of a normal gastrula (the Spemann-Mangold experiment), namely, cells expected from the fate map to make ventral tissues, such as epidermis, coelom, and blood, now form dorsal axial structures if they are near the graft.

Despite its major role in gastrula organization, as demonstrated by the development of these embryos without and with an organizer, there is no evidence that the organizer provides detailed information for local pattern and cytodifferentiation. Its signals probably establish only global organization, i.e. the place, orientation, and scale of development of large groups of cells, a point borne out by recent molecular analysis. The induced neighboring cells then take over the subsequent more local patterning. The chordate organizer is distinctive, not by virtue of its capacity to release inductive signals, which many cell groups do in the course of development, but by the large scope of its effect on embryonic organization. Only meso-endoderm induction, by which the organizer and competence groups are established in the first place, might be said to rival this scope.

THE ORGANIZER'S THREE FUNCTIONS

Organizer functions include induction, morphogenesis, and self-differentiation, all occurring at the gastrula stage. The organizer's inductions are often called primary inductions to distinguish them from secondary and tertiary inductions occurring after gastrulation and involving cell groups derived from the organizer or from tissues the organizer had previously induced to form. These later inductions are not discussed here. The progression from primary to secondary inductions is smooth, and distinctions are sometimes difficult to make; for example, the inductions in the post-neurula tail bud may represent continued inductions of the organizer as well as new ones (Tucker & Slack 1995).

In discussing organizer function, it is important to recognize the role of the rest of the embryo, composed of the large competence groups of ectoderm, mesoderm, and endoderm, i.e. the prospective germ layers. These groups surround the organizer and receive its signals. All were established by meso-endoderm induction at the blastula stage. Each group contains cells identical in their receptiveness to inducers and their variety of possible developmental responses. The equivalence of cells was shown in classical embryological studies of removing or interchanging cells of the early gastrula, with no effect on later development. This is true except for the organizer itself. As said before, transplantation of these cells has great non-autonomous effects on the development of neighboring

cells. Although all cells of a competence group have the capacity to respond, only some will receive the signal to do so, and hence cells of a group will eventually lose equivalence.

Self-Differentiation

Cells of the organizer eventually differentiate a variety of mesodermal and endodermal tissues. Mesodermal derivatives include the notochord and prechordal plate head mesoderm (head mesenchyme and cartilaginous rods of the skull floor), whereas endodermal derivatives include pharyngeal endoderm (including gill slits) and anterior gut tissues such as the liver. In some species, especially chick, the medial portion of somites may come from organizer (node) cells, as does the floor plate of the neural plate, the only ectodermal derivative. The floor plate of urodeles may also come from the organizer. Finally, the tail bud is formed partially from cells of the organizer. Many of these differentiations occur autonomously in explanted organizer tissue left *in vitro* for several days to differentiate (Ban-Holtfreter 1965, Keller & Danilchik 1988).

Morphogenesis

Gastrulation transforms the organization of the cellularized egg into that of the definitive embryo. In most metazoa, gastrulation involves the internalization of mesoderm and endoderm from the surface layer of cells, but chordates

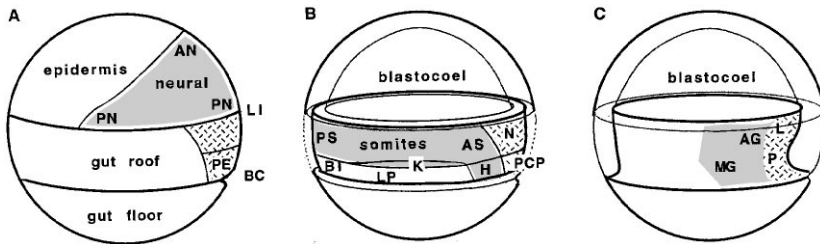


Figure 6 The spatial extent of the organizer's inductive influence. Various layers of cells of a *Xenopus* early gastrula are shown. The organizer is located at the equatorial level to the right (*cross hatching*). (A) Surface view: The ectodermal competence group occupies the upper half, and the endodermal competence group, the lower half. The neural plate (gray) is induced in the ectoderm by signals from the organizer. AN, prospective anterior neural plate (prospective forebrain-midbrain); BC, line along which bottle cells form; PE, pharyngeal endoderm; LI, limit of involution of endoderm; PN, posterior neural plate (prospective hindbrain-spinal cord). (B) The mesoderm competence group, located internally: Cut-away view, after surface ectoderm and endoderm of panel A removed. Cells of the gray shaded area are reached by signals from the organizer. AS, anterior somites; BI, blood islands; H, heart; K, kidney; LP, prospective lateral plate; PS, posterior somites. (C) The deep endoderm: Further cut-away view after mesoderm of panel B removed. These are large yolky cells internal to the mesoderm. The gray shaded area is reached by signals from the organizer. AG, anterior gut; L, liver; MG, midgut; P, pancreas.

engage in larger cell displacements during gastrulation than do other groups. The early gastrula fate map of chordates is impressively convoluted with regard to antero-posterior and dorso-ventral axes. Notice in Figure 6A that the region of prospective posterior somites is located opposite the organizer in the early gastrula, as far away as physically possible, yet the posterior somites and notochord will be adjacent in the neurula. Also the prechordal head mesoderm lies far from the anterior neural plate in the early gastrula. Extensive morphogenesis brings the organizer into the vicinity of other tissues. If morphogenesis is blocked by agents such as microtubule-depolymerizing drugs, embryos achieve only short-range induction (Lane & Keller 1997). Thus it is likely that morphogenesis is necessary if the organizer's inducers are to reach distant targets.

However, not all morphogenesis in the gastrula depends on the organizer. Organizer-independent movements include the widespread involution of the latero-ventral marginal zone of the amphibian gastrula, as shown by the fact that ventralized *Xenopus* embryos succeed in internalizing endoderm and closing the blastopore (Scharf & Gerhart 1983). Similar movements occur in chick embryos from which Hensen's node has been removed and not regenerated (Schoenwolf et al 1992). Mesoderm and endoderm cells still undergo waterfall ingression through the streak in nearly normal numbers.

