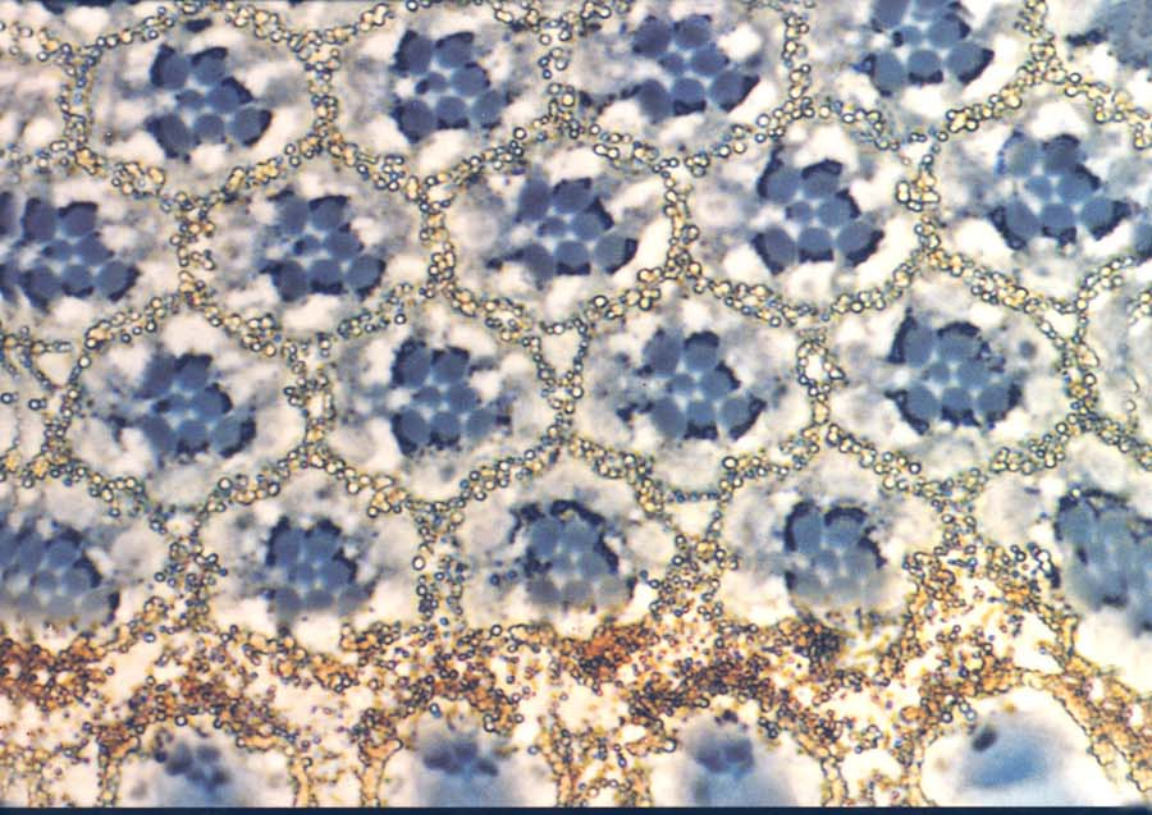

Current Topics in Developmental Biology

Volume 39

Edited by

Roger A. Pedersen

Gerald P. Schatten

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Cover photograph: Phase contrast photomicrograph of tangential sections through adult wild-type retina. See Chapter 4 by Treisman and Heberlein for more details.

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Preface

This volume continues the custom of this series in addressing developmental mechanisms in a variety of experimental systems. The conceptual sequence of topics begins with "The Murine Allantois," a contribution from Karen M. Downs at the University of Wisconsin that considers this understudied structure and its importance in both fundamental developmental biology and clinical fetal therapy. Richard L. Gardner from the University of Oxford highlights the newest discoveries on axis specification during mammalian development in his chapter "Axial Relationships between Egg and Embryo in the Mouse." In the "Maternal Control of Pattern Formation in Early *Caenorhabditis elegans* Embryos," Bruce Bowerman of the University of Oregon describes the vital importance of maternal gene families in normal and abnormal development. Jessica E. Treisman of the New York University Medical Center and Ulrike Heberlein from the University of California, San Francisco, report on a fascinating topic in "Eye Development in *Drosophilia*: Formation of the Eye Field and Control of Differentiation." William J. Moody of the University of Washington contributes the chapter "The Development of Voltage-Gated Ion Channels and Its Relation to Activity-Dependent Developmental Events." Santosh R. D'Mello from the University of Connecticut considers a topic of great fundamental, as well as clinical, importance in "Molecular Regulation of Neuronal Apoptosis." Chapter 7 by J. Parrington and K. Swann from University College in London and F. A. Lai from the MRC National Institute for Medical Research in London, titled "A Novel Protein for Ca^{2+} Signaling at Fertilization," considers the still controversial field of precise molecular signals leading to the ionic cascade during egg activation. This volume concludes with "The Development of the Kidney" by Jamie A. Davies and Jonathan B. L. Bard from Edinburgh University, a chapter underscoring the virtues of fundamental research for solving human disorders.

Together with the others in this series, this volume provides a comprehensive survey of major issues at the forefront of modern developmental biology. These chapters should be valuable to researchers in the fields of mammalian and nonmammalian development, as well as to students and other professionals who want an introduction to current topics in cellular, molecular, and genetic approaches to developmental biology and neurobiology. This volume in particular will be essential reading for anyone interested in fetal development; axis specification; pattern formation; apoptosis; visual, neuronal, and renal development; and ion channels.

This volume has benefited from the ongoing cooperation of a team of participants who are jointly responsible for the content and quality of its material. The authors deserve the full credit for their success in covering their subjects in depth, yet with clarity, and for challenging the reader to think about these topics in new ways. We thank the members of the Editorial Board for their suggestions of topics and authors. We thank Liana Hartanto and Michelle Emme for their exemplary administrative and editorial support. We are also grateful to the scientists who prepared chapters for this volume and to their funding agencies for supporting their research.

Gerald P. Schatten
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1

The Murine Allantois

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I. Introduction

The focus of this chapter is the development of the mouse allantois. The murine allantois is the future umbilical component of the chorioallantoic placenta. Its primary function is to fuse with the chorion and vascularize, thereby serving as the vital connection between mother and fetus for the interchange of nutrients, gases, and metabolic wastes during much of mammalian gestation. Whereas much effort has been devoted to investigating the role of the mature placenta, studies of the development of its component structures, the chorion and the allantois, have been minimal.

Until now, there have been no reviews on the murine allantois, probably because few studies have focused on it. This may account for the fact that even where there has been modest opportunity to increase understanding of allantoic growth and morphogenesis through gene expression studies or examination of transgenic mice mutant in vasculogenesis, the allantois has been treated superficially or ignored altogether. There has been little context for gene expression

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and transgenesis, the significance of which can only be appreciated in any developing system if the fate and state of differentiation of its component cells are known.

The aim of this review is to synthesize what is known about early development of the murine allantois. Topics to be addressed include the embryonic origin of the allantois, how the allantois acquires its unusual shape, and the mechanism of chorioallantoic fusion, which establishes the early placenta. A model is proposed for how the murine allantois may employ its unique morphology to undergo vasculogenesis. Whether the allantois plays a role in the formation of the future germ line will also be discussed. The chapter will conclude with the author's long-term view that the allantois, with its direct connection to fetal circulation, may be manipulated to deliver therapeutic factors to certain developmentally compromised fetuses.

II. Fetal Membranes: Overview

In the development of any vertebrate embryo, only part of the egg's cleavage cell mass will form the actual embryo; the other part will elaborate the extraembryonic structures, referred to especially in mammals as fetal membranes. The set of fetal membranes comprises the yolk sac, amnion, chorion, and allantois. Fetal membranes eventually are shed at birth by natural methods and discarded.

The yolk sac is the most variable fetal membrane of animals (Hamilton *et al.*, 1947). The mammalian yolk sac, although not functional in the sense of storing yolk, is so similar in many details to the reptilian yolk sac as to be homologous (Snell and Stevens, 1966). In mammals, the yolk sac plays a significant role in the uptake and transfer of nutrients to the embryo by way of the exocoelomic cavity (Wislocki *et al.*, 1946). Combined with the observation that the yolk sac vascularizes very early and engages in erythropoiesis, the yolk sac functionally is an organ of nutrition and gas exchange and is considered a proper placenta. In most marsupials it is the only placenta (Pijnenborg *et al.*, 1981). The yolk sac is also thought to seed the fetus with hematopoietic stem cells [reviewed in Morrison *et al.* (1995)]. In humans, yolk sac function diminishes very early, but the yolk sac itself persists through the remainder of gestation as a rudimentary organ, a constituent of the mature umbilical cord (Boe, 1951; see the following). In contrast, in small rodents, the yolk sac is an important nutritive organ during the full period of gestation (Everett, 1935), although the chorioallantoic placenta supersedes it in physiological importance.

The chorioallantoic placenta is formed by the union of two initially well-separated structures, the chorion and the allantois. The most significant feature of the allantois is that it vascularizes. Development of the allantois varies between animal species (Panigel, 1993). The allantois was evolved by reptiles and birds as a temporary sac for the storage of urinary waste [Arey, 1965; a comparative

anatomy of the allantois has been reviewed in Steven and Morriss (1975)]. In these animals, fusion of the outer wall of the allantoic sac with the chorion produces a structure in direct contact with the porous shell. The allantois develops a vasculature, and oxygenation takes place in the vascularized wall of the sac. The allantoic sac continues to serve as a reservoir for kidney excreta, and part of the allantoic wall assists in the absorption of egg albumen.

Monotremes [egg-laying mammals such as the echidna (or spiny anteater) and platypus] possess an allantoic sac whose function is comparable to that of reptiles and birds. In most marsupials, the allantois fails to grow far enough to reach the chorion, which is found in most marsupials and consists of ectodern and avascular somatic mesoderm. Nitrogenous waste therefore is retained in the allantois until parturition (Morriss, 1975). Only one marsupial (*Perameles* or bandicoot) possesses a vascular chorioallantoic unit, which is apposed to the uterine mucosa (Panigel, 1993). In this species, the chorioallantois is important in hematrophic nutrition of the fetus (Morriss, 1975).

In humans, the allantois consists of endoderm and mesoderm and is the scaffolding upon which the mature umbilical cord is formed. The mature human umbilical cord is a composite structure, formed when the allantois-derived connecting stalk and vitelline duct of the yolk sac are bound together by the expanding amnion (Larsen, 1993). An umbilical vein and two arteries develop within the connecting stalk and vascularize the chorionic disk. In mice, the allantois is wholly mesodermal (Snell and Stevens, 1966); allantoic mesoderm differentiates into a major artery and vein and surrounding connective tissue. This simple outcropping of the anteroposterior axis (see the following) becomes the umbilical component of the murine chorioallantoic placenta.

III. Development of the Exocoelomic Cavity

Development of the murine allantois is intimately associated with the development of the exocoelomic cavity, or exocoelom, which contains the yolk sac, amnion, chorion, and allantois, all of whose correct morphogenesis and function are essential for fetal health, survival, and development during the second half of gestation [Fig. 1; see Jolly and Férester-Tadié (1936) and Bonnevie (1950) for meticulously detailed classical papers on the development of the murine exocoelom]. The yolk sac is the first site of hematopoiesis [see Haar and Ackerman (1971) for a detailed morphological description of the early development of the yolk sac], and the amnion serves a protective function and permits the fetus to move freely, thus aiding in growth and development (Moore, 1982). The chorion and the allantois together comprise the major components of the placenta.

The exocoelom develops during gastrulation, the time during which the embryonic epiblast lays down the basic body plan of the fetus [for a classic review, see Beddington (1983); Lawson *et al.*, 1991; Lawson and Pedersen, 1992]. At the

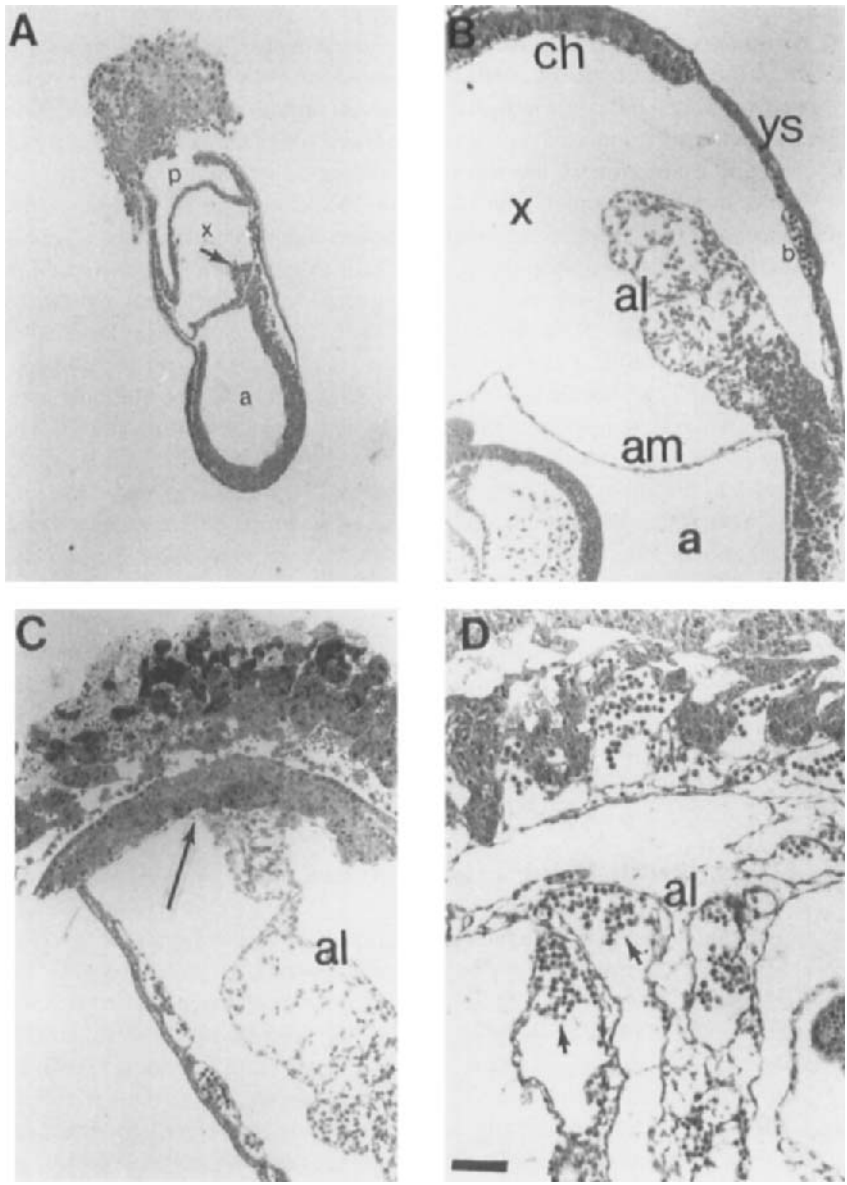

Fig. 1 Allantoic development. (A–D) Histological sections of PO conceptuses (Kelly and Rossant, 1976; Downs and Gardner, 1995) were prepared for visualization of the four phases of allantoic development. (A) Formation of the allantoic bud. Neural plate stage (7.25–7.5 dpc). The arrow indicates the allantoic bud. (B) Growth of the allantois. Early somite stage (8.0–8.25 dpc). (C) Chorioallantoic fusion (8.5 dpc), seven somite stage. The arrow points to the chorioallantoic fusion junction. (D) Vascularization of the allantois. Conceptus (9.5 dpc) showing overt vascularization of

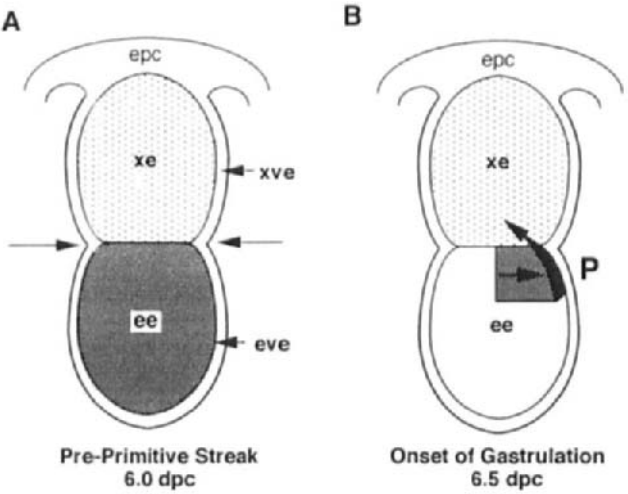

Fig. 2 Schematic diagram of the mouse egg cylinder just before and after the onset of gastrulation. The mouse egg cylinder is divided into two major regions just before (A) and after (B) the onset of gastrulation. In A, the unlabeled arrows separate extraembryonic ectoderm (xe) from embryonic ectoderm (epiblast or ee). Both the extraembryonic and embryonic ectoderms are surrounded by a single layer of visceral endoderm: xve, extraembryonic visceral endoderm; eve, embryonic visceral endoderm. Other abbreviations: epc, ectoplacental cone. In B, gastrulation has begun as indicated by formation of the primitive streak (solid black area in the embryonic portion of the egg cylinder) adjacent to embryonic visceral endoderm. The appearance of the streak indicates the posterior (P) region of the future fetus. The proximal epiblast indicated just below the embryonic–extraembryonic junction is ingressing (horizontal arrow) into the posterior primitive streak and emerging from the latter (arrow going into the extraembryonic region) as extraembryonic mesoderm.

onset of gastrulation, approximately 6.5 days postcoitum (dpc), the conceptus consists of two major areas, the extraembryonic and embryonic regions [Fig. 2A; described in Snell and Stevens (1966); Hogan *et al.*, 1994; also, a morphological staging system of gastrulation is found in Downs and Davies (1993)]. The extraembryonic region encompasses multiple tissue types, but the two important to the formation of the exocoelom are the ectoderm of the future chorion and the endoderm of the future yolk sac. The embryonic region primarily consists of a pluripotent epithelium called the "primitive ectoderm" or "epiblast," which is surrounded by a layer of (extraembryonic) visceral endoderm (Fig. 2A).

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the umbilical component of the chorioallantoic placenta. Arrowheads indicate red blood cells. Abbreviations: a, amniotic cavity; al, allantois; am, amnion; b, yolk sac blood island; ch, chorion; p, ectoplacental cavity; ys, yolk sac; x, exocoelomic cavity. Scale bar: 100 μm (A, B); 50 μm (C); 68 μm (D). Methods: (A and D) Bouin's-fixed, paraffin-embedded conceptuses were sectioned at 7 μm and stained in hematoxylin and eosin. (B and C) Glutaraldehyde-fixed, araldite-embedded conceptuses were sectioned at 1 μm and stained in toluidine blue.

The key feature of gastrulation is diversification of the epiblast. The epiblast alone is responsible for laying down all of the tissues of the definitive mouse embryo (Gardner and Rossant, 1979; Gardner *et al.*, 1985). In addition, the epiblast forms the extraembryonic mesoderm and all of the amnion. The general sequence of epiblast diversification is that it lays down first the primitive streak (Fig. 2B; Bonnevie, 1950), second the extraembryonic mesoderm (Lawson *et al.*, 1991), and third the three embryonic germ layers, ectoderm, mesoderm, and endoderm, as well as the ectoderm of the amnion (Lawson *et al.*, 1991; Lawson and Pedersen, 1992; Lawson and Hage, 1994). The germ layers subsequently will interact according to their level relative to the streak and set off the cascade of events necessary for the formation of all of the definitive organs.

Although the details are far from clear, mesoderm is formed by the ingression of epiblast into the primitive streak (Beddington, 1981, 1982; Copp *et al.*, 1986; Tam and Beddington, 1987; Lawson *et al.*, 1991; Lawson and Pedersen, 1992). As gastrulation proceeds, the primitive streak lengthens anteriorly, and extraembryonic, lateral plate, paraxial, and axial mesoderms are specified by the ingression of epiblast cells into progressively more anterior levels of the streak (Tam and Beddington, 1987).

Germane to this review is extraembryonic mesoderm. Extraembryonic mesoderm is formed as a result of ingression into the posterior primitive streak by proximal embryonic epiblast, which is that epiblast situated just below the embryonic–extraembryonic junction of the egg cylinder [Fig. 2B; Lawson *et al.*, 1991; reviewed in Boucher and Pedersen (1996)]. Extraembryonic mesodermal cells emerge from the streak as a bulge overlying and continuous with it. Anteriorly, a smaller bulge of extraembryonic mesoderm forms. Cavitation within these masses of extraembryonic mesoderm results in the formation of two amniotic folds that fuse, thereby forming the exocoelom. This results in the partitioning of the egg cylinder into distinct regions dominated by three cavities: amniotic, exocoelomic, and ectoplacental (Beddington, 1983).

In the exocoelom, extraembryonic mesoderm complexes with extraembryonic ectoderm to form the chorion and with extraembryonic endoderm to form the yolk sac. Eventually the amnion will appear; it consists of ectoderm and mesoderm and will comprise the floor of the exocoelom. The allantois will form at the angle between the yolk sac and amnion. It consists wholly of extraembryonic mesoderm.

The sequence of events by which extraembryonic mesoderm contributes to the components of the exocoelom has not been described. However, results of fate mapping of the epiblast and the posterior primitive streak at the onset of gastrulation and through the three-somite stage (approximately 8.25 dpc) have tentatively suggested that the extraembryonic mesoderm may contribute to exocoelomic structures in three successive waves. The first wave occurs at the onset of gastrulation; the first extraembryonic mesoderm contributes to all four components of the exocoelom. This is quite clear, because descendants of labeled proximal

epiblast cells were found in the mesoderm of the chorion, yolk sac, amnion, and allantois at the experimental endpoint of fate-mapping experiments (Lawson *et al.*, 1991). In the second wave (mid-to-late streak stages; Downs and Davies, 1993; K. Downs, preliminary observations), extraembryonic mesoderm arising from the posterior streak may contribute only to the allantois and amnion. In the third wave (neural plate and headfold stages; Beddington, 1982; Copp *et al.*, 1986; Tam and Beddington, 1987), the majority of extraembryonic mesodermal cells emerging from the posterior streak were found largely in the allantois, although a few grafted cells were also identified in yolk sac mesothelium (Tam and Beddington, 1987). Colonization of the yolk sac at this time may be explained by contamination with the donor graft during transplantation (Downs and Harmann, 1997).

Once the addition of extraembryonic mesoderm to the allantois ceases by three somite pairs (Tam and Beddington, 1987; Downs and Gardner, 1995), formation of the exocoelom is complete. This putative sequence of mesoderm deposition into components of the exocoelomic cavity may be critical for elucidating the mode of action of the *Brachyury* mutation of the allantois (discussed in Section V.B).

IV. Development of the Allantois

Little is known about allantoic development, but allantoic morphology and behavior suggest a convenient quadraphasic sequence of events: (A) formation of the allantoic bud (Fig. 1A), (B) allantoic growth (Fig. 1B), (C) chorioallantoic fusion (Fig. 1C), and (D) overt vascularization of the allantois (Fig. 1D).

A. Formation of the Allantoic Bud

The timing of appearance of the allantoic bud has been reported in two mouse strains. In the first, the allantoic bud is visible shortly after cavitation of extraembryonic mesoderm [late streak stage, approximately 7.0 dpc, F_2 generation of (C57BL/6 $\times$ CBA) hybrid intercrosses; Kaufman, 1992]. In the second strain, the bud is apparent after fusion of the proamniotic folds and formation of the amnion (Fig. 1A; early neural plate stage, approximately 7.25 dpc, closed-bred Swiss-derived albino; Beddington, 1983; Downs and Davies, 1993). Despite these slight strain-dependent differences in timing of appearance, the cellular composition of the allantois is not thought to differ between strains.

The allantoic bud lies suprajacent to and is continuous with the posterior primitive streak. The outer cells of the bud will differentiate into a layer of mesothelium that surrounds an inner core of extraembryonic mesoderm. In the rat, the apical surface of the mesothelial layer contains no modifications, but the

basal membrane of these cells bears small processes that project into the subjacent extracellular space (Ellington, 1985).

How the allantoic bud is formed is not known, but one possibility is that it does so through a decline in the rate of proliferation of the first wave of extraembryonic mesoderm once it has become complexed with extraembryonic ectoderm and endoderm. This would result in the production of excess mesoderm by continued ingression of proximal epiblast into the posterior primitive streak. That surplus extraembryonic mesoderm becomes the allantois.

B. Growth of the Allantois

The allantois appears to enlarge by the addition of mesoderm from the posterior streak until the three somite stage (Tam and Beddington, 1987). After this time, a combination of mitosis and distal cavitation may be responsible for its continued growth in the exocoelom (Ellington, 1985).

During growth, the allantois is cylindrical, approximately 0.5 mm long, 0.18 mm wide at the base, and tapering to 0.08 mm wide near the tip (Tamarin and Boyde, 1976). Nevertheless, some variation is observed between conceptuses at similar developmental stages (Downs and Gardner, 1995). Also during growth, the base of the allantois becomes hollowed out, and the inner surface consists of a discontinuous pavement of cells containing multiple processes (Tamarin and Boyde, 1976). The significance of these morphological features is not known.

The outer mesothelium is most conspicuous during the allantoic growth phase; it is continuous with the mesodermal layer of the yolk sac, chorion, and amnion. In the rat, the mesothelial layer acquires desmosomes and exhibits bulbous protrusions whose significance is unclear. Distally, these protrusions may be involved in chorioallantoic fusion (Ellington, 1987), and proximally, they resemble the blood islands of the yolk sac (Ellington, 1985; Tamarin and Boyde, 1976).

