Cell, Vol. 100, 41–55, January 7, 2000, Copyright ©2000 by Cell Press

The Molecular Metamorphosis of Experimental Embryology

Review

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Most embryologists have been drawn to the field by the beauty of the developing embryo and the mystery of its structure emerging from a single fertilized egg. The classic publications in the field of experimental embryology illustrate the power of describing cell behavior (cf. lineages, movements) and perturbing the embryo to test hypotheses of the underlying mechanisms. This tight coupling between observation, hypothesis, and perturbation has extracted significant insights from relatively simple experimental designs. For example, by analyzing eggs that had been fertilized by more than one sperm, Boveri showed the importance of a full set of chromosomes to normal development and thus established the chromosomes as the source of genetic material (Boveri, 1907). Simple perturbation experiments designed to destroy the genetic information offered some of the first evidence that information in the chromosomes depended on intact DNA (Boveri, 1904). Thus, the basic approach of the experimental embryologist—an integrative cycle between description, proposal, and experimentation—has generated insights that are amazing in their accuracy and depth.

The explosion of progress in the fields of cell biology, biochemistry, and molecular biology has offered new motivations and new technologies with which to explore developmental mechanisms. The integrative approach of experimental embryology offered the perfect complement to these reductionistic fields, fueling rapid progress. As a result, embryology has recaptured its cutting edge status, which many argued had been lost. In contrast to the overly etched "black/white" statements in which a single molecule or mechanism (e.g., the only one that could then be studied by that laboratory) drives all of development, the field is no longer satisfied with simple-minded, linear, and absolute pathways. Now it is phrasing its questions in shades of gray, asking how the many different influences on a given developmental event are integrated to give appropriate developmental patterning. The major challenge for the next decade will be to develop even more powerful tools for asking questions and collecting data in a fashion that fully embraces the complexity of the intact system.

It is, admittedly, impossible for any article or two (see Scott, 2000) to review all of the progress in developmental biology. Rather than attempt an encyclopedic overview of even a narrowly prescribed subarea, we will draw examples from a few of the areas in which experimental embryology has recently advanced. This will allow us to discuss the revitalization of experimental embryology, to illustrate the accelerating rate of progress in the field, and to consider some of the challenges and opportunities that will be offered to embryologists in the coming decades.

Fate Maps and the Study of Diversification

Development involves groups of cells undergoing morphogenetic movements and phenotypic differentiation. Given that these cells once appeared indistinguishable from one another, the generation of diversity must be considered the central issue of developmental biology. Whether studied at the tissue, cell, or molecular level, progress requires reliable knowledge of two closely related topics: the fate map of the embryo and the cell lineage of single precursor cells. Fate maps are depictions of what cells in various regions of an embryo will become during normal development. Cell lineage studies can identify the range of phenotypes that arise from single cells. Construction of a fate map requires a means of following a cell (or distinct group of cells) from a defined region of the embryo, and scoring the final phenotypes and positions of their progeny. In some cases, the embryo provides a unique cellular marker, in the form of a cytoplasmic inclusion (e.g., the yellow crescent of some ascidian embryos) that eases this challenging task. In other cases, the experimental embryologist must introduce a label to follow a cell lineage or construct a fate map. The approaches for labeling cell lineages and their relative merits are well defined (Fraser, 1992). While none of the techniques offer all of the desired attributes (neutrality, indelibility, and targeting ability), they each have offered windows into the cell lineages that construct the embryo. Although fate maps offer critical information, it is important to keep in mind what a fate map or cell lineage study cannot tell us. They show the fate of the cells if left in their context in a normal embryo, which is not the same as what the cells are specified to become. For example, it is possible that the cells have little or no information about their eventual fate but are carried by morphogenetic movements to the position where a specific instruction is provided. Similarly, cell lineage analyses demonstrate what phenotype a cell adopted, not the full range of phenotypes it is capable of achieving. Although maps cannot by themselves tell us whether cells are committed to generate a given tissue or cell type, fate maps are the critical first step in analyzing the mechanisms of cell fate determination, embryonic induction, and tissue morphogenesis.

In the absence of direct evidence concerning cell lineages and movement, workers sometimes substitute a "molecular fate map," the set of cells that express a gene characteristic of a later differentiated cell type or of a distinct region of the later embryo. Space does not allow a detailed treatment of the huge number of cases in which such analyses have been misleading. In virtually every instance, detailed examination of expression domains show only rough correspondence to the fate map. Cells from inside the marker gene expression domain leave to populate other tissues, or cells from outside the expression domain move into the region and differentiate appropriately for the new domain (two of many

examples: Birgbauer and Fraser, 1994; Joubin and Stern 1999). The change in gene expression of the cells moving into and out of the domain merely masks the presence of the cells that are in disagreement with the fate map. Thus, marker gene expression data cannot serve as a substitute for a fate map.

Fate maps and lineage studies are critical to the design and interpretation of experiments, but due to uncertainties in labeling and in scoring cell position and phenotype, they are best viewed as imperfect "works in progress" rather than definitive and complete statements. This requires that they be used with some caution. For example, a recent reanalysis of the fate map of the frog embryo shows that the progenitors of the blood are much more broadly distributed in the embryo than previously expected (Lane and Smith, 1999). Such revisions of the fate map position of the blood might require a reevaluation of mechanisms proposed for patterning in the frog embryo. In addition, it should hasten a careful reanalysis of the fate maps and patterning mechanisms proposed for other species by analogy with the frog. Fortunately, in addition to pointing out past misconceptions, such dramatic recasting of the fate maps also suggests new experimental interpretations and directly motivates more decisive experimental designs.

Is Development Guided by Intrinsic or Extrinsic Cues?

A central question of developmental biology has been whether the cues that guide the embryo are set up by intrinsic or extrinsic information. This question has taken several forms over the years, with some phrasing it as the difference between mosaic and regulative modes of development. The most evocative description has been ascribed to Sydney Brenner, asking whether development proceeded according to the European plan or the American plan. In the European plan, the fates of the cells are dictated by their ancestry; cytoplasmic determinants and intrinsic cues would play the dominant role in the cell's choice of phenotype. In contrast, cells developing by the American plan would be driven by their interactions with their neighbors, either due to some drive toward conformity (keeping up with the Joneses) as proposed more recently by the community effect, or due to competition between neighbors through lateral inhibition. To test between the American and European plans, the experiments are obvious: isolation of a subregion of the embryo or even of single cells to eliminate neighbor interactions, and grafting of cells to a new site to confront cells with a different set of neighbors. In their most crude form, these experiments can involve bisecting an embryo to ask if the two halves can each make a complete embryo. The test question reduces to whether the cells remain true to their original fates (mosaic development) or if they adopt new fates to compensate for the missing neighbors (regulative development).

The tests for mosaic versus regulative modes of development have often generated somewhat contradictory experimental results. About a century ago, Roux argued for the mosaic nature of developmental patterning based on the half-embryo that was obtained after killing one of the two blastomeres of an amphibian at first cleavage. In contrast, Driesch offered evidence against mosaic development by demonstrating that some isolated blastomeres can develop into small but complete embryos. His concept of "entelechy" carried this to an extreme, positing that any subset of the embryo possesses a vital force that drives it toward completeness. As quaint and amusing as stories of conflicting findings and personalities might be, this one carries with it an important lesson. Both studies were performed without adequate knowledge of the fate map of the embryo. As shown by the later experiments of Horstadius (1939), experiments guided by knowledge of the normal fates of the cells permit much more balanced and definitive experimental interpretations. Armed with a fate map, Horstadius demonstrated that complete miniature embryos resulted only from those partial embryos that contained representatives of each of the tiers of cells in the animal-vegetal axis. Grafting experiments in the sea urchin embryo have demonstrated that moving the most vegetal cells (the micromeres) to an ectopic site can have profound effects on the new neighbors, inducing the formation of a second gut (Davidson et al., 1998). More recent molecular studies offer ample evidence for both the necessity and the sufficiency of extrinsic interactions with the micromeres, and equally strong evidence for the intrinsic differences in the micromeres. Thus, as might be expected from any simplified, "either-or" choice, the answer appears that the embryo develops by both the European plan (micromeres) and the American plan (neighboring vegetal plate cells, which are induced by the micromeres).

Ascidians: from Cell Lineage toward an Identified Cytoplasmic Determinant

The embryogenesis of the sea squirt (ascidians, tunicates) has occupied a central position in experimental embryology for more than a century. Given recent advances, it offers one of the best examples of the progression from observational embryology to experimental embryology to molecular biology and finally to a synthesis of these fields. The embryos are readily available from sessile adults, and the adults themselves are straightforward to collect. They progress from fertilization to larvae with a well-defined yet simple body plan in less than a day and are made up of a manageable number of cells ($\sim$2500) with sufficiently distinct morphologies to simplify both observational and experimental analysis. Furthermore, in contrast to the sessile morphology and lifestyle of the adult, the larva displays a notochord, a dorsal neural tube, longitudinal muscles along the notochord, as well as other characteristics making it clear that ascidians are chordates. As Charles Darwin wrote in the *Origin of Species*, ". . . Mr. Kovalevsky has lately observed that the larvae of ascidians are related to the Vertebrata, in their manner of development, in the relative position of the nervous system, and in possessing a structure closely like the chorda dorsalis of vertebrate animals; . . . and should his results be well established, the whole will form a discovery of the very greatest value. Thus, if we may rely on embryology, ever safest guide in classification, it seems that we have at last gained a clue to the source whence the Vertebrata were derived."