Organizer-dependent morphogenesis consists of the movements of organizer cells themselves, and the movements they induce in neighbors, especially in the somitic mesoderm. Anterior organizer cells, comprising mostly the prospective prechordal plate cells (part of the head organizer), engage in a spreading migration. These cells move as a multicellular cluster on the wall of the blastocoel toward the animal pole, sensing the direction of fibers in the extracellular matrix (Winklbauer & Keller 1996, Boucaut et al 1996), whereas individual cells fail to move directionally.

Posterior organizer cells, comprising prospective notochord cells (the trunk-tail organizer), engage in a very different morphogenesis, a convergent extension of the cell population leading to a great backward extension of the progressively forming notochord, eventually entering the vicinity of prospective posterior somites. The cell population is initially trapezoid shaped and several cells thick; it lengthens and narrows into the notochord, a long rod one cell wide, by a process mostly involving cell repacking, not cell shape change. (A cell shape change to give the final stack of coins morphology occurs in late differentiation.) Convergent extension is the driving force of node regression in amniotes. The node, which is a locus of cell proliferation, is pushed posteriorly on the tip of the elongating notochord, to which it continuously adds new cells. The notochord extends along the center of the streak, eventually reaching the posterior end of the embryo.

The somites, which are induced from latero-ventral mesoderm by signals from the organizer, themselves engage in convergent extension. Even without

the successful differentiation of a notochord (but with a remaining population of organizer cells), the embryo can elongate as in lightly UV-irradiated *Xenopus* embryos (Youn & Malacinski 1981), or in the no-tail mutant (Halpern et al 1993) or floating head mutant (Talbot et al 1995) of zebrafish, or a chick embryo from which the node has been removed just before regression (Darnell & Schoenwolf 1992, 1995), or the zebrafish embryo from which the shield has been removed (Shih & Fraser 1996). Also, activin-treated animal cap cells, which eventually differentiate muscle but fail to form notochord, engage in a convergent extension-like morphogenesis at a time equivalent to gastrulation (Symes & Smith 1987).

The mechanism of convergent extension has been well described at the cell population level in *Xenopus* (Keller et al 1992, Shih & Keller 1992a,c). Individual cells (prospective notochord and somite) become elongated and bipolar in shape and display rampant protrusive activity at the two poles. At the midgastrula stage, they begin to align themselves in parallel (an alignment zone) by processes initially requiring microtubules, but not thereafter as the zone spreads upward in the marginal zone (Lane & Keller 1997). Cells exert traction on each other in both directions and interdigitate, driving a medio-lateral convergence and extension of the population. If a cell chances to expose a pole at the boundary, which forms between the notochord and somite territories, that pole loses protrusive activity and spreads. The monopolar boundary cell, however, continues to pull internal cells to the boundary. Internal cells are successively captured at the boundary, which lengthens. Eventually all notochord cells have both poles on a surface, as the population narrows to a single file, and they become immobile. The notochord boundary is maximized. When the organizer is explanted, it tends to generate a new boundary of somite precursors on its cut edges and then convergent extension occurs in vitro (Keller & Hardin 1987). Convergent extension may require continuous signaling and responding of members of the population and their sequential entry into the interdigitation process. The signaling has not been analyzed.

Inductive Signaling

All three germ layers are affected by signals from the organizer. The mesoderm is considered first, then the ectoderm, and finally the endoderm.

DORSALIZATION OF THE MESODERM Organizer signals give rise to heart, kidney, and somites (from which the sclerotome, bone and cartilage; myotome, muscle; and dermatome, dermis, derive by secondary inductions). Although several dorsalizing signals are now known, it is not known if all parts of the organizer are equally effective in dorsalization.

Dorsalization is not needed for the development of lateral plate coelomic mesoderm from which derive visceral and parietal coelomic mesoderm, or

for the establishment of blood cells, or for germ cells in urodeles (a ventral mesodermal differentiation in this amphibian order). These differentiations occur in ventralized embryos, and the initial meso-endoderm inductions of the blastula period are probably sufficient to set marginal zone cells on this path, where they are kept by BMP and Wnt signals they generate themselves (autocrine-paracrine induction).

NEURAL INDUCTION OF THE ECTODERM This gives rise to the antero-posterior and dorso-ventral organization of the neural plate. The recent identification of neural-specific marker genes has revealed the early events of neural induction. By the midgastrula stage, for example, the *Xotx2* gene may be expressed in the approximate region of the neural plate (Blitz & Cho 1995), and in the late gastrula, the *engrailed* gene is already expressed in a stripe at the position of the prospective mid-hindbrain boundary while the neural plate is fully open (Hemmati-Brivanlou et al 1991). Antero-posterior pattern includes the regions of fore-, mid-, and hindbrain and spinal cord. The pituitary gland (a neuroendocrine gland; *Xanf2* as a marker) is induced at the anterior-most end (Mathers et al 1995). In the dorso-ventral dimension, a stripe of cells expressing HNF3-beta arises at the midline (floorplate) (Ruiz i Altaba & Jessell 1992). Also, various homologues of Achaete/Scute (Xash), E(spl), Delta, and Notch (for example see Chitnis et al 1995 and reviews by Harland 1996 and Sasai & De Robertis 1997) are expressed in several stripes lateral to the midline. Finally the *slug* and *twist* homologues are expressed at the border of the plate where neural crest cells will appear (neural crest does not form at the anterior end of the plate). Patterning is quite elaborate even before the neural plate starts to close, although certain aspects of this pattern are still labile (see below). The induction of seven placodes anterior and lateral to the neural plate should perhaps be included, as should the induction of the hatching gland and cement gland, the latter anterior to the plate. Neural crest may be formed by a secondary induction, i.e. by an interaction of neural plate with nearby epidermis because crest cells are formed when two such pieces of tissue are apposed experimentally (Moury & Jacobson 1990, Selleck & Bronner-Fraser 1995).