How the allantoic mesothelial cells become morphologically distinct from underlying core cells is unknown, but it may be by virtue of their external position, similar to the positional differentiation of trophectoderm cells in the morula (Tarkowski and Wroblewska, 1967; Barlow *et al.*, 1972). Electron micrograph studies do not report a basement membrane subjacent to the mesothelial cells (Ellington, 1985), which suggests that sustained growth of the mesothelium during the growth phase may be due to intercalation of cells from the allantoic core rather than self-propagation of the outer mesothelial cell layer.

During growth, distal inner core allantoic mesoderm becomes loosely organized with the distance between cells increasing, with some cell contacts but no specialized junctions (Ellington, 1985). Cells in the proximal allantois remain densely packed and always continuous with the posterior streak. The line of demarcation between the base of the allantois and the subjacent primitive streak

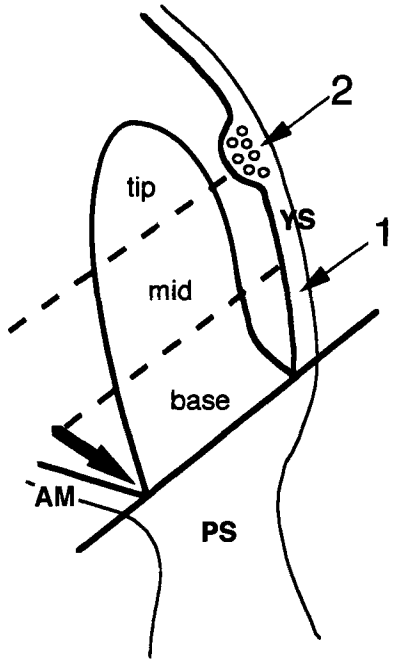


Fig. 3 Schematic diagram of the headfold-stage allantois. The arrow indicates the insertion of the amnion (AM) used to delineate the base of the allantois from the subjacent primitive streak (PS; Oździenski, 1967). The bold line surrounding the allantois, which is continuous with the yolk sac, amnion, and chorion (not shown), represents the mesodermal lining of the exocoelom. The headfold-stage allantois was subdivided (dashed lines) into three major regions, base, midregion, and tip, for studies of allantoic potency (Downs and Harmann, 1997). Other abbreviations: YS, yolk sac.

is typically taken as the level of the amnionic extension from the base of the allantois (Oździenski, 1967; Fig. 3).

A few gene expression studies have revealed that some biochemical differences may be found between the base and the distal two-thirds of the allantois. For example, *Brachyury* is expressed in the base of the allantois in the headfold and early somite stages (Clements *et al.*, 1996), whereas CD44, the major receptor of hyaluronan (Aruffo *et al.*, 1990), appears everywhere but in the core of the base at this time (Fig. 4). *VCAM1*, required for chorioallantoic fusion (Yang *et al.*, 1995), is expressed in the distal two-thirds of the allantois as early as what appears to be the four-somite stage (Kwee *et al.*, 1995). There is also a small population of alkaline phosphatase-positive cells in the base of the allantois, which may contribute to somatic lineages (e.g., the mature allantois), the future germ line, or both (Oździenski, 1967; discussed in Section V.C).

Addition of extraembryonic mesoderm to the allantois ceases by three somite pairs. This was demonstrated in experiments where the posterior primitive streak

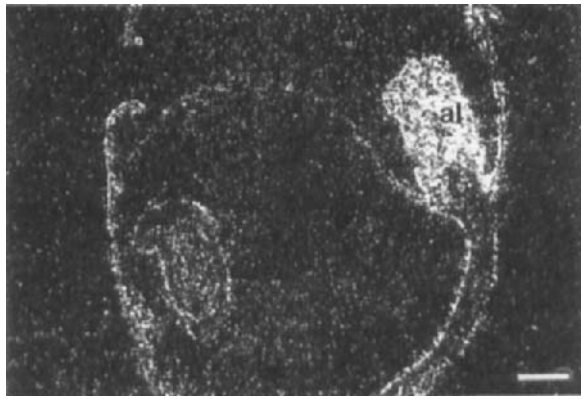


Fig. 4 Expression of CD44 mRNA in the allantois of an early somite stage conceptus. Histological section showing mRNA hybridization *in situ* of an early somite-stage conceptus to CD44 cDNA. Arrow indicates the allantois. Note that the base of the allantois exhibits relatively little, if any, positive signal for CD44, whereas the distal allantois is strongly positive. Scale bar: 100 μm. Methods: Hybridization was originally described in Downs (1992). A CD44 cDNA probe (gift of Dr. E. Shtivelman, Systemix, Palo Alto, CA) was restriction-digested with appropriate restriction enzymes, gel-purified, and random-primed with [^{33}P]-dATP to specific activity of 10^8–10^9 cpm following the instructions in the Boehringer-Mannheim Random-Primed DNA Labelling Kit ([^{33}P]-dATP, sp. act. 3000 Ci/mmol, NEN Dupont, UK). Paraffin sections (6 μm) of mouse embryos in early somite stages were prepared as described by Wilkinson and Green (1990) with the following exceptions: (1) Histoclear (National Diagnostics, Manville, NJ) was used to clear the tissue instead of toluene (less toxicity) before embedding. (2) 0.85% NaCl was eliminated from the dehydration ethanols and the several rinse steps. Sections were hybridized for 3 days at 42°C under clean cover slips at a probe concentration of 0.5 ng/μl and washed as previously described (Downs *et al.*, 1989). The slides were then dehydrated as described, coated once with NTB-2 emulsion (diluted 5 parts emulsion to 7 parts 1% glycerol), and left to dry vertically in air for 1 hr. Slides were then exposed for 8–20 days in sealed, light-tight boxes in the presence of desiccant at 4°C and brought to room temperature for development. The tissue was stained in Mayer's hematoxylin alone or counterstained in eosin (and cover-slipped in DPX mounting medium). All photographs were taken on a Zeiss Axioplan and processed for identical exposure times. The control probe was α-fetoprotein and was applied to alternating serial sections as previously described (Downs *et al.*, 1989).

was orthotopically transplanted at the late neural plate–early headfold stage and the fate of donor cells followed. No contribution of grafted cells was observed in the allantois beyond three somite pairs (Tam and Beddington, 1987). Also, in experiments where the allantois had been removed, a new allantois regenerated and fused with the chorion before, but not after, this developmental time (Downs and Gardner, 1995).

The rate of mitosis within the rodent allantois declines steadily until fusion (Ellington, 1985). There does not appear to be a proliferating center within the allantois itself, although the fine region-specific mapping required to find such a center has not been carried out.

C. Chorioallantoic Fusion

By the 6–7 somite stage (approximately 8.25 dpc), the allantois meets the chorion and attaches to it (Theiler, 1989; Downs and Gardner, 1995). This first step in placental morphogenesis is mediated by the mesothelial surfaces of the allantois and chorion (Ellington, 1985; Downs and Gardner, 1995). Fusion occurs shortly thereafter. The vital link between fetus and mother has thus been forged.

The events that take place during fusion are far from clear. In most rat embryos examined, the tip of the allantois settles into a depression in the chorion (Ellington, 1985). This has also been described in less detail in the mouse (Tamarin and Boyde, 1976). However, in a few rat embryos, the allantois first comes into contact with the posterior peripheral region of the chorion and then spreads along the surface of the chorion until contact and fusion are made in the central region of the chorion (Ellington, 1985). In the central chorionic depression, the cells are rounded up. This morphological feature may be of important functional significance, as chorioallantoic fusion has never been observed in areas of flattened chorionic mesoderm (Ellington, 1987).

What happens subsequent to chorioallantoic fusion is not clear. Attachment of the allantois to the chorion does not appear to be required for the formation of allantoic blood vessels or the infiltration of red blood cells into the allantois (Downs and Gardner, 1995). However, secretion of hepatocyte growth factor–scatter factor by the allantois subsequent to fusion with the chorion may stimulate the growth of trophoblastic cells in the labyrinth region of the chorionic disk (Uehara *et al.*, 1995).

D. Vasculogenesis in the Allantois

The mature murine umbilical cord consists of a single artery and vein. The allantois is not overtly vascularized until some time after chorioallantoic fusion, although it appears that allantoic vasculogenesis begins before this time (Yamaguchi *et al.*, 1993; Ellington, 1985; Downs and Harmann, 1997). Thus, angioblast formation and coalescence into rudimentary blood vessels do not depend upon interaction with the chorion (Jolly and Férester-Tadié, 1936; Downs and Gardner, 1995; Downs and Harmann, 1997).

By about 10 dpc in the rat (approximately four somite pairs in the mouse), rudiments of the allantoic vascular system appear as long, thin cytoplasmic processes joining allantoic cells together, forming small vesicles that later coalesce to form blood vessels (Ellington, 1985). Also during this time in the mouse, *flk*-1, a tyrosine kinase receptor whose ligand is vascular endothelial growth factor (VEGF), is expressed in individual cells of the allantois (Yamaguchi *et al.*, 1993). VEGF appears to be involved in many functions of endothelial cells, including proliferation and vascular permeability [reviewed in Mustonen and Alitalo (1995); Shibuya, 1995].

Studies designed to discover directly when angioblasts are present in the allantois were carried out by heterotopic transplantation. These experiments revealed that angioblasts are present in the allantois as early as the headfold stage (approximately 8.0 dpc; Downs and Harmann, 1997). The source of the angioblasts is likely to be the allantois itself, as cells from the yolk sac, all of which are red blood cells, do not infiltrate the allantois until possibly as late as the 10 somite stage. This is approximately 22 hr after transplantations at the headfold stage were carried out (K. Downs, S. Gifford, and M. Blahnik, preliminary data). A model for how angioblasts are formed in the allantois will be discussed in Section VI.A.

V. Unique Characteristics of the Allantois

A. The Mechanism of Chorioallantoic Fusion

The allantois grows into the exocoelomic cavity and attaches to and fuses with the chorion. Fusion is defined here as a union of tissue through mutual adhesion; there is no evidence or belief that actual cell fusion occurs. The mechanism of chorioallantoic fusion can be envisioned by one of three models: (1) directed growth of the allantois, where initial contact between the allantois and the chorion would be sufficient to stimulate the expression of appropriate cell adhesion molecules; (2) selective adhesion involving molecules specific to the allantois and chorion that might be expressed constitutively on both of these structures during their growth phase; or (3) selective adhesion involving expression of the requisite molecules but that might be developmentally regulated during maturation of one or the other structure.

The models were then tested (Downs and Gardner, 1995). Experiments were carried out in which distal halves of donor allantoises were placed individually into the exocoelomic cavity of similar-stage hosts whose own allantois had been removed (Fig. 5). This manipulation allowed the donor allantois the freedom to select the exocoelomic surface to which it most preferred to adhere and revealed how early fusion could occur if the opportunity presented itself.

In *ex vivo* and cultured control conceptuses, chorioallantoic fusion began at about four somite pairs and reached a maximum by six somite pairs (Downs and Gardner, 1995). Maximal fusion by six somite pairs was in agreement with a previous report (Theiler, 1989). Thus, the use of whole embryo culture to study chorioallantoic fusion was validated.

In the operated conceptuses, most donor allantoic tips attached solely to the chorion, revealing that fusion was specific. Chorioallantoic attachment occurred first between the mesothelial surfaces and was followed by the intimate juxtaposition of allantoic and chorionic cells. Further, attachment occurred at the appropriate developmental stage, beginning at four somite pairs and reaching a

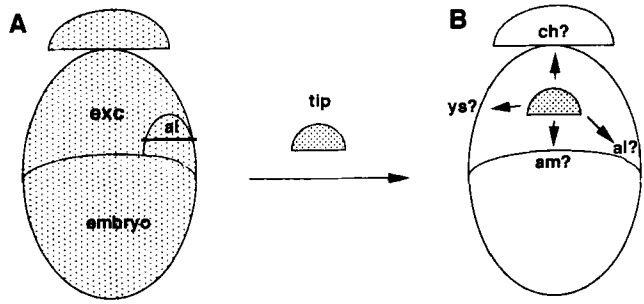


Fig. 5 Schematic diagram of the microsurgical technique used to discover the mechanism of chorioallantoic fusion. (A) The allantoic tip of a [^{3}H]methylthymidine-labeled conceptus was introduced into (B) the exocoelomic cavity from which the host allantois had been removed. The operated conceptus was then cultured for various times and scored for fusion of the tip to component tissues of the exocoelom. This microsurgical technique has been described in Downs and Gardner (1995). Abbreviations: al, allantois; am, amnion; ch, chorion; exc, exocoelom; ys, yolk sac.

maximum rate by six somites (Downs and Gardner, 1995). Thus, even in isolation and without continuity with the basal portion, donor allantoic tips behaved as they would *in situ*, fusing at the appropriate developmental stage. It appeared that a clock had been set to go off in the tip at the correct developmental time prior to loss of its connection with the base.

These experiments resolved the three possible models of chorioallantoic fusion. Although the donor allantoic tip was often found wedged in between the amnion and chorion before four somite pairs were attained by the host, no enduring attachment of the allantois to these structures had occurred, demonstrating that cell–cell interactions between the allantois and chorion do not trigger expression of the required cell adhesion molecules. Thus, the model of directed growth of the allantois as the driving mechanism of chorioallantoic fusion was eliminated. Moreover, expression of the requisite adhesion molecules was not constitutive, or the allantois and chorion would have fused prematurely because they were often positioned to do so. This observation eliminated the second model. Rather, chorioallantoic adhesion was selective and involved gradual acquisition of the specific adhesion molecules, beginning at about four somite pairs and increasing over the next several hours. This was borne out in heterochronic transplantations, where the allantois was either younger or older than the host chorion. Fusion was dependent upon the developmental maturity of the allantois, whereas the chorion was always receptive to a mature allantois.

The host embryos, in which the allantois had been removed, revealed two important properties. Where the allantois did not regenerate a significant new allantois, fetuses nonetheless were able to continue development over the next 34 hr, acquiring the same morphological landmarks as the unoperated controls (Downs and Gardner, 1995). In addition, axial rotation of the deleted mouse

embryos did not appear to be affected in the absence of an allantois, although one report has suggested that chorioallantoic fusion may be required for determining the sidedness of tail rotation in the rat (Fujinaga and Baden, 1993).

Thus, at least in the mouse, a mature chorioallantoic placenta does not appear to be essential for gross fetal development during the headfold stage, through about 18 somite pairs. The presence of the rodent yolk sac, which serves as a functional placenta early in gestation, makes the living conceptus amenable to manipulation outside of the mother during gastrulation [reviewed in Freeman (1990)].

1. Genetic Control of Chorioallantoic Fusion

Several gene deficiencies appear to affect the ability of the murine allantois to engage in fusion with the chorion. These deficiencies may involve defective proliferation of the allantois, preventing it from growing far enough to reach the chorion to fuse with it, for example, *LIM1* (Shawlot and Behringer, 1995), or they may affect allantoic morphogenesis, as in *Brachyury* mutants (Beddington *et al.*, 1992; discussed in Section V.B). The gene products of vascular cell adhesion molecule (*VCAM1*) and its receptor, the α4 subunit of integrin (α4-integrin) have been implicated as principal players in chorioallantoic fusion (Gurtner *et al.*, 1995; Kwee *et al.*, 1995; Yang *et al.*, 1995). Transgenic mice deficient in these protein products failed to exhibit chorioallantoic fusion and died at approximately 11.0–11.5 dpc.

Integrins are involved in many cellular processes, including cell proliferation, cell differentiation, cell migration, cytoskeletal organization, and cell polarization (Yang *et al.*, 1995). Integrins are heterodimers that contain α- and β-subunits. α4-integrin is expressed on hematopoietic stem cells and several types of leukocytes. *VCAM1* is expressed on the endothelium of blood vessels during inflammation and mediates leukocyte migration from blood into tissues. This receptor–ligand complex also appears to play a role in myogenesis, as α4-integrin is expressed on primary myotubes and *VCAM1* is expressed on secondary myoblasts, both of which will fuse to form skeletal muscle (Rosen *et al.*, 1992).

During chorioallantoic development, *VCAM1* and α4-integrin are expressed in the allantois and chorion. *VCAM1* is expressed in the allantois as early as the four-somite stage (Kwee *et al.*, 1995), and its receptor, α4-integrin, is expressed constitutively on the chorion (Yang *et al.*, 1995; Kwee *et al.*, 1995). These expression data would appear to fulfill the predictions of the mechanism of acquired adhesion described in the previous section (Downs and Gardner, 1995). However, the earliest time at which *VCAM1* is expressed is not known, and it is not clear why *VCAM1* is expressed in the distal two-thirds of the allantois instead of on the mesothelium alone. It may be that *VCAM1* is also involved in vasculogenesis of the allantois, which, during the allantoic growth phase, appears

to take place in the distal two-thirds of the allantois (Downs and Harmann, 1997; Section VI.A).

VCAM1 and α4-integrin may not be the only genes required for chorioallantoic fusion. When null alleles of these genes were created in transgenic mice, chorioallantoic fusion was not prevented in all cases. This lack of penetrance suggests that other genes are involved.

2. Allantoic Proliferation vs Allantoic Adhesion in Embryos Defective in Chorioallantoic Fusion

Without microsurgical intervention, it is impossible to know whether the inability of the allantois to fuse with the chorion in certain genetic mutants, such as *Brachyury* (Glueksohn-Schoenheimer, 1944), *Csk1* (Thomas *et al.*, 1995), or *LIM1* (Shawlot and Behringer, 1995) is the result of inadequate proliferation of the allantois or defective expression of adhesion molecules required for attachment and/or fusion with the chorion. The microsurgical technique described here to discover the mechanism of chorioallantoic fusion may be invaluable for such studies. For example, appropriately labeled mutant allantoises could be excised from donor embryos and introduced into the exocoelomic cavity of wild-type hosts, and the operated hosts could be cultured until the time of fusion and subsequently analyzed for adhesion to the chorion. These experiments would reveal whether the allantois is defective in adhesion to the chorion. In reciprocal experiments, wild-type allantoises could be placed into the exocoelomic cavity of mutant hosts to discover whether the absence of fusion is due to defective functioning of the chorion.

B. Allantoic Morphology

It is not known how the allantois acquires its unique morphology, but rodlike extension into the exocoelom does not appear to be an intrinsic property of the allantois. This is because removal of the allantois at the headfold and early somite stages followed by growth in roller culture results in the rapid loss of its fingerlike shape and transition into a spherical mass of cells (Fig. 6A; Downs and Harmann, 1997). The mass undergoes vasculogenesis only rarely (Downs and Harmann, 1997). From this, it is tempting to speculate that the characteristic shape of the allantois may be the result of its proximity to and wedging between the amnion and yolk sac, which together place restraints upon the allantois and mold it into a rod during the growth phase (Fig. 6B). Some morphological evidence that the allantois makes "traction" on the amnion during growth, utilizing what appear to be the bulbous projections on the mesothelial surface of the allantois, has been reported previously (Downs and Gardner, 1995). The adhe-

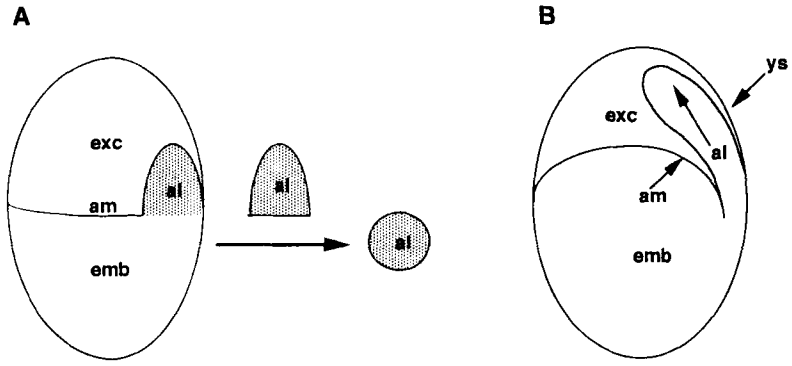


Fig. 6 Hypothetical mechanism for shaping the allantois. (A) Schematic diagram depicting the results of culture of the allantois in isolation (Downs and Harmann, 1997). Headfold-stage allantoises (stippled marks) were removed via yolk sac puncture and cultured for 24 hr, after which they assumed the shape of nearly perfect spheres. (B) Schematic diagram of the hypothetical mechanism proposed by the author to account for the shape of the allantois. The intact allantois is molded by contact with the yolk sac and amnion. During this time, the allantois may make transitory contacts with the amnion (Downs and Gardner, 1995). Abbreviations: emb, embryo; see Fig. 5 legend.

sion between the allantois and amnion does not appear to be enduring because attachment between donor allantoic tips and the host amnion is a rare event (Downs and Gardner, 1995).

1. Genetic Control of Allantoic Morphology: *Brachyury*

The only mutation known to severely affect the morphogenesis of the allantois is *Brachyury (T)*. *Brachyury* was recognized about 70 years ago because mice with one copy of this mutation have short, kinked tails (Dobrovolskaia-Zavadskaia, 1927). Further analysis of this mutation revealed that embryos with two copies of *Brachyury (T/T)* died during midgestation (about 10.5 dpc). The major defects were found in the posterior region of the embryos, notably in the primitive streak, the notochord, and the allantois. In homozygotes, the allantois fails to grow substantially into the exocoelomic cavity and fuse with the chorion (Gluecksohn-Schoenheimer, 1944; Fig. 7).

The allantoic defect of *Brachyury* has not been studied, but it appears that it may arise from the abnormal deployment of allantoic mesodermal cells emerging from the primitive streak (Beddington *et al.*, 1992). The situation may be more complex, however, because fate mapping has demonstrated that the yolk sac and chorionic mesoderms are deployed from the same proximal epiblast tissue as the allantois (Lawson *et al.*, 1991), and yet these tissues do not appear to be affected in *Brachyury*, at least at the gross morphological level.

Several new lines of evidence have provided clues regarding the allantoic defect in *Brachyury* mutants and the apparent escape of the defect by the yolk sac

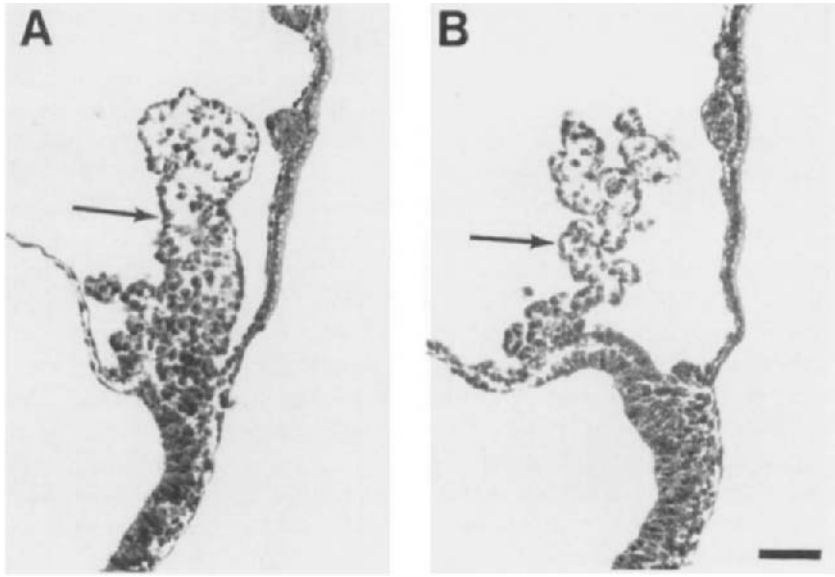


Fig. 7 Comparison of wild-type and putative *Brachyury* allantoises. Matings between heterozygous *T/+* adult mice produced a litter in which the number of embryos displaying normal (A) and defective (B) allantoises (arrows) appeared in the expected ratio. The arrow points to the allantois. Please see Fig. 1 for other morphological features. Scale bar: 53 μm (A); 50 μm (B). Methods: Conceptuses from a single litter of a *T/+* intercross were removed from their implantation sites at nominal day 8.0 postcoitum, and Reichert's membrane was reflected. The conceptuses were then fixed in Bouin's solution for 24 hr, embedded in paraffin, sectioned at 7 μm, dewaxed, and stained in hematoxylin and eosin.

and chorionic mesoderms (Clements *et al.*, 1996). Transgenic mice expressing the *lacZ* reporter gene under the control of a *Brachyury* promoter were used to recapitulate normal *Brachyury* expression in the primitive streak during gastrulation. This sensitive method revealed that *Brachyury* was expressed in the base of the future allantois at the earliest stages that extraembryonic mesoderm forms, i.e., during the mid-to-late streak stage. Previously, this expression had gone unreported (Herrmann, 1991), but careful scrutiny of the expression patterns shown in Figures 1A and 1E of Herrmann (1991) clearly reveal that the base of the allantois expresses *T* as early as the late streak stage.

When a small deletion was included in the promoter of the *lacZ*-tagged *Brachyury* gene and a transgenic mouse line was made from this construct, a somewhat surprising change in the expression pattern of *Brachyury* was observed in gastrulating embryos: the earliest extraembryonic mesoderm to arise from the posterior streak expressed lacZ at the midstreak stage, but later emerging mesodermal cells did not. In particular, expression of the transgene was conspicuously absent from the base of the allantois.

These observations fit in very well with the author's hypothesis that formation of the components of the exocoelomic cavity occurs in three waves and may explain why the yolk sac and chorionic mesoderm may not be affected in *Brachyury* mutants but the allantois is. They suggest that the Brachyury protein product, although expressed, may not be required for the first wave of formation of extraembryonic mesoderm. However, it does appear to be necessary for continued proliferation of extraembryonic mesoderm shortly thereafter (Clements *et al.*, 1996). This would coincide with the second wave of formation of extraembryonic mesoderm, which goes into the amnion and allantois and may explain why the allantois and possibly the amnion are morphologically and functionally defective in *Brachyury* mutants (Rashbass *et al.*, 1991).