The Yellow Crescent as a Marker and Cytoplasmic Determinant. Work from the early part of the century

exploited the clarity of the embryo and the intrinsic colors of up to five subregions of cytoplasm to follow the cell lineages that construct the ascidian embryo (Conklin, 1905). Cytoplasmic motions in the fertilized egg create a "yellow crescent" that is later split by first cleavage. By following the cells that inherited the yellow crescent, Conklin was able to demonstrate that these cells were fated to become muscle cells. Based on this finding, and the distinct mixture of fates adopted by the cells with the different colored cytoplasms, he concluded that the embryo was largely mosaic in its development (the fates of the cells in the early embryo were highly deterministic). Because the fates were defined in these studies by following the cells that inherited the distinctly colored cytoplasm, it should come as no surprise that the colored cytoplasm itself was thought to contain factors that specified the fates of the cells that inherited it. For example, the critical factor of the yellow crescent has been termed the myoplasm.

Experimental embryology built upon these careful observations, attempting to test the conclusion that the development of the ascidian followed mosaic rules. For example, surgically separating the cleavage stage embryo into subregions shows that cell fates are unequally assigned in the embryo, consistent with the inheritance of distinct cytoplasms. If the blastomeres that normally inherit the myoplasm are isolated, they develop into muscle cells; no other region of the embryo does so. Regions of the embryo that would not have developed into muscle do so when the normal segregation of the yellow crescent is perturbed (see review: Whittaker, 1987). These findings offered experimental evidence in support of the proposals of deterministic or mosaic development, based upon the inheritance of cytoplasmic determinants (see Satoh, 1994).

Cell Lineage and Steps toward a Molecular Determinant. In more recent years, the lineages of the cells have been examined in greater detail, and the mosaic nature of the embryo has been shown to be less absolute. Fate mapping studies by the application of chalk particles or by intracellular injection of a marker have validated the observational fate map of Conklin but also have demonstrated that these early fate maps captured only a portion of the cells that contribute to the muscle (see review: Nishida, 1997). Of the 36 cells that contribute to the muscle in one species, only 28 come from the cells that inherit the myoplasm; the other 8 come from neighboring lineages. Blastomere isolation and recombination experiments indicate that the contribution of these cells to the muscle lineage requires instructional interactions between cells. Similar secondary lineage contributions have been observed in other tissues such as the notochord, suggesting the parallel operation of deterministic and regulative mechanisms of developmental patterning.

Recent experiments have used the *cis*-regulatory domain of a muscle-specific gene to identify an excellent candidate for the active ingredient of the myoplasm. This was made possible by the development of a technique for introducing genes into the ascidian by electroporation (Corbo et al., 1997) and thereby obtaining expression of marker genes (*lacZ*; *GFP*). This allowed Erives et al. (1998) to isolate a sequence flanking the coding region for *Ci-snail*, a gene expressed strongly in the muscle of the ascidian, and to identify a minimal enhancer of 262 bp that was adequate to drive expression of GFP in muscle cells. Within this minimal element, they have identified five recognizable binding sites for transcription factors (three HLH-binding sites and two T box sites). Mutational analysis showed that these sites were necessary for the minimal enhancer to function. Such progress, distilling one of the long-standing questions of experimental embryology down to a short *cis*-regulatory domain, demonstrates the power of combining classical approaches with the most incisive molecular biology. In short, it appears certain that the problem of the myoplasmic determinant, posed at the outset of the century, will be solved soon after its close.

C. elegans*: A Collaboration of Mosaic and Regulative Mechanisms*

The recent lessons from research on the nematode, *Caenorhabditis elegans* offers an intriguing counterpoint to the recent progress on the ascidian embryo. The results show clearly that invariant cell lineages do not imply purely deterministic development or the absence of important roles for intercellular signaling. Although much of the early effort in the nematode paralleled the experimental approach used in most other systems, the genetic tools have been more completely developed, allowing a distinct mode of progress. In a very short time, the nematode *C. elegans* has lived up to the promise outlined in a letter to Max Perutz from Sydney Brenner written some 35 years ago (see Wood, 1988). Because the major problems of molecular biology seemed to Brenner to be either solved or at least predictable in nature, he argued that it was time to move on to more complex issues such as developmental biology. Based on the rapid development, small size, and manageable number of cells in the embryo and the adult, Brenner argued that the nematode offered a system well poised for both descriptive developmental biology and genetics. This made it the appropriate system to attack his goal of a complete description of the cell lineages in an embryo and the genes that guide them. From our current vantage point, in which the lineages that generate the 558 cells of the embryo and the 959 somatic cells of the adult hermaphrodite are well described, and in which the complete genome of about 19,099 genes is sequenced, one can only marvel at the rapidity of the progress and the insight of Brenner's proposal.

Development by the European Plan? At one time, the reliable patterns of early cleavage and the defined cell lineages of the *C. elegans* embryo and larva were taken as evidence that the nematode executed a very deterministic, lineage-based, developmental program. In fact, a scan of the current textbooks shows that it is used as the major example of mosaic development (few exceptions to this mosaic rule are mentioned). However, the very reliability of the cleavage patterns and cell movements make it impossible to discern between the operation of a mosaic (European) plan of development (as all suspected) or a regulative (American) plan of development. Given that the cells appeared to always lie in the same location, patterning based on intrinsic cues segregated by cell lineage and patterning based on extrinsic cues from reliably positioned neighbors would not be discernable by observation alone. The first ablation experiments, intended to challenge the developmental

patterning mechanisms by eliminating the neighbors that might normally give patterning information to a cell, yielded results that were taken as supportive of a European plan of development. Viewed in hindsight, the evidence offered by these experiments appears less conclusive (see Sulston and White, 1980).

Convincing early evidence for an important role of cell interaction in the developmental patterning of *C. elegans* came from studies of the postembryonic development of the vulva, the opening in the hypodermis that allows offspring to exit the hermaphrodite. Observation suggested a key role of the gonad's anchor cell in the formation of the vulva. If the anchor cell was ablated before it contacted the cells of the hypodermis, the vulva did not form, while manipulations that displaced the anchor cell resulted in an ectopic vulva (Kimble, 1981; Thomas et al., 1990). Thus, following the classical approach of experimental embryology, the anchor cell was shown to be both necessary and sufficient for the formation of the vulva. The genetic tools available in *C. elegans* allowed these observations to be rapidly expanded upon. The distinctive "bag of worms" phenotype created when there is no vulva for larvae to leave the hermaphrodite (leaving them inside to consume the parent), allowed the isolation of several key mutants. Assembly of the primary mutations and the suppressors and enhancers of these mutations has defined the nematode homolog of the EGF receptor system of vertebrates (see review: Sternberg and Han, 1998). As an example of the best of modern experimental embryology, this story of cell interaction in *C. elegans* development rapidly grew from observational studies and fate mapping, to experimental and genetic manipulation and finally to a full molecular characterization.

Given the great success of the vulval development story, it may seem surprising that evidence for cell interactions in the early embryo was not quickly forthcoming. Instead, the data seemed firmly in support of mosaic development in the early embryo. For example, the lineage diagram shows an early and rather complete segregation of the cells that contribute to the endoderm, as might be expected in a mosaic organism. In addition, careful analyses of the lineage patterns showed an unanticipated degree of variation in the timing of the divisions and in the cell–neighbor relationships (Schnabel et al., 1997). Such variations perform an "experiment in nature" similar to a classical test of cell commitment. Even though cells are displaced by variation in the timing of their division, they follow normal fates, suggesting something other than the neighbors of the cells guides their developmental fates. Finally, the germline of *C. elegans* offers some of the best evidence for a cytoplasmic determinant: the P granules. The P granules segregate to the posterior of the embryo and are inherited by the most posterior blastomere for the first several rounds of division. Disruption of the segregation of the P granules eliminates the germ line. Recent work has confirmed that at least one of the components in the P granules is essential. If the gene encoding the component is mutated, the cells execute a normal set of cleavages, but the cell that normally inherits the P granules can no longer give rise to germ cells (Kawasaki et al., 1998). Judged against such dramatic findings, it is not surprising that cell interactions during cleavage stages were not considered a significant aspect of developmental patterning.

Reliable Fates from the American Plan. It has now become clear in several systems that reproducible lineages cannot be taken as strong evidence that cytoplasmic determinants define cell fate. In cases in which the cells do not move during early development, a simple set of cell interactions can result in such regular outcomes that it might appear as if inherited determinants are driving development. Even the sea urchin embryo, which offered some of the best and earliest evidence for regulative development, displays a regularity in its fate map and patterns of early gene expression that could be mistakenly taken as evidence for mosaic development. It is only through experimental manipulations that the critical role for cell interactions can be shown to influence the fate map and the gene expression domains (Davidson et al., 1998). In *C. elegans*, cell relationships do change as the early embryo develops, but the contacts progress in a very stereotyped fashion, due to the regular cleavage patterns and the constraints imposed by the eggshell.

Several different experiments have now challenged the role of cell signaling in the regular cell lineages of *C. elegans* by either eliminating or displacing a potential signaling partner. These experiments demonstrate the powerful synergy possible between genetics and experimental embryology. Issues such as the exact stage of a required cell interaction, the time required for the interaction to take place, and the position of the key interaction (Goldstein, 1993) would be difficult if not impossible to address by genetics alone. Similarly, the experimental embryology would be lost without the tools and insights offered by genetics. In some elegant recent experiments, gentle pressure was applied with a micropipet tip on the outside of the eggshell, flipping the axis of a single division. The reoriented division inverts the position of contact with neighboring blastomeres; the inversion of the neighboring blastomere fates offers clear evidence for cell interactions in dorsoventral patterning (Lin et al., 1998). Similar reorientation experiments show a critical role for cell interactions in the right-left handedness of the embryo (Bergmann and Wood, 1997). Now the molecular bases of these interactions are being defined. The interaction that patterns the ABp blastomere via contact with the P2 blastomere is mediated by Notch signaling (Moskowitz and Rothman, 1996). The P2 cell expresses Apx-1, a member of the Delta family of ligands, on its surface; the ABp blastomere expresses Glp-1, a member of the Notch family. However, there is an unusual wrinkle to the story, as the mRNA for each of these is present in all cells. The asymmetry is created by differential translation of the message in the AB and P2 lineages that is itself dependent on cell interactions.