From the work of Nieuwkoop and of Saxen & Toivonen in the 1950s and 1960s, neural induction is generally accepted as having two steps: neuralization (or activation of the ectoderm) and caudalization (or transformation or posteriorization of the neuralized ectoderm). Hence, the organizer releases two kinds of signals important for complete neural induction: a neuralizer(s) and a caudalizer(s). The second only modifies the effects of the first and has no neuralizing effect on its own (like the earlier dorsal competence factor). Without caudalization, neuralization leads to the differentiation of only anterior neural tissue, i.e. fore- and midbrain, pituitary gland and perhaps cement gland and hatching gland (a so-called archencephalic induction). The subsequent formation of head

neural crest (e.g. chondrogenic crest) is presumably dependent on this signal insofar as anterior neural tissue can then secondarily interact with epidermis (Sive & Bradley 1996). Caudalization results in the neuralized ectoderm's development of hindbrain and spinal cord. As a further antero-posterior difference, the posterior neural plate engages in a strong convergent extension, whereas the anterior plate does not. The formation of trunk neural crest (non-chondrogenic) presumably depends on the secondary interaction of the hindbrain-spinal cord level of neural tissue with nearby epidermis.

The two neural plate regions specified by neuralization alone, or by the combination of neuralization and caudalization, match with the two major domains of expression of certain homeobox-containing genes: The *otx*, *emx* genes are expressed in the neuralized region (fore- and midbrain), whereas the *HOX* genes are expressed in the caudalized region (hindbrain-spinal cord). *HOX* expression seems to require more in the way of induction than does *otx*, *emx* expression. The great antiquity of this distinction of homeotic domains is seen by the facts that in *Drosophila*, the homologous *otd*, *ems* genes are expressed in the head and the *HOM* genes in the thorax and abdomen, and that *Tribolium* mutants lacking the entire *HOM* cluster of genes express head appendages (antennae of the *otd*, *ems* expression domain) on all segments.

Neuralization starts at stage 10 and continues until stage 12.5 (Albers 1987). Although it is often accepted that the involuted dorsal mesoderm of the organizer imprints its entire pattern on the overlying ectoderm by vertical induction, Nieuwkoop & Koster (1995) have argued that the initial signal for neuralization must be presented vertically by the first involuted part of the organizer, but then it can spread anteriorly and laterally by a propagated (homeogenetic) planar induction within the ectoderm. According to these authors, the location of the border of the neural plate is set by the time-dependent cessation of the ectoderm's competence to respond to the spreading signal, not by the signal being too dilute at the low end of a gradient. Caudalization continues until stage 16 when the neural tube has been closed (Nieuwkoop & Albers 1990), and this signaling is also thought to be transmitted first by the vertical and then by the planar path.

Neural induction has been further examined in the Keller explant, which is an excised piece of the dorsal sector of a *Xenopus* early gastrula, including the organizer and the prospective neural ectoderm (Doniach et al 1992, Keller et al 1992). The explant is kept flat under a glass coverslip to preclude vertical interactions of the mesoderm with the ectoderm. Signals are confined to spreading by the planar path across the end-to-end border of the mesoderm and ectoderm. In such explants, the ectoderm is not autonomous for neural differentiation at the time of removal from the embryo but becomes so within a few hours in vitro. Eventually the ectoderm can differentiate extensive antero-posterior and dorso-ventral arrays of neural markers (Doniach et al 1992, Papalopulu &

Kintner 1993). A ventral piece of ectoderm can be grafted into planar contact with the mesoderm and it too differentiates neural markers, which it otherwise would not (Doniach et al 1992). These studies indicate that even the initial neuralizing and caudalizing signals can reach the ectoderm by a planar path, and certainly that the signals can spread thereafter by this path. They do not exclude that vertical interactions occur in the embryo, and it remains possible that transmission by both paths operates there. Also, other amphibians (*Rana*, urodeles) may make less use of planar induction (Saint-Jeannet & Dawid 1994).

ANTERIORIZATION OF THE ENDODERM Anterior endoderm (in *Xenopus*) such as the pancreas and liver (and perhaps lung) seem to require signals from the organizer, a recent insight (Gamer & Wright 1995, Henry et al 1996, Sasai et al 1996) gained by the use of new markers (IFABP for small intestine, Xlhbbox8 for pancreas, 4G6 epitope for midgut, and endodermin for gut in general). Holtfreter (studying urodeles) thought that endoderm was fully patterned by the start of gastrulation and did not require organizer signals (Holtfreter & Hamburger 1955), although perhaps such signals reached the endoderm by that time. The pharyngeal endoderm (head endoderm including gill slits) differs from the trunk endoderm in its induction history, being induced largely by meso-endoderm induction at the blastula stage and comprising part of the organizer.

RECENT INSIGHTS INTO THE ORGANIZER'S INDUCTIONS

Within the last four years several secreted proteins have been isolated that are probably among the organizer's true secreted signals, namely, Noggin, Follistatin, Xnr3, Chordin, Cerberus, Frzb, and eFGF. Their identification is a historic event in the 73 years of study of the organizer. (Shh is produced later by notochord and prechordal plate cells and is not included in the early set.) Interestingly, receptors have not been found for most of these newly discovered molecules, and it is considered likely that they act as anti-ligands by binding to other secreted proteins (the ligands BMP 2, 4, and 7, and Wnt8), disrupting their signaling by blocking their binding to specific receptors. BMP and Wnt ligands are produced by cells of the large competence groups outside the organizer, and if those cells bind these ligands and transduce these signals, they remain on paths of ventral and posterior development. From recent studies, the organizer seems to be a source of anti-ventralizing signals that create a BMP- and Wnt-free zone near the organizer in which cells of the three germ layers express their inherent, but otherwise self-suppressed, capacity for dorsal and anterior development. This is a new perspective, one only obliquely implied in the old embryological literature.

Table 1 Candidate signals of the organizer

Secreted protein	Dorsalization of mesoderm	Neuralization of ectoderm	Anteriorization of endoderm	Inducer provided as
Noggin K_d (BMP4) = 0.02 nM	+(1 nM)	+(10 nM)	+	Protein, mRNA, DNA
Chordin K_d (BMP4) = 0.3 nM	+(1 nM)	+(1 nM)	+	Protein, mRNA, DNA
Follistatin	+	+	?	mRNA
Xnr3	+	+	?	mRNA, DNA
Cerberus ¹	—	+	+	mRNA
Frzb ²	+/-	—	?	mRNA
FGF (eFGF)	—	+ or caudalization	—	Protein, mRNA

References. Noggin: Lamb et al 1993, Smith et al 1993; Sasai et al 1996; Chordin, Sasai et al 1994, 1995, 1996; Follistatin, Hemmati-Brivanlou et al 1994, Wilson & Hemmati-Brivanlou 1995; Xnr3, Smith et al 1995, Hansen et al 1997; Cerberus, Bouwmeester et al 1996; Frzb, Leyns et al 1997, Wang et al 1997; FGF, Isaacs et al 1992, 1994, Kengaku & Okamoto 1995, Lamb & Harland 1995. ¹Cerberus blocks dorsalization. ²Frzb enhances dorsalization.