The Brachyury gene product appears to be a DNA-binding protein (Kispert *et al.*, 1995). The downstream genes regulated by *Brachyury* have not been identified, but they may be part of the family of adhesion molecules. This is because the cell surface appears to be altered in *Brachyury*, as *T/T* mutant cells form smaller aggregates than their normal counterparts (Yanagisawa and Fujimoto, 1977). Ultrastructural examination of the embryonic portion of the conceptus, and not the allantois, has also revealed that the extracellular matrix in *T/T* embryos is greatly decreased (Jacobs-Cohen *et al.*, 1983). Chimeric studies in which the deployment of *T/T* mutant ES cells was examined revealed a buildup of *T/T* mutant cells in the posterior region and overall reduced motility of these cells, in accordance with the theory that the surface of *Brachyury* mutant cells is altered (Rashbass *et al.*, 1991; Beddington *et al.*, 1992). *Brachyury* allantoises form, but they are misshapen and adhere to the amnion, suggesting that *Brachyury* allantoises have altered surface properties the allow them to adhere to the amnion or that the amnion is affected as well.

C. The Base of the Allantois and Primordial Germ Cells

It is well-established that the germ line is formed from the epiblast of the mouse gastrula (Gardner and Rossant, 1979; Gardner *et al.*, 1985) and that the precursors of the germ line, called the primordial germ cells (PGCs), are found in the hindgut and then migrate along the dorsal mesentery to colonize the gonads by about 11.5 dpc (Mintz and Russell, 1957; Gomperts *et al.*, 1994). Alkaline phosphatase (AP) activity has long been considered to be diagnostic of primordial germ cells because, during migration to the gonads, a large number of cells along the dorsal mesentery express alkaline phosphatase activity that is maintained in the gonads (Mintz and Russell, 1957). The most convincing evidence that these alkaline-phosphatase-positive cells are the PGCs derives from a comparison between wild-type embryos and embryos mutated in the genes for dominant white spotting (*W*) and steel (*Sl*). The mutant embryos revealed a severe deficiency in AP^+ cells along the dorsal mesentery and in the gonads (Mintz and

Russell, 1957). Further convincing support for AP reactivity as diagnostic for migrating PGCs was obtained from the demonstration that the AP^+/SSEA-1 cells emigrating from the hindgut become tethered to each other via cytoplasmic processes along the dorsal mesentery, allowing them to link up to form exclusive networks that culminate in their aggregation in the gonads (Gomperts *et al.*, 1994).

The location of the primordial germ cells before migration is less clear. Alkaline-phosphatase-positive cells have been traced back from the dorsal mesentery to the yolk sac of the conceptus at about 8.5 dpc (Chiquoine, 1954) and to the base of the allantois, which is particularly rich in AP^+ cells, at about 8.0 dpc (Oździenski, 1967). Oździenski (1967) proposed two hypotheses to explain the significance of AP^+ cells in the base of the allantois. Either they are the PGCs, having been set aside there and eventually returning to the fetus to populate the gonads, or they will contribute only to somatic lineages. The hypothesis that AP^+ cells in the base of the allantois are the primordial germ cells is complicated by the observation that many cells and tissue types, including the epiblast, exhibit alkaline phosphatase activity during gastrulation (MacGregor *et al.*, 1995). Further, because tissue-nonspecific alkaline phosphatase, expressed in migrating PGCs, is not required for formation of the germ line (MacGregor *et al.*, 1995), it is impossible to identify histologically which population of AP^+ cells, if any, are the PGCs.

The only method for resolving whether the cells in the base of the allantois are the PGCs is to fate map this region. If cells residing in the base of the allantois return to the fetus and colonize the gonads, then it is likely that the base of the allantois contains the precursors of the mammalian germ line. It would also be useful to discover the mechanism of expansion of the allantois to demonstrate whether it is at all feasible that a small cluster of AP^+ cells can remain fixed in the base of the allantois from the midstreak to the early somite stage, approximately 42 hr, as previously suggested (Ginsberg *et al.*, 1990).

Preliminary steps were taken to discover the fate of cells in the base of the allantois (Downs and Harmann, 1997). Orthotopic transplantation revealed that cells in the base do not return to the fetus (Downs and Harmann, 1997). Rather, although relatively pluripotent, they appear to contribute only to the allantois and move distally during allantoic growth, contributing to the mature allantois.

However, the theory that the base of the allantois nevertheless contains the future germ line cannot be ruled out on several grounds. The first is that there is no precise morphological feature that clearly defines where the base of the allantois begins. This was apparently the reason that Copp *et al.* (1986) included the entire allantoic bud in orthotopic transplantations designed to elucidate the location of the primordial germ cells in gastrulation. There is also the possibility that the germ cells failed to survive transplantation. This seems unlikely, however, as previous studies have shown that cells taken from this region survive culture and extensively contribute to somatic chimeras (Matsui *et al.*, 1992). In

one instance, these cultured cells even contributed to the germ line (Labosky *et al.*, 1994). Finally, although the base of the allantois had been defined according to Oździenski (Fig. 3) in the transplantation studies, it had previously been demonstrated that allantoic tissue can be regenerated following aspiration of the headfold-stage allantois (Downs and Gardner, 1995). This argues that removal of the allantois via aspiration is either incomplete or, if it is complete, cells beneath the allantois retain the potential to regenerate an allantois (Downs and Gardner, 1995). Therefore, any future studies designed to analyze fate in the base of the allantois will need to precisely define the allantoic–posterior primitive streak region.

VI. Function of the Allantois

A. Vasculogenesis in the Allantois

Formation of large embryonic blood vessels and the vitelline vasculature is thought to occur by a uniquely embryonic process called vasculogenesis [reviewed in Risau and Flamme (1995)]. Pluripotent cells differentiate *in situ* into angioblasts, which subsequently coalesce to form a rudimentary endothelial scaffolding that will be remodeled until the mature blood vessels are formed. The other type of vessel formation, angiogenesis, occurs by sprouting of extant endothelium and occurs throughout the life of the organism.

How the murine umbilical vasculature forms is not known. Given that vascularization is thought to involve mesodermal–endodermal interactions, it is far from clear how the allantois, which is wholly mesodermal, vascularizes.

Some aspects of allantoic vasculogenesis have come to light. In particular, the fate and developmental potency of allantoic mesoderm at the headfold stage have been analyzed in a series of transplantation experiments (Downs and Harmann, 1997). Results of these studies demonstrated that angioblasts are present in the allantois as early as the headfold (presomite) stage. The headfold stage was chosen because it is the earliest time at which the allantois could be subdivided into thirds along its proximodistal axis (Figs. 3 and 8). The tip was thought to be specialized for chorioallantoic fusion, and the base was thought to protect or induce the future germ line (Section IV.C). On the basis of these suppositions, it was envisioned that the allantois may exhibit distinct cellular properties along its proximodistal axis.

lacZ-expressing allantoises were subdivided into three regions, the base, midportion, and tip, and clumps of cells from each region were placed into three sites in the conceptus (Fig. 8). When placed into the fetus, none of the allantoic regions colonized paraxial (somitic) mesoderm, but all three regions colonized the endothelium of the dorsal aorta and adjacent mesenchyme. The allantoic midportion colonized the dorsal aorta most avidly. Only the base exhibited a

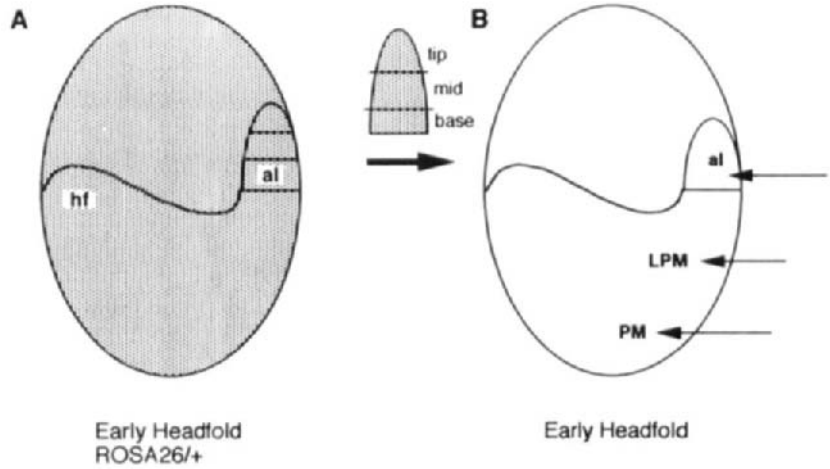


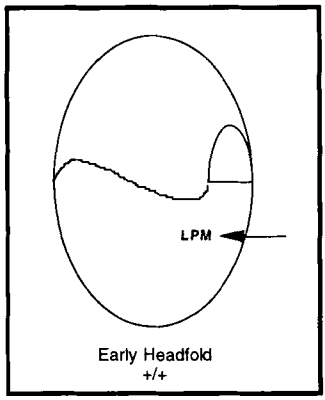
Fig. 8 Sites of transplantation. Donor allantoises from headfold-stage ROSA26* *lacZ*/+ hemizygous donor conceptuses (A) (Downs and Harmann, 1997) were removed and subdivided into three regions: tip, middle portion (mid), and base (see also Fig. 3). Clumps from each allantoic region were transplanted into each of three headfold-stage host sites in a nontransgenic host (B): the base of the allantois (al), the primitive streak at the level of prospective lateral plate mesoderm (LPM), and the primitive streak at the level of prospective paraxial mesoderm (PM). Other abbreviations: hf, region of the headfolds.

tendency to colonize a few more cell types, including lateral plate mesoderm, surface ectoderm, and the endoderm of the future coelom. These data are summarized in Figs. 9 and 10.

Transplantation of all three allantoic regions back into the allantois revealed that a gradient of differentiation may exist along the proximodistal axis of the headfold-stage allantois (Fig. 11). This was reflected in the observation that, despite being placed into the base of the hose allantois, all three donor allantoic regions displayed colonization patterns of the host allantois that were distinctly different from each other. Donor basal cells typically moved only as far as the midregion of the host allantois, whereas tip cells most often returned to the tip (Fig. 12).

These studies suggested that the allantois undergoes vasculogenesis rather than angiogenesis because allantoic angioblasts solely contributed to large vessel endothelium. Preliminary transplantation experiments of the yolk sac have tentatively confirmed this hypothesis, revealing that there is no contribution of the yolk sac to the allantois until at least the 10 somite stage (K. Downs, S. Gifford, and M. Blahnik, unpublished data). Thus, the allantois may be the exception to the rule that angioblast formation is dependent upon mesodermal interactions with endoderm.

If angioblasts are formed *de novo* within the allantois, then how do the extraembryonic mesodermal cells that comprise the allantoic bud differentiate into



Summary of Grafts into the Primitive Streak at the Level of Prospective Lateral Plate Mesoderm

	Orthotopic: Prospective Lateral Plate Mesoderm	Heterotopic: Allantois to Prospective Lateral Plate Mesoderm		
Tissue Grafted→ Tissue Colonized ↓	LPM	Base	Mid-Portion	Tip
Lateral Plate Mesoderm		+		
Intermediate Mesoderm				
Somites				
Neural Tube				
Capillaries				
Endoderm of Future Coelom				
Surface Ectoderm		+		
Endothelium of Intersegmental Vessels				
Endothelium of Aorta				
Mesenchyme Adj to Dorsal Aorta				
Umbilical Endothelium at All/Am Junction		+		
No. Chimeras with Unincorporated Donor Cells	0	0	5	2
Initial Stage of Host Embryo	Headfold, 2-3	Headfold	Headfold, 3,4	Headfold
Final No. Somite Pairs	11-15	10-12	10-16	11-16
Total Number of Incorporated Transgenic Cells	1925	2789	2769	995
Number Chimeras (% of Total Injected)	10 (52.6%)	4 (23.5%)	12 (54.4%)	5 (27.8%)

angioblasts? One model that can be evoked is dependent upon spatial cues. For example, extraembryonic mesoderm destined for the allantois emerges from the primitive streak in a relatively pluripotent state, but as it is pushed distally by sustained addition of nascent extraembryonic mesoderm into the allantoic base, it moves out of the sphere of influence of the primitive streak and differentiates into angioblasts. Movement farther into the tip entails further differentiation, into either chorioadhesive cells or possibly specialized umbilical endothelial cells.

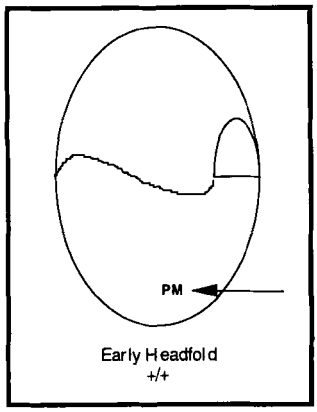
The second model is a temporal one. The fate of allantoic cells may be dependent upon the amount of time they reside in different allantoic levels, the length of which may in turn be dependent upon the rate at which cells are recruited into the base of the allantois and/or the number of cell cycles completed by allantoic cells before distal translocation.

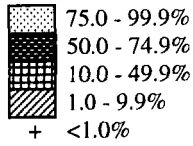
B. Erythropoietic Potential of the Allantois

Although red blood cells are obvious in the allantois shortly after allantoic vasculogenesis begins, it has been assumed that these come from either the yolk sac or the fetus. Bloom and Bartelmez (1940) reported profiles of hematopoietic cells in the nascent human allantois, but concluded on the basis of histological evidence that these were the result of "abortive" blood cell formation in belly stalk (umbilical) vessels. Gene expression studies that localize erythropoietic transcription factors to component tissues of the exocoelom during gastrulation have offered few clues as to whether the allantois may be an erythropoietic organ, for focus is typically on the yolk sac.

It was reported that allantoises cultured in isolation do contain cells that stain positively for benzidine, which is indicative of the presence of hemoglobin (Downs and Harmann, 1997). However, because the allantoises had been removed through the yolk sac, contamination by yolk sac blood islands or induction of allantoic hemoglobin synthesis by factors emanating from the broken yolk sac could not be ruled out. If the latter is correct, the allantois may possess the developmental potential to become erythropoietic under appropriate conditions and could, at the very least, turn out to be an attractive system for studying the genetic control of erythropoiesis.

Fig. 9 Summary of allantoic transplantation into the prospective primitive streak at the level of lateral plate mesoderm. Control orthotopic and experimental heterotopic grafts of allantoic tissue into the prospective lateral plate mesoderm are summarized. The total number of grafted cells for each series is reported at the bottom of the figure. The filled-in boxes represent the percentage of grafted cells found in each of the host's tissues described in the leftmost portion of the figure. Original data are in Downs and Harmann (1997).





Summary of Transplants into the Primitive Streak at the Level of Prospective Paraxial Mesoderm

	Orthotopic: Prospective Paraxial Mesoderm	Heterotopic: Allantois to Prospective Paraxial Mesoderm		
Tissue Grafted→ Tissue Colonized ↓	PM	Base	Mid-Portion	Tip
Somites				
Pre-Somitic Mesoderm				
Capillaries (in neurectoderm)				
Neural Tube				
Notochordal Plate				
Surface Ectoderm	+			
Endothelium of Intersegmental Vessels				
Endothelium of Dorsal Aorta				
Mesenchyme Adj to Dorsal Aorta				
No. Chimeras with Unincorporated Donor Cells	1	0	4	1
Initial Stage of Host Embryos	Headfold, 3-4	Headfold	Headfold, 1	Headfold
Final Number Somite Pairs	10-14	9-11	8-13	11-13
Total Number of Incorporated Transgenic Cells	1021	136	1060	340
Number Chimeras (% Total Injected)	7 (33.3%)	2 (10.5%)	6 (28.6%)	4 (30.8%)

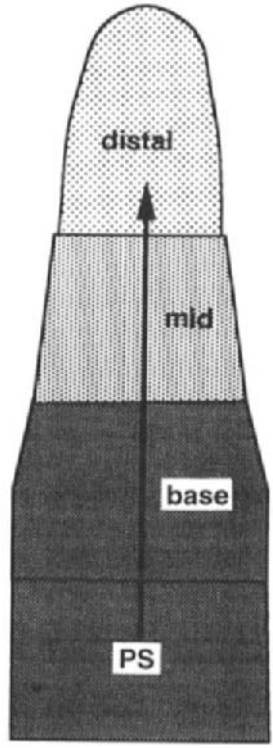
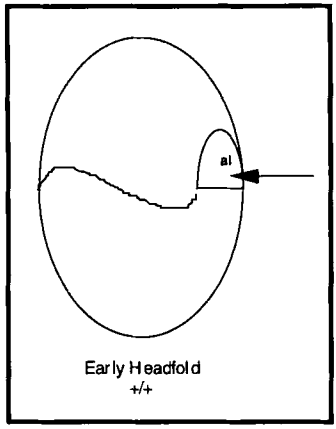
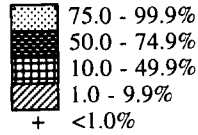


Fig. 11 Gradient of differentiation in the allantois. Schematic diagram depicting the topographical arrangement of pluripotent mesodermal cells and angioblasts in the headfold-stage allantois (Downs and Harmann, 1997). The allantois is continuous with the primitive streak (PS). Cells from the epiblast ingress into the primitive streak where they are transformed into pluripotent extraembryonic mesodermal cells. These pluripotent cells move distally into the allantoic base (indicated by the arrow extending from the PS to the distal allantoic region). As more extraembryonic mesodermal cells are added from the streak to the allantois, the basal cells move distally, first into the midregion where they become differentiated into angioblasts and second into the distal region, where some cells may continue to differentiate into angioblasts and others into cells specialized for fusion with the chorion or endothelium at the chorioallantoic fusion junction.

←

Fig. 10 Summary of allantoic transplantation into the prospective primitive streak at the level of paraxial mesoderm. Control orthotopic and experimental heterotopic grafts of allantoic tissue into the prospective paraxial mesoderm are summarized. The total number of grafted cells for each series is reported at the bottom of the figure. The filled-in boxes represent the percentage of grafted cells that was found in each of the host's tissues described in the leftmost portion of the figure. Original data are in Downs and Harmann (1997).





Summary of Grafts into the Base of the Allantois			
Region Grafted→ Region Colonized ↓	Base	Mid-Portion	Tip
Base	+	+	0
Mid-Region			
Distal Third			
Vitelline Omphalomes Artery	+	+	
No. Chimeras with Unincorporated Donor Cells	1	2	0
Initial Stage of Host Embryos	Headfold	Headfold, 3-5	Headfold, 3
Final Number Somite Pairs	10-16	8-16	11-16
Total Number of Incorporated Transgenic Cells	5652	9715	2501
Number Chimeras (% Total Injected)	21 (60.0%)	16 (76.2%)	12 (85.7%)

Fig. 12 Summary of allantoic transplantation into the base of the allantois. The key to this figure is similar to that for Figs. 9 and 10, with the exception that there is one less column, as the control orthotopic grafts were of the base of the allantois. Original data are in Downs and Harmann (1997).

VII. The Allantois in Fetal Therapy

The principal function of the allantois is to form the vascular connection between mother and fetus. The umbilical vessels provide a direct gateway to fetal circula-

tion and a potentially invaluable system for the continuous delivery of blood-borne therapeutic factors to the fetus during gestation. Data have been presented in this chapter that demonstrate that, with little effort, the murine allantois can be manipulated extensively without compromising the development of the fetus. These results, along with others that have demonstrated gene expression from endothelial cell-specific promoters (Korhonen *et al.*, 1995; Schlaeger *et al.*, 1995), suggest that the umbilical cord has the potential to express therapeutic factors via the endothelial cells that comprise its blood vessels, with subsequent delivery of the factor to fetal circulation. The mouse may be an ideal system for testing the soundness of umbilical therapy.

The success of fetal therapy via the umbilical cord rests upon obtaining allantoic cells, either primary cells or allantoic cell lines whose properties are similar enough to intact allantoic cells that they can colonize the umbilical cord. Because the location of pluripotent allantoic cells and angioblasts is now more clear (Downs and Harmann, 1997), these cells may be exploited for umbilical therapy. These cells or cell lines derived from them may be reintroduced into the umbilical cords of developmentally compromised fetuses as wild-type cells in cases where the therapeutic factor of interest is produced normally in endothelial cells and in sufficient quantity to rescue the fetal defect via secretion of the factor into the fetal bloodstream. Alternatively, where the gene is not expressed normally in endothelial cells or where large amounts of the gene product are needed, allantoic cells may be transfected with a therapeutic gene of interest under the regulation of a robust endothelial cell-specific promoter. Such genetically engineered cells would then be placed into the umbilical cord, assimilate, differentiate into endothelial cells, and express the gene of interest, secreting it into the circulation (Fig. 13).

Umbilical therapy may be critical in cases where the therapeutic factor may be toxic to the mother, cannot cross the placental barrier, or whose half-life is so short as to make intermittent injection into the umbilical cord impractical and costly. Delivery of recombinant factors in fetal life, while the immune system is developing, may permit some therapeutic factors to be tolerated by the immune system later in life. One potentially attractive benefit to umbilical therapy is that because the placenta will be shed at birth, there will be no unforeseen deleterious effects of this approach on the adult.

Key to the success of this therapy are considerations of fetal diagnosis early in pregnancy, the source of the umbilical cells, and the possibility of tissue rejection, as well as the number of cells required for successful umbilical chimerism. None of these parameters have been considered here.

An example of a disease for which umbilical therapy may be beneficial is hemophilia A. Hemophilia A affects 1 in 5,000–10,000 males in all populations and is caused by a defect in clotting Factor VIII. What makes hemophilia A particularly difficult to treat is that many patients will develop inhibitors against recombinant Factor VIII (Bi *et al.*, 1995). Therefore, a possible means for pre-

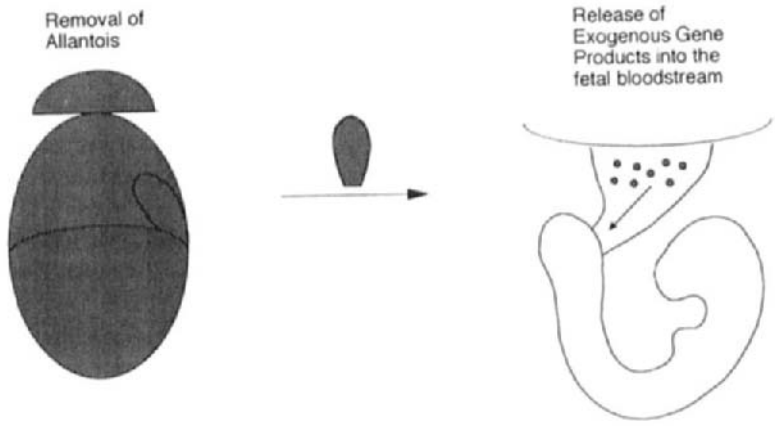


Fig. 13 Umbilical gene therapy. Allantoic cells are removed from an appropriately staged conceptus and introduced into the allantois or umbilicus of a developmentally compromised fetus. There they assimilate into the umbilicus and differentiate into endothelial cells that can deliver either sufficient amounts of wild-type therapeutic factor to the fetal bloodstream or high levels of the therapeutic factor if the allantoic cells have been genetically altered to overproduce the factor. At birth, the chimeric umbilical cord will be shed.

venting the formation of these inhibiting antibodies would be to begin therapy *in utero* as the immune system is developing. This would ensure that Factor VIII is not recognized as a foreign protein when given after birth. However, because Factor VIII does not cross the placental barrier (Lozier and Brinkhous, 1994) and the half-life of Factor VIII is only 10 hr (Brownlee, 1995), delivery of Factor VIII to the fetus via the mother or by injection into the umbilical cord is impossible or impractical.

Factor VIII is produced in the liver, although its exact site of production is still controversial (Sadler and Davie, 1994). Factor VIII mRNA has been detected in extrahepatic tissues such as the spleen, lymph nodes, and kidneys (Brownlee, 1995). In the liver, Factor VIII is processed in the endoplasmic reticulum and Golgi, where proteolytic cleavage produces a heavy and a light chain held together by metal ions. The Factor VIII heterodimer is then secreted as a glycoprotein into the blood, where it circulates as a complex with von Willebrand factor, itself a multimer. Although Factor VIII requires the presence of von Willebrand factor for stability (Brownlee, 1995), previous studies have revealed that the endothelial cells of the umbilical cord are the principal producing cells of von Willebrand factor (Dichek and Quertermous, 1989). Therefore, Factor VIII should be stable in the system proposed here, as the chimeric umbilical cord will express both Factor VIII and von Willebrand factor.

VIII. Conclusions

The allantois, heretofore little studied, may provide fresh approaches to old problems. Because the principal function of the allantois is to vascularize, the allantois is a very attractive organ for the study of vasculogenesis, especially as it can be isolated free of contamination from embryonic and extraembryonic tissues. The mechanism by which differentiation along the proximodistal axis of the allantois is established may shed new light on how morphogenetic gradients are set up in the mammal. If the allantois possesses erythropoietic potential, then it may be an ideal system for studying the genetic control of erythropoiesis. Ultimately it is hoped that the results of basic research combined with the privileged position of the allantois in fetal development will stimulate advances in methods of fetal therapy.

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Axial Relationships between Egg and Embryo in the Mouse

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"All the evidence thus converges to the conclusion that the polarity of the egg is of universal occurrence among animals. Its fundamental promorphological significance lies in the fact that the axis of the egg shows a definite relation to that of the gastrula of the later embryo, and of the adult body: and this relation, broadly considered, appears to be constant throughout the Bilateralia, though it is often disguised in later stages through processes of asymmetrical growth such as are strikingly seen in the metamorphosis of echinoderms or, in a simpler form in the platodes, annelids or molluscs."

(E. B. WILSON, 1928)

"Except for acoel turbellarians and mammals, where no evidence for localization exists, the localization phenomenon is found to occur in all areas of the phylogenetic map."

(E. H. DAVIDSON, 1976)

I. Introduction

Much of the excitement that developmental biology currently enjoys stems from a growing conviction that the very rich diversity of modes of development found among metazoa is underpinned by unexpected and remarkable conservatism in

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their genetic basis. This conviction has emerged from the investigation of constituent processes at the molecular level. Hence, when a group of organisms such as mammals is held to differ markedly from the norm in certain aspects of their early development, it is pertinent to note that this conclusion was reached principally on the basis of experiments that were done well over a decade ago when diversity rather than unity was the dominant theme. The issue to be explored here is whether, as is generally true in metazoa, the egg or the zygote in mammals may contain information that is instrumental in specifying one or more embryonic axes.