By combining the tools of experimental embryology with those of genetics and molecular biology, it now appears that cell interactions, rather than segregated cytoplasmic determinants, play roles at nearly all lineage branchpoints. For example, Wnt-signaling, acting shortly after mitosis, may be the major determinant of anteroposterior differences of nearly all divisions in the early embryo (Lin et al., 1998). Even the gut lineage, which is cleanly segregated to the descendants of the

E blastomere, appears to be the product of cell interactions rather than cytoplasmic determinants. Interaction between the parent EMS blastomere and the P2 blastomere is critical: in the absence of contact from P2, neither EMS cell daughter produces gut descendants; instead of one E cell and one MS cell, both daughters make the muscle and other cell types characteristic of the MS daughter. Forcing contact with the P2 cells is sufficient to cause the MS cell to execute an E lineage. Blastomere recombination experiments together with genetic analyses demonstrate that this interaction is mediated by a Wnt pathway signal, with P2 making the Wnt family member, and EMS responding via an Fz receptor and wormadillo (Thorpe et al., 1997).

Primary Axis Formation

While research on *C. elegans* has led to a departure from the traditional view of mosaic development toward a current understanding of regulative development dependent on cell interactions, research on the amphibian embryo has been progressing in the opposite direction. Recent advances have identified cytoplasmic determinants that are essential for early pattern formation, so that amphibian development is now understood to result from a mixture of mosaic and regulative mechanisms.

Discovery of the amphibian organizer ranks as one of the most influential events in embryology in the century (Hamburger, 1988). While testing a hypothesis that different regions of the embryo are predisposed to form different tissues, Hilde Mangold grafted different regions to ectopic locations. Not only did she show that dorsal mesoderm was stably determined, but by using marked embryos, Spemann and Mangold also showed that the grafted organizer induced neighboring tissues to follow new and well-organized fates. These experiments highlighted the importance of the dorsal mesoderm during gastrulation, when "Spemann's Organizer" becomes the center of signaling to produce the definitive vertebrate body plan. However, earlier work by Spemann had traced the difference between dorsal and ventral organization back to early cleavage stages. In the classic experiments where half-embryos were produced by ligating embryos with a loop of baby hair, the dorsal side (with the gray crescent) contained enough information to produce most of the structures of the tadpole; in contrast, the ventral side produced only a "belly piece" (Spemann, 1938; Hamburger, 1988). Later work by Nieuwkoop and others showed that during these early cleavage stages, the yolky vegetal cells were already different between dorsal and ventral sides in their ability to induce different mesodermal structures from naïve ectoderm (Harland and Gerhart, 1997). Thus, an early cleavage event imposed differences on the apparently cylindrical symmetry of the egg.

Experimental embryology was successful in defining many of the developmental events in amphibian embryos, as well as the signaling centers and responsive tissues. Simplistically, the early events were those of endomesoderm induction and dorsal signaling from a "Nieuwkoop center;" the later events were those of endomesoderm patterning and neural induction from Spemann's organizer (Gerhart et al., 1989; Gerhart et al., 1991; Harland and Gerhart, 1997). A molecular level of understanding has flourished in the last fifteen years, building on the methods and concepts developed in the previous eighty years.

The Challenge of Breaking Symmetry

Most eggs are spherical, though in most cases there is an obvious animal-to-vegetal axis, reflecting the asymmetric distribution of yolk and other cytoplasmic components. A single axis of rotational symmetry is not enough to instruct the cleaving egg to develop dorsoventral, anteroposterior, and left–right axes. In some embryos, maternally deposited information can direct dorsoventral pattern. This is true in the fly embryo, where the follicle surrounding the egg locally processes the Spätzle ligand to activate the Toll receptor (Anderson, 1998). The *Drosophila* embryo is somewhat unusual in having two almost unrelated sets of determinants that direct anteroposterior polarity and dorsoventral polarity, while other embryos set up these coordinates starting with just one maternally deposited axis (as with the amphibian animal–vegetal axis) or perhaps even no strong bias (as in the mammalian egg). How do these animals break their symmetry and elaborate pattern from a limited amount of prelocalized information?

Symmetry Breaking in Amphibian Eggs. The amphibian egg demonstrates properties of both mosaic and regulative development. The egg starts with cylindrical symmetry, but with an axis organized from animal to vegetal pole. Cylindrical symmetry is broken by the relative motion of two sets of informational molecules. One of these appears to be a prelocalized mRNA coding for the VegT transcription factor (see below). The other, which activates Wnt signaling, has not yet been formally defined, though many of its properties have been established. As outlined in the following sections, once symmetry is broken, the maternal determinants initiate a cascade of cell–cell interactions, where each step builds complexity from the previous step.

In normal development, amphibian embryos break symmetry before first cleavage by polymerizing microtubules in the outer cortical layer of the egg, with the direction of polymerization biased by the location of sperm entry (see Figure 1; Gerhart et al., 1989). As cortical microtubules polymerize, they provide tracks for a cytoplasmic shear, in which the central ball of cytoplasm rotates relative to the outer cortex. Any initial asymmetry in the shear (for example, provided by the sperm astral microtubules) will tend to be self-reinforcing, and with rotation, the microtubules progressively align in a parallel array. The aligned microtubule array is thought to provide tracks for components that activate the Wnt signaling pathway; these are smeared out from an initial vegetal location to become distributed along the future dorsal meridian (Harland and Gerhart, 1997; Moon and Kimelman, 1998).

The selective binding and movement of Wnt signaling components on the microtubules provides asymmetry in early cleavage. If all determinants were bound to the microtubule array, then no useful complexity would result. However, another crucial cytoplasmic determinant, the mRNA coding for the T box transcription factor VegT, remains vegetally localized. When VegT mRNA is ablated, most of the mesoderm and endoderm is lost, demonstrating that VegT is a classical cytoplasmic determinant (Zhang et al., 1998). Since activin-like TGFβ

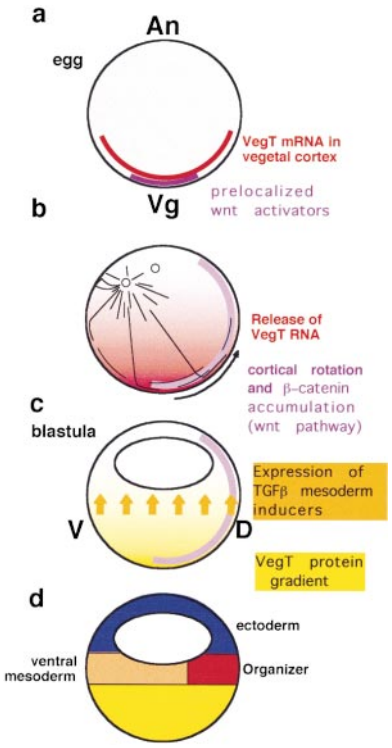

Figure 1. Breaking Symmetry in the Amphibian Egg

The egg contains prelocalized components that are used differently to generate asymmetry in the animal–vegetal axis and dorsal–ventral axis.

(a) In the egg, VegT mRNA is prelocalized in the cortex (red), and activators of Wnt signaling are also vegetally localized (purple).

(b) During the first cell cycle after fertilization, cortical rotation occurs. The sperm enters on the left, and the sperm aster forms in the animal hemisphere. The arrow indicates the 30° rotation of the cortex, which aligns microtubules in a parallel array close to the cortex. Cytoplasmic materials (purple) originating near the vegetal pole are transported along the array to determine the prospective dorsal side. The cylindrical symmetry of the unfertilized egg has now changed to bilateral symmetry.

(c) Cross-section of the mid–blastula stage (4000 cells), as mesoendoderm induction occurs in response to the graded distribution of VegT protein (yellow). A blastocoel space has formed in the animal hemisphere. Gene expression has just begun. Orange arrows indicate general mesoendoderm inducers released from the vegetal half, as a result of zygotic transcription.

(d) The zygotic response of adjacent animal hemisphere cells establishes the marginal zone of prospective ventral–posterior mesoderm and endoderm, encompassing the blastula. The elevated level of β-catenin (purple in [c]) modifies dorsal competence. When cells respond to the combination of general mesoendoderm inducers and the competence modifier, they form the organizer (red) in the dorsal marginal zone.

signals are the next step in the cascade of signals that results in mesoderm and endoderm induction (Harland and Gerhart, 1997), VegT must be required for the activation of these signals.

While the vegetal-to-animal gradation of VegT activity could account for the germ layer organization of vegetal endoderm, and equatorial mesoderm, the sheared Wnt components can account for dorsal-to-ventral organization. Here dorsal is used to describe the dorsal location of prospective notochord and prechordal mesoderm, with ventral being the rest. Wnt signals cause stabilization of β-catenin on the dorsal side. Just as maternal VegT mRNA is essential for the germ layer organization of the embryo, β-catenin mRNA is essential for dorsal development. When free β-catenin associates with transcription factors of the LEF/Tcf family, the Tcf factors are converted from their basal transcriptional repressing state into transcriptional activators. They then act either autonomously to turn on dorsal-specific transcripts such as *siamois* and *Xnr3*, or they cooperate with activin-like signals to turn on genes such as *goosecoid* (Moon and Kimelman, 1998).

Even though the process of forming dorsal mesoderm relies on the interplay of dorsally activated Wnt signaling and equatorial activin-like signaling, complexity is already built into the detailed pattern of gene activation (Harland and Gerhart, 1997). Radial differences in gene expression (from inside to outside) become evident as gastrulation starts, perhaps relying on other prelocalized components that have yet to be identified. The differences in gene expression from superficial to deep layers of the marginal zone become greater during gastrulation, and with the morphogenetic movements of gastrulation, these inside-to-outside differences become anteroposterior differences.