Noggin and Xnr3 were isolated from cDNA libraries of gastrula/neurula sequences by an axis-induction assay (Smith & Harland 1992, Smith et al 1995); several others inducers were obtained by differential screening of organizer libraries for sequences specifically expressed in the organizer, and tested later for axis-duplicating properties (Sasai et al 1994, Bouwmeester et al 1996). Others were isolated as homologues of interesting proteins with activities in other assays (Wang et al 1997). Several of these putative inducers meet two minimal criteria (never met before in the history of attempts to isolate inducers) set by classical embryological experiments: (a) They are present at right time and place in the embryo, namely in the organizer at the gastrula stage (at least the mRNAs are, for the proteins have not yet been detected in the embryo). (b) The purified protein, when presented to a normal responsive tissue at the right time, elicits a normal response (see Table 1). For example, the latero-ventral mesoderm of the early gastrula can be dorsalized by Noggin or Chordin to produce muscle, or the gastrula ectoderm can be neuralized by these same proteins to produce anterior brain (Lamb et al 1993, Smith et al 1993, Sasai et al 1994, Bouwmeester et al 1996, Piccolo et al 1996). This is discussed further below.

Several of the proteins are not available in purified form, so they have been tested in the responsive tissue by injecting the egg with the relevant mRNA. Although all the candidate inducers tested in this way have effects on axis formation, this procedure has the problem that the protein is produced long before the normal time, and usually at ectopic sites as well, and may have misleading

side effects. A better procedure is to express the gene for the protein from injected DNA, which is not transcribed until at least the midblastula stage. Thus Xnr3, Noggin, and Chordin proteins expressed from injected DNA dorsalize ventral mesoderm and induce anterior neural tissue (Lamb & Harland 1995, Sasai et al 1995, Hansen et al 1997). However, expression can be non-uniform, and so effects may be missed. Expression in transgenic *Xenopus* gives more uniform expression but has not been used extensively as yet (Kroll & Amaya 1996).

A third criterion is the most demanding, and most of the candidates have not yet been tested for technical reasons. This criterion holds that if the protein is essential, its removal must result in a deficiency in organizer function, such as a failure of neural induction, dorsalization, or anteriorization of the endoderm. Techniques for elimination of the endogenous protein now include knockout of the gene (in mouse and zebrafish), knockout of the endogenous protein's mRNA by experimentally introduced anti-sense RNA, and knockout of the endogenous protein's function by an experimentally introduced dominant-negative form of the protein. By these means, a few results have been obtained. The *dino* mutant of zebrafish is a *chordin* mutant—it is partially ventralized, with a reduced neural tube, small somites, and an expanded ventral region (Schulte-Merker et al 1997). The *noggin*^{-/-} mouse knockout mutant gives a rather normal neural tube at early stages (hence neural induction occurs) and somites (hence dorsalization occurs), but it develops severe neural patterning and skeletal problems (McMahon et al 1997). The *follistatin*^{-/-} knockout mouse shows hardly any alterations of early development (Matzuk et al 1995), and from comparative studies, follistatin is found expressed in lateral plate mesoderm of the chick, not the node (Albano et al 1994). Thus although Chordin and Noggin meet the criterion at least partially, Follistatin does not. However this criterion of essentiality may be too severe if one protein partially overlaps another in function. The effect of its absence would then have to be studied in compound knockout mutants or under developmental conditions where its function is less overlapped by that of others.

Five insights are now available from the study of these molecules.

Newly Recognized Regions of the Organizer

When the organizer is defined as the population of cells expressing the genes encoding these secreted signals, it includes not only the territories recognized by tissue transplantation (the prospective prechordal mesoderm, notochord, and pharyngeal endoderm) but also the surface endoderm below the blastopore and the deep yolk endoderm prospective for liver (Figure 6C). The *cerberus*-expressing deep endoderm is not inductive in the usual grafting tests, but it appears that *cerberus* expression is not maintained in the explanted piece (Bouwmeester et al 1996), or perhaps only works in combination with other

inducers. The organizer may thus be larger and deeper than had been classically recognized.

Same Signal, Different Responses

The different competence groups, which are the products of meso-endoderm induction at the blastula stage, differ in their responses to inducers. As shown in Table 1, when gastrula ectoderm is treated with Noggin protein, it develops anterior neural differentiations including dorso-ventral anterior differences (Knecht et al 1995), whereas when latero-ventral mesoderm is treated with this same protein, it develops somites (scored as muscle) (Smith et al 1993). Dorsalization or neural induction is also obtained when these respective cells are treated with Chordin (Sasai et al 1994) or Follistatin (Hemmati-Brivanlou et al 1994). Follistatin treatment (via mRNA injection) of the ectoderm allows more advanced neuron differentiation than does Noggin or Chordin treatment. Chordin and Noggin have also been tested for endoderm anteriorization and both are effective inducers, leading to the expression of genes specific for pancreas and liver (Sasai et al 1996). Thus Chordin and Noggin are candidate inducers of all three germ layers.

The common basis for the action of Noggin, Chordin, and Follistatin is the capacity of these molecules to form tight complexes with various BMPs and to block their binding to their receptors, and hence inhibit their signaling. This has been directly demonstrated for Noggin complexing with BMP4 ($K_d = 20$ pM) and BMP2, and less strongly with BMP7 (Zimmerman et al 1996), and for Chordin complexing with BMP4 ($K_d = 300$ pM) (Piccolo et al 1996). These complexes are formed with higher affinity than BMP4 has for its receptor ($K_d = \approx 900$ pM). Follistatin is well known to complex with activin, a TGF- β family member, but the affinity of Follistatin for BMPs is unclear, although it may have affinity for BMP7 (Yamashita et al 1995). There is evidence that some of the BMP heterodimers are more active than homodimers in signaling (Aono et al 1995), but the BMP heterodimer distribution in the embryo is unknown. From the current data, Noggin and Chordin seem equally qualified as candidate signals of the organizer and at least partially overlapping in their function, perhaps differing mostly in their diffusibility in the embryo. Noggin, for example, can dorsalize the entire embryo when expressed locally in a small ventral sector, whereas Chordin acts more locally (Smith & Harland 1992, Sasai et al 1994).