That cells typically contain both a single nucleus and a centrosomal complex has long been recognized as a basis for regarding polarity as a universal feature of their organization (Van Beneden, 1883). Eccentric positioning of the nucleus within cells is usually the most obvious manifestation of the axis of polarity. The eggs of almost all metazoa are visibly polarized (Denis, 1996), even though in many species they lack a centrosome prior to fertilization. Introduction of the terms "animal" and "vegetative" (or vegetal) to denote the two poles of the egg axis has been attributed to Robert Remak (Wilson, 1928), who recognized the tendency of these regions to form structures whose vital activities are concerned with sensory and locomotory versus nutritive functions (Kume and Dan, 1988). In practice, the animal pole of the egg is recognizable in many species because it is invariably the site where the polar bodies are formed during the very unequal cell divisions of meiosis. The vegetal pole can seldom be differentiated in relatively nonyolky eggs and thus is simply taken as being diametrically opposite the animal pole. However, in eggs that are well-endowed with yolk, it is often distinguishable as the focus of accumulation of such material. In some species unfertilized eggs show additional morphological differentiation along the animal–vegetal (AV) axis through local accumulation of cytoplasmic inclusions that differ in size or color. More often, as will be discussed later, such zonation of eggs along the AV axis is only established definitively after rather than before fertilization. Regardless of when inclusions become localized, it is evident from centrifugation experiments that perturbing their distribution is not usually of any consequence for normal development. Nevertheless, studies on the developmental potential of egg fragments or isolated blastomeres that differ with respect to such inclusions have often identified them as convenient markers of regional ctyoplasmic differentiation that is instrumental in embryonic patterning. Unfortunately, however, developmentally significant cytoplasmic localization is accompanied only occasionally by corresponding morphological differentiation that is discernible in living eggs or zygotes.

Mammals are no exception to the rule that eggs are polarized cells because for the first and second meiotic divisions that occur, respectively, before and after sperm penetration the chromosomes are assembled on spindles that are localized cortically. Furthermore, properties in the region of the surface of the egg overlying these chromosome sets differ from those elsewhere (Eager *et al.*, 1976;

Nicosia *et al.*, 1977; Phillips and Shalgi, 1980; Wolfe and Ziomek, 1983; Maro *et al.*, 1984, 1986; Longo and Chen, 1985; Longo, 1987; Calarco, 1991). However, whereas this arrangement confers an axis of polarity on the egg, its significance does not necessarily extend beyond the necessity of minimizing the loss of egg cytoplasm at each of the two meiotic divisions. Likewise, the eccentricity of the germinal vesicle nucleus in primary oocytes (Dalcq, 1957; Kruip *et al.*, 1983) need not reflect a stable underlying polarity. That polarity may be conserved throughout oogenesis is evident from studies in the holothurian echinoderm, *Synaptula hydriformis*, in which the AV axis of the mature oocyte was found to correspond with the apicobasal axis of its epithelial precursor cell (Frick and Ruppert, 1996; Frick *et al.*, 1996). In the case of mammals, both the timing and mode of origin of the AV axis during oogenesis remain obscure, and the cytoplasm of the egg does not show consistent regional differentiation either before or after fertilization [reviewed in Gardner (1996a)]. However, as noted earlier, neither feature of the cytoplasm is a prerequisite for localization within the egg of information for patterning the embryo.

What is the significance of the AV axis of the metazoan egg or zygote for subsequent development? Information for patterning of the early embryo can be distributed along the AV axis either in gradient form or as localized "determinants." The fact that the planes of early cleavage divisions normally show a consistent orientation in relation to the AV axis will serve to ensure that such information is partitioned to blastomeres in a regular way. Often, the first two cleavages are parallel to the AV axis (meridional) but perpendicular to each other, and the third is orthogonal (equatorial) to it, as is illustrated graphically in sea urchins. This pattern dictates that from the eight-cell stage no blastomeres will contain cytoplasm from all axial levels of the egg. In the case of sea urchins, this stage corresponds with obvious restriction in the developmental potential of blastomeres, notably those of the animal as opposed to the vegetal quartet (Horstadius, 1973). Moreover, the developmental potential of eight-cell blastomeres closely corresponds with that of the region of the egg with whose cytoplasm they were endowed (Murayama *et al.*, 1985). However, the first cleavage can be oblique to the AV axis, as exemplified by the marine shrimp *Sicyonia ingentis* (Wang *et al.*, 1997), or even orthogonal to it as in nematodes (Goldstein and Hird, 1996). The relationship of the axes of the future embryo to the AV axis of the egg or zygote varies both between and, in some cases, also within species, as has been demonstrated particularly clearly in sea urchins (Cameron *et al.*, 1989; Henry *et al.*, 1992; Ruffins and Ettensohn, 1996; Summers *et al.*, 1996). In insects, the animal pole of the egg equates with the embryo's anterior and the vegetal pole with the embryo's posterior, whereas in cephalopod molluscs the animal pole of the egg becomes the dorsal and the vegetal pole the ventral surface of the embryo.

Insect and cephalopod eggs are unusual in exhibiting an obvious bilaterally symmetrical morphology well before fertilization (Wilson, 1928). More com-

monly, bilateral symmetry originates more subtly thereafter through sperm penetration or subsequent male pronuclear migration inducing the displacement of one or more cytoplasmic regions from a symmetrical distribution about the AV axis. Cortical rotation, which results in the establishment of the dorsoventral axis in amphibians, is a particularly well-studied example of the breaking of radial symmetry by sperm penetration (Slack, 1991). In this case it is clear from experiments rooted in embryological antiquity that the sperm is dispensible [see Morgan (1927) for a review of early studies]. Although ways of interfering with this process and thus reproducibly generating radially symmetrical embryos have been found, so far they have yielded little insight into what cortical rotation entails in molecular terms. Nevertheless, the associated eccentric displacement of material that is localized at the vegetal pole before egg activation is clearly indispensible for subsequent patterning of the embryo (Yuge *et al.*, 1990; Fujisue *et al.*, 1993; Holowacz and Elinson, 1993; Kikkawa *et al.*, 1996). Interestingly, transcripts of several genes implicated in such processes have been found to be localized vegetally prior to fertilization (Melton *et al.*, 1989; Lustig *et al.*, 1996; Zhang and King, 1996; Stennard *et al.*, 1996). Whereas the plane of meridional first cleavage, which accords with the site of sperm penetration, often coincides with the bilateral plane of the future gastrula in amphibians, it frequently departs from it widely.

More complex cytoplasmic rearrangements accompany migration of the pronucleus of the fertilizing sperm in ascidians, and these have also been implicated in the bilateral localization of several putative determinants (e.g., Jeffery, 1992; Nishida, 1994a,b, 1996; Yoshida *et al.*, 1996). First cleavage corresponds much more rigidly with the plane of bilateral symmetry in this group than in amphibians and yields an early embryo that is obviously bilaterally symmetrical. All subsequent cleavages respect this plane, the second being transverse to it in both left and right blastomeres and the third, frontal (see Slack, 1991). However, in ascidians it remains unclear what determines the observed patterns of cytoplasmic rearrangement and especially, given the variability in site of sperm penetration (Roegiers *et al.*, 1995), what drives the male pronucleus in its predictable but irregular peregrinations. Among vertebrates, lineage studies suggesting that sequential specification of embryonic axes may occur during the first three cleavages in the zebrafish remain more contentious (Strehlow and Gilbert, 1993; Strehlow *et al.*, 1994; Wilson *et al.*, 1993; Kimmel and Law, 1985; Kimmel and Warga, 1987).

In the foregoing examples, the fertilizing sperm plays a role in redistributing cytoplasmic localizations that were already established in the egg before fertilization. This clearly differs from the situation in the nematode, *Caenorhabditis elegans*, in which no asymmetries in the distribution of either determinants or cytoskeletal organization are evident in the oocyte. Asymmetry is first discernible shortly before fertilization with movement of the oocyte's nucleus toward one pole, the future animal pole, which normally becomes the anterior of the embryo.

Sperm penetration and development of the male pronucleus normally occur at the opposite vegetal pole, the embryo's presumptive posterior, and appear to induce the flow of cytoplasm, including P granules, toward this region and drive cortical F actin away from it. If sperm penetration is made to occur at the animal pole of the egg, as defined by eccentricity of its nucleus, then the AV polarity of the zygote and hence the AP axis of the embryo are reversed (Goldstein and Hird, 1996). Sperm penetration normally occurs at the pole where the polar bodies are formed in some other nematodes, and in these species the animal pole of the zygote becomes the posterior of the embryo. In yet other species, the site of sperm entry seems to be essentially random, and even when lateral to the egg axis it still directs cytoplasmic flow toward and cortical F actin away from it. The fact that the male pronucleus eventually migrates to the pole closest to the site of sperm penetration in these cases is attributed to the axial elongation of the egg, which is consolidated by secretion of a tough chitinous shell shortly after fertilization. Hence, the orientation of the AP axis of the embryo is held to depend on this constraint on the shape of the AV axis of the egg and its polarity on the pole to which sperm penetration is closest (Goldstein and Hird, 1996). The implication is that if the nematode egg were maintained as a sphere from before fertilization through to the completion of first cleavage, both the orientation and polarity of the AP axis of the embryo would be specified by the site of sperm penetration as this would be expected to dictate where the male pronucleus formed.

In hydrozoan coelenterates like *Phialidium gregarium*, which are radially symmetrical about their AP axis, the location of the zygotic nucleus seems to be the critical factor in specifying the AP axis of the future embryo by determining the orientation of first cleavage, on which it depends. Here, the germinal vesicle comes to lie in the region of the oocyte closest to the external epithelium so that the polar bodies are produced here, thereby establishing it as the embryonic pole. Because sperm penetration can only occur where the polar bodies are formed and the female pronucleus remains at this site, the zygote nucleus is also located at the animal pole in normal circumstances. That the first cleavage furrow is dictated by the location of the zygotic nucleus has been demonstrated by using centrifugation to alter its location in fertilized eggs in which the site of polar body formation was marked [see Freeman (1990) for details].

Hence, it is evident from this necessarily very brief survey [see Wilson (1928), Davidson (1986), and Wall (1990) for a more comprehensive treatment of this subject] that both the timing and mode of establishment of patterning information in one or two dimensions differ markedly among metazoa, ranging from relatively early in oogenesis, through various stages of fertilization, to shortly before the onset of cytokinesis. Indeed, in the case of mammals, the polarization hypothesis envisages a radial patterning process that is dependent on asymmetrical contacts between blastomeres that is not set up until after the third cleavage (Johnson, 1980; Johnson *et al.*, 1981). However, it is only in *Drosophila* that a detailed understanding has begun to emerge of how information established in

the egg specifies axial patterning of the embryo at the molecular level. Here, it is evident that the somatic cells of the ovary play a very significant role [see Denis (1996) for a review]. It is not practicable to do justice to this work here, nor does it seem necessary in view of the fact that it has been the subject of numerous reviews. It is, furthermore, still far from clear to what extent findings in an organism that retains a syncytial organization throughout cleavage can be extrapolated to those in which development is strictly cellular *ab initio*. It is, however, interesting to note in this context that extensive regional differentiation can take place within a common cytoplasm following the suppression of cytokinesis in a normally cellular embryo, as exemplified spectacularly by the study of Lillie on the annelid *Chaetopterus* [Lillie, 1906; summarized in Wilson (1928)].

A final point that should be kept in mind when considering the situation in mammals is that the early specification of embryonic axes may be labile rather than rigid and thus susceptible to later regulation. Hence, there may be a marked disparity between what occurs during normal unperturbed development and following experimental intervention. This is illustrated particularly instructively in relation to specification of the oral–aboral (OA or ventrodorsal) axis in sea urchins. Lineage labeling of one blastomere at the two-cell stage has revealed that this axis bears a consistent relationship to the plane of first cleavage in a variety of sea urchins. In some species, including *Strongylocentrotus purpuratus*, the OA axis is normally 45° clockwise to this plane, whereas in others it may coincide with or lie orthogonal to it (Cameron *et al.*, 1989; Henry *et al.*, 1992). Furthermore, correspondence of the oral–aboral axis of the adult with the AV axis of the egg has been established in a species of sea urchin that develops directly rather than via a pluteus larval stage (Morris, 1995). Collectively, such findings show that the OA axis can be specified before the first cleavage in undisturbed development, even though the eggs of sea urchins, unlike those of certain other echinoderms, show no morphological signs of bilaterality before fertilization. However, various experimental findings argue that bilateral symmetry may, nevertheless, already be present in a cryptic form. Thus, eggs activated artificially can yield bilateral larvae and, more significantly, the pairs of meridional halves of eggs bisected before fertilization may differentiate as right–left or oral–aboral twins (Horstadius, 1973). Furthermore, treatment of eggs with certain substances before fertilization can lead to the formation of radialized larvae. Nevertheless, such early bilaterality is clearly labile because, if eggs are centrifuged before fertilization, the side that happens to lie centrifugally tends to form the oral surface of the resulting larva (Czihak, 1963; Brandriff and Hinegardner, 1975). Additionally, by stretching or constricting eggs in a meridional plane, it is possible to make them form biventral larvae. These experiments imply that information specifying the future oral surface of the embryo is susceptible to relocation and that once activated it exerts an inhibitory influence that diminishes with distance.

Other experimental studies entailing embryo bisection or aggregation clearly

show that specification of the OA axis is provisional until shortly before gastrulation. Thus, both halves of 16-cell stages that were bisected meridionally could form plutei with essentially normal bilateral symmetry. Significantly, by vitally staining the newly exposed surfaces, reversal of polarity of this axis in one half could often be demonstrated [reviewed in Horstadius (1973)]. When meridional halves are exchanged between different embryos with their AV axis in the same orientation, complete regulation commonly occurs despite the fact that the halves will often be disparate with respect to the direction of their OA axis. This suggests, in accordance with the notion developed from stretching or constricting eggs, that establishment of a ventral side suppresses ventral development elsewhere unless, as when eggs are stretched or constricted or embryos aggregated, part of the surface is placed beyond its sphere of influence (Horstadius, 1973). Aggregation of pairs of 32-cell stages after they have been opened out so that both have their AV axis in the same orientation can result in the entire spectrum of outcomes from normal larvae through to those with partially or completely duplicated axes. Hence, the lesson from sea urchins that needs to be borne in mind when considering the situation in mammals is that the possession of marked regulative ability does not constitute a valid basis for discounting early specification of axes in normal unperturbed development.

II. Terminology

To minimize confusion in the following pages, it is worth dwelling briefly on this issue before embarking on a detailed consideration of the specification of axes in mammals. The preimplantation phase of development in mammals is primarily concerned with the precocious differentiation of two issues, the trophectoderm and the primitive endoderm or hypoblast, which make no cellular contribution to the fetus itself. Even the persisting cluster of pluripotent primitive ectoderm or epiblast cells from which the fetus eventually originates also gives rise to additional extraembryonic tissues following implantation (Gardner, 1985). Hence, in mammals, a considerable interval occurs following fertilization before the process of embryogenesis proper begins. Nevertheless, there is an almost universal tendency for workers in the field to refer to all stages of mammalian development, whether pre- or postimplantation, as embryos on the implicit understanding that the specific meaning depends on the particular stage of development to which the term is applied. However, this often necessitates prefixing embryo with "definitive" to distinguish the nascent fetus from its various extraembryonic components. Concerted efforts to redress such ambiguities in terminology in the United Kingdom were motivated by debate on the use of early stages of human development for research purposes rather than the need to overcome confusion among workers in the field. Thus, the argument that the use of preprimitive streak stages was acceptable because organization of the future individual had not yet

begun was liable to be undermined by referring to the developing entity as an embryo before this. Hence, less emotive appellations like proembryo or preembryo were suggested for these stages, but neither has been adopted universally.

Other recommendations for a more comprehensive revision of terminology in early mammalian development have been advocated since then (e.g., Johnson, 1996; Johnson and Selwood, 1996), but with little impact so far. In addressing the topic of this chapter, it is necessary to differentiate unequivocally between the axes and polarity of the fetus and of the entire entity derived from the fertilized egg. To achieve this distinction, I have chosen to follow Smith (1980, 1985) in using the time-honored term "conceptus" to denote the sum of total embryonic and extraembryonic tissues or their precursors at all stages of pre- and postimplantation development except the fertilized egg prior to cleavage, for which "zygote" is reserved. As will emerge later, it is a major theme of this review that the conceptus has both axes and polarities that are independent of those of the fetus and that may, indeed, specify the latter.

A second area of potential confusion relates to the two tissues of the inner cell mass (ICM) in the late blastocyst. The deep or inner fetal precursor tissue is variously known as the primary ectoderm, the primitive ectoderm, or, in view of its established similarity in developmental potential to its presumed homologue in the chick, the epiblast. Because epiblast now seems to be the preferred name for this tissue, I have used it here. However, there seems to be a persisting reluctance to be consistent and thus apply the term hypoblast to the adjacent endoderm, which continues to be referred to most widely as primitive endoderm, a practice to which I have adhered here.

III. Conventional View of Specification of Axes during Early Development in the Mouse

Both the mature oocyte and the zygote are recognizably polar cells, principally because of the very asymmetrical cell divisions during meiosis and the associated mosaicism in both the membrane and the cortex [reviewed in Gardner (1996a)]. While at least some of these features appear to be induced by the proximity of chromosomes that are not contained within a nuclear membrane, this does not seem to be true of all. Thus, an axially restricted and radially symmetrical arrangement of mitochondria has been observed in the mature oocyte by confocal microscopy (Calarco, 1995). According to the location of surviving polar bodies, first cleavage is generally regarded as meridional. While clearly it is not invariably parallel to the AV axis of the zygote, a claim that it bears no consistent relationship to this axis (Evsikov *et al.*, 1994) fails to account for the fact that surviving polar bodies typically lie in the interblastomeric groove at the two-cell stage. Rotation of one $\frac{1}{2}$ blastomere through roughly a right angle with respect to the other, either during or immediately following the second cleavage, accounts

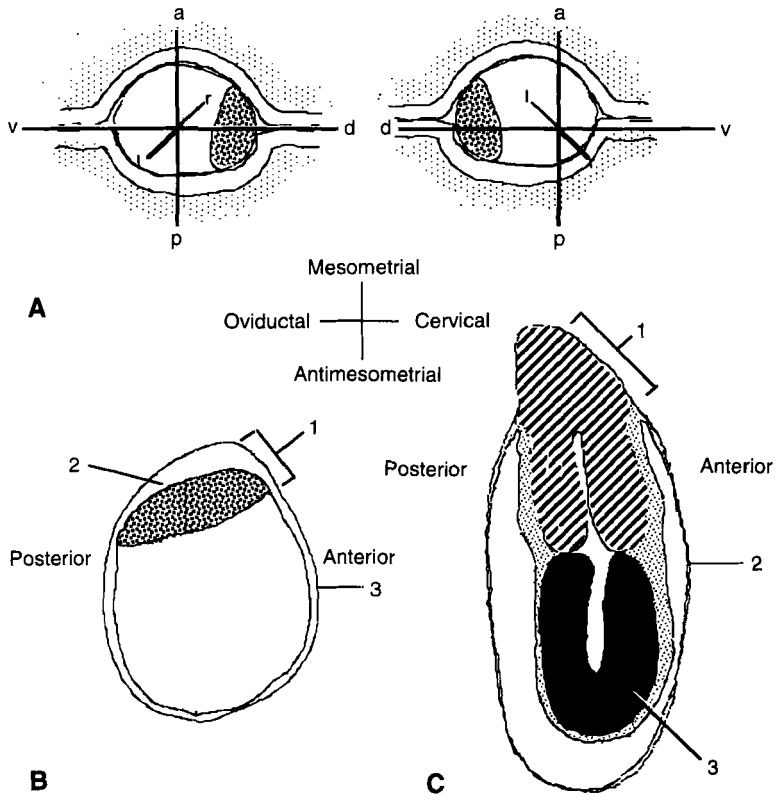

Fig. 1 Diagrammatic summary of Smith's (1980, 1985) observations on asymmetry of the blastocyst and early postimplantation conceptus based on specimens that were sectioned serially *in utero*. All conceptuses are oriented correctly relative to the uterine axes shown in the middle of the figure. (A) Unattached blastocysts showing the two opposing orientations of their sets of axes relative to dorsoventral (mesometrial–antimesometrial) and anterior–posterior (oviductal–cervical) axes of the uterine horn. (B) Early-implanting blastocyst oriented with its Em–Ab axis parallel to the mesometrial axis of the uterus showing more marked bilateral symmetry. Thus, the anterior surface is distinguishable from the posterior by (1) obvious upward tilting of the PT–ICM complex, (2) greater thickness of the PT, and (3) bulging outward of the mural trophectoderm. (C) Postimplantation conceptus showing asymmetries similar to those of the implanting blastocyst in B, the most obvious of which (1) is tilting of the ectoplacental cone and proximal region of the egg-cylinder. Outward bulging of the parietal yolk sac (2), and displacement of the distal tip of the egg cylinder toward the side where the ectoplacental cone is farthest from the Ab pole (3) were also recorded by Smith (1980, 1985). Legend: heavy stippling, ICM; light stippling, visceral endoderm; cross-hatching, polar trophectoderm derived ectoplacental cone (projecting dorsally) and extraembryonic ectoderm.

for the fact that blastomeres often show a tetrahedral arrangement at the four-cell stage. Because of both this arrangement and the onset of compaction after the third cleavage, it is very difficult to determine how cleavage planes relate to the

AV axis of the egg beyond the second cleavage, although Gulyas (1975) has concluded that the third cleavage is equatorial. Once compaction has occurred in the latter part of the eight-cell stage, the conceptus is regarded as a spherically symmetrical structure until the gradual accumulation of fluid, first intracellularly and then eccentrically and extracellularly (Garbutt *et al.*, 1987; Aziz and Alexandre, 1991), heralds the beginning of the blastocyst stage. Once this fluid has been consolidated in a single cavity, the conceptus is once more a clearly polarized entity, with the focal attachment of its internal ICM cells defining its so-called embryonic (Em) pole and the blastocoele its abembryonic (Ab) pole (Fig. 1). How this axis of polarity is specified remains obscure (Surani and Barton, 1984; Garbutt *et al.*, 1987; Aziz and Alexandre, 1991) but is of interest because it endures throughout the remainder of development, and the early fetus's ventral surface is invariably directed toward the Ab pole and its dorsal surface toward the Em pole.

The conceptus is generally regarded as being radially symmetrical about its Em–Ab axis until gastrulation when bilaterality is imposed with the formation of the primitive streak (PS). Various earlier studies hinted at the possibility that asymmetry may be discernible morphologically in the presumptive embryonic region shortly before the primitive streak forms in the mouse (e.g., Bonnevie, 1950), but, unlike in the rabbit (Viebahn *et al.*, 1995), this is neither obvious nor consistent. However, as will be discussed later (Section IX), the patterns of expression of several genes lend support to the notion that breaking of radial symmetry occurs in the embryonic region before gastrulation in the mouse.

To summarize, according to the conventional view the mouse is unusual among the metozoa whose embryology has been studied in detail in that the DV axis of the embryo is defined, if not specified, well before its AP axis. It is assumed that the locus of the primitive streak is specified only shortly before it becomes evident morphologically (e.g., Rogers and Varmuza, 1996), although the questions how and when have not yet been addressed experimentally at either the cellular or molecular level. One consistent finding is that the AP axis of the early fetus is approximately transverse to the long axis of the uterus (Snell and Stevens, 1966). Because mouse conceptuses explanted before implantation can yield morphologically normal-looking early somite stage fetuses *in vitro* (Hsu *et al.*, 1974), the maternal uterine environment clearly is not required to induce the formation of a primitive streak. It might, nonetheless, normally provide cues for specifying its location.

Thus, the notion is accepted that specification of the DV axis of the fetus depends on an asymmetry that is established in the conceptus before the epiblast even forms, let alone embarks on embryogenesis. It is therefore curious why serious consideration has not been given to the possibility that other fetal axes might also be specified early, particularly in view of the growing body of evidence that the conceptus develops additional asymmetries before it implants.

IV. Doubts about the Conventional View

Smith (1980, 1985) was the first to challenge seriously the view that the mouse conceptus remains radially symmetrical about its Em–Ab axis until the onset of gastrulation. From a very detailed analysis of material sectioned *in utero* in which the problem of preparative artifacts was both recognized and addressed, she concluded that the blastocyst is already bilaterally symmetrical before it implants. The analysis is complicated because, by the time departure from radial symmetry is clearly discernible, the blastocyst has established an intimate relationship with the uterus that shows complementary asymmetries. Immediately prior to attachment to the uterine epithelium, the blastocyst still lies with its Em–Ab axis roughly parallel to the floor of the uterine lumen and aligned with the long axis of the latter. As discussed earlier, the Em–Ab axis of the blastocyst corresponds with the DV axis of the future fetus. At this juncture, Smith (1980) recognizes two additional axes that are perpendicular to both this axis and to each other (see Fig. 1A). One is parallel to the mesometrial–antimesometrial (DV) axis of the uterus, and the other is roughly parallel to its left–right axis. Similar but more pronounced asymmetries are apparent in blastocysts at 4.5 days postcoitum (dpc) when implantation is well advanced, leading Smith to conclude that all three axes are conserved as the blastocyst attaches to the investing uterine crypt and then become reoriented with the embryonic pole directed mesometrially.