Organizer—A Source of Blocking Signals. All the tools of experimental embryology were brought to bear to investigate the mechanism of induction by Spemann's organizer, but the questions were ahead of their time (Witkowski, 1985); it has only been in the postcloning era that the question has become tractable at the molecular level.

One of the surprises has been the finding that the organizer is the source of molecules that block signaling pathways. Previously, the most attractive view was that the organizer would be a source of active signals that induce dorsal fates. However, the first secreted organizer molecules found turned out to be potent antagonists of the ventralizing effects of bone morphogenetic proteins (BMPs); other organizer molecules are antagonists of Wnt signaling (Harland and Gerhart, 1997). Of course, these findings do not change the logic of the system, where the organizer is the source of patterning signals. So long as the lack of signal transduction can be interpreted by a cell, it is as informationally rich as the presence of signal transduction. While dorsal fates are normally blocked by BMP and Wnt signals, it is the antagonism of these signals—inhibition of the inhibitor—that allows dorsal fates to be realized (Harland and Gerhart, 1997; Wilson and Hemmati-Brivanlou, 1997).

Head and Tail Organizers. Gastrulation reorganizes the embryo to produce the germ layers of ectoderm, mesoderm, and endoderm, and to produce dorsal–ventral organization. Anteroposterior pattern also appears during gastrulation and is intimately linked to dorsal–ventral organization. From the first experiments of Spemann and Mangold, it was clear that the head-to-tail organization of the embryo can be induced by organizer grafts. This poses the question of whether a single signaling center patterns the head, trunk, and tail or if the grafted organizer is heterogeneous, with separable head, trunk, and tail organizing abilities. Most experiments suggest that head induction is a property of a complete organizer; if the organizer is incomplete (or

weak), posterior patterning appears dominant (Gerhart et al., 1989). Morphogenesis is also implicated in head and tail inductions; in the absence of movement, a prospective head would remain within range of the dominant trunk-signaling center and be converted into trunk. Mechanistically, the difference between head and tail inducers may be the difference between neural induction in the presence or absence of signals that suppress trunk mesoderm formation (Piccolo et al., 1999). In the normal animal, the head signals are restricted to the organizer, and the trunk/tail inducer may be spread over the rest of the mesoderm (Woo and Fraser, 1997).

In the mouse, there is good support for a partially separable inducing center that is required for head development (Beddington and Robertson, 1999): the anterior visceral endoderm (AVE). Mutations in several genes expressed in the AVE result in loss of head development; however, it is not yet clear whether the AVE has an instructional role or is only required for the maintenance of a complete and vigorous organizer. A symptom of the present uncertainty is that some of the best evidence for an independent head inducing activity in mammals comes from grafts into the chick epiblast, where the rabbit (but not chicken) AVE results in anterior inductions (Knoetgen et al., 1999).

While analysis of head and tail organizer mechanisms is already difficult enough, the neural tube is much more elaborately patterned along the anteroposterior axis. Although there is evidence that juxtaposition of head and tail can result in the production of intermediate fates, the basis for detailed patterning of the neural tube remains poorly understood (Lumsden and Krumlauf, 1996).

The Cascade of Inductions Continues through Neurulation. The organizer acts on the ectoderm, mesoderm, and endoderm to induce neural, paraxial, and anterior gut fates, respectively. The organizer also undergoes self-differentiation into axial tissues (prechordal plate and notochord) and leads morphogenetic movements during gastrulation and neurulation. Following gastrulation, the embryo has achieved an impressive amount of new complexity. There are anteroposterior, dorsal–ventral, and left–right axes. The increased complexity is exploited to achieve yet further patterning. Each new tissue or boundary seemingly becomes a new signaling center. For example, in amniotes, the somite (paraxial mesoderm) is patterned by signals from the notochord and floor plate of the neural tube, the roof of the neural tube, the surface ectoderm, and the lateral plate mesoderm. Each of these tissues is newly specified during gastrulation and early neurulation and expresses a characteristic array of genes, including signaling molecules. Interestingly, the array of signals appears to remain fairly constant in a variety of early decisions and includes members of the TGFβ, Wnt, Hedgehog, and FGF families (Cossu et al., 1996; Marcelle et al., 1997). The individual responses of the target tissue must therefore depend very much on previous restrictions in fate determination. Presumably, such restrictions depend on the array of transcription factors, receptors, and transduction components that were turned on by earlier signals and determinants. Such progressive restrictions in the choice of fates that a cell can adopt are referred to as changes in competence, and these changes are particularly poorly understood in vertebrates.

How Are Genes Used to Generate Pattern?

The molecular metamorphosis of our understanding of embryology has relied on the identification of genes that control development. In vertebrates, many of these genes have been discovered through cell biological or embryological assays for function (e.g., FGFs, *noggin*; Harland and Gerhart, 1997; Slack, 1998), through positional cloning in mice (the protooncogene *Wnt1*, Nusse et al., 1984; or the T box transcription factor Brachyury, Herrmann et al., 1990), or through differential cloning, where a transcript is expected in one tissue but not another (e.g., the myogenic factor MyoD; Davis et al., 1987). Perhaps the greatest impact to understanding embryos has come from genetic screens in *Drosophila*, particularly the screen for defects in embryonic patterning by Wieschaus and Nüsslein Volhard (Nüsslein-Volhard and Wieschaus, 1980). As detailed in the accompanying review (Scott, 2000), many genes and gene networks are highly conserved. Therefore, the cloning of genes mutated in the fly screens not only provided the raw material for a mechanistic understanding of fly development, but through homology cloning has also had enormous impacts on vertebrate embryology. In this section, we discuss our understanding of how territories of protein expression are read out as different gene expression thresholds, and ultimately as different tissues.

Setting up Thresholds of Gene Activation–Transcription Factors and the Syncytial Blastoderm of Drosophila. Whether setting up the notochord-somite boundary in a vertebrate or setting up cleanly specified segments in a fly, one of the most challenging problems in developmental biology is how initial broad distributions of positional information are exploited to define sharp thresholds of response. Can thresholds be defined in one step, or are they determined by secondary interactions, to amplify a signal or to repress it? The answer may depend on just how sharp a threshold of gene activity is set. In cases where the threshold is very sharp, feedback by repression may be a universal mechanism; for broader thresholds of activation, secondary signals may not be necessary. Whatever the mechanism for threshold responses to a signal, the general assumption has been that the signal is graded. Although well-documented examples of graded signals are rare, the question of how graded signals can specify multiple responses is central in many areas of embryology. In some systems, the signals appear to be diffusible molecules acting over a field of cells; in others, they may be graded levels of a transcription factor acting in a syncytium. Obviously, some of the details must differ, but many of the principles may be the same. The greatest progress in understanding the mechanism has come from studies of *Drosophila* embryos, demonstrating that *cis*-acting elements of genes can act to provide sharply defined patterns.

Threshold Response to Bicoid and Dorsal. The blastoderm stage in *Drosophila* has provided two very clear examples of gradients in development: the graded concentration of the transcriptional activating protein Bicoid in the anteroposterior axis and the graded nuclear distribution of the Dorsal activator protein in the dorsoventral axis. It has been argued that threshold responses to

such gradients could be explained by the following three mechanisms.

First, the affinity of individual transcription factor–binding sites may modulate the concentration of Bicoid that is needed to activate transcription (St Johnston and Nüsslein-Volhard, 1992). Genes with very high-affinity binding sites can be activated by low concentrations of Bicoid; higher concentrations would be necessary for the activation of genes with low-affinity Bicoid-binding sites. To experimentally test this idea, different classes of Bicoid-binding sites from the *hunchback* promoter were independently multimerized and used to create a reporter gene. In the fly, the low- and high-affinity reporters showed different domains of activity, although the principle has yet to be shown to act in a normal Bicoid-responsive enhancer. In contrast, sites of different affinity to Dorsal have been found in genes expressed in response to different nuclear concentrations of Dorsal. The proximal enhancer of *twist* has low-affinity binding sites that respond only to the highest Dorsal concentrations. If the Dorsal-binding sites in the reporter are converted to higher affinity sites, such as those from *rhomboid*, the domain of expression expands as expected if lower concentrations were able to activate the gene (Jiang and Levine, 1993).

A second mechanism involves the cooperative interaction of factor-binding sites. For example, if Bicoid-bindings sites are arranged in tandem, cooperative interactions should result in a more avid apparent binding constant and a steeper dose–response curve as the sites are multimerized. This offers a means to "tune" the concentration that activates transcription, and to generate very sharp thresholds. The Bicoid target, *knirps*, is activated at much lower Bicoid concentrations than *hunchback* or *orthodenticle*, and the Bicoid-binding sites are indeed arranged in tandem in the *cis*-regulatory domain of *knirps* (Burz et al., 1998).

The third mechanism involves synergy between two transcriptional activators as exemplified by the enhancer of the Dorsal target *rhomboid*. In such cases, the production of a second weak transcriptional activator is required for expression (Rusch and Levine, 1996). In the case of *rhomboid*, the high concentration of Dorsal protein in the ventral nuclei activates the transcription of *twist;* Twist then binds near Dorsal to cooperatively activate *rhomboid* expression.

Repression as a Mechanism to Restrict and Sharpen Domains of Gene Activity. While positive activation at different thresholds may suffice to explain the broad domains of gene activity that are set up initially, secondary repression is a crucial element of boundary sharpening. Localized repression acts in both the specification of segments along the anteroposterior axis, and the specification of mesoderm in the dorsoventral axis (Mannervik et al., 1999). In the case of dorsal–ventral organization, the gradient of nuclear localization of Dorsal is crucial in setting up territories of epidermis (at low concentration of Dorsal), nonneural ectoderm (at intermediate concentration), and mesoderm (at high concentration) (see Figure 2; Huang et al., 1997). Dorsal alone can activate many genes, but at high doses, Dorsal activates the transcriptional repressor, Snail. Transcription of genes like *rhomboid*, which would otherwise be active throughout the ventral domain, is repressed by Snail and therefore restricted to the neurogenic ectoderm.