Cerberus protein differs from the others in its range of effects. When expressed from injected mRNA, Cerberus is a strong inducer of anterior neural tissue, cement gland, and anterior endoderm such as liver, but is not a dorsalizing inducer of mesoderm (Bouwmeester et al 1996). In fact, Cerberus appears to block mesoderm formation as it induces anterior endoderm and neural

development. It is not yet known if its mode of action is by way of binding to BMP2, -4, or -7, or a specific heterodimeric complex of two of these. The action of Xnr3 may be different, perhaps binding to a BMP as a dominant-negative ligand or to a BMP receptor as an antagonist; but in either case, blocking BMP signaling (Hansen et al 1997).

The Frzb protein, which resembles the extracellular domain of members of the frizzled-like family of Wnt receptors, has effects different from those of the BMP antagonists (Leyns et al 1997, Wang et al 1997). It is a Wnt antagonist that complexes with certain Wnt proteins (Wnt 1 and 8), thereby blocking their receptor binding. On its own, Frzb does not act as a neural inducer of ectoderm or a meso-endoderm inducer of animal cap cells. It may weakly dorsalize mesoderm, because partial secondary axes can be formed at equatorial sites of local ectopic expression, but mostly Frzb counteracts limits set by locally secreted Wnts on the effects of other signals from the organizer. Thus, when Frzb is widely overexpressed in *Xenopus* embryos, they develop enlarged notochords (and concomitantly reduced somites and *myoD* expression) and enlarged anterior neural parts (heads). Thus Frzb protein seems to potentiate dorsalizing and neuralizing signals (Leyns et al 1997, Wang et al 1997). This action is consistent with the possibility that Wnts themselves normally act, on one hand, in the marginal zone to limit mesoderm dorsalization to somite specification, thereby preventing notochord formation, and on the other hand, in the ectoderm as caudalizing agents, preventing anterior neural development. Frzb secretion by the head organizer would ensure that only anterior neural induction occurs in its vicinity. In summary, although several of the candidate inducers overlap in function, others show germ layer specificity and complex interdependent effects. Combinations of these agents have not been tested to simulate the mix, order, and spatial distribution that might be released by the organizer.

Inducers as Derepressors

The signals, as now understood, are locally provided derepressors of dorsal development, antagonizing the BMP and Wnt ligands, which themselves are inhibitors of dorsal and anterior development and maintainers of ventral and posterior development. Neural development can be considered the default path of development by the ectoderm, and somite/notochord development the default path of the mesoderm (although it should be appreciated from the detailed analysis of *Drosophila* development that the particular default has validity only under the particular experimental conditions). The opposite default may be found for other conditions. If the BMP and Wnt effects are considered short-range (autocrine/paracrine) inductions, the epidermis would be the induced state of the ectoderm, lateral plate and blood would be the induced state of

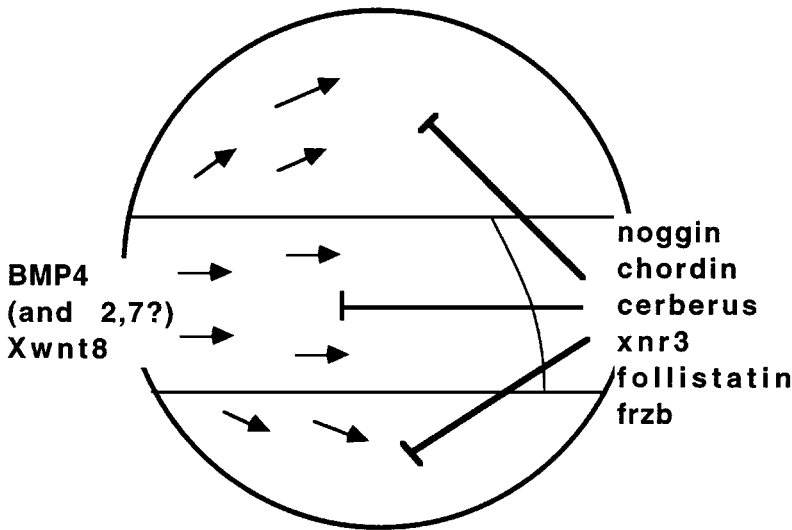


Figure 7 Organizer signals antagonize the effects of BMPs and Wnts released by non-organizer cells. *Short arrows* indicate BMP4 (also BMP2 and -7) and Xwnt8 released from ventral cells. *Long heavy lines* indicate antagonists from the organizer, blocking the effects of BMPs and Wnts. The organizer appears to promote dorsal development in all three germ layers by releasing signals that antagonize ventralizing agents.

the mesoderm, and the hindgut would be the induced state of the endoderm. The dorsal and anterior paths of development would then be uninduced states (Figure 7). The evidence for this new perspective is fourfold.

AUTONEURALIZATION Barth discovered in the early 1940s, and Holtfreter confirmed and extended the findings, that the gastrula ectoderm of urodeles or *Rana pipiens* can be made to differentiate neural tissue simply by shocking it with extreme pH or high salt conditions that tended to disaggregate cells briefly and to upset their internal ion balances. When the cells were restored to normal culture conditions, they reaggregated and developed later to muscle, nerve, pigment cells, ciliated epithelium, and a wide variety of cell types, depending on the particular conditions (Barth 1941, Barth & Barth 1974). Clumps of ectoderm cells would form brain vesicles with multiple eyes or nasal pits (Holtfreter 1947). Thus cell types and local organization could be formed without specific organizer signals, which revealed the ectoderm's inherent capacity for neural development.

Holtfreter (1951) proposed that the noxious conditions (causing "sublethal cytolysis," as he called the effect on cells) led to the release of a latent neuralizing

agent from a complex with an endogenous inhibitor, and hence autoneuralization occurred. Normal neural induction would, in his view, involve the breakup of the complex by agents linked to organizer signals. Holtfreter was very close to the current idea of neural induction as a derepression, except that in his proposals the self-repression was imposed by intracellular rather than intercellular means. Similar observations of autoneuralization have been made recently for *Xenopus* ectoderm (Grunz & Tacke 1989, Godsave & Slack 1991). Nerve cells frequently differentiate from gastrula ectoderm following a brief disaggregation to single cells (and without extreme shock). In retrospect, autoneuralization may have involved the disruption of intercellular BMP signaling.