Several consistent features of blastocysts sectioned *in utero* led Smith (1980) to conclude that they were bilaterally rather than radially symmetrical orthogonal to their Em–Ab axis by the onset of implantation. The first, and most notable, was that the polar trophectoderm–ICM (PT–ICM) complex was typically tilted in relation to this axis. The second was that the surface of the PT–ICM complex appeared wedge-shaped, being blunter where it was farthest from the abembryonic pole than where it was closest to it. The third feature was the shape of the mural trophectoderm. This also differed in correspondence with the direction of the tilt of the PT–ICM complex, often appearing more rounded on the side of the blastocyst where this complex was farthest from the abembryonic pole than on the side where the complex was closest to this pole. Of these three features, which are illustrated schematically in Fig. 1B, tilting of the PT–ICM complex was both the most conspicuous and the most consistently observed. Collectively, they serve to define an obviously polarized axis of bilateral symmetry in the blastocyst. In extending her analysis, Smith (1980, 1985) observed asymmetries in successive postimplantation stages of precisely the sort to which those of the late blastocyst would be expected to give rise. The most conspicuous was consistent tilting of the ectoplacental cone (epc) and proximal region of the egg cylinder with respect to the Em–Ab axis of the remainder of the conceptus (Fig. 1C). Less frequently, the parietal yolk sac appeared more rounded on the

side opposite the direction of tilt than that toward it. Additionally, the distal tip of the egg cylinder was sometimes clearly not located centrally in the yolk cavity, but displaced in the direction opposite the proximal tilt.

Obvious tilting of the proximal region of the postimplantation conceptus persists well into gastrulation, thereby enabling the relationship between the axis of bilateral symmetry of the conceptus and the AP axis of the nascent fetus to be determined ((Fig. 1C). Smith concluded from her examination of appropriately staged material sectioned *in utero* that the two axes have not only a common orientation but also the same polarity, with the primitive streak (PS) consistently lying at the side of the epiblast to which the proximal tilt of the conceptus is directed. It is for this reason that she names the axis of bilateral symmetry of the conceptus its AP axis and the end of this axis toward which the tilt is directed its posterior. Obviously, if the fetus and conceptus share both common AP and DV axes, their RL axes must also be the same, and, accordingly, the third axis of the conceptus is so named by Smith (1980). The implication of this body of work is that all three axes of the fetus are anticipated by the establishment within the conceptus of a corresponding three-dimensional system of positional information more than 2.5 days before the PS first appears (Fig. 1).

In view of the implications of Smith's work for both the timing and mode of specification of the AP axis of the fetus, it is appropriate to attempt to evaluate her findings in some detail. Smith's conclusion that all three axes of the fetus correspond with those of the conceptus depends on her claim that the primitive streak is aligned with the posterior surface of the conceptus. Because the conceptus' posterior is the aspect toward which the epc–proximal region of the egg cylinder is tilted, such a relationship should, in principle, be relatively simple to determine. In practice, however, this can only be done reliably on material sectioned *in utero* where sectioning is strictly frontal with respect to the uterus. Because no uterine lumen is present in close proximity to conceptuses at this stage, deviations from this plane are less readily detected than at implantation. It was for this reason that the relationship was checked on conceptuses dissected from the uterus, from which the parietal yolk sac had been reflected before the direction of tilt was assessed. Tilting of the proximal region relative to the distal was routinely discernible in such conceptuses recovered early in gastrulation (Gardner *et al.*, 1992). This showed that such asymmetry is a feature of the conceptus itself and is not simply a consequence of its being reversibly deformed by the surrounding uterine tissue. Once the posterior surface had been determined, it was marked by injecting horseradish peroxidase into visceral endoderm cells before the conceptuses were sectioned transversely to their Em–Ab axis so that the circumferential relationship between the PS and the posterior of the conceptus could be established by computer-aided reconstruction. It was thus found that whereas AP axes of fetus and conceptus shared a common orientation, they were as often of the opposite as the same polarity (Gardner *et al.*, 1992). Hence, according to these marking experiments, the so-called AP axis of the

conceptus may specify only the orientation of the corresponding axis of the fetus and not its polarity.

The striking disparity between the findings on the relationship between the AP axes of the conceptus and fetus deduced from the marking experiments on recovered specimens versus those sectioned *in utero* has yet to be explained. To ensure that the circumferential position of the base of the tilt could be localized as accurately as possible, only those conceptuses whose proximal region was conspicuously tilted were used in the marking experiments. However, because these amounted to about two-thirds of the conceptuses recovered, they cannot be dismissed as an unrepresentative minority. As noted earlier, it is undoubtedly easier to determine the direction of the tilt in isolated conceptuses than in specimens sectioned *in utero* at the early gastrula stage because of the difficulty of detecting deviations in the plane of sectioning. However, were this the problem, one would expect Smith to have failed to find any consistent relationship between the two axes rather than to identify one that differed systematically from that found in the marking experiments.

While this issue clearly warrants further investigation, the two studies concur in showing that the AP axes of the fetus and conceptus have a common orientation. Obviously, if the two axes also have the same polarity, then, in principle, all three axes of the fetus could be specified at or before implantation. If not, the specific choice between two possible sites for the location of the PS, and the consequent specification of the fetus' left versus right, would have to depend on other factors, which might be either intrinsic or extrinsic to the conceptus. Either way, it is pertinent to examine in greater detail the evidence regarding both the origin and conservation of the AP axis of the conceptus. This obviously requires trying to determine whether the establishment of an axis of bilateral symmetry in the conceptus at the blastocyst stage can be disentangled from the process of implantation.

Subsequent to Smith's (1980, 1985) work, observations on living blastocysts examined both immediately upon recovery from the uterus and after a period of culture *in vitro* established that their bilateral symmetry was not simply due to their being deformed *in utero*. Obvious tilting of the PT–ICM complex was consistently discernible not only in blastocysts recovered during implantation but in those from females in which implantation had been prevented by ovariectomy, even after such delayed-implant blastocysts had been cultured for up to 20 hr following recovery from the uterus (Gardner, 1990). However, differences in shape of the mural trophectoderm between where the PT–ICM was farthest from versus closest to the abembryonic pole were not always obvious in the living blastocysts, sometimes appearing the opposite of what Smith (1980) found *in utero* [see Fig. 2A in Gardner (1990)]. Furthermore, the PT–ICM complex was not found to be obviously wedge-shaped in any blastocysts recovered from the uterus, even when examined shortly thereafter. However, an additional feature of bilateral symmetry was recorded routinely in blastocysts that were flushed from

the uterus after they had begun to implant. This was that the proximal boundary of morphologically transformed mural trophectoderm (Dickson, 1963) was closest to the PT/ICM complex at what Smith (1980) defines as the posterior surface of the blastocyst and farthest from it at the opposite, anterior surface [e.g., see Fig. 1B in Gardner *et al.* (1992)]. This process of morphological giant transformation of the mural trophectoderm, whose significance remains obscure, invariably begins at the abembryonic pole in normal circumstances. However, evidence that this site is not predetermined was obtained from the results of short-term experiments in which second ICMs were transplanted into blastocysts so as to remain separate from host ones. Here it was found that, regardless of the location of the donor ICM relative to the host one, giant transformation always began at the site that was most remote from both (Gardner, 1977, and unpublished observations). This implies that tilting of the PT–ICM complex in advanced blastocysts is a consequence of a shift in position of the embryonic rather than the abembryonic pole.

It is therefore evident, particularly from the observations on *ex utero* delayed-implant blastocysts, that the conceptus has developed a fairly stable axis of bilateral symmetry before it implants. Two questions immediately stem from this finding. First, is this axis conserved through to gastrulation? Second, how is it specified in the first place? At present, there is little that can be said about the first question because the marking experiments on which a definitive answer must depend have yet to be completed. Nevertheless, considering how profoundly bilateral symmetry affects the structure of the early-implanting blastocyst, it is difficult to envisage how similar tilting of the derivative of the PT–ICM complex could occur in a different direction once the conceptus had become closely invested by uterine tissue. Hence, although still unproven, it is nevertheless very likely that the AP axis of the conceptus is conserved between the blastocyst and the gastrula stage.

Regarding the second question, Smith (1980), in accepting the prevailing notion that the origin of bilateral symmetry could not be rooted in stable heterogeneities in the cytoplasm of the egg, looked to asymmetries in the uterine environment as a source cues for its generation. Whatever information the uterus might provide, evidently it cannot be very specific because, as noted earlier, preimplantation conceptuses are able to develop into normal-looking early somite stages *in vitro* (Hsu *et al.*, 1974). Prior to implantation, blastocysts sectioned *in utero* have their Em–Ab axis horizontal (McLaren, 1970; Kirby, 1971; Smith, 1980), arguing that they move along the horns in this orientation during their initial spacing. According to Smith (1980), during this period the Em–Ab is also parallel to the long axis of the horn with either of its poles leading and with the future posterior surface of the conceptus downmost and its anterior surface uppermost. Clearly, there is no prospect of this differentiation being imposed on blastocysts by the different luminal surfaces of the uterus to which their opposing sides are exposed, if they are free to rotate about their Em–Ab axis during this

period. In discussing how rotation might be avoided, Smith (1980) draws attention to two morphological studies, one in the mouse and the other in the rat, in which flattening of blastocysts along one axis had been noted. Whereas the occurrence of flattening is simply stated without the provision of any details in the mouse study (Enders, 1971), it is supported by detailed measurements on blastocysts sectioned *in utero* in the rat (Huber, 1915). Specifically, Huber concluded that preimplantation rat blastocysts are normally oval rather than circular in profile orthogonal to the Em–Ab axis. Given that blastocysts are closely invested by the luminal epithelium even during the preattachment period when they are being distributed along the uterine horns (Potts, 1966; Reinius, 1967; Enders and Schlafke, 1969), such flattening might well restrict their rotation. The shape of blastocysts about their Em–Ab axis is a subject that will be discussed further.

As noted earlier, Smith's choice of heterogeneity of the uterine environment to account for the origin of bilateral symmetry of the blastocyst rests on the assumption that its dependence on determinants in the egg can be discounted by the large body of data obtained from various experimental embryological manipulations on preimplantation conceptuses in mammals. However, as discussed at length elsewhere (Gardner, 1996a), all such experiments have been concerned with elucidating the basis of diversification of cells during preimplantation development rather than facets of patterning such as the specification of axes. When the case for discounting the egg from playing a role in patterning is examined in detail, it is far from compelling. While it is not appropriate to rehearse the various arguments here, one point is worth emphasizing: none of the experimental manipulations undertaken have been carried out in such a way as to exclude the possibility that retention of specific regions of the cortex or cytoplasm of the egg is required for normal development. This is largely because living mammalian eggs, unlike those of many other species, appear to be devoid of regional cytoplasmic differences that could serve as reference points in such studies. However, the reason for not dismissing the notion that the origin of bilateral symmetry of the conceptus is rooted in egg organization is no longer simply that the evidence against it is not compelling. In the next section, new data will be discussed that directly support such a possibility.

V. Relationship between Axes in the Zygote and Early Blastocyst

Throughout the metazoa, the animal pole of the egg is defined as the site where the polar bodies (PBs) produced during each of the two meiotic divisions are formed (e.g., Balinsky, 1970). The diametrically opposite point on the surface of the egg is termed the vegetal pole because this is where yolk is concentrated in eggs, which, unlike those of mammals, are well-endowed with such nutritive

material. The line extending between these poles is therefore known as the egg's animal–vegetal (AV) axis. Hence, as a result of the peripheral migration of the meiotic apparatus and the consequent very unequal partitioning of the egg mass at each meiotic division, mature eggs are polarized cells regardless of whether they contain localized yolk. Therefore, provided that polar bodies remained at their site of production, they could serve as markers of the axis of polarity of the egg for as long as they survived. The possibility of exploiting PBs for this purpose in mammals seems to have been neglected for two reasons. First, their survival is held to be limited (e.g., Tarkowski and Wroblewska, 1967), even though in an early morphological study of mouse development more than one-half of a limited series of living morulae and early blastocysts were found to possess one intact PB, presumably the second (Lewis and Wright, 1935). Second, it was concluded from time-lapse cinemicrographic recordings in several mammals that PBs move about on the surface of the conceptus during cleavage (e.g., Borghese and Cassini, 1963). However, neither objection appeared justified from a study of preimplantation stages of development in mice of the PO (Pathology, Oxford) closed-bred albino strain (Gardner, 1997). Almost two-thirds of early blastocysts in this strain had at least one intact PB which, according to its pattern of chromatin staining, was usually the second. Most interestingly, the distribution of both intact and degenerated PBs in relation to the Em–Ab axis of such blastocysts was highly nonrandom, with the majority being in the midregion at or close to the junction between the polar and mural trophectoderm. With the additional finding that living early blastocysts are typically oval rather than round when viewed along this axis came the discovery that the distribution of PBs was even more tightly circumscribed (Gardner, 1997). Thus, not only did most PBs lie at or close to the junction between the polar and mural trophectoderm, but they also were typically aligned with the blastocyst's greater diameter perpendicular to its Em–Ab axis (Fig. 2).

Such a strikingly nonrandom distribution of PBs in early blastocysts is very difficult to reconcile with the claim that these bodies move about on the surface of the conceptus during cleavage and, indeed, attempts to detect their movement using blastomere labeling and other approaches yielded negative results. Further evidence against their being free to move came from examining how they were attached to the conceptus. At all stages between the zygote and early blastocyst, PBs did not merely adhere to conceptuses but were bound to them by a very thin, extensible, weakly elastic, tether (Gardner, 1997). From both its appearance and properties and the finding that dye transfer could occur routinely between conceptus and PB at the zygote stage, this tether was most likely to be the persisting intercellular bridge formed when the second PB was abstricted. While dye transfer was observed only exceptionally beyond the zygote stage, ionic coupling between the PB and conceptus could still be detected in a proportion of cases even in early blastocysts (Gardner, 1997). Collectively, these various morphological and experimental findings are most readily explained by persisting attach-

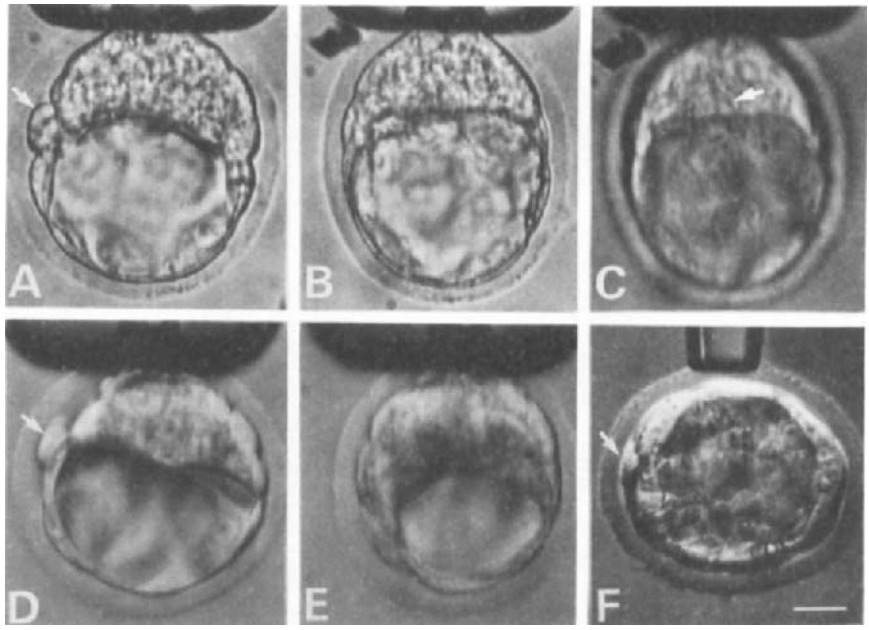

Fig. 2 Photographs showing flattening of early blastocysts about their Em–Ab axis and alignment of the polar body with greater diameter (GD). (A) Side view of a blastocyst with its GD horizontal. Note that the polar body lies at the junction between the polar and mural trophectoderm in the plane of the GD. (B) Same blastocyst as in A rotated through ca. 90° around its Em–Ab axis with the focus on the midplane to show its lesser diameter (LD). (C) Same blastocyst as in A and B with focus on the polar body (arrow). (D, E) Side view of another early blastocyst showing the GD in D and the LD in E. Again, the polar body (arrow in E) lies at the polar–mural junction and is aligned with the GD. (F) View of a third early blastocyst from the Em pole showing its oval shape and alignment of the polar body with the GD (from Gardner, 1997).

ment of the second PB to the conceptus via the intercellular bridge that formed during its production. That intercellular bridges between sister blastomeres can retain patency for unusually long periods during early development in the mouse is evident from other studies on preimplantation conceptuses in this species (see Goodall and Johnson, 1984). If the continuing attachment of the second PB is mediated in this way, it means that this body remains at its site of production following sperm penetration right through to the blastocyst stage. Obviously, this is contrary to the conclusion drawn from various time-lapse cinemicrographic studies. It should be noted, however, that although they show that PBs are capable of undergoing dramatic changes in shape, such studies provide no compelling evidence that they actually move relative to the surface of the conceptus during cleavage [see, for example, Borghese and Cassini (1963)]. Rather, the periods of intense membrane activity of PBs may reflect the activity of a cyto-

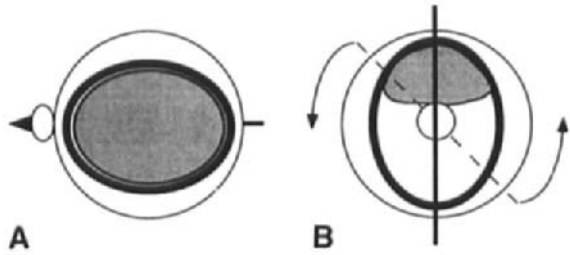

Fig. 3 Diagrams illustrating how, using the second polar body as a marker, the axes of the early blastocyst (thick outline) map onto the zygote (thin outline). (A) View from the blastocyst's Em pole showing alignment of its bilateral axis (GD), which is not discernibly polarized at this stage, with the AV axis of the zygote (thick line with outline of a polar body and an arrowhead denoting the animal pole). (B) View from the zygote's animal pole showing that not only is the Em–Ab axis of the blastocyst (continuous thick line) orthogonal to its AV axis but, according to the results of lineage labeling ½ blastomeres, bears no consistent relationship to the first cleavage plane (dashed line).

plasmic clock that has been implicated in the timing of successive cleavages (Waksmundzka *et al.*, 1984).

If the second PB is not free to move about on the surface of the conceptus but remains tethered at its site of production, it provides an enduring marker of the animal pole of the zygote. This makes it possible to determine whether there is any regularity in the way in which the AV axis of the zygote maps onto the structure of the blastocyst. Such a regularity emerged with the finding noted earlier, which was anticipated by Huber's (1915) observations on material sectioned *in utero* in the rat that, whether freshly recovered from the uterus or grown *in vitro* from the two- to four-cell stage, living early blastocysts are bilaterally rather than radially symmetrical about their Em–Ab axis in the mouse. It seems most unlikely to be fortuitous that the axis of bilateral symmetry of the early mouse blastocyst should typically be aligned with the AV axis of the zygote (Fig. 3A). It is therefore difficult to avoid the conclusion that the establishment of this axis depends on information that is present in the zygote before it embarks on cleavage. While both the nature and significance of such information have yet to be determined, there are several additional points that can be usefully considered at this juncture.

First, this study extends Smith's work in showing that bilateral symmetry is already evident at the early blastocyst stage, even in conceptuses that were grown *in vitro* from early cleavage and therefore were not exposed to the uterine environment. This finding, like those of Hsu *et al.* (1974) discussed earlier, argues that if the development of bilateral symmetry does depend on environmental factors, they cannot be specific to the female reproductive tract. However, the bilateral axis of the early blastocyst differs from that of the later one in that it is not polarized. With expansion of the blastocoele, this early bilateral axis is no longer discernible as blastocysts temporarily become circular rather than oval about their Em–Ab axis.

It has yet to be established whether the orientation of the bilateral axis is nonetheless conserved between the early and late blastocyst stages. If this is found to be the case, it will be interesting to know whether the polarity of the AP axis of the conceptus is fixed in relation to the AV axis of the zygote. If it is not, then it is conceivable that the polarity of the AP axis of the fetus rather than the conceptus may be specified by the AV axis of the zygote. The shape of conceptuses, though variable, can seldom be described as spherically symmetrical before the blastocyst stage. Precompacted eight-cell stages are typically nonspherical (see Graham and Deussen, 1978), and morulae frequently appear somewhat flattened (R. L. Gardner, unpublished observations). Were this flattening to correspond with both the plane of first cleavage and that of the axis of bilateral symmetry of the blastocyst, the boundary between the clonal descendants of a labeled $\frac{1}{2}$ blastomere and its unlabeled partner should consistently be parallel to the Em–Ab axis of the blastocyst (see Fig. 3B). No such regularity in the orientation of this boundary has been observed (Gardner, 1997, and unpublished observations).

Obviously, further work is needed to determine the significance of the finding that the axis of bilateral symmetry of the early blastocyst is aligned with the axis of polarity of the zygote. One possibility is that establishment of the bilateral axis is independent of axial information in the zygote, with the latter simply providing enduring cues that serve to orient it during normal undisturbed development. If such zygotic cues were perturbed or negated as a result of the experimental manipulation of conceptuses during cleavage, the bilateral axis would still form with its orientation dictated either randomly or by other, possibly environmental, cues. Examples of the regulation of axes whose orientation is normally specified very early in development following experimental manipulation at a later stage can be found in various metazoan species including, as discussed earlier, the highly regulative embryos of sea urchins (see Section II). Another possibility is that information in the zygote is essential for both the establishment and orientation of bilateral symmetry of the blastocyst. Such a possibility might seem very unlikely given the very considerable capacity of preimplantation stage mouse conceptuses to regulate their development following the gain, loss, or rearrangement of blastomeres. Indeed, as noted earlier, this dramatic regulative capacity is the principal reason why a developmental role for egg organization has generally been discounted in mammals. The argument here, which warrants closer examination, would be that axial information should be missing from partial conceptuses and both multiplied and malaligned in composites formed by morula aggregation.

Regarding partial conceptuses derived from single blastomeres or incomplete sets of blastomeres, the critical consideration is whether information from all or only part of the AV axis of the zygote is required for normal development (Gardner, 1996a). Because of the apparent lack of an enduring marker of this axis, no studies on the developmental potential of isolated blastomeres that bear on this question have been undertaken so far. If Gulyas (1975) is correct in asserting

that the first two cleavages are meridional and the third is equatorial in mammals, notwithstanding the rotation of one ½ blastomere before or during second cleavage, then the eight-cell will be the first stage when no individual blastomeres will be endowed with cytoplasm from all axial levels of the zygote. That normal development has never been obtained from individual ⅛ blastomeres in the mouse is generally attributed to their inability to produce sufficient cells before blastulation to form blastocysts rather than trophectodermal vesicles (Tarkowski and Wroblewska, 1967). However, even in species like the rabbit, sheep, goat, and cow in which conceptuses attain a sufficiently high cell number before blastulation to enable ⅛ blastomeres to form blastocysts, only a minority continue to develop normally thereafter (Prather and Robl, 1991). The fact that any do so argues against the notion that material from all axial levels of the zygote must be present, provided, as noted earlier, that the third cleavage is consistently equatorial with respect to the AV axis of the zygote. What such studies leave unanswered is whether animal ⅛ blastomeres differ from vegetal in the extent to which they are able to continue to develop in isolation. In this context, it would be interesting to determine whether the second PB could also be exploited as an enduring marker of the animal pole of the zygote in these other more tractable mammals.

Although offspring obtained from aggregates of two or more morulae are almost invariably entirely normal, it is noteworthy that a substantial proportion are nonchimeric (Mullen and Whitten, 1971). Very little information exists regarding the fate of cells of the other morula(e) in such cases. In addition, from the limited data on comparison of the rate of development of chimeric and standard conceptuses of the same genotype, the performance of chimeras may be inferior, despite their markedly enhanced cell number (Gardner, 1996a). Hence, there are at least indications that the development of composite embryos is not necessarily an entirely harmonious process and may be accompanied by substantial loss or displacement of cells of one genotype as well as reduced fitness. In focusing more specifically on the consequences of having two or more sets of axial determinants within a composite conceptus, several points need to be borne in mind. First, most of the relatively limited prenatal studies on morula aggregation chimeras have concentrated on the immediate postimplantation period prior to gastrulation when size regulation occurs. Nevertheless, were partial (conjoined or siamese) or complete twins within a common amnion regular features of the development of morula aggregation chimeras, they should have been observed. However, the presence of axial patterning information in the zygote is not precluded by the failure to detect such anomalies. As discussed in Section II, it is clear from studies on other developmental systems that the presence of two conflicting sets of axial information need not lead inexorably to axial duplication (Horstadius, 1973; Azar and Eyal-Giladi, 1981). Where more than one set of information is present, either assimilation or inhibition may occur, thereby ensuring that only a single axis is formed. Polembryony can be obtained comparatively

easily in the avian embryo in which the epiblast is relatively large and flat at the blastoderm stage (Lutz, 1948; Waddington, 1952). Given the comparatively small size and rounded structure of the mouse conceptus and the fact that even composites are reduced to within the normal size range before gastrulation (Buehr and McLaren, 1974; Lewis and Rossant, 1982; Rands, 1985, 1986), assimilation or inhibition would only need to operate over small distances to enable the formation of supernumerary axes to be suppressed. A further peculiarity of mammals that might assist any necessary remodeling is that cell death is a normal facet of development prior to gastrulation (El-Shershaby and Hinchliffe, 1974; Handyside and Hunter, 1986; Hardy *et al.*, 1989; Pierce *et al.*, 1989). However, it would be idle to carry such speculation further at this juncture. The point to be made is that there are mechanisms that are known to operate in other developmental systems that could account for axial regulation in mammals even if egg organization were a *sine qua non* for the establishment of one or more axes.