Enhancers Are Modular. The local integration of information on enhancers is another general principle that allows multiple different patterns of gene expression. An individual transcription unit can be driven by numerous enhancers; in many cases, each enhancer operates independently, so that repressors bound to one enhancer do not affect transcriptional activation driven by a distant enhancer. This requires that such repressors suppress activity of transcriptional activators over a short range of a few hundred base pairs. Thus, the several enhancers that drive the stripes of expression of the *even-skipped* gene integrate transcription factor activity independently so that repressors bound to one enhancer do not affect the activity of a DNA element a few kilobases away.

Sequence-Specific Transcription Factors Can Be Either Activators or Repressors. The effect of transcription factor binding is influenced by other factors bound nearby. Indeed, transcription factors may even have opposite effects when in different environments and be converted (as with Dorsal) from transcriptional activators to transcriptional repressors. The binding of Dorsal to the *zen* enhancer relies on cooperative binding with two other sequence-specific transcription factors. In this particular context, the complex of bound proteins recruits the potent corepressor Groucho. In this way, *zen* transcription is repressed wherever Dorsal is in the nucleus, permitting *zen* transcription only in the dorsal epidermis. This example also illustrates the diversity of transcriptional repressing mechanisms. Unlike the local integration by local repression on the DNA discussed above, some repressors can act over a long range (many kb) by recruitment of a strong corepressor such as Groucho. Independent of activator binding at distant enhancer sites, such a strong repressor will prevent transcription (Mannervik et al., 1999).

In all these experiments on threshold responses in the fly embryo, the models developed can be subjected to rigorous evaluation not just for what principles might apply, but also for what mechanisms are actually used in the developing embryo. The requirements for specific DNA elements can be assessed by mutation, and the requirement for individual transcription factors can be tested by crossing transgenic reporter DNA constructs into a mutant background. In contrast, it has been considerably more difficult to elucidate the principles that apply to developmental regulation of transcription in vertebrate genes. Often, enhancers that regulate complex gene expression occupy many megabases of DNA (e.g., DiLeone et al., 1998), and it is far from easy to test large numbers of transgenes for their expression, let alone test them in a mutant background. Very few developmentally complex genes have been analyzed in vertebrates; instead, most work has sensibly focused on tissue-specific enhancers, which are often set up to stay active by positive feedback. The *hox* genes are an example where considerable progress has been made in delineating control elements that direct expression along the anteroposterior axis, but the understanding has been hampered by not knowing definitively what the primary signals in induction of expression are, and what the primary *cis*-acting response elements are (Lumsden

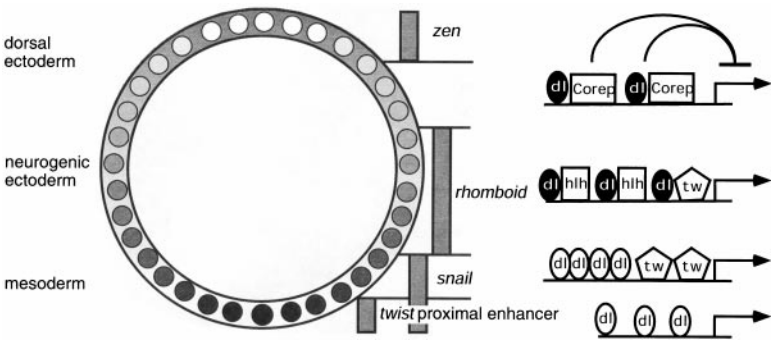

Figure 2. Multiple Thresholds of Gene Activity Responding to the Gradient of Nuclear Concentration of Dorsal

Cross-section through the blastoderm, illustrating the progressive nuclear concentration of Dorsal that results from signaling through the Toll receptor. Some of the various thresholds are illustrated (after Huang et al., 1997). Ventrally, the highest concentration of Dorsal is able to bind to weak Dorsal-binding sites (low-affinity Dorsal binding is illustrated in open ellipses [dl]). Peak levels are able to activate the *twist* proximal enhancer. At lower levels of Dorsal, cooperation between Dorsal binding to low-affinity sites and Twist binding (tw pentagons) activates *snail* expression throughout the mesoderm. Snail in turn binds to other enhancers, such as that of *rhomboid* (sites not illustrated) to exclude *rhomboid* expression from the mesoderm. High-affinity Dorsal-binding sites (closed ellipses [dl]) can act at intermediate nuclear concentrations of Dorsal, along with twist and ubiquitous basic helix-loop-helix activators (hlh squares) to promote expression of *rhomboid* in the neurogenic ectoderm. At the lowest levels of nuclear Dorsal, ubiquitous activators promote expression of *zen*. However, *zen* expression is kept off in all but the dorsal ectoderm by recruitment of Dorsal to high-affinity binding sites, where its normal activating property is reversed by cooperative interaction with sequence-specific corepressors (Corep in rectangles).

and Krumlauf, 1996; Duboule, 1998). Nonetheless, threshold responses of genes in vertebrate cells have been analyzed, though more with the tools of experimental embryology, and some of the same principles are emerging. In contrast to the syncytial fly embryo (but like the cellular blastoderm embryo), threshold activation of gene activity is measured in response to external signals.

Setting up Thresholds of Gene Activation in the Amphibian Embryo

In the amphibian, the principles that might govern threshold response have been studied by experimental embryology. Many such experiments rely on the excellent survival and differentiation of explants, or even of single cells. The demonstration that embryonic cells could respond to very small differences in growth factor concentration by differentiating into either mesoderm or epidermis was important (Green and Smith, 1990). This led to the dual challenges of determining how tiny differences in concentration could be measured by cells, and whether this mechanism is used in normal development.

It has become progressively clearer that the threshold response of tissue explants is not immediate but involves cell autonomous feedback as well as nonautonomous mechanisms. The initial response of cells is scalar, in response presumably to quantitative activation of cell surface receptors (Gurdon et al., 1998). An immediate-early set of genes responds, including transcriptional inhibitors like Goosecoid, which is induced by high levels of activin-like signals and subsequently downregulates the expression of other immediate-early genes such as *brachyury* (Latinkic and Smith, 1999). This kind of response can lead to multiple cell-autonomous thresholds of gene activity. However, just as important is the positive feedback, or maintenance effect, provided by secreted molecules such as FGF, which maintain the expression of *brachyury*. Another example of interacting and competing signaling pathways comes from the induction of *noggin*, which inhibits the ventralizing effects of BMPs and therefore reinforces dorsal fates (Harland and Gerhart, 1997). Since these are each secreted molecules, their effect is not cell autonomous, and therefore, the net effect can be integrated over a field of cells. These few examples illustrate that feedback can sharpen initially broad ranges of gene activation into more defined patterns.

Because the amphibian blastula has been so experimentally tractable, it has been tempting to conclude that the principles elucidated from experiments with added signals must also apply to normal development. However, evidence for graded activity of endogenous signals has been difficult to amass, and graded activity of activin-like factors in particular may not describe the initial pattern of expression of mesoderm-specific genes well. From experiments with graded addition of added activin, it is clear that a cellular response can be graded; however, many genes show initial territories of expression that are binary, either on or off (Harland and Gerhart, 1997). Here the combined input of two separate transduction pathways better explains the onset of gene expression. The dorsally restricted Wnt pathway is superimposed on an activin-like pathway in the dorsal domain of the embryo, and the two pathways converge at individual promoters like the *goosecoid* promoter to efficiently activate gene transcription in a narrow dorsal domain (Kimelman and Griffin, 1998; Moon and Kimelman, 1998). The more complex pattern of gene expression that emerges during gastrulation relies on secondary interactions. Such secondary signaling is mediated by members of the BMP, FGF, and Wnt families, as well as on the local production of their antagonists (Harland and Gerhart, 1997).

Diffusion and Distribution of Secreted Protein Morphogens

In considering the likely distribution of signals, and particularly graded signals, it has been difficult to relate how the range of activity of artificially added secreted proteins compares to the physiological range of action in normal embryos. Some experimental assays show that proteins can diffuse over long distances (McDowell and Gurdon, 1999), but these assays probably employ nonphysiological levels of signaling protein. Under normal physiological conditions, it might be equally likely that proteins only act over very short ranges. The challenge of manipulating normal gene expression experimentally has made it difficult to address the range of

physiological signaling in vertebrates. In contrast, some very compelling work has been done in the genetically tractable fly embryo, particularly in the imaginal disk and in the precursors of the abdomen (Strigini and Cohen, 1999). However, even here the physiologically effective concentrations of secreted proteins are well below the technical limits to detection, requiring that the presence of the signal be inferred from the physiological output. A compelling demonstration of diffusion of a physiological signal is in the *Drosophila* abdomen, where hedgehog signaling induces different cuticular patterns at different doses. The Hedgehog receptor, Patched, appears to limit the range of diffusion, since removal of *patched* in a clone of cells allows the range of *hedgehog* activity to expand. Although still an indirect proof, this is a solid piece of data taken to suggest that a ligand can diffuse over a distance. This is because it is difficult to come up with any mechanism other than one in which the presence of the Patched receptor binds to and limits the range of Hedgehog.

As the ligand diffuses away from its source, it appears to induce different fates. In the case of the imaginal disc, the different fates can be read out several hours after signaling by DPP as the induction of *spalt* at high doses, and *optomotor blind* at lower doses. In experiments using cell-autonomous activation of the DPP pathway with an activated receptor, these genes are expressed strictly cell autonomously, while the expression of the normal ligand induces the genes at a distance of up to 30 cells, a long-range effect (Strigini and Cohen, 1999).