Autodorsalization of explanted pieces of mesoderm (even without cell disaggregation) was so readily obtained in newts, nay unavoidable, that it was impossible to do specification tests of the gastrula mesoderm (Holtfreter & Hamburger 1955). The differentiations of the explanted piece of mesoderm always exceeded in variety those expected from the fate map. For example, a piece of the prospective somite territory of the early gastrula would develop somite muscle, notochord, neural tube, and epidermis, organized in a small bilateral axis. The piece probably gained organizer activity on isolation and engaged in neural induction, since a neural tube was formed. Hence, this piece was thought to be part of the trunk-tail organizer. This too could be the effect of the disruption of autocrine/paracrine BMP and Wnt signaling. Holtfreter (1951) concluded from the results that inducing signals of the organizer provide very little information and that most of the specificity comes from the responding tissue in which these paths of development are latent, waiting to be released. He would be delighted with the new evidence of organizer signals as derepressors.

EXPERIMENTAL DISRUPTION OF BMP SIGNALING Disruption of BMP signaling results in dorsal development in prospective ventral regions. BMP4 and 7 mRNAs are present in the early and midgastrula except in the vicinity of the organizer and part of the neural plate (perhaps where cortical rotation had brought Wnt-related vegetal materials in the first cell cycle) (Fainsod et al 1994, Hawley et al 1995). BMP4 is initially expressed even in the organizer region but is quickly downregulated (Re'em-Kalma et al 1995). The proteins have not yet been located by antibodies. As shown in Figure 4D, E, *Wnt8* mRNA is present mostly in the latero-ventral marginal zone (Smith & Harland 1991, Christian & Moon 1993). At the same time, the mRNAs for the BMP4 receptor (Graff et al 1994) and the Wnt8 receptor (Yang-Snyder et al 1996) are ubiquitously present in the embryo from early stages as maternal proteins. BMP and Wnt signaling can in principle occur over large areas of the embryo.

If BMP signaling is locally disrupted by a dominant-negative receptor produced in the ventral marginal zone by local mRNA injection, a partial secondary

axis develops at the ventral site (Graff et al 1994, Suzuki et al 1994). Nearly normal ventral gastrulation begins at the injection site, and *gsc* is hardly expressed, as if ventral development was initiated but not maintained. The secondary axis lacks notochord and the most anterior axial parts, but dorsalization and neural induction have occurred. It has not been determined how much of the effect is directly from BMP disruption and how much owes to signals from organizer tissue arising from the BMP disruption. Signaling has also been disrupted by injections of antisense RNA to the mRNA of the BMP4 ligand or to the receptor, or by injection of mRNA for a dominant-negative ligand (Hawley et al 1995, Steinbeisser et al 1995, Suzuki et al 1995, Xu et al 1995), and partial secondary axes also result in the injected area. In injected animal caps, anterior neural tissue develops in the absence of mesoderm.

Several years ago, related results were obtained with a dominant-negative activin receptor (produced from injected mRNA), namely, the formation of neural tissue in injected embryos lacking mesoderm. These results were the first to suggest that a TGF- β family member is active in suppressing neural development. These results are now understood in terms of the capacity of the particular dominant-negative form to interact promiscuously with other TGF- β receptors such as those of BMPs (Hemmati-Brivanlou & Melton 1992).

A dominant-negative Wnt8 ligand, expressed from injected mRNA, has been used to disrupt signaling in the embryo (Hoppler et al 1996), and this interferes with the expression of genes normally transcribed in the latero-ventral marginal zone (*MyoD* and *Xpo*). Ectopic overexpression of *frzb*, an antagonist of Wnt8, leads to a dorsalization of the embryo (reduced ventral structures) in which the organizer appears to form normally but then the chordin gene is more broadly expressed (Leyns et al 1997). The current conclusion from this work is that both Wnt and BMP signaling are used to maintain ventral development, and the experimentalist can disrupt the signaling by inactivating receptors, to attain dorsal development, in parallel to the organizer's use of anti-ligands to disrupt signaling. It is not yet known how the ventral maintenance is divided between BMPs and Wnts, but it is thought that Wnts may have shorter-range effects more restricted to the marginal zone than BMPs.

ECTOPIC EXPRESSION OF BMPs AND WNTs When BMP4 is produced ectopically in the dorsal marginal zone by mRNA injections, dorsal development is suppressed and ventral development results (Dale et al 1992, Jones et al 1992). Axis formation is reduced, as if the organizer had exerted a weaker inductive effect. Still, the *goosecoid* gene activates normally and a dorsal blastopore lip forms, but then organizer activities such as convergent extension and induction fail (Jones et al 1996c). Thus even though the organizer starts normally, it remains ventralizable. When BMP4 protein is provided at low levels (10 pM),

the neuralization normally caused by partial disaggregation of gastrula ectoderm cells is blocked, and their epidermal development is favored (Wilson & Hemmati-Brivanlou 1995). Also, when Wnt8 is expressed in the dorsal marginal zone at the gastrula stage, axial development is blocked (Christian & Moon 1993), and overexpression of the Frzb protein in the region can prevent this blockage (Leyns et al 1997, Wang et al 1997).

SIMILARITY TO THE SOG/DPP INTERACTION IN *DROSOPHILA* As shown by recent molecular genetic analysis, Sog (short gastrulation) is a Chordin-like protein produced in the neurogenic ectoderm region of the cellular blastoderm embryo, and Dpp is a BMP-like protein produced in the dorsal ectoderm region of this stage. When Sog activity is eliminated by mutation, less neurogenic ectoderm develops and dorsal ectoderm expands to fill the space. When Dpp is eliminated, the reverse effect occurs; the dorsal ectoderm and amnioserosa do not form, and neurogenic ectoderm expands to fill the space as the default path of this tissue. The *sog/dpp* double mutant has the same phenotype as the *dpp* mutant alone, indicating that Sog protein acts through Dpp and has no additional effect of its own (Ferguson & Anderson 1992, Holley et al 1996). The evidence strongly supports the conclusion that Sog inhibits Dpp binding to its receptor and that the dorso-ventral dimension of *Drosophila* is organized by this interaction of secreted agents produced in neighboring regions. There are other protein mediators of this interaction yet to be analyzed (e.g. shrew and tollid). The Dpp/BMP interaction with Sog/Chordin may be an ancient means of dorso-ventral patterning predating the evolutionary branching of arthropods and chordates. Both groups have the nervous system centered in the Chordin/Sog zone. The only difference would be that in chordates, this zone faces up (dorsal) and in arthropods it faces down (ventral), relative to the earth (Nübler-Jung & Arendt 1994, De Robertis & Sasai 1996).