A noteworthy finding is that exposure of cleaving mouse embryos to lithium (LI^+) can perturb aspects of patterning of the nascent fetus (Rogers and Varmuza, 1996). Effects of this ion on development have been documented for many years, particularly in amphibians (Kinoshita and Asashima, 1995) and echinoderms (Ghiglione *et al.*, 1996). In amphibians, exposure of embryos to this monovalent cation during cleavage has become a standard way of dorsalizing larvae. Pertubation of patterning has also been reported in zebrafish embryos following their exposure to Li^+ between the 8- and 400-cell stages (Stachel *et al.*, 1993). In the mouse, exposure of 2- or 8-cell conceptuses to Li^+ at concentrations of up to 90 m*M* for 5 hr or 300 m*M* for as little as 5 min was found to reduce the expression of *brachyury* during gastrulation and result in a range of axial defects thereafter (Rogers and Varmuza, 1996). Although treated conceptuses were claimed to form normal blastocysts, morphological abnormalities clearly were not confined to epiblast derivatives. Starting with the premise that cytoplasmic determinants can be discounted in mammals, the authors argue that the effect of Li^+ may be to cause epigenetically heritable modifications of chromatin that alter the competence of cells to respond to positional cues that arise later. They envisage this operating through the cation's disruption of the inositol pathway (Busa and Gimlich, 1989), thus activating protein kinase C and, thereby, the chromatin-associated topoisomerases I and II. They suggest, furthermore, that the well studied effects of Li^+ on amphibian embryos may also be mediated in this way. However, in stating that . . . "no positional information can be observed in the preimplantation embryos," Rogers and Varmuza (1996) neglect the point discussed elsewhere (Gardner, 1996a) that no systematic attempt has yet been made to look for it during this phase of development. Regardless of how Li^+ acts, the fact that exposure to it early in development results in defects in axial patterning in mammals as well as in lower vertebrates suggests that certain underlying processes are conserved.

VI. Basis of Bilateral Symmetry of the Blastocyst?

Clues about the nature of the information that might be provided by the egg or zygote for the establishment of bilateral symmetry in the blastocyst depend on obtaining a clearer understanding of the basis of the latter. Observations on the growth of the trophectoderm have proved illuminating in this respect. Early studies using blastocyst dissection and reconstitution revealed that whether trophectoderm cells continued to proliferate beyond the blastocyst stage or embarked on giant transformation and polyteny depends on their position in relation to the ICM (Gardner and Beddington, 1988). Thus, by the late blastocyst stage, the increase in cell number in the tissue is confined to its so-called "polar" region overlying the ICM. The fact that nontransformed mural tissue can be converted into polar by placing an ICM inside it argues that the ICM is normally the source of a stimulus that is required to sustain the proliferation of overlying polar trophectoderm cells. The consequence of a decline in growth of the mural trophectoderm relative to the smaller polar region is that there is a net movement of cells from the latter into the former during the later stages of growth of the blastocyst before it implants. Such cell movement was initially deduced from a comparison of cell numbers and mitotic and dead cell indices in the two regions (Copp, 1978) and was subsequently confirmed by cell-labeling experiments (Copp, 1979; Cruz and Pedersen, 1985; Dyce *et al.*, 1987; Gardner, 1996b). What remains contentious is whether the polar trophectoderm grows entirely from the cells of which it was composed at the time of blastulation or by continuing recruitment from the ICM (Gardner and Nichols, 1991; Gardner, 1996b). Regardless, the assumption has been that its growth results in a radially symmetrical flow of cells from the polar to the mural region, so that more recent recruits to the latter lie progressively closer to the ICM and the cells that have been in the mural region the longest lie at the abembryonic pole where giant transformation begins. However, experiments in which horseradish peroxidase (HRP) was used to lineage label either the most central polar trophectoderm cell or a peripheral one produced results that are incompatible with this simple scheme. Thus, whereas the clones formed by labeled central cells were typically displaced toward or into the mural trophectoderm during subsequent culture of the blastocysts, those formed by peripheral polar cells were not. The majority of the latter retained their position, with the remainder being as often displaced toward the central polar region as away from it (Gardner, 1996b).

This pattern of deployment of clones is clearly not consistent with a radially symmetrical flow of cells from the polar to the mural trophectoderm. Rather, it argues for an anisotropic pattern of flow through the restriction of egress of polar cells to only part of the junction between the two subregions of the trophectoderm. This has been substantiated by experiments in progress in which endocytosis of strongly fluorescent microspheres has been used to label selectively the entire polar trophectoderm. Here, following further growth of the blastocysts,

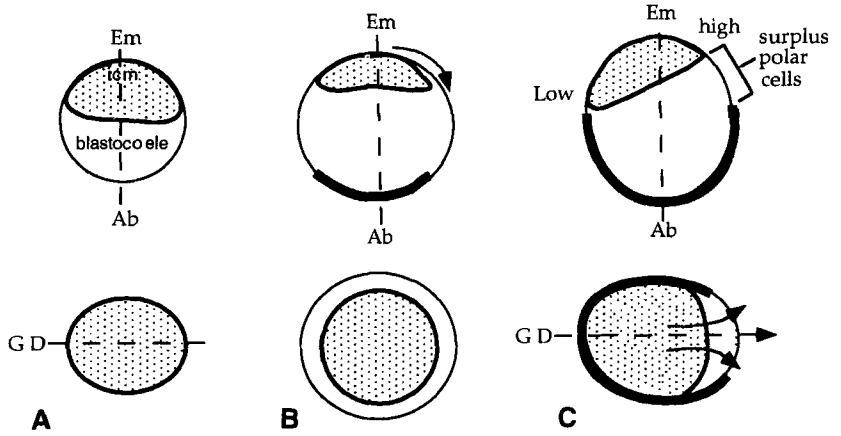

Fig. 4 Diagrammatic representation of early (A), expanding (B), and late (C) blastocysts viewed from the side (upper row) and from the embryonic pole (lower row). The early blastocyst (A) is oval about its Em–Ab axis with its axis of bilateral symmetry, which is not discernibly polarized, as indicated by the dashed line marked GD. As the blastocyst expands it temporarily becomes circular about its Em–Ab axis (B). In the late blastocyst (C), an axis of bilateral symmetry is evident once again, but is now clearly polarized through tilting of the ICM–polar trophectoderm complex relative to the Em–Ab axis. It is proposed that this tilting is due to the anisotropic growth of the polar trophectoderm (Gardner, 1996b) being directed along the bilateral axis, so that surplus cells spread into the mural trophectoderm surrounding the blastocoelic cavity at the blastocyst's anterior surface. Because, as denoted by the difference in line thickness, the region composed of newly recruited cells is more deformable than the remainder of the mural trophectoderm, it is more readily stretched as the blastocoele expands, thereby rotating the anterior surface of the polar trophectoderm and attached ICM toward the embryonic pole.

bead-labeled cells are seen to extend from the polar into the mural trophectoderm in a broad arc whose distal limit may be as much as two-thirds of the way down the latter. Significantly, diametrically opposite where the bead-labeled cells have spread most distally into the mural region, they typically remain confined to the polar trophectoderm (R. L. Gardner, unpublished observations). This argues that egress of cells from the polar region is maintained in the same direction throughout the approximately 24-hr period of additional growth between labeling and scoring. Because the polar trophectoderm was usually still fully labeled at the time of analysis, the possibility that this phenomenon is simply due to relative movement between the ICM and trophectoderm can be discounted. What acts to restrict the flow of cells out of the polar trophectoderm circumferentially is not known, although anchorage of junctional cells via the processes they extend over the blastocoelic surface of the ICM is an obvious possibility (Gardner, 1996b). Of direct interest here is that were this flow aligned with the bilateral axis of the early blastocyst, it would not only argue for conservation of this axis during growth of the blastocyst but could also explain how it becomes polarized. Ac-

cordingly, the aspect of the surface of the blastocyst from which cells flow from the polar to the mural trophectoderm would be its future anterior. As the cells emerge they spread laterally as well as distally. Because trophectoderm cells seem to become less deformable the longer they have resided in the mural region, possibly through their deposition of an extracellular matrix on their blastocoelic surface, the increasing hydrostatic pressure of the blastocoele will tend to push out the region populated by the more recently recruited cells. This could account for all facets of blastocyst asymmetry, including tilting of the PT–ICM complex, the tendency of the anterior surface to be more rounded than the posterior surface, and displacement of later giant transformation toward the posterior (Fig. 4).

To summarize at this juncture, the blastocyst is already bilaterally rather than radially symmetrical from its earliest stage and its bilateral axis is typically aligned with the AV axis of the zygote. Flow of surplus polar cells into the mural trophectoderm that occurs during blastocyst growth is polarized rather than, as was formerly supposed, radially symmetrical. As the blastocyst expands, bilateral symmetry is temporarily superceded by radial symmetry, before reasserting itself in polarized form at the advanced blastocyst stage. The structure most obviously affected by bilateral symmetry in the late blastocyst, the PT–ICM complex, shows the expected asymmetry in its postimplantation derivatives. This asymmetry clearly persists past the beginning of gastrulation, thus enabling the relationship between the bilateral axis of the nascent fetus and the conceptus to be determined.

VII. Axial Relations between Conceptus and Uterus

From her examination of peri-implantation conceptuses sectioned *in utero,* Smith (1980) also concluded that blastocysts initially attach to the luminal epithelium equally frequently in one or other of just two orientations. Thus, about half attach to the left wall of a horn with their abembryonic pole directed toward the cervix and their embryonic pole toward the oviduct (type L orientation), and the remainder attach to the right wall with their abembryonic pole directed toward the oviduct and their embryonic pole toward the cervix (type R orientation). Furthermore, the uterine wall to which the blastocyst attached first exhibited a local concavity that served to differentiate it from the opposite wall, which remained relatively straight. Neither attachment to the left wall with the abembryonic pole toward the oviduct nor attachment to the right wall with the abembryonic pole toward the cervix was observed. That the distinction might be between medial and lateral rather than left and right was discounted because the same two orientations were recorded in both horns. A further conclusion reached by Smith (1980) was that blastocysts implanting in both the type L and type R orientations invariably have their right side toward the wall of the uterus to which

they initially attach. Accordingly, Smith concluded that before axial rotation, fetuses developing from conceptuses implanting in type L mode should have their anterior pointing toward the left side of the horn and posteriorly and those implanting in the type R mode should have their anterior pointing to the right and anteriorly. As discussed earlier, this conclusion is based on the assumption that the polarity of the AP axis of the early fetus and conceptus is invariably the same, which does not seem to be the case (Gardner *et al.*, 1992). If, as the marking experiments suggest that, despite sharing a common orientation, the two axes are as often of the opposite as the same polarity, then fetuses facing left anterior and right posterior should also be expected. So far, however, none of Smith's findings regarding the orientation of implantation have been followed up. Nevertheless, that the AP axis of early fetuses is oriented across rather than along the uterine horns is beyond dispute, although deviations of up to 45° may occur (Snell and Stevens, 1966). The fact that this orientation occurs consistently suggests that it is of significance for the success of development. There are, however, no data bearing on the questions of whether the development of maloriented fetuses is impaired or why this particular orientation is favored when the decidual tissue surrounding each conceptus appears to be radially symmetrical, both morphologically and with regard to the expression of genes that have been shown to be of morphogenetic significance in other contexts (Manova *et al.*, 1992; Albano *et al.*, 1994).

Transcripts of growth factors of the TGFβ family, which have been implicated in mesoderm induction in other vertebrates, have been found to be localized in the decidual tissue surrounding the pre- and early gastrulation mouse conceptus rather than in the conceptus itself (Manova *et al.*, 1992; Albano *et al.*, 1994). The TGFβ2 protein detected in the visceral endoderm (Slager *et al.*, 1991) may therefore, as suggested by Manova *et al.* (1992), have been acquired from the decidua. None of the growth factors whose expression has been detected in decidua have been found to exhibit expression that is restricted circumferentially within them (Albano *et al.*, 1994) and which might therefore provide a local inductive stimulus. Given that the conceptus is surrounded by maternal blood sinusoids, it is difficult to envisage how their protein products could act in a spatially restricted manner, even if their expression were localized in decidua. Thus, whereas a facilitatory or permissive role might be ascribed to such agents, an instructive one in embryonic patterning would seem most unlikely, unless the future embryonic region was already prepatterned in its responsiveness to them. The need to invoke dependence of siting of the streak on uterine cues to account for the orientation of the fetus with respect to the uterus arises from the traditional view that the conceptus is radially symmetrical until the PS forms. Evidence that the conceptus is bilaterally symmetrical before it implants raises the prospect of its attaching in an orientation that ensures that its AP axis, and hence the AP axis of the early fetus, is transverse to the uterus *ab initio*. What is required in this case is circumferential differentiation of the mural trophectoderm through which

initial attachment to the uterus is made. It is noteworthy that initiation of giant transformation is displaced toward the posterior surface of the blastocyst, i.e., where initial attachment to the uterus may occur (Smith, 1980).

VIII. Where Does Information for Specifying the Locus of the PS Reside before Gastrulation? Clues from Epiblast Growth and Molecular Asymmetries prior to Gastrulation

The initial period of development in the mouse is essentially one of coherent clonal growth in which there is very limited cell mixing (Garner and McLaren, 1974; Kelly, 1979; Balakier and Pedersen, 1982). However, it is evident from the extraordinary degree of dispersal and intermingling of clones in chimeras that coherent growth must break down in the epiblast early in, if not before, gastrulation (Gardner, 1986; Soriano and Jaenisch, 1986; Lawson *et al.*, 1991). It has been argued that the epithelial organization adopted by the epiblast shortly after implantation should militate against extensive cell mixing in the tissue prior to gastrulation (Rossant *et al.*, 1983; Rossant, 1985; Gardner, 1986). The only postimplantation chimeras previously analyzed before gastrulation with an *in situ* marker yielded results in accord with this view. However, early mixing is likely to be underestimated in morula aggregation chimeras where the cells of the component genotypes presumably form sizeable coherent patches in the epiblast initially, especially when they are derived from different species of mouse (Rossant *et al.*, 1983; Rossant, 1985). That this is indeed the case is suggested by the finding of Lawson *et al.* (1991) that clonally related cells identified *in situ* by spreading of a macromolecular label between them are frequently apart in the epiblast of immediately preprimitive streak stage conceptuses.

Analysis of chimeras produced by transplanting single founder epiblast cells from blastocysts carrying the ROSA26 transgene to nontransgenic host blastocysts at a range of early postimplantation stages supports a very different conclusion from the foregoing studies on interspecific chimeras. That is, mingling and dispersal of cells begins in the epiblast coincident with its epithelialization and leads to complete dissipation of coherent clonal growth in the tissue by the beginning of gastrulation (Gardner and Cockroft, 1998). Coherent growth was sustained in corresponding clones in the visceral endoderm, except in the embryonic region where the visceral endoderm is replaced by definitive endoderm during gastrulation (Lawson *et al.*, 1986, 1987). This argues that the behavior of clones in the epiblast is a tissue- rather than a genotype-specific phenomenon. Furthermore, *in situ* analysis of the spatial relationship between sister cells identified by spread of an intracellularly injected macromolecular label gave results in accord with the chimera data. Thus, sister cells were usually adjacent to each other in the epiblast on the sixth day when the tissue is transforming from a solid

ball of cells into an epithelium. However, by the seventh day when this transformation is complete, they were frequently well apart before completing the cycle in which they were born. Together, these findings argue that there is such extensive dispersal and intermingling of the clonal descendants of founder cells in the epiblast shortly before gastrulation as to render conservation of positional information within this tissue most unlikely.

In considering where else information for specifying the AP axis of the fetus might reside, the visceral endoderm is an attractive candidate for several reasons. First, it continues to grow coherently until the beginning of gastrulation when, in the embryonic region specifically, it is gradually replaced by definitive fetal endoderm (Gardner, 1984, 1985; Lawson *et al.*, 1986, 1987). Second, it forms a very intimate relationship with the epiblast, which includes specialized close cell contacts between the two tissues via discontinuities in the basal lamina that separates them (Takeuchi and Takeuchi, 1987). Third, its avian counterpart, the hypoblast, has been implicated in specifying the locus of the PS in the chick (Azar & Eyal-Giladi, 1981). Fourth, in parthenogenetic mouse conceptuses the extent of fetal development appears to correlate with the degree of differentiation of the visceral endoderm, which is quite variable in such conceptuses (Sturm *et al.*, 1994). Fifth, the disruption of two genes whose early expression is largely or entirely confined to the primitive or visceral endoderm has been found to perturb subsequent development of the epiblast. Thus, conceptuses that are homozygous for *HNF-4* (Duncan *et al.*, 1994) fail to progress beyond the early primitive streak stage in gastrulation (Chen *et al.*, 1994), evidently because the visceral endoderm does not express several genes that are markers of its advanced differentiation at an appropriate level (Duncan *et al.*, 1997). Homozygosity for disruption of the *Hβ58* gene, whose early expression is also principally in the visceral endoderm, results in gross perturbation of the development of the epiblast during gastrulation without obviously affecting the subsequent differentiation of the visceral endoderm itself (Radice *et al.*, 1991; Lee *et al.*, 1992). Expression of both of these genes appears throughout the visceral endoderm rather than being restricted to part of it and seems to be required for the normal progression of gastrulation rather than its initiation. The situation regarding a third gene, *Evx-1*, whose early expression is purportedly limited to extraembryonic endoderm, is less clear. Its disruption results in very early postimplantation lethality in homozygotes, which, because the mural trophectoderm is also affected, has prompted the conclusion that the extraembryonic endoderm is needed to sustain this tissue as well as the epiblast (Spyropoulos and Capecchi, 1994). This is patently incorrect because trophectoderm vesicles that are completely devoid of ICM tissue form healthy outgrowths *in vitro* and also survive to beyond midgestation *in vivo* (Gardner, 1972; Snow, 1973). Indeed, individual cells from morulae can differentiate directly into typical mural trophoblastic giant cells in complete isolation (Sherman and Atienza-Samols, 1979). Hence, it is likely that this gene is normally expressed in, and necessary for the survival of, the trophectoderm

as well as the extraembryonic endoderm. That *in situ* hybridization does not always reveal the full range of tissues in which a gene is expressed is illustrated particularly clearly by the experience with Krox-20 (Topilko *et al.*, 1994). Finally, as discussed below, the visceral endoderm exhibits both morphological and molecular signs of regional differentiation by, or even before, the beginning of gastrulation that bear a consistent relationship to the location of the future PS.

A growing number of genes whose disruption affects AP patterning of the epiblast have been found to be expressed early in the differentiation of the visceral endoderm. An additional gene that is expressed before gastrulation, *Fgf8*, is of particular interest inasmuch as its transcripts, which are present in the epiblast as well as the endoderm, are restricted to the prospective posterior of the embryo (Crossley and Martin, 1995). An additional intriguing regional differentiation of the visceral endoderm has been revealed at the molecular level using an antiserum against an N-terminal peptide of mature bovine FGF2. Because localized binding of the antiserum was not impaired by depleting its reactivity against the immunogen, it evidently recognizes an epitope that is not present in FGF2 itself. Staining for this VE-1 antigen is first discernible on the sixth day postcoitum when it extends throughout the entire proximodistal extent of the visceral endoderm as a stripe on the prospective anterior surface of the embryo (Rosenquist and Martin, 1995). By the onset of gastrulation, staining is limited to the visceral endoderm of the embryonic region, where it is centered diametrically opposite the PS and extends from the junction with the extraembryonic region to the distal tip of the egg cylinder and about one-third round its circumference. Morphological evidence of differentiation of the prospective anterior of the mammalian embryo before the PS forms has been obtained in the rabbit. Thus, Viebahn and his colleagues (1995) have identified a localized thickening of both the epiblast and visceral endoderm in whole-mount and semithin sagitally sectioned rabbit blastoderms, which lies at the opposite end to where the PS will makes its appearance shortly thereafter. This "anterior marginal crescent," which had been noted in a classical morphological description of rabbit development published a century earlier (Assheton, 1895), is thus the earliest marker of the AP axis of the fetus in this species. Given the relative simplicity of separating and recombining blastoderm tissues in this species compared to the mouse, it is to be hoped that further work on the rabbit may establish unequivocally whether, as in the avian embryo, the primitive endoderm or hypoblast specifies the fetal AP axis in mammals.

That early differentiation of the anterior end of the fetal axis may also be true in the mouse is suggested by various molecular studies. Thus, expression of both *Otx-2* and *Lim-1*, whose disruption has been shown to lead to truncation of the the anterior end of the neuraxis of the fetus (Acampora *et al.*, 1995; Shawlot and Behringer, 1995), is discernible by the onset of gastrulation, specifically in the anterior region of the visceral endoderm overlying the epiblast and in certain of

its derivatives thereafter (see Varlet *et al.*, 1997). Transcripts of *Hesx1/Rpx* (Thomas and Beddington, 1996; Hermesz *et al.*, 1996) are also evident locally in the visceral endoderm by the onset of gastrulation where they show an even more circumscribed distribution. Thus, initially they are evident in a patch of about 20 cells in the anterior region of the tissue lying some five cell diameters below the extraembryonic–embryonic junction before also appearing in the adjacent epiblast. Although the consequences of disrupting this gene have not yet been reported, Thomas and Beddington (1996) have shown that elimination of the early expressing endoderm cells not only impairs subsequent expression of this gene in the epiblast but also perturbs patterning of the anterior CNS. Anterior truncation is also seen in embryos that are homozygous for the 413.d proviral integration (Conlon *et al.*, 1991; Iannaccone *et al.*, 1992), which has been shown to have disrupted *nodal*, a member of the TGFβ family of genes (Conlon *et al.*, 1994; Zhou *et al.*, 1993). However, in this case although the overriding importance of wild-type visceral endoderm rather than epiblast for normal development has been demonstrated by means of ES cell chimeras, the expression of the gene is much weaker in the endoderm than in the epiblast and does not seem to be localized in the former tissue. The slightly more obvious staining in a thickened prospective anterior patch than elsewhere in the tissue is attributed to a higher density of cells rather than a higher concentration of the LacZ reporter protein (Varlet *et al.*, 1997).

In summary, there is a growing body of data that is not only consistent with the possibility that the visceral endoderm may be responsible for specifying the AP axis of the fetus but which suggests that it may do so simultaneously and, to some extent, independently at both ends.

IX. Concluding Remarks

Despite the surge of interest in the early development of mammals, particularly gastrulation, it is not known either when or how any axis of the conceptus or fetus is specified. The very few relevant experimental studies that have been undertaken relate principally to the Em–Ab axis of the blastocyst. Seemingly, because fetal development begins only after a protracted period that is devoted entirely to the production of extraembryonic tissues, it has been assumed that specification of its AP axis cannot depend on information that is localized originally in the egg or zygote and that it begins only shortly before the primitive streak forms. Collectively, the various findings discussed in this chapter provide strong grounds for questioning this view.

Even though early development is clearly directed to the differentiation of extraembryonic tissues, there is no reason to discount the possibility that patterning information residing in the zygote might be partitioned to an extraembryonic tissue, from which it could act later to impose asymmetry on the epiblast. Support for a role for extraembryonic tissues in patterning of the vertebrate embryo

is provided by studies on the hypoblast in birds (Waddington, 1952; Khaner and Eyal-Giladi, 1989) and on the yolk cell in teleosts (Oppenheimer, 1936; Kostamarova, 1968).

However, although making the traditional view of specification of the AP axis of the fetus untenable, recent findings fall far short of providing a coherent picture of what this entails in the mouse or, indeed, in any other mammal. What is needed now is critical and imaginative use of the techniques of experimental embryology to define both when and how the relevant patterning information is established and deployed. Studies at the molecular level are now beginning to provide real insight into certain developmental processes, such as morphogenesis of the vertebrate limb, because they rest on a secure foundation of work at the cell or tissue level. In the case of the limb, this experimental embryological work served to define the location and properties of such vital components of the early bud as the apical ectodermal ridge, the progress zone, and the zone of polarizing activity. Without this foundation, the molecular findings would have been unintelligible.

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3

Maternal Control of Pattern Formation in Early *Caenorhabditis elegans* Embryos

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Genetic screens for recessive, maternal-effect, embryonic-lethal mutations have identified about 25 genes that control early steps of pattern formation in the nematode *Caenorhabditis elegans*. These maternal genes are discussed as belonging to one of three groups. The par group genes establish and maintain polarity in the one-cell zygote in response to sperm entry, defining an anterior/posterior body axis at least in part through interactions with the cytoskeleton mediated by cortically localized proteins. Blastomere identity group genes act downstream of the par group to specify the identities of individual embryonic cells, or blastomeres, using both cell autonomous and non-cell autonomous mechanisms. Requirements for the blastomere identity genes are consistent with previous studies suggesting that early asymmetric cleavages in the *C. elegans* embryo generate six "founder" cells that account for much of the *C. elegans* body plan. Intermediate group genes, most recently identified, may link the establishment of polarity in the zygote by par group genes to the localization of blastomere

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identity group gene functions. This review summarizes the known requirements for the members of each group, although it seems clear that additional regulatory genes controlling pattern formation in the early embryo have yet to be identified. An emerging challenge is to link the function of the genes in these three groups into interacting pathways that can account for the specification of the six founder cell identities in the early embryo, five of which produce somatic cell types and one of which produces the germline.

I. The Early *Caenorhabditis elegans* Embryo

One fascinating question in developmental biology, put simply, is how does it all get started? How does a single cell, such as an oocyte or a zygote, initiate the processes that generate a specific and complex multicellular body? For many years, the fruit fly *Drosophila melanogaster* was the only metazoan used for large-scale genetic screens to study the early steps in pattern formation (St. Johnston and Nusslein-Volhard, 1992). However, genetic screens in the nematode *Caenorhabditis elegans* and in *Arabidopsis thaliana* have begun to broaden our view of early development by identifying additional regulatory loci in another animal and in a plant embryo (Jurgens, 1995; Kemphues and Strome, 1997; Schnabel and Priess, 1997). Mechanistic comparisons of pattern formation in these and other early embryos may reveal not only how different life forms develop but perhaps also how they evolve.