While it has been a simple assumption that morphogens diffuse passively through a tissue (as may happen from an artificial source of a signaling protein), intercellular communication may take place by other means. For example, in the sea urchin embryo, thin, dynamic filopodia have been observed between cells at two of the major signaling regions of the embryo. The filopodia appear to mediate an inhibitory interaction between the primary and secondary mesenchyme cells and a patterning event between the skeletogenic mesenchyme and the ectoderm (Miller et al., 1995). These filopodia grow rapidly enough ($\sim$10 μm/min) and long enough to mediate intercellular interactions over the same distances and times as diffusion might. Recently, alternative mechanisms of active transport have also been invoked for some signals in *Drosophila*, and cellular processes (or cytonemes) that may extend over several cell diameters have been observed. These observations once again raise the possibility that presentation of morphogens might not rely on diffusion but could be due to direct contact between the presenting cell and the recipient cell (Pfeiffer and Vincent, 1999; Ramirez-Weber and Kornberg, 1999).

The Challenge of Integration. The presence of graded responses of genes to signals, combined with feedback loops, both positive and negative, allows for considerable specificity of gene response. Although there have been dramatic advances, the mechanistic details remain elusive. As detailed above, we do not know definitively what mechanism is used to pass a protein signal across a field of cells. In addition, the integration of responses has not been studied in detail, though the responses of cells reinforce and regulate the pattern. For example, a set of thresholds that is set up by a morphogen gradient is not fixed in space, since secondary interactions occur between cells to change relative growth rates. In the fly blastoderm, the dose of *bicoid* can be changed from one copy to six, with dramatic effects on the location of different thresholds, yet the whole embryo regulates the growth of different regions to produce a viable larva (St Johnston and Nüsslein-Volhard, 1992). Similarly, in experiments where pieces are cut out of the imaginal disc, confrontation between different parts of the imaginal disc results in regulation to produce all the elements that would normally be in between the remaining bits (Bryant and Fraser, 1988). Such secondary responses of cell proliferation and differentiation, which are crucial in refining pattern in the normal embryo, remain poorly understood. New tools will need to be developed to visualize the signaling process and the responses of genes to the signals as they take place. Understanding how these signaling systems work will therefore occupy embryologists for some time to come.

Signaling Centers and Embryonic Fields

It is striking that many developmental fields are organized from discrete signaling centers. This did not have to be so, as one can certainly imagine that a field could be organized by repeated local interactions, or by graded interactions over the whole field. However, animals are not designed: they have evolved by exploiting whatever mechanism works well. Signaling from boundaries or from discrete centers must therefore provide a robust mechanism for organization.

The Vertebrate Limb

The organization of the vertebrate limb provides an example of how classical embryological approaches have laid a groundwork of knowledge of signaling centers, then molecular biology built on the classics to provide more mechanistic explanations of formation of the limb axes.

Observations of the unusual shape of the chick limb bud, with its raised apical ectodermal ridge (AER), prompted microsurgical removal of the ridge. The ridge turned out to be crucial in promoting continued outgrowth and was therefore a source of factors that promote proximodistal growth. A second wave of molecular anatomical description of where individual signaling molecules were expressed revealed that FGFs are prominently expressed in the ridge and can substitute for its activity (Johnson and Tabin, 1997). This theme, of knowing where a signaling center is located from classical embryology, and then finding a gene expressed precisely in that location, has often been repeated.

One of the great experiments in experimental embryology, the finding that the posterior mesenchyme of the limb bud has organizing activity, was prompted by observations that it was a center of cell death. In a test of whether posterior mesenchyme was an instructive center of cell death, Saunders found instead that it was a center of organizing activity, able to induce a complete mirror image duplication of the limb when grafted to an anterior position (see Saunders, 1998). This posterior zone of polarizing activity (ZPA) was extensively studied and mapped, but the molecular mechanism remained elusive. Success came with the finding that implantation of a bead soaked in retinoic acid could mimic the activity

of the ZPA. The idea that retinoic acid was an endogenous morphogen, acting to instruct different fates at different concentrations as it diffused away from its source, became accepted. However, inadequacies in the model appeared, as it became clear that retinoic acid induced a new transplantable organizing center, which is difficult to explain by transplantation of cells that carry the diffusing retinoid. Perhaps the idea that retinoic acid is the ZPA molecule would not have crumbled so quickly had it not been for the emergence of a much better candidate. This candidate, *sonic hedgehog*, like so many crucial signaling molecules, was isolated using knowledge from the Wieschaus and Nüsslein-Volhard genetic screens in *Drosophila* and is one more illustration of the conservation of signaling mechanisms. As so often happens, vertebrate homologs of fly genes were cloned, and the expression was described; expression patterns can immediately suggest hypotheses as to the mechanism of action. In the case of *sonic hedgehog*, the pattern of expression in the midline suggested a role in patterning the neural tube and somite, while the expression in the ZPA suggested that Sonic hedgehog (SHH) was the important informational component of the ZPA. This prediction was confirmed both by addition experiments and by removal of the SHH signal (Johnson and Tabin, 1997; Vogt and Duboule, 1999).

Identification of Other Molecular Signaling Components. Similar tactics have paid off with other conserved signaling components, the Wnts, where *wnt7a* is locally expressed in the dorsal ectoderm and is important in dorsal–ventral patterning of the limb (Johnson and Tabin, 1997). With roles for Hedgehog, Wnt, and FGF families, there surely had to be a role for the TGFβ family. This family is well represented in expression patterns by various BMPs in the limb (Hogan, 1996). Indeed, BMPs were first isolated in attempts to understand signaling mechanisms in cartilage and bone formation. BMPs are expressed in various domains of the limb, suggesting possible modes of action for different family members. However, their activity appeared destructive rather than instructive, causing regression of the AER and arrest of distal outgrowth. Nevertheless, as long as their activity is regulated, in this case by BMP antagonists that are expressed in the mesenchyme just below the AER, the AER prospers and is able to maintain regulated outgrowth of the limb (Vogt and Duboule, 1999).

Not surprisingly, as more data is obtained, the puzzles deepen. For example, loss of SHH does not lead to a simple loss of anteroposterior polarity in the limb, but rather to an arrest of distal outgrowth. This finding shows that the simple view that the limb has the three independent axes, proximodistal, anteroposterior, and dorsal–ventral, which has been a convenient construct for the experimenter, may not have biological significance. Instead of developing with three tidily independent axes, the results show that interactions between the various signaling centers makes the maintenance of the AER depend on the maintenance of the ZPA. Thus, outgrowth and patterning requires a (somewhat) complete set of signaling centers as proposed by the complete circle rule of the polar coordinate model (French et al., 1976) or by the cooperation of compartments model of Meinhardt (1983). The challenge for the future will be to determine whether the nature and mechanism of the interactions are as proposed by these models and to ascertain whether the proposed coupling of the signaling centers is sufficient to explain the extent of limb outgrowth.

The mechanisms by which the final differentiated structures arise in the limb are quite obscure. Although processes such as the deposition of cartilage and bone can be mimicked by application of BMPs, there is no obvious prepattern of BMP mRNA expression that matches the initial cartilage condensations (Hogan, 1996). As with many developmental processes, we now have a superficially satisfying explanation for the pattern of the limb, but a deep ignorance remains of the mechanisms used for the execution of pattern formation. One can hope that further insights will be gained from mutations, but so far the relevant mutations have yielded surprisingly different insights. For example, a mutation that at first sight deletes the radius and ulna (the *Hoxa11, Hoxd11* double mutant) does not affect the initial condensation of cartilage but rather affects its rate of growth after condensation. This example illustrates the importance of knowing the immediate effects of signaling. The examination of structures long after the primary signaling event has occurred ignores the extent to which intermediate signaling and growth may affect the final outcome of deleting or altering the signaling (Johnson and Tabin, 1997; Vogt and Duboule, 1999).

The limb continues to provide a wealth of experimental opportunity, understanding how initially broad domains of gene activity are refined into signaling centers, and how those centers direct the formation of biological structure. The limb also provides a fascinating example in which developmental biology should provide insights into evolution, since the tetrapod limb has adapted to new environmental challenges in so many ways with so many shapes. While we assume that the detailed deployment of signaling molecules will explain these different patterns, we currently have no idea how the signals are regulated differently in different animals.

The Next Era of Experimental Embryology

As the tools of molecular biology and experimental embryology have been combined, the pace of progress in the field of developmental biology has exploded. It has become a field with sufficient breadth to fruitfully compare developmental mechanisms across taxa, and with sufficient depth to attack patterning questions at the tissue, cellular, and molecular levels. While the few vignettes offered above can only begin to illustrate some of the advances in the field and fall short of reviewing even a fraction of the recent progress, they can illustrate the power of modern experimental embryology. The best of the studies combine knowledge of the key events in the embryos and their fate maps with insights into the molecular biology of the system. Such a combination of experimental techniques can show the need for cell interactions, a means for the cells to recognize one another, an intracellular signaling cascade, and the *cis*-regulatory machinery that regulates the responsive genes. These successes highlight not only the significant progress but also several areas that require further attention if the experimental embryology of the next decades is to continue to accelerate.

A major goal of the field must be to refine techniques

for identifying more of the components involved in developmental patterning. While the focused molecular screens and the cloning of candidate factors have been fruitful thus far, the experimental embryology of the future will need to more fully embrace and become integrated with genetics. One need only compare the rate of progress in the field of insect developmental biology before and after the saturation screen of Wieschaus and Nüsslein-Volhard for early lethal mutations to appreciate the power of the genetic approach. This set of mutations helped to establish the hierarchy of interactions between cells, suggested new embryological experiments, and provided the baseline tools for an entire generation of investigations in all animals. Evidence of rapid advance in the nematode and the zebrafish because of the fruitful collaboration of genetics and experimental embryology offers similar evidence for the power of the approach. Because the forward genetic approach centers on phenotypes, the approach offers distinct advantages to the genomics approach that the sequencing effort now permits. Given this, it is heartening to see the engagement of several laboratories in genetic screens of species ranging from the mouse (Skarnes, 1999), the ascidian (Nakatani et al., 1999), and now even a frog species (Bronchain et al, 1999). These efforts require hard work before they bear fruit, but the insights gained have great implications for the entire community.