In light of these new ideas, it is noteworthy that in 1950, Yamada (1950) proposed that neural induction of the ectoderm and dorsalization of the mesoderm are basically the same. Not only did he coin the term dorsalization of the mesoderm, but also called neural induction a dorsalization of ectoderm, to stress the similarity.

Caudalization of Neurectoderm

As noted above, the neural-inducing proteins encoded by genes expressed in the organizer all evoke the development of anterior neural structures but not posterior ones. That is, they seem to act as neuralizing but not caudalizing signals. The trunk-tail-inducing part of the organizer is thought to release caudalizing as well as neuralizing signals, in effecting the development of hindbrain and spinal cord. In normal gastrulation, this part of the organizer converges and extends

under the ectoderm that will soon form the posterior neural plate. To analyze caudalization in the embryo, Nieuwkoop & Albers (1990) transplanted pieces of anterior neural plate to more posterior positions in the plate and scored the extent of their caudalization. Caudalization was detectable until stage 16. The more posterior the piece was placed, and the longer it was there before stage 16, the more posterior was its eventual neural differentiation.

The organizer and notochord may not be unique in producing caudalizing signals. The posterior somites, which also lie under the posterior neural plate, may be another source (Hemmati-Brivanlou et al 1990). When animal cap cells are induced at the blastula stage with low levels of activin that suffice for their developing muscle, they are simultaneously induced to gain neural-inducing activity, i.e. they induce posterior neural structures in gastrula ectoderm, indicating that they release both neuralizing and caudalizing signals even though they eventually only form muscle. At intermediate levels of activin, the treated caps can induce posterior and anterior neural tissue in gastrula ectoderm, and they eventually differentiate notochord and muscle. Finally at high activin levels, the treated caps are able to induce head neural structures, as if no caudalization occurs, and they differentiate only anterior mesoderm, perhaps prechordal plate. Thus the presence of posterior dorsal mesoderm, including somites, correlates with a capacity for caudalization (Green et al 1997). From the opposite side, when the *Xlim1* gene is expressed in animal cap cells via mRNA injection, the cells induce only anterior neural marker genes in gastrula ectoderm (Taira et al 1997), and they differentiate no muscle or notochord. When *Xlim1* and *Xbra* are co-expressed in these cells, they induce posterior neural marker genes and form muscle, and hence must release a caudalizing factor. They express the eFGF gene, consistent with eFGF being the factor.

The first attempts to simulate the action of a caudalizing signal in vitro have been made with FGF. In the *Xenopus* embryo, the gene encoding eFGF (a secreted form of FGF) is expressed at the correct time and place (Isaacs et al 1995), namely, in the trunk-tail-inducing part of the organizer (see Figure 5C). If anterior neural plate, which has been neuralized in the embryo, is explanted and treated with bFGF protein, it differentiates posterior neural tissue (Cox & Hemmati-Brivanlou 1995), indicating FGF's capacity to caudalize. If gastrula ectoderm is treated with Noggin and bFGF proteins, it expresses an extensive antero-posterior range of neural markers (Lamb & Harland 1995), indicating FGF's capacity to caudalize the neuralizing effects of Noggin. Care was taken to show that no mesoderm was formed in these experiments, as evidence that the FGF effects were direct. Thus the caudalizing effect of FGF in vitro seems well supported. Does FGF also neuralize? If blastula animal cap cells are aged in vitro to the gastrula stage and then treated with bFGF, they indeed differentiate neural tissue of a posterior type, expressing markers of the hindbrain and spinal cord

(Lamb & Harland 1995, Kengaku & Okamoto 1995). This result suggests that FGF, as a single molecule, can neuralize as well as caudalize. Interestingly, the longer the tissue was aged before FGF treatment, the less posterior the markers it expressed, as if ectoderm competence to respond was lost in a posterior-to-anterior direction with age (Lamb & Harland 1995). These provocative results contradict the two-step model in which caudalizing agents only modify neuralizing agents and cannot neuralize independently. The alternative is a two-inducer model in which FGF would both neuralize and caudalize, whereas other agents such as Noggin would only neuralize. However, it is not certain that FGF really neuralizes the ectoderm (and in current thinking, FGF is not thought to antagonize Wnts or BMPs). During the aging period, the tissue was kept in a low Ca^{2+} , Mg^{2+} medium before FGF treatment, and this medium reduces cell association, perhaps causing autoneuralization that FGF could then caudalize (Lamb & Harland 1995). If a normal non-dissociating medium is used during aging, FGF has no effect. At the same time, the ectoderm quickly becomes refractory to neural-inducing signals from the organizer (Sive 1989). However, even if FGF does not neuralize, its *in vitro* caudalizing action seems clear.

The role of FGF in neural induction in the embryo is currently disputed, based on the neural development of transgenic *Xenopus* embryos producing a dominant-negative FGF receptor to disrupt FGF signaling. These produce the altered receptor after the midblastula transition, and normal FGF receptor function is disrupted thereafter. Such embryos develop anterior neural parts such as brain, indicating the dispensability of FGF for the neuralizing step of neural induction. Surprisingly however, posterior neural development is also achieved, as seen by the expression of hindbrain and spinal cord markers in a split nerve cord along the lateral edges of the still open blastopore (Kroll & Amaya 1996). Thus FGF seems unnecessary for caudalization in the embryo. Because the notochord and most somite tissue are absent, the mesodermal source of neural-inducing signals is unknown. If the FGF receptor is to be considered the mediating receptor for caudalization in the embryo, one would have to argue that the dominant-negative does not block all signaling by FGF or by non-FGF ligands, such as Ig-containing surface proteins (see P Doherty & F Walsh, this volume). Alternatively, other signaling systems may mediate caudalization *in vivo*. At this time, the *in vivo* role of FGF in caudalization is unclear.