For a comparison of early development, the insect and nematode embryos are impressively different. In *Drosophila*, the embryo is a 500-μm-long syncytium in which a peripheral monolayer of nuclei share a common cytoplasm until completion of the 13th round of mitosis (St. Johnston and Nusslein-Volhard, 1992). Diffusion of transcriptional and translational regulators from localized sources forms morphogenetic gradients of positional information that pattern large fields of nuclei. In dramatic contrast, the 50-μm-long *C. elegans* embryo is completely cellularized, and the early events that control patterning must negotiate the plasma membranes that partition all nuclei (Sulston *et al.*, 1983). Not surprisingly, mechanisms very different from those in *Drosophila* appear to pattern the early *C. elegans* embryo (see the following).

Another notable difference between flies and worms is the relative importance of pattern formation during oogenesis versus after fertilization. In an early insect embryo, many of the events that establish anterior–posterior and dorsal–ventral asymmetry begin during oogenesis, with fertilization activating previously localized regulators (St. Johnston and Nusslein-Volhard, 1992). In *C. elegans*, the body axes form not during oogenesis but sequentially during embryogenesis, with sperm entry establishing an anterior–posterior axis (see below). Such seemingly dramatic differences in early development are not unusual. For example, in *Xenopus* embryos the localization of maternal mRNAs in the oocyte, the point of sperm entry, and rotation of the zygote's cytoplasmic cortex all appear necessary

for specifying the body axes. One largely unmet challenge in developmental biology is to understand how and perhaps even why early embryos begin development in such remarkably different ways. As one route to a broader understanding, the genetics and sequenced genome of *C. elegans* provide rapid access to the molecules and mechanisms that initiate pattern formation in a cellularized animal embryo.

A. Maternal Control of Early Embryogenesis

Although the fly and worm embryos seem very different, they are alike in developing rapidly and in depending extensively on maternally supplied regulatory factors to control early steps in pattern formation. As with *Drosophila*, extensive genetic screens for recessive, maternal-effect, embryonic-lethal mutations in *C. elegans* have identified many regulatory factors that govern the early steps of patterning. Thus, for the genes discussed here, homozygous mutant mothers become fertile adults but then produce broods in which all the embryos die. The mutant embryos differentiate well, but exhibit highly penetrant and specific defects in pattern formation. In almost all cases, the mutations are strictly maternal: mating wild-type males into homozygous mutant mothers to produce $-/+$ embryos does not rescue the defect, as strictly maternal genes must be expressed by the mother during oogenesis. In this chapter, I refer to the embryos produced by self-fertilization in homozygous mutant mothers as mutant embryos or mutants.

In *C. elegans*, maternal genes are transcribed in the germline nuclei that line the syncytial distal arm of the tubular nematode ovary, sharing a common cytoplasmic "core" (Fig. 1). *In situ* hybridization studies have shown that maternal mRNAs are transcribed in germline nuclei and accumulate in the cytoplasmic core of the distal arm (Seydoux and Fire, 1994). As the ovary bends back on itself to form the proximal arm, single nuclei arrested in meiosis become cellularized to form a short, single row of oocytes (Schedl, 1997). Each oocyte appears to acquire maternal gene products from the core cytoplasm that is pinched off with a nucleus during cellularization. Nutritional yolklike factors appear to be secreted by intestinal cells, which are in close proximity to the ovary, and taken up in oocytes by endocytosis. Oocytes enlarge as they near the proximal end of the ovary and eventually enter the spermatheca, where fertilization occurs. Sperm entry initiates the completion of meiosis by the maternal pronucleus, the rapid production of a tough chitinous eggshell secreted by the zygote, and an extensive cytoskeleton-dependent reorganization of the maternally derived cytoplasm, setting the stage for maternal gene products to initiate different cell fate programs in different regions of the embryo (Kemphues and Strome, 1997; see the following).

Recessive, nonconditional, embryonic-lethal mutations that specifically affect pattern formation have been identified in about 25 maternally expressed genes in

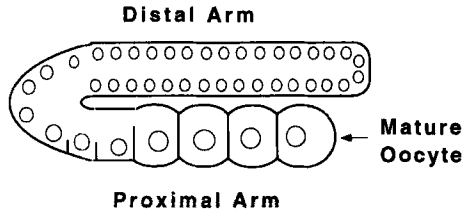

Fig. 1 One arm of the bilaterally symmetrical *C. elegans* ovary. The distal arm of the ovary is a syncytium of nuclei maintained in a mitotic state near the tip of the ovary but that enter into meiosis as they move proximally (Schedl, 1997). At the bend of the ovary, individual nuclei become cellularized, acquiring maternal gene products from the common cytoplasmic core of the ovary. As oocytes approach the spermatheca (not shown) at the proximal end of the ovary, the oocyte pronucleus moves to the side of the oocyte opposite the spermatheca. In this and all subsequent figures, see the text for additional details and references.

C. elegans (Table I). Mutations in these genes result in patterning defects without affecting more general cellular processes such as mitosis, metabolism, or even differentiation. Because homozygous mutant mothers must survive to adulthood to reveal maternal-effect phenotypes, and because many maternal genes also have essential zygotic functions, it is likely that important regulatory loci have been missed in spite of extensive screening for nonconditional maternal-effect mutations.

The ability to efficiently eliminate maternal gene functions from *C. elegans* by using antisense RNA has provided a new and powerful tool for the identification of regulatory loci (Guo and Kemphues, 1996a,b). By microinjecting antisense RNA into the syncytial ovary of wild-type hermaphrodites, one can test maternal requirements for genes that mutate to give zygotic-lethal phenotypes or for genes in which no mutations have been identified in *C. elegans* but that are related by sequence to known regulatory genes in other organisms. The use of antisense RNA microinjection has led to the identification of additional genes required for pattern formation in the early embryo and is certain to play an increasingly important role with the nearly complete sequence of the worm genome now available. Finally, genetic screens for temperature-sensitive mutations and molecular and genetic screens for loci that interact with previously identified genes should continue to identify new regulatory factors and clarify how developmental regulators interface with more general cellular machineries to pattern the early embryo.

B. Three Groups of Maternal Genes in *C. elegans*

For heuristic purposes, it is useful to group the maternal-effect mutants identified in *C. elegans* on the basis of their shared phenotypic traits. In fact, very few of the mutants identified thus far have identical or even nearly identical phenotypes,

Table I Maternal Loci in *C elegans*: Gene Names and Molecular Identities (See Text for References)

Gene	Name	Molecular identity
	Par Group Genes	
let-99	*Let*hal	?
par-1	*Par*titioning-defective	Ser–Thr kinase; binds a nonmuscle myosin
par-2	Same	Novel; ATP-binding site
par-3	Same	Novel; two PDZ domains
par-4	Same	Ser–Thr kinase
par-5	Same	?
par-6	Same	?
mes-1	*M*aternal-e*ff*fect *s*terile	?
	Blastomere Identify Group Genes	
P_1 subgroup		
pal-1	*P*osterior *al*ae defective	Homeodomain protein; putative transcription factor
pie-1	*P*harynx and *i*ntestine *e*xcess	TIS-11-like Zn^{2+} finger ptn
skn-1	*Skn* excess	bZIP-like putative transcription factor; lacks a leucine zipper
pop-1	*P*osterior *p*harynx defective	HMG domain protein; putative transcription factor
mom-1	*M*ore *m*esoderm	Porcupine homologue; ER protein required for Wnt secretion
mom-2	Same	Wingless/Wnt homologue; putative secreted glycoprotein ligand
mom-3	Same	?
mom-4	Same	?
mom-5	Same	Frizzled homologue; putative receptor for Wnt ligands
AB subgroup		
aph-2	*A*nterior *ph*arynx defective	Novel membrane-associated extracellular protein
apx-1	*A*nterior *p*harynx *ex*cess	Delta-like transmembrane protein; putative GLP-1 ligand
glp-1	*G*erm*l*ine *p*roliferation defective	Notchlike transmembrane protein; putative receptor
	Intermediate Group Genes	
mex-1	*M*uscle *ex*cess	TIS-11-like Zn^{2+} finger ptn
mex-3	Same	Two KH domains; putative RNA-binding protein
pos-1	*P*osterior localized mRNA	TIS-11-like Zn^{2+} finger ptn

but for this review I have grouped all maternal genes into one of three groups. The first, the par group, consists of eight genes, *par-1* through *par-6*, *let-99*, and *mes-1*. Mutational inactivation of any one of these genes results in losses of

asymmetry in early embryos and widespread defects in cell fate patterning. As summarized here, the *par* genes are essential for establishing or maintaining anterior–posterior polarity in the one-cell zygote. Inactivation of maternal genes in the second blastomere identity group causes much more specific defects in the fates of individual blastomeres, without causing the more general defects in asymmetry and cell fate patterning characteristic of par group mutants. These genes include *aph-2*, *apx-1*, *glp-1*, *pal-1*, *pie-1*, five *mom* genes, and *skn-1*. The mutant phenotypes caused by inactivation of these genes fall into several distinct subgroups, as reflected by the different gene names. The genes in this group thus appear to regulate development more specifically and probably act later than the *par* genes. The third and smallest category consists of only three genes: *mex-1*, *mex-3*, and *pos-1*. Mutations in these intermediate group genes result in phenotypes with a pleiotropy in between those observed for the par group and blastomere identity group mutants. The intermediate group genes may link the establishment and maintenance of polarity in the one-cell zygote to the specification of individual blastomere identities in the early embryo.

C. Polarization of a Dynamic Cytoskeleton and the Establishment of Anterior–Posterior Asymmetry

Genes in the par group provide at least a glimpse of the machinery that polarizes a *C. elegans* zygote after fertilization. This usage of the term polarization specifically refers to the generation of asymmetry within individual cells and therefore is distinct from another common usage of polarity, which is in reference to morphogenetic patterning across a field of cells. Because the polarization of single cells in different organisms appears to require a functional cytoskeleton (Drubin and Nelson, 1996), an overview of our understanding of cytoskeletal dynamics and polarity in the early *C. elegans* embryo is prerequisite to a discussion of the par group genes.

As in *Drosophila*, studies of pattern formation in *C. elegans* indicate that the polarization of a dynamic cytoskeleton is an early and fundamental step for generating polar asymmetries (Grunert and St. Johnston, 1996; Guo and Kemphues, 1996b). In wild-type *C. elegans* embryos, three early asymmetries are evident at the one-cell stage, and all three require functional microfilaments. One obvious early asymmetry is the posterior displacement of the first mitotic spindle, which results in the production of two daughters with very different fates: a smaller posterior blastomere called P_1 and a larger anterior blastomere called AB (Fig. 2). This polar asymmetry is lost upon treatment of wild-type embryos with cytochalasin D for only brief intervals to transiently disrupt the actin cytoskeleton during part of the first cell cycle (Hill and Strome, 1988, 1990). Cytochalsin D treatment results in some embryos having apparently reversed polarity, with a smaller P_1-like cell located anteriorly and a larger AB-like cell located

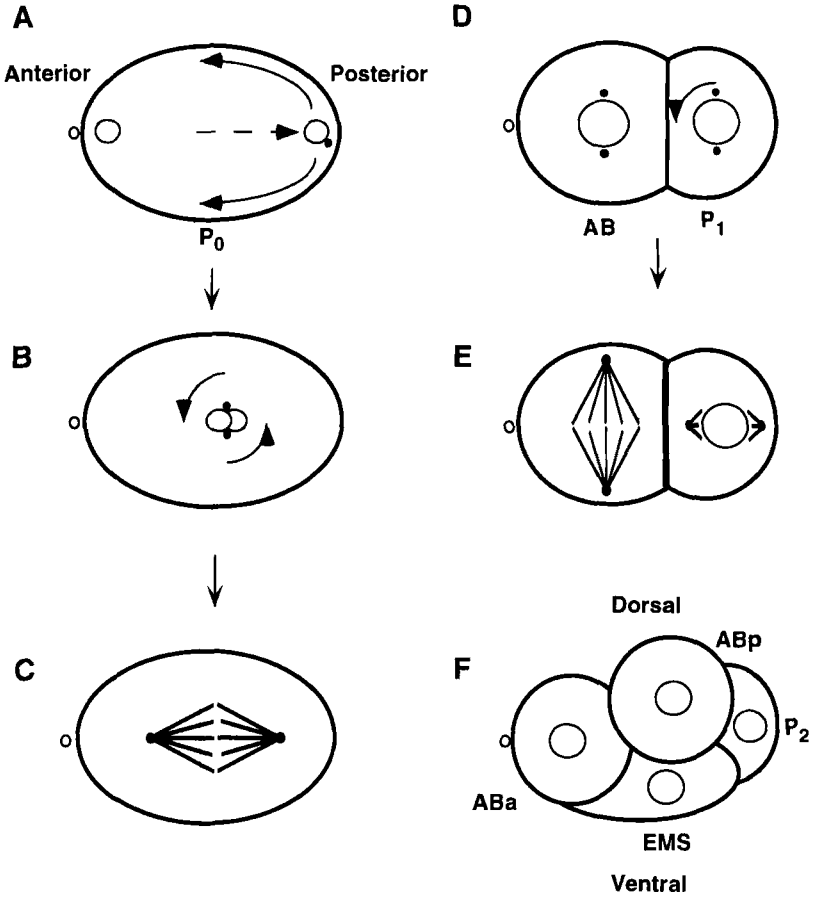

Fig. 2 Early asymmetries in the *C. elegans* zygote at the one-cell (A–C), two-cell (D, E), and four-cell stages (F) of embryogenesis. (A) The position of the sperm pronucleus and its accompanying centriole define the posterior pole of the zygote and initiate cytoplasmic flux in the posterior half of the zygote (curved and dashed arrows). (B) After congression of the pronuclei, the initially transverse spindle, indicated by solid dots to represent centrosomes, rotates to lie along the long axis (curved arrows). (C) The first mitotic spindle becomes displaced slightly to the posterior, resulting in the production of a smaller posterior daughter called P_1 and a large anterior daughter called AB. (D) Both mitotic spindles in the two-cell stage embryo initially set up transversely, but the P_1 spindle rotates before the completion of mitosis to lie along the long axis (curved arrow). (E) P_1 divides slightly after AB, again with a posteriorly displaced spindle, while AB divides transversely and equally. (F) In a four-cell stage embryo, the two daughters of AB, called ABa and ABp, are of equal size and initially have equivalent developmental potential. The two P_1 daughters, called P_2 and EMS, are of different size and are born with different fates.

posteriorly. In other cases, two equally sized P_1-like cells or two equally sized AB-like cells resulted, with the similarities to P_1 and AB based on the orientation of their mitotic spindles during subsequent mitosis (Fig. 2). Although the cell fate patterns produced by P_1 and AB were not examined further in these experiments, the cytochalsin D treatments appeared to severely disrupt AP axis formation.

The discovery of cytoplasmic structures called P-granules provided the first example of molecular asymmetry in the early *C. elegans* embryo (Strome and Wood, 1983; Wolf *et al.*, 1983). Although their function remains largely unknown, P-granules are ribonucleoprotein complexes present specifically in germline precursors (Strome and Wood, 1983; Seydoux and Fire, 1994; Draper *et al.*, 1996; Guedes and Priess, 1996). Ultrastructurally, P-granules resemble polar granules, which are present in the *Drosophila* germline and are known to be important for germline specification (Wolf *et al.*, 1983; St. Johnston, 1993). Mutations in a *C. elegans* gene called *pgl-1* were found to result in the loss of detectable P-granules and sterility, providing the best genetic evidence that P-granules are important for germline development (Susan Strome, personal communication). P-granules initially are present throughout the cytoplasm of the oocyte and the one-cell zygote, but after fertilization they are actively segregated to the cortical cytoplasm at the posterior pole before the first embryonic mitosis, localizing most P-granules to P_1. P-granules continue to be segregated to germline precursors at each subsequent division until the birth of the final germline progenitor, P_4, at the 24-cell stage. Treatment of one-cell-stage wild-type embryos with brief pulses of cytochalasin D prevents P-granule segregation, indicating that this asymmetry also requires functional microfilaments (Hill and Strome, 1990).

A third asymmetry in early embryos is a cytoplasmic flux that occurs posteriorly shortly after fertilization and the ensuing completion of meiosis (Fig. 2). By using time-lapse videomicroscopy to observe the movements of individual cytoplasmic "yolk" droplets—visible throughout the cytoplasm of all early blastomeres—one can readily detect cortical cytoplasm flowing anteriorly while more internal cytoplasm flows posteriorly (Hird and White, 1993; Hird, 1996). This cytoplasmic flux occurs in the posterior half of the one-cell zygote, and similar cytoplasmic fluxes may occur in P_1 and AB. Cytochalasin D treatment prevents the cytoplasmic flux from occurring, although disruption of microtubules with nocodazole treatment does not (Hird and White, 1993).

Intriguingly, the asymmetric positioning of the first mitotic spindle, the localization of P-granules, and the posterior flux of cytoplasm all require functional microfilaments. Moreover, the time period in which cytochalasin D treatment can disrupt spindle positioning and P-granule localization precisely corresponds to the time of cytyoplasmic flux; treatment with pulses of cytochalsin D before or after the flux does not affect positioning of the first mitotic spindle or the localization of P-granules (Hill and Strome, 1990; Kemphues and Strome, 1997). These results suggest that, after fertilization, actin-dependent processes generate

a cytoplasmic flux required for localizing P-granules and possibly for posteriorly displacing the first mitotic spindle.

D. Sperm Entry Provides the Initial Cue for Anterior–Posterior Polarity

The importance of the actin cytoskeleton in establishing anterior–posterior asymmetry begs the obvious question: What factor(s) initiate establishment of these asymmetries and distinguish the anterior and posterior poles of the oblong zygote? Observations of developing oocytes and fertilization provide some clues. In the ovary of a wild-type worm (Fig. 1), the occytes line up in a single row that terminates at the spermatheca, a socklike structure that houses hermaphrodite and male sperm. As an oocyte approaches the spermatheca, its pronucleus becomes displaced toward the prospective anterior pole, and a sperm usually enters at the prospective posterior pole, the end that first engages the spermatheca (Figs. 1 and 2). This sequence of events typically results in the anterior end of an embryo being marked by two polar bodies extruded during the completion of meiosis by the maternal pronucleus. However, polar bodies are present at the anterior end in only about 90% of embryos with the remainder positioned laterally or even posteriorly, suggesting that the position of the oocyte pronucleus is not an accurate predictor of polarity in the zygote (Albertson, 1984; Bowerman *et al.*, 1993).

Rather than having prepatterned oocytes, sperm entry appears to provide the initial asymmetric cue that polarizes the *C. elegans* zygote and thereby determines the anterior–posterior body axis (Goldstein and Hird, 1996). This conclusion follows from experiments using genetically feminized nematodes that lack sperm. In these female nematodes, oocytes enter the spermatheca before fertilization. Consequently, if such females are then mated, male sperm contact the oocyte in a more random manner, sometimes entering at the side or even at the same end occupied by the maternal pronucleus. Wherever the sperm enters, its pronucleus and accompanying centriole appear to generate a cytoplasmic flux that appears to push the male pronucleus and centriole to the nearest pole of the oblong embryo, with that end becoming the posterior pole. Thus, if the oocyte does possess any polarity, it can be overridden by altering the position of sperm entry. This finding suggests that, upon entering an oocyte and taking up residence at one end, the sperm-donated pronucleus or centriole, or some other sperm-donated product(s), defines the posterior pole and initiates the cytoplasmic flux that redistributes maternally provided factors such as P-granules along the AP axis. Genetic studies show that sperm in fact do donate a factor(s) critical for early embryogenesis, beyond their genetic material and mitotic spindle apparatus. The paternally expressed *spe-11* gene encodes a novel protein associated with the sperm pronucleus that is required for development to proceed beyond the one-cell stage (Browning and Strome, 1996). The function of *spe-11* remains

unknown but can also be provided by using a transgene to express *spe-11* in oocytes.

One important but unresolved issue is how the circulating cytoplasm in an early *C. elegans* zygote results in localization of P-granules to the posterior cortex. Presumably, anchoring molecules active only at the posterior pole trap P-granules as they pass by. If so, the localization of such anchoring complexes presumably would precede that of P-granules and might require a different mechanism. It is conceivable that the hydrodynamic properties of the cytoplasm imposed by the narrowing of the eggshell at the pole could result in localized sheer forces mechanically activating ubiquitously distributed anchors. Alternatively, the sperm might donate or activate anchoring molecules that accompany the sperm pronucleus and centriole in moving to the future posterior pole. In these latter two models, the localization of P-granules and the localized function of anchoring molecules would represent two different responses to one event, the sperm-induced flux of cytoplasm.

II. The Par Group of Maternal Genes and Polarization of the Embryonic Cytoskeleton

Tantalizing insights into how regulation of the cytoskeleton might polarize the *C. elegans* zygote have come from the identification of six maternally expressed *par* genes, *par-1* through *par-6* (Kemphues *et al.*, 1988; Guo and Kemphues, 1996b; Watts *et al.*, 1996). For reviews of the *par* genes, see Guo and Kemphues (1996b) and Kemphues and Strome (1997). Mutational inactivation of *par* genes causes defects in cytoplasmic reorganization after fertilization, a failure to partition P-granules properly, and abnormal positioning of mitotic spindles (Kemphues *et al.*, 1988; Kirby *et al.*, 1990). The first mitotic spindle fails to move posteriorly in all but *par-4* mutant embryos, producing equal-sized posterior and anterior two-cell-stage daughters (Fig. 3). In all six *par* mutants, the two-cell-stage blastomeres divide synchronously and equally, in contrast to the asynchronous and unequal divisions of P_1 in wild-type embryos (Fig. 3). Cytoplasmic flux appears defective to some degree in *par-1* through *par-4* mutants, with *par-1* and *par-4* perhaps exhibiting less severe defects (Kirby *et al.*, 1990). Because the first mitotic cleavage is equal in *par-1* but unequal in *par-4* mutants, the relationship between cytoplasmic flux and positioning of the first mitotic spindle is not clear. Finally, the characteristic orientations of two-cell-stage mitotic spindle axes in *par* mutant embryos define three *par* subgroups: (i) In *par-1* mutant embryos, P_1 and AB have longitudinally and transversely oriented spindles, respectively, as in wild-type embryos. But unlike wild-type embryos, both two-cell-stage blastomeres in *par-1* embryos divide synchronously. (ii) In *par-2* and *par-5* mutant embryos, both spindles divide transversely and synchronously, and

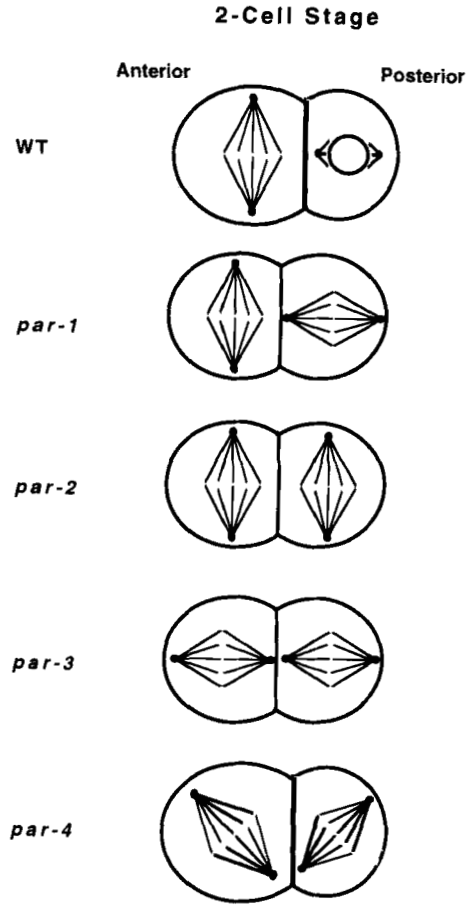

Fig. 3 Two-cell-stage mitotic spindle orientations in *par* mutant embryos. In *par-1* mutant embryos, the posterior blastomere (for convenience called P_1) and the anterior blastomere (for convenience called AB) divide with normal spindle orientations, but synchronously instead of, as in wild-type embryos, asynchronously. In *par-2* and *par-5* mutant embryos, P_1 and AB both divide transversely and synchrononously. In *par-3* and *par-6* mutant embryos, both divide longitudinally and synchronously. In *par-4* mutant embryos, P_1 and AB divide with random orientations; one arbitrary example is shown. In all but *par-4* mutant embryos, the first cleavage produces two blastomeres roughly equal in size. In *par-4* mutants, the first cleavage produces a smaller posterior and larger anterior blastomere, as in wild type, but both blastomeres subsequently divide synchronously.

(iii) in *par-3* and *par-6* mutants, both divide longitudinally and synchronously (Kemphues and Strome, 1997).

Because *par* mutants are defective in cytoplasmic flux, P-granule localization, and spindle positioning—the same processes disrupted by cytochalasin D treatment of wild-type embryos—the *par* genes may regulate pattern formation at

least in part by interacting with and perhaps polarizing the actin cytoskeleton (Guo and Kemphues, 1996b). Consistent with this hypothesis, studies have shown that the C-terminus of PAR-1 binds a conventional nonmuscle myosin required for proper polarization of the early embryo (Guo and Kemphues, 1996a). Inactivation of this myosin by RNA microinjection results in the production of embryos with transversely oriented two-cell-stage spindles, in addition to defects in cytokinesis and oogenesis. Affinity column chromatography has been used to identify 17 oocyte proteins that bind filamentous actin (Aroian *et al.*, 1997). Antibodies to three such proteins show distinct localization patterns in the zygote and early embryo. CABP1 localizes to the actin-rich cortex throughout the zygote and in all early blastomeres. CAPB14 is dynamic, cycling from the nucleus during prophase, to the cortex during metaphase, and to the cleavage furrow during cytokinesis. Perhaps most intriguing, CABP11 is localized to the cortex but only in the anterior part of the embryo, indicating that asymmetries involving proteins that physically interact with actin microfilaments exist as early as the time of pronuclear congression in the one-cell zygote. The powerful molecular, genetic, and biochemical methods now available for identifying and inactivating gene products in *C. elegans* make it seem likely that the discovery of important mechanistic links between the PAR proteins, the cytoskeleton, and embryonic polarity in *C. elegans* are close at hand.