While the phenotype-driven aspect of forward genetics guarantees insights, one cannot ignore the successes of reverse genetics. Dominant-negative constructs have often yielded the first clues as to what molecules or mechanisms are used in normal development. Homologous recombination in the mouse has been used to make mutations that can confirm the importance of a gene in a developmental process or may yield a new and surprising insight. Also, there is the surprising methodology of RNAi in the nematode, in which even bathing animals in dsRNA can be sufficient to inactivate the cognate gene in their progeny. Although the mechanism of RNAi remains a mystery, its dramatic effects have even become a useful screening technique. These techniques not only yield important or unexpected insights, but also pose the challenge of understanding why so many highly conserved genes appear dispensable for apparently normal development.

The cis *Regulatory Domain as a Rosetta Stone*

Given that embryonic development requires the regional and temporal activation of specific genes, it should come as little surprise that the analysis of the control of gene expression has offered important insights. Because it is the *cis*-regulatory domain of developmentally regulated genes that must integrate a variety of inputs to control the timing and position of gene expression, it in many ways provides a "rosetta stone" linking the inputs and the outputs of developmental biology. Analyses of the transcriptional activation of genes in several different systems have revealed a modular organization, breaking the problem into two parts. First, how do the occupancy of regulatory sites and the posttranslational modification of the transcription factors activate or repress a given module of the *cis*-regulatory domain? Second, how are the states of these different modules integrated by the transcriptional apparatus? In yeast, a combination of tools including DNA chips is allowing detailed dissections of promoters (Cosma et al., 1999), offering hope for the needed assays in developing embryos. The key nature of these questions demands new analytical tools to determine the occupancy of *cis* regulatory sites in vivo, and to examine the interactions of proteins with their targets in developmental time.

Imaging of Cellular and Molecular Events

With few exceptions, research in the field relies upon comparing different specimens, fixed at different times, to reconstruct a likely time course of developmental and molecular events. In recent years, the refinement of laser scanning confocal microscopy and other advanced imaging tools has helped make key internal features in embryos visible. However, in only a small fraction of the systems under study have these techniques been used to follow events in living, developing embryos. The power of tools available for the manipulation of gene expression in the mouse embryo would be rendered far more productive if there were a means to follow the gene expressions and cellular events in the embryo over time. Embryo culture techniques have offered a window of a day or two into the developmental events, but given the prolonged times required for many events, it is clear that this is inadequate. Novel labeling techniques together with imaging tools such as microscopic MRI (Jacobs and Fraser, 1994; Jacobs et al., 1999) or ultrasound (Gaiano et al., 1999) might be best suited for noninvasive in utero imaging throughout development. Imaging tools that can obtain cellular or subcellular resolution in such demanding settings would increase both the quality and the significance of the findings.

Similarly, the spatial relationships of the many molecular species involved in any one developmental event are currently deduced by comparing the expression patterns between specimens collected at the same stage and processed individually. Time course data are obtained by comparing different specimens collected at different stages. Since the visualization methods are qualitative, key developmental episodes occur rapidly, and data from different specimens are difficult to align, such approaches are destined to give only a crude cartoon of the actual developmental events. A hindrance to quantitative analysis is that most of the data currently available comes from in situ hybridization. Given the posttranscriptional and posttranslational mechanisms for controlling the amount and activity of proteins, and their demonstrated modulation during some key developmental events, such approaches are not adequate. The advent of green fluorescent protein and other reporters offers an important adjunct to the usual approaches based on in situ hybridization. However, these will need to be extended to the optically difficult environment of the intact embryo. Perhaps new approaches based on "smart" MRI contrast agents that are responsive to reporter genes (Moats et al., 1997) will help resolve this challenge. No matter which technique is used, there will be two very significant challenges: sensitivity and quantitation. Many key signaling molecules are active at concentrations that are well below the detection limits of direct visualization techniques. The enzymatic amplification approach, which might increase the size of the signal to be imaged, can also make quantitation more challenging. Once these dual challenges are solved, the remaining challenge will be the tools to image

more than one gene product at a time and the computational tools to adequately align and compare imaging data.

Morphogenesis: The Next Frontier?

Perhaps no area of embryology is so poorly understood, yet so fascinating, as how the embryo develops form. Certainly the efforts in understanding gene regulation have occupied embryologists, and it has always been an assumption that once we understand what building blocks are made, we will be able to attack the question of how they are used. Mutations and gene manipulations have given insight into what components are employed for morphogenesis, but surely this is one example where we need to use dynamic imaging to assess how cells behave, and what components are interacting to drive cell movements and shape changes.

Analytical Tools to Interpret Complexity

As the sophistication of the data collection improves, so does the challenge of fully harvesting the fruits of these efforts. The results to date show a dizzying array of signaling systems acting within and between cells. Such networks both diverge, with a single effector controlling a number of downstream targets, and converge, with multiple inputs impinging on a single target. In such settings, intuition can be inadequate, often giving incomplete or incorrect predictions. For example, in a system with both activating and inhibiting interactions between neighboring cells, experimentally adding the activating substance might in some instances inhibit both cells (Meinhardt, 1993). Thus, any simple model that reduces a network of interacting factors to a linear set of players linked by arrows is destined to be incorrect. In the face of such complexity, computational tools must be employed as a tool for understanding. The purely theoretical attempts of a few years ago are now becoming increasingly constrained by data and may finally be gaining real utility to experimentalists. However, there is still a great distance to go. Beyond some simple attempts to show how signaling networks can be robust in the face of biological variation (Alon et al., 1999), computational models have only begun to embrace biological reality, including the small sizes and numbers of any given factor or receptor (McAdams and Arkin, 1999). The experimental embryology of the future deserves computational tools equal in sophistication to the molecular tools being used.

Of course, we cannot anticipate what technical breakthroughs will bring the next revolutions in developmental biology and make our current understanding seem naïve and quaint. But as Spemann (1938) concluded, "When progress is as rapid as it is at present in this domain of science, it is not too difficult to wait patiently until . . . step by step a firm footing may be gained".

Acknowledgments

The thinking represented in this review draws a great deal from the lectures, discussions, and arguments that surround the embryology course of the Marine Biological Laboratory, Woods Hole. The authors wish to thank, in addition to those in their home laboratories, the many faculty, students, and organizers of the course over the past decade for helping to shape the synthesis attempted here. While the credit for the concepts presented here should go to this diverse and interactive group, the authors must accept responsibility for any inaccuracies or misconceptions.

References

Alon, U., Surette, M.G., Barkai, N., and Leibler, S. (1999). Robustness in bacterial chemotaxis. Nature *397*, 168–171.

Anderson, K.V. (1998). Pinning down positional information: dorsal–ventral polarity in the *Drosophila* embryo. Cell *95*, 439–442.

Beddington, R.S., and Robertson, E.J. (1999). Axis development and early asymmetry in mammals. Cell *96*, 195–209.

Bergmann, D., and Wood, W.B. (1997). Establishing the handedness of left/right asymmetry in *C. elegans*. Dev. Biol. *186*, S56.

Birgbauer, E., and Fraser, S.E. (1994). Violation of cell lineage restriction compartments in the chick hindbrain. Development *120*, 1347–1356.

Boveri, T. (1904). Ergebnisse über die Konstitution der chromatischen Substanz des Zellkerns, (Jena, Germany: G. Fischer).

Boveri, T. (1907). Zellenstudien VI: Die Entwicklung dispermer Seeigeleier. Ein Beitrag zur Befruchtungslehre und zur Theorie des Kernes. Jena. Zeitschr. Naturw. *43*, 1–292.

Bronchain, O.J., Hartley, K.O., and Amaya, E. (1999). A gene trap approach in *Xenopus*. Curr. Biol. *9*, 1195–1198.

Bryant, P.J., and Fraser, S.E. (1988). Wound healing cell communication and DNA synthesis during imaginal disc regeneration in *Drosophila*. Dev. Biol. *127*, 197–208.

Burz, D.S., Rivera-Pomar, R., Jackle, H., and Hanes, S.D. (1998). Cooperative DNA-binding by Bicoid provides a mechanism for threshold-dependent gene activation in the *Drosophila* embryo. EMBO J. *17*, 5998–6009.

Conklin, E.G. (1905). The organization and cell lineage of the ascidian egg. J. Acad. Nat. Sci. *13*, 1–119.

Corbo, J.C., Levine, M., and Zeller, R.W. (1997). Characterization of a notochord-specific enhancer from the Brachyury promoter region of the ascidian, *Ciona intestinalis*. Development *124*, 589–602.

Cosma, M.P., Tanaka, T., and Nasmyth, K. (1999). Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle– and developmentally regulated promoter. Cell *97*, 299–311.

Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996). How is myogenesis initiated in the embryo? Trends Genet. *12*, 218–223.

Davidson, E.H., Cameron, R.A., and Ransick, A. (1998). Specification of cell fate in the sea urchin embryo: summary and some proposed mechanisms. Development *125*, 3269–3290.

Davis, R.L., Weintraub, H., and Lassar, A.B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell *51*, 987–1000.

DiLeone, R.J., Russell, L.B., and Kingsley, D.M. (1998). An extensive 3′ regulatory region controls expression of Bmp5 in specific anatomical structures of the mouse embryo. Genetics *148*, 401–408.

Duboule, D. (1998). Vertebrate hox gene regulation: clustering and/or colinearity? Curr. Opin. Genet. Dev. *8*, 514–518.

Erives, A., Corbo, J.C., and Levine, M. (1998). Lineage-specific regulation of the *Ciona* snail gene in the embryonic mesoderm and neuroectoderm. Dev. Biol. *194*, 213–225.

Fraser, S.E. (1992). *In vivo* analysis of cell lineage in vertebrate neurogenesis. Sem. Neurosci. *4*, 337–345.