There is also evidence that certain Wnt ligands can caudalize. When Wnt3a is produced alone in animal cap cells (prospective epidermis), these do not subsequently differentiate neural markers. When Noggin is expressed alone in these cells, anterior neural markers are expressed. However, when both are expressed in these cells, posterior neural tissue differentiates, indicating that Wnt3a has a caudalizing action (McGrew et al 1995). This posteriorization

presumably works through β -catenin stabilization and nuclear localization, the same pathway that had been used earlier for the dorsal modification of meso-endoderm induction. Indeed, overexpression of β -catenin and Noggin in these cells leads to the expression of posterior neural marker genes. Presumably this caudalization is mediated by the β -catenin activation of the LEF-1, Tcf transcription factor.

Intermediate Mesoderm

Although current evidence supports the view that the dorsalization of mesoderm within the latero-ventral marginal zone involves a derepression of an inherent capacity for dorsal development (as in somite development), and also supports the view that ventral development of mesoderm within this region involves continued repression of dorsal development (as in lateral plate and blood development), the question remains of the inductive mode of specification of intermediate mesodermal tissues such as kidney and heart mesoderm. These intermediates form near the boundary of somites and lateral plate (see Figure 6). Has a partial derepression occurred in these tissues? In the earlier discussion of meso-endoderm induction, a similar issue of intermediate states was raised and deferred since it was concluded that only two parts are specified in the marginal zone at that time: the organizer dorsal mesoderm and the ventral mesoderm. Intermediate states were deferred to a later stage when organizer signals would be present. Somites are incontrovertibly specified by organizer signals at the gastrula stage, but is this true for heart and kidney? There are two interpretations at present. In the first, the various antagonists diffuse from the organizer in a gradient and set up a counter-gradient of active BMPs and Wnts. Mesoderm cells then have different responses, including heart and kidney responses, to the different concentrations of, or durations of exposure to, BMPs and Wnts. In the second view, heart and kidney are not specified in the gastrula stage, but in the neurula stage when new signals are secreted by competence groups established in the gastrula stage. In this view, the specification of intermediate mesoderm is deferred again.

Evidence bearing on these distinctions remains ambiguous. As exemplified in Figure 4, patterns of gene expression change rapidly in the marginal zone mesoderm of the late blastula and early gastrula stages, probably reflecting the changing exposure and response of cells to organizer signals. These changes continue through the gastrula stage. As a test for a role of BMP gradients, the quantity of BMP or the quantity of organizer signals such as Noggin or Chordin has been increased by ectopic expression from injected mRNA or DNA. For genes whose encoded products reflect the specification of somites and ventral mesoderm, the patterns of expression shift in ways consistent with the expectation that a graded range of active BMP determines the size and location of the

domain of somite specification (Re'em-Kalma et al 1995, Dosch et al 1997), and reciprocally of the adjacent domain of ventral mesoderm specification (lateral plate and blood). Altered levels of Wnt8 also affect the expanse of the somite response (Christian & Moon 1993).

However, the expression of marker genes of heart and kidney specification has been examined in less detail, and it is uncertain how much of their specification can be ascribed to organizer signals. The organizer alone does not evoke heart development from lateral mesoderm in recombinates with it (Nascone & Mercola 1995), nor does Noggin protein (W Smith & R Harland, unpublished data). A combination of organizer mesoderm and anterior endoderm must be present to induce heart in lateral mesoderm (Nascone & Mercola 1995). Thus heart development and, perhaps, kidney may require secondary inducers released at the neurula stage by various kinds of cells established and brought together in gastrulation, rather than being induced directly by gradients of organizer signals at the gastrula stage.

The somites themselves provide a lesson about intermediate mesoderm. Cells of the somite region are exposed to secondary signals after the gastrula stage: to Shh from the notochord and floor plate, to BMPs from the lateral plate, and to other signals from the epidermis and dorsal neural tube. These inductions lead to the development of sclerotome and dermatome in addition to myotome (Pourquie et al 1996), the first two of which are sometimes considered intermediate mesoderm. Their specification is thus only partially ascribable to the direct effects of organizer signals.

Finally, in considering gradients of organizer signals, the caudalization of trunk mesoderm (reflected in antero-posterior somites differences; Donoghue et al 1992) has received little attention. In Keller explants, the organizer mesoderm appears to establish an antero-posterior order of a few expressed genes without contact with other regions (Doniach et al 1992).

CONCLUSIONS

Cells at the equatorial level of the amphibian blastula form Spemann's organizer as a response to two signals: (a) a meso-endoderm inducer widely distributed around the egg's equator, probably a TGF- β family member; and (b) a dorsal modifying signal, probably a Wnt-signaling pathway intermediate transported from the vegetal pole to one sector of the equator during the first cell cycle after fertilization. The group of blastula cells able to release both kinds of signals, and hence induce the organizer, is called the Nieuwkoop center. The signals are thought to be maternal in origin; their exact identity is not yet known. They may induce only a portion of the organizer and that part may subsequently induce the rest. The organizer can be recognized at the late blastula stage as a cell

population uniquely expressing certain genes encoding particular transcription factors and secreted proteins.

The recent identification of certain proteins secreted by the organizer and others secreted by the surrounding competence groups has greatly advanced studies of inductive signaling and patterning in the amphibian gastrula. As understood so far, all inducers from the organizer antagonize short-range (autocrine/paracrine) signals initially produced by cells of the competence groups and needed by them to remain on paths of ventral posterior development. The antagonists, which are anti-ligands of BMP4 and Wnt8, derepress those cells to take dorsal and anterior paths of development: neural induction of the ectoderm, dorsalization of the mesoderm, and anteriorization of the endoderm. Intermediate mesoderm development may involve a partial release of dorsal development, but this remains to be established. Caudalization of the neuralized ectoderm is plausibly a modification of neuralization, but the organizer's caudalizing signals have not yet been identified (although FGFs and Wnts are candidates), and the basis of the modification remains to be understood. Other proteins secreted by the organizer may yet be found, and it remains to be seen if some will act via their own receptors in promoting dorsal development. The near future should bring new information on these points and extend the analysis to other chordates such as mouse and zebrafish.

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