French, V., Bryant, P.J., and Bryant, S.V. (1976). Pattern formation in epimorphic fields. Science *193*, 969–981.

Gaiano, N., Kohtz, J.D., Turnbull, D.H., and Fishel, G. (1999). A method for rapid gain-of-function studies in the mouse embryonic nervous system. Nat. Neurosci. *2*, 812–819.

Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Rowning, B., and Stewart, R. (1989). Cortical rotation of the *Xenopus* egg: consequences for the anteroposterior pattern of embryonic dorsal development. Development *107*, 37–51.

Gerhart, J., Doniach, T., and Stewart, R. (1991). Organizing the *Xenopus* organizer. In Gastrulation: Movements, Patterns, and Molecules, R.E. Keller, W.J. Clark, and F. Griffin, eds. (New York: Plenum Press), pp. 57–77.

Goldstein, B. (1993). Establishment of gut fate in the E lineage of *C. elegans*—the roles of lineage-dependent mechanisms and cell interactions. Development *118*, 1267–1277.

Green, J.B., and Smith, J.C. (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. Nature *347*, 391–394.

Gurdon, J.B., Dyson, S., and St Johnston, D. (1998). Cells' perception of position in a concentration gradient. Cell *95*, 159–162.

Hamburger, V. (1988). The Heritage of Experimental Embryology: Hans Spemann and the Organizer (New York: Oxford University Press).

Harland, R.M., and Gerhart, J.C. (1997). Formation and function of Spemann's organizer. Annu. Rev. Cell Dev. Biol. *13*, 611–667.

Herrmann, B.G., Labeit, S., Poustka, A., King, T.R., and Lehrach, H. (1990). Cloning of the T gene required in mesoderm formation in the mouse. Nature *343*, 617–622.

Hogan, B.L. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. *10*, 1580–1594.

Horstadius, S. (1939). The mechanics of sea urchin development studied by operative methods. Biol. Rev. *14*, 251–479.

Huang, A.M., Rusch, J., and Levine, M. (1997). An anteroposterior Dorsal gradient in the *Drosophila* embryo. Genes Dev. *11*, 1963–1973.

Jacobs, R.E., Ahrens, E.T., Meade, T.J., and Fraser, S.E. (1999). Looking deeper into vertebrate development. Trends Cell. Biol. *9*, 73–76.

Jacobs, R.E., and Fraser, S.E. (1994). Magnetic resonance microscopy of embryolic cell lineages and movements. Science *263*, 681–684.

Jiang, J., and Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the dorsal gradient morphogen. Cell *72*, 741–752.

Johnson, R.L., and Tabin, C.J. (1997). Molecular models for vertebrate limb development. Cell *90*, 979–990.

Joubin, K., and Stern, C.D. (1999). Molecular interactions continuously define the organizer during the cell movements of gastrulation. Cell *98*, 559–571.

Kawasaki, I., Shim, Y.H., Kirchner J., Wood, W.B., and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. Cell *94*, 635–645.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *Caenorhabditis elegans*. Dev. Biol. *87*, 286–300.

Kimelman, D., and Griffin, K.J. (1998). Mesoderm induction: a postmodern view. Cell *94*, 419–421.

Knoetgen, H., Viebahn, C., and Kessel, M. (1999). Head induction in the chick by primitive endoderm of mammalian, but not avian origin. Development *126*, 815–825.

Lane, M.C., and Smith, W.C. (1999). The origins of primitive blood in *Xenopus*: implications for axial patterning. Development *126*, 423–434.

Latinkic, B.V., and Smith, J.C. (1999). Goosecoid and mix.1 repress Brachyury expression and are required for head formation in *Xenopus*. Development *126*, 1769–1779.

Lin, R.L., Hill, R.J., and Priess, J.R. (1998). POP-1 and anterior–posterior fate decisions in *C. elegans* embryos. Cell *92*, 229–239.

Lumsden, A., and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. Science *274*, 1109–1115.

Mannervik, M., Nibu, Y., Zhang, H., and Levine, M. (1999). Transcriptional coregulators in development. Science *284*, 606–609.

Marcelle, C., Stark, M.R., and Bronner-Fraser, M. (1997). Coordinate actions of BMPs, Wnts, Shh and noggin mediate patterning of the dorsal somite. Development *124*, 3955–3963.

McAdams, H.H., and Arkin, A. (1999). It's a noisy business! Genetic regulation at the nanomolar scale. Trends Genet. *15*, 65–69.

McDowell, N., and Gurdon, J.B. (1999). Activin as a morphogen in *Xenopus* mesoderm induction. Semin. Cell Dev. Biol. *10*, 311–317.

Meinhardt, H. (1983). A boundary model for pattern-formation in vetebrate limbs. J. Embyol. Exp. Morphol. *76*, 115–137.

Meinhardt, H (1993). A model for pattern-formation of hypostome, tentacles, and foot in hydra—how to form structures close to each other, how to form them at a distance. Dev. Biol. *157*, 321–333.

Miller, J., Fraser, S.E., and McClay, D. (1995). Dynamics of thin fliopodia during sea urchin gastrulation. Development *121*, 2501–2511.

Moats, R.A., Fraser, S.E., and Meade, T.J. (1997). A "smart" magnetic resonance imaging agent that reports on specific enzymatic activity. Angew Chem. Int. Edit. *36*, 726–728.

Moon, R.T., and Kimelman, D. (1998). From cortical rotation to organizer gene expression: toward a molecular explanation of axis specification in *Xenopus*. Bioessays *20*, 536–545.

Moskowitz, I.P.G., and Rothman, J.H. (1996). lin-12 and glp-1 are required zygotically for early embryonic cellular interactions and are regulated by maternal GLP-1 signaling in *Caenorhabditis elegans*. Development *122*, 4105–4117.

Nakatani, Y., Moody, R., and Smith, W.C. (1999). Mutations affecting tail and notochord development in the ascidian *Ciona savignyi*. Development *126*, 3293–3301.

Nishida, H. (1997). Cell lineage and timing of fate restriction, determination and gene expression in ascidian embryos. Semin. Cell Dev. Biol. *8*, 359–365.

Nusse, R., van Ooyen, A., Cox, D., Fung, Y.K., and Varmus, H. (1984). Mode of proviral activation of a putative mammary oncogene (int-1) on mouse chromosome 15. Nature *307*, 131–136.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. Nature *287*, 795–801.

Pfeiffer, S., and Vincent, J.P. (1999). Signalling at a distance: transport of Wingless in the embryonic epidermis of *Drosophila*. Semin. Cell Dev. Biol. *10*, 303–309.

Piccolo, S., Agius, E., Leyns, L., Bhattacharya, S., Grunz, H., Bouwmeister, T., and DeRobertis, E. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature *397*, 707–710.

Ramirez-Weber, F.A., and Kornberg, T.B. (1999). Cytonemes: cellular processes that project to the principal signaling center in *Drosophila* imaginal discs. Cell *97*, 599–607.

Rusch, J., and Levine, M. (1996). Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. Curr. Opin. Genet. Dev. *6*, 416–423.

Satoh, N. (1994). Developmental Biology of Ascidians. (New York: Cambridge Univ. Press).

Saunders, J.W. (1998). Apical ectodermal ridge in retrospect. J. Exp. Zool. *282*, 669–676.

Schnabel, R., Hutter, H., Moerman D., Schnabel, H. (1997). Assessing normal embryogenesis in *Caenorhabditis elegans* using a 4D microscope: variability of development and regional specification. Dev. Biol. *184*, 234–265.

Scott, M.P. Development: the natural history of genes. Cell *100*, 27–40.

Skarnes, W.C. (1999). A resource of mouse mutations in secreted and membrane proteins. Dev. Biol. *210*, 206–206.

Slack, J.M.W. (1998). Egg and Ego: An Almost True Story of Life in the Biology Lab (Berlin: Springer Verlag).

Spemann, H. (1938). Embryonic Development and Induction (New Haven: Yale University Press).

St Johnston, D., and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. Cell *68*, 201–219.

Sternberg, P.W., and Han, M. (1998). Genetics of RAS signaling in *C. elegans*. Trends Genet. *14*, 466–472.

Strigini, M., and Cohen, S.M. (1999). Formation of morphogen gradients in the *Drosophila* wing. Semin. Cell Dev. Biol. *10*, 335–344.

Sulston, J.E., and White, J.G (1980). Regulation and cell autonomy during postembryonic development of *Caenorhabditis elegans*. Dev. Biol *78*, 577–597.

Thomas, J.H., Stern, M.J., and Horvitz, H.R (1990). Cell interactions coordinate the development of the Caenorhabditis elegans egg-laying system. Cell *62*, 1041–1052.

Thorpe, C.J., Schlesinger, A., Carter, J.C., and Bowerman, B. (1997). Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm. Cell *90*, 695–705.

Vogt, T.F., and Duboule, D. (1999). Antagonists go out on a limb. Cell *99*, 563-566.

Whittaker, J.R. (1987). Cell lineages and determinants of cell fate in development. Am. Zool. *27*, 607–622.

Wilson, P.A., and Hemmati-Brivanlou, A. (1997). Vertebrate neural induction: inducers, inhibitors, and a new synthesis. Neuron *18*, 699–710.

Witkowski, J. (1985). The hunting of the organizer: an episode in biochemical embryology. Trends Biochem. Sci. *10*, 379–381.

Woo, K., and Fraser, S.E. (1997). Specification of the zebrafish nervous system by nonaxial signals. Science *277*, 254–257.

Wood, W.B. (1988). The Nematode *Caenohabditis elegans*. (Cold Spring Harbor, N.Y.: Cold Spring Harbor Press).

Zhang, J., Houston, D.W., King, M.L., Payne, C., Wylie, C., and Heasman, J. (1998). The role of maternal VegT in establishing the primary germ layers in *Xenopus* embryos. Cell *94*, 515–524.