A. Polarized Distribution of the PAR Proteins in the One-Cell Zygote and in Germline Precursors

At a molecular level, the DNA sequences of *par-1*, *par-2*, *par-3*, and *par-4* are known, and all four of the proteins they encode are present in the cytoplasm and enriched in the cytoplasmic cortex. PAR-1 contains a predicted N-terminal Ser–Thr kinase domain and a C-terminal domain that interacts with the nonmuscle conventional myosin (Guo and Kemphues, 1995, 1996a). Before fertilization, PAR-1 is not polarized in its cortical distribution, but after fertilization and cytoplasmic flux, by the time the maternal and paternal pronuclei meet, PAR-1 is present at the cortex only posteriorly in the one-cell zygote, called P_0 (Fig. 4). Homologues of *par-1* have been identified in yeast and in mammals (Levin *et al.*, 1987; Levin and Bishop, 1990; Drewes *et al.*, 1997). The involvement of these related kinases in regulating polarity in yeast and microtubule stability in mammalian cells suggests that the functions of the *par* genes are likely to be of general importance in studies of cell polarity and the cytoskeleton. PAR-2 is a protein of unknown function with a putative ATP-binding site and a zinc-binding domain of the "RING finger" class (Levitan *et al.*, 1994). Like PAR-1, PAR-2 is enriched in the cortex but only in the posterior part of the zygote shortly after fertilization (Boyd *et al.*, 1996). PAR-3 is a novel protein with three PDZ repeats that presumably mediate protein–protein interactions. PAR-3 is present cortically

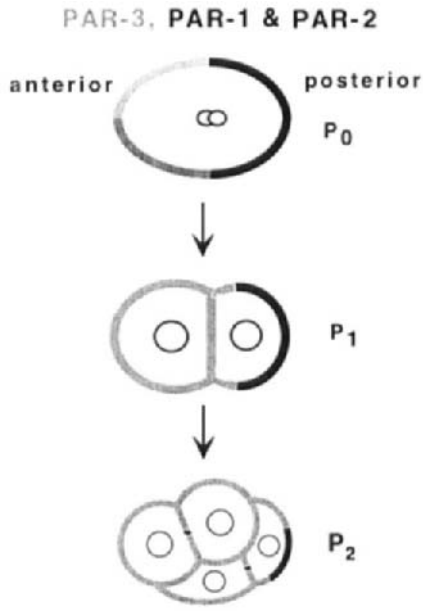

Fig. 4 Polarized distributions of PAR-1, PAR-2, and PAR-3 cortically localized proteins. PAR-1 and PAR-2 are both enriched in the cytoplasmic cortex and only posteriorly shortly after fertilization in P_0 (dark lines). PAR-3 is also enriched cortically but only in the anterior portion of P_0 (gray lines). PAR-1, PAR-2, and PAR-3 continue to show a polarized distribution in the germline precursors, P_1 and P_2, at the two-cell and four-cell stages of embryogenesis, respectively. This polarized distribution is also maintained in the P_3 daughter of P_4 (not shown, but see Fig. 15). PAR-1 and PAR-2 are absent from somatic blastomeres, while PAR-3 is enriched cortically throughout the entire cortex of all somatic blastomeres.

only in the anterior cortex of the one-cell zygote; its posterior boundary precisely coincides with the common anterior boundary of the cortical domains of PAR-1 and PAR-2 (Etemad-Moghadam and Kemphues, 1995). PAR-4 also contains a Ser–Thr kinase domain different from that in PAR-1. PAR-4 is unique in having a nonpolarized distribution, localized to the cortex throughout the zygote and early blastomeres (J. Watts and K. Kemphues, personal communication). The polarized distributions of PAR-1, PAR-2, and PAR-3 are inherited by the germline precursors P_1, P_2, and P_3, but not by P_4, the final germline progenitor (Guo and Kemphues, 1996b; Kemphues and Strome, 1997; see Fig. 4). It is not known whether the PAR proteins are required for maintaining polarity in germline precursors. However, the temperature-sensitive periods for mutant alleles of *par-2* and *par-4* are both over by the end of the one-cell stage, consistent with *par* function being required only during the first zygotic cell cycle (Kemphues and Strome, 1997).

Studies of PAR protein distributions in *par* mutant embryos indicate that interactions among the *par* genes are important for regulating embryonic polarity. Four interactions have been noted. First, the cortical localization of PAR-1 requires *par-2* function: In *par-2* mutant embryos, PAR-1 is present throughout the cytoplasm with no enrichment at the cortex (Boyd *et al.*, 1996). Furthermore, PAR-2 and PAR-3 each depends on the other for their polarized distributions (Etemad-Moghadam and Kemphues, 1995; Boyd *et al.*, 1996; Kemphues and Strome, 1997). In *par-2* mutants, cortical PAR-3 extends posteriorly, and in *par-3* mutants cortical PAR-2 extends anteriorly. Finally, the cortical localization of PAR-3 requires *par-6* function, and in turn *par-3* function is required for proper cortical localization of CABP11, an actin-binding protein present in oocytes that shows a distribution similar to that of PAR-3 (Watts *et al.*, 1996; Aroian *et al.*, 1997). Finally, none of the other PAR proteins require *par-4* function for their polarized distribution, and PAR-4 appears normal in all other *par* mutants (Kemphues and Strome, 1997).

Genetic studies, together with the known distributions of the PAR proteins just described, suggest that the only function of *par-2* may be to limit the posterior extension of PAR-3. This conclusion is based on the observation that eliminating one copy of *par-6* rescues *par-2* mutant embryos: *par-6(−)/par-6(+); par-2(−)/par-2(−)* mothers produce viable embryos (Watts *et al.*, 1996). As cortical PAR-3 in *par-2* embryos forms a gradient that fades posteriorly, one possible explanation for the suppression of *par-2* is that reducing the *par-6* function by one-half causes the loss of enough cortical PAR-3 to reduce its posterior extension sufficiently for normal development to occur. If so, then the only function of *par-2* might be to restrict cortical PAR-3 to the anterior portion of the zygote (Watts *et al.*, 1996). Another observation consistent with *par-2* functioning only to define the posterior boundary for cortical PAR-3 is that *par-1*, but not *par-2*, mutants fail to partition P-granules to P_1; *par-2* embryos do fail to partition P-granules during subsequent divisions (Kemphues *et al.*, 1988). Thus *par-2* embryos partition P-granules to P_1 even though cortical PAR-1 is absent from *par-2* embryos (discussed earlier). Therefore, the cortical localization of PAR-1 is not necessary to partition P-granules to P_1, and the loss of cortical PAR-1 in *par-2* mutant embryos may not be relevant to the *par-2* mutant phenotype. This result raises the important caveat that cortical localization of a PAR protein may not be important for its function. Instead, it is possible that cortical localization could be a secondary consequence of cytoplasmic PAR functions that more directly regulate polarity in the zygote. The relative importance of cytoplasmic and cortical pools of the PAR proteins remains unknown.

B. *par* Gene Functions: A Network, Not a Pathway?

The observations that proper cortical localization of some PAR proteins requires some *par* gene functions suggest that the *par* genes interact to regulate polarity.

Additional evidence for *par* genes functioning in linear pathways has come from studies of *par-2* and *par-3* and their requirements for proper orientation of the mitotic spindles in P_1 and AB (Kemphues *et al.*, 1988; Cheng *et al.*, 1995). In wild-type two-cell-stage embryos, both the P_1 and AB initially set up transversely oriented mitotic spindles (Fig. 2). AB continues to divide transversely, slightly ahead of P_1 in timing. Just before P_1 divides, however, its mitotic spindle rotates to lie along the longitudinal axis. Rotation of the P_1 spindle appears to involve the capture of astral microtubules emanating from either end of the P_1 spindle by a largely uncharacterized complex present in the P_1 cortex near the center of P_1's border with AB (Hyman and White, 1987; Hyman, 1989). Other studies have shown that microtubules, actin, and actin-capping protein colocalize to this cortical site during the time that the P_1 spindle rotates (Waddle *et al.*, 1994). Whatever its nature, something associated with the anterior cortex of P_1 can trap and pull one end of the P_1 spindle toward the center of P_1's border with AB. This attachment can be severed by delivering pulses from a laser microbeam in between the cortex and the attached centrosome (Hyman, 1989). Severing of the connection causes the rotation to stop, but it then resumes in one or the other direction upon recapture of a centrosome. On the basis of analogies to bud site selection and orientation of the mitotic spindle in *Saccharomyces cerevisiae* (Drubin and Nelson, 1996), it is tempting to speculate that a spindle-rotating complex in *C. elegans* might assemble in association with proteins left upon the termination of cytokinesis at the birth of P_1 and AB.

As illustrated in Fig. 3, both spindles orient transversely in two-cell-stage *par-2* mutants while they both orient longitudinally in *par-3* mutants, suggesting that *par-2* and *par-3* interact to regulate the longitudinal and transverse orientations of wild-type P_1 and AB mitotic spindles (Kemphues *et al.*, 1988). Because both spindles orient longitudinally in *par-2;par-3* double mutants, *par-3* is epistatic to *par-2* with respect to spindle rotation in two-cell-stage blastomeres, and neither *par-2* nor *par-3* is required for spindle rotation (Cheng *et al.*, 1995). Rather, *par-3* is required to prevent spindle rotation in AB while *par-2* appears to prevent *par-3* from functioning in P_1, thereby permitting some other process to rotate the P_1 spindle.

Models in which PAR-3 directly prevents spindle rotation in AB received support from immunohistochemical studies showing that *par-2* restricts cortical PAR-3 mostly to the AB blastomere (Etemad-Moghadam and Kemphues, 1995; Boyd *et al.*, 1996). On the basis of these genetic and molecular studies of *par-2* and *par-3*, it has been proposed that cortical PAR-3 may stabilize spindle axes by interacting with astral microtubules in AB (Guo and Kemphues, 1996b; Kemphues and Strome, 1997). The presence of cortical PAR-3 only at the anterior of P_1 presumably would be insufficient to prevent spindle rotation in P_1. Mutations in *par-2* result in PAR-3 being present throughout the cortex in both P_1 and AB, consistent with stabilization of the transverse spindles in both two-cell-stage blastomeres in *par-2* mutant embryos. However, mutations were identified in another maternal gene, named *let-99*. In about 50% of *let-99* embryos, the AB

spindle orients longitudinally and the P_1 spindle orients transversely, the opposite of the pattern observed in wild type (Rose and Kemphues, 1997). Remarkably, PAR-1, PAR-2, and PAR-3 all show a normal polarized distribution in *let-99* mutant embryos (Rose and Kemphues, 1997). Therefore, spindle rotation in AB is not prevented by PAR-3, and the P_1 spindle can be stabilized without altering the distribution of PAR-3, calling into question the notion that stabilization of the spindle by cortical PAR-3 is either necessary or sufficient to prevent spindle rotation. Alternatively, *par-2* and *par-3* might function to polarize the distribution of other factors, resulting in only P_1 acquiring the machinery necessary for spindle capture and rotation. Consistent with this latter possibility, mutations in *par-3* also are epistatic to mutations in *par-2* with respect to the distribution of SKN-1, a putative transcription factor (see the following). As in wild-type embryos, SKN-1 is present at high levels only in the posterior blastomeres in *par-2* mutant embryos, but in *par-3* and *par-3;par-2* double mutant embryos SKN-1 is present at equal levels in anterior and posterior blastomeres (Bowerman *et al.*, 1997). Thus, it may be simpler to explain the functions of *par-2* and *par-3* in terms of the polarized distributions of other maternal factors, rather than in terms of PAR-3 mediating interactions between the cortex and the mitotic spindle.

While the epistatic relationship between *par-2* and *par-3* indicates that some par group genes function in linear pathways, the *par* genes more often appear to act independently of each other to regulate pattern formation. This conclusion is based in part on the dissimilar phenotypes of most par group mutants and on the distributions of four regulatory proteins in *par-1*, *par-2*, *par-3*, and *par-4* mutant embryos (Crittenden *et al.*, 1996; Bowerman *et al.*, 1997). These four proteins, discussed in more detail later, are distributed asymmetrically in wild-type embryos. Two, GLP-1 and MEX-3, are present at high levels only in anterior blastomeres at the two-cell and four-cell stages. Two others, SKN-1 and PAL-1, are present at high levels only in posterior blastomeres at the four-cell stage. Because the maternal mRNAs for all of these proteins are distributed throughout the early embryo, either translational regulation or regional differences in protein stability must account for their asymmetric distributions. Remarkably, no correlation is seen in how the distributions of these four proteins respond to mutations in the *par* genes (Fig. 5). For example, mutations in *par-1* result in SKN-1 being present in all four-cell-stage blastomeres, but also result in a complete absence of PAL-1. Furthermore, while mutations in *par-2* do not affect MEX-3 distribution, GLP-1 is present in all four-cell-stage blastomeres in many *par-2* mutants. Thus, mutations in the *par* genes extensively uncouple the mechanisms that localize different regulatory molecules. Perhaps the *par* genes represent components of a network of polarizing factors that in turn regulate the spatial distribution of translational or proteolytic regulators, ultimately localizing more specifically acting regulators like GLP-1, MEX-1, PAL-1, and SKN-1 to specific blastomeres. Whereas some of these events may involve linear pathways using

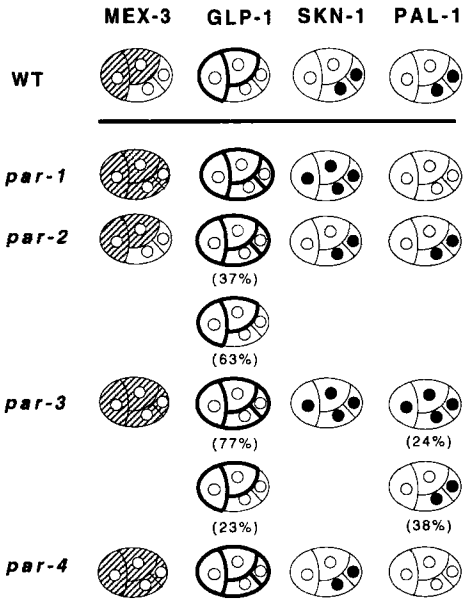

Fig. 5 Summary of the distributions of MEX-3, GLP-1, SKN-1, and PAL-1 in *par* mutant embryos. Wild-type distributions are shown in the upper row, with the distributions in *par-1*, *par-2*, *par-3*, and *par-4* mutant embryos summarized below.

two or more par group genes, the extensive uncoupling of cell fate specification pathways in *par* mutant embryos and the diversity of the *par* mutant phenotypes suggest that the *par* genes also act independently of each other during the regulation of polarity and pattern formation.

C. The *mes-1* Gene and a Reversal of Polarity during Germline Development

The maternal gene *mes-1* also warrants consideration as a par group member. The most obvious defect in *mes-1* mutant embryos is the equal cleavage of the germline precursor P_3 into two daughters that produce body wall muscle (Strome *et al.*, 1995). Thus, *mes-1* mutants make two D-like blastomeres and lack the germline progenitor P_4. However, *mes-1* mutants probably have defects in the development of C, the sister of P_3, and the earliest detectable defect occurs in P_2 when the P_2 mitotic spindle fails to orient properly. Studies by Schierenberg suggest that the germline undergoes a polarity reversal in P_2: If P_1 is extruded from the eggshell and allowed to divide, P_2 is born posteriorly, but then P_2 divides to produce a smaller P_3 anteriorly and P_3 makes a smaller P_4 anteriorly (Schierenberg and William, 1985). When constrained at the narrow end of a rigid

eggshell, the germline daughters P_3 and P_4 are forced to adopt more ventral and anterior positions relative to their somatic sisters, even though their parents divide along a reversed AP axis relative to P_1 and P_0. Thus, it is possible that the defective positioning of the P_2 spindle in *mes-1* mutant embryos reflects a specific defect in a second phase of "reversed" germline polarity. If so, then *mes-1* might be considered a late-acting example of a par group gene, reversing polarity within germline precursors perhaps after the roles of the other par group genes are complete.

III. The Blastomere Identity Group of Maternal Genes in *C. elegans*

Before a discussion of maternal genes that act more specifically than those in the par group to control pattern formation, it is useful to briefly review the names of early embryonic cells, or blastomeres, and the characteristic cell types they each produce. Cleavage of the one-cell zygote generates the smaller P_1 blastomere and the larger anterior blastomere, AB. AB then divides before P_1 to produce two daughters of equal size, ABa and ABp, which subsequently divide synchronously. P_1 and its descendants undergo a series of asymmetric divisions to produce blastomeres differing in size and in the timing of their cell cycles (Sulston *et al.*, 1983). These early unequal cleavages produce a group of six so-called founder cells, born from the 2-cell to the 24-cell stage. Five founder cells, called AB, MS, E, C, and D, produce somatic cells, while P_4 is the germline progenitor. The descendants of each founder cell have characteristic and somewhat synchronous cell cycle times and in sum produce the 558 surviving cells that form a hatched larva. Founder cell descendants are named according to their position at birth relative to their sisters. For example, ABpr is the right-hand daughter of ABp, and ABp is the posterior daughter of AB (Sulston *et al.*, 1983).

Each founder cell produces an essentially invariant pattern of cell fates, with one or two cell types sufficing to distinguish each of the six founder fates (Sulston *et al.*, 1983). Three founder cells produce descendants that all share roughly the same fate: P_4 divides to make Z2 and Z3, the two progenitors of the germline; D produces only body wall muscle cells; and E makes all of the worm's intestinal cells. The remaining founder cells produce more complex patterns of cell fate. For example, MS generates body wall muscle and the somatic gonad in addition to several cell types that form the posterior part of the pharynx, an organ in the head of the animal used for feeding. C produces body wall muscle cells and most of the dorsal epidermis. ABa makes many neurons, some epidermal cells, and the anterior half of the pharynx. ABp also makes many neurons and some epidermal cells, and a number of specialized cell types, including the excretory cell and cells associated with the rectum and anus.

As described earlier, the position of sperm entry determines the anterior–posterior body axis in *C. elegans*. However, a dorsal–ventral axis is not evident until P_1 and AB are nearly done dividing (Priess and Thomson, 1987). As the spindle of AB elongates, it becomes longer than the eggshell is wide and skews to one side or the other, forcing P_1 to become skewed in a complementary fashion (Fig. 5). This sequence of events results in one AB daughter, ABa, being the anterior-most four-cell-stage blastomere, while ABp becomes the dorsal-most. The asymmetric division of P_1 produces the smaller germline progenitor P_2, the posterior-most blastomere in a four-cell-stage embryo, and EMS the ventral-most blastomere. Thus, the dorsal–ventral axis appears to be defined by the positions of two four-cell-stage blastomeres, ABp and EMS, the fates of which are specified using largely independent mechanisms (see the following). Left–right differences become apparent at the eight-cell stage, when for unknown reasons the left-side daughters of ABa and ABp adopt positions more anterior than their right-side sisters (Wood, 1991); the constraints of the eggshell and the left–right skewing of the EMS spindle may contribute to this asymmetry.

A. P_1 and AB Descendants: Mechanisms Controlling the Specification of Blastomere Identities

As noted earlier, the first cleavage of the *C. elegans* embryo produces two daughters, P_1 and AB, with dramatically different fates, indicating that mosaic mechanisms play an important role in patterning the early embryo, beginning with the first cleavage (Schnabel and Priess, 1997). The different developmental potentials of AB and P_1 can be demonstrated by separating them upon birth and examining their abilities to develop in isolation (Priess and Thomson, 1987). If AB is physically removed from a two-cell-stage embryo, P_1 still produces many if not all of the cell types it normally makes. Thus, P_1 appears to inherit a largely intrinsic ability to develop, suggesting that localization of embryonic determinants to P_1 might specify its fate, with cell signals from AB descendants apparently playing at most a minor role. In contrast, if P_1 is removed and AB develops in isolation, AB descendants fail to produce many cell types, including the anterior pharyngeal cells normally made by ABa and the intestinal–rectal valve cells characteristic of ABp fate. While AB development appears to depend extensively on cell signals from P_1 descendants, presumably factors that act cell-autonomously in AB and its descendants are also important for their proper development.

The relative importance of cell-autonomous and non-cell-autonomous mechanisms for patterning the fates of P_1 and AB descendants was demonstrated most clearly by a landmark experiment in 1987 that has shaped much mechanistic thinking about embryogenesis in *C. elegans* for the past 10 years. Priess showed that if one uses a micromanipulator to switch the positions of ABa and ABp just

as they are being born, a normal embryo results with an expected reversal in left–right asymmetry (Priess and Thomson, 1987). This one simple experiment showed that ABa and ABp are born with equivalent developmental potentials. Because ABa and ABp can adopt appropriate fates according to their positions at the beginning of the four-cell stage, cell–cell interactions must distinguish their fates. Furthermore, the ability to switch ABa and ABp without disrupting embryogenesis indicates that the DV axis in *C. elegans* can be reversed at the beginning of the four-cell stage: The blastomere switch puts ABa in the region formerly occupied by EMS and forces EMS to move dorsally to the former position of ABp. A second experiment by Priess in 1987 suggested that P_1 descendants develop using more mosaic mechanisms. After switching the positions of P_2 and EMS, each P_1 daughter appeared to produce its normal complement of cell types but in the wrong place, resulting in morphologically abnormal embryos that fail to form a worm or hatch (Priess and Thomson, 1987). In summary, both cell-autonomous and non-cell-autonomous mechanisms pattern the early embryo, with AB descendants apparently relying more on cell signaling and P_1 descendants relying more on the asymmetric segregation of development potential during early cleavages.

Over the past 10 years, genetic studies have in large part supported the conclusion that P_1 and AB descendants develop using substantially different mechanisms (see the following). These differences in P_1 and AB development make it convenient to discuss members of the blastomere identity group by referring to two subgroups of maternal genes, a P_1 subgroup and an AB subgroup.

B. Maternal Genes That Specify the Identities of P_1 Descendants

1. *skn-1*, *pal-1*, and *pie-1*: Cell-Autonomous Control of Posterior Blastomere Identities

The three maternal genes *skn-1*, *pal-1*, and *pie-1* all encode putative transcriptional regulators that act in P_1 descendants to initiate the different cell fate programs that define the fates of the four P_1-derived somatic founder cells called MS, E, C, and D (Bowerman *et al.*, 1993; Hunter and Kenyon, 1996; Mello *et al.*, 1996). Whereas other genes clearly are essential for the specification of these four fates (see the following), *skn-1*, *pal-1*, and *pie-1* may play the most direct and early roles of perhaps any maternal genes in specifying these blastomere identities. This conclusion is based on the observation that the elimination of both *skn-1* and *pal-1* functions from early embryos results in P_1 failing to produce any differentiated somatic cell types and instead producing many small and apparently undifferentiated descendants (Hunter and Kenyon, 1996). This is the only combination of all the mutants discussed in this review that results in the production of small, undifferentiated cells instead of in trans-fating. As described in the following, *pie1* appears to separate the functions of *skn-1* and *pal-1* in

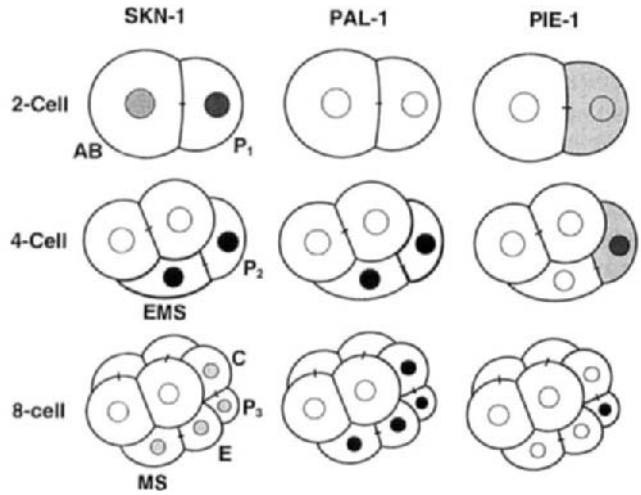

Fig. 6 Distributions of SKN-1, PAL-1, and PIE-1, three putative transcriptional regulators that specify the fates of P_1 descendants in the early embryo. SKN-1 accumulates to higher levels in P_1 than in AB and persists in P_1 descendants until the eight-cell stage. PAL-1 is detectable first at the four-cell stage, only in P_2 and EMS, and persists at high levels in P_1 descendants until the eight-cell stage and beyond. PIE-1 is mostly cytoplasmic at the two-cell stage but becomes localized to the nuclei of germline precursors in subsequent stages.

space and time and may specify germline fate in the remaining founder cell derived from P_1, the germline progenitor P_4.

Before a discussion of the genetic evidence that *pal-1*, *pie-1*, and *skn-1* specify the fates of P_1-derived somatic founder cells, it is helpful first to describe the distributions of the proteins encoded by these three genes (Fig. 6). SKN-1 is a sequence-specific DNA-binding protein with an unusual C-terminal DNA-binding domain related to the basic region of bZIP transcription factors (Bowerman *et al.*, 1992, 1993; Blackwell *et al.*, 1994). However, SKN-1 lacks the leucine zipper motif C-terminal to the basic region in bZIP proteins that is essential for dimerization and bZIP DNA-binding activity. Instead, SKN-1 terminates immediately after its C-terminal basic region and binds DNA as a monomer (Blackwell *et al.*, 1994). NMR studies indicate the SKN-1 DNA-binding domain by itself in solution may be an unstructured "molten globule" and that upon binding DNA it may use a novel fold of short α-helices to position an extended bZIP-like α-helix in the major groove of the DNA-binding site (Keith Blackwell, personal communication). SKN-1 is first detectable at very low levels in the maternal and paternal pronuclei at the end of the one-cell stage (Bowerman *et al.*, 1993). By late in the two-cell stage, SKN-1 accumulates to substantially higher levels in the nucleus of P_1 than in that of AB, and SKN-1 levels peak midway through the four-cell stage in the two P_1 daughters, EMS and P_2 (Fig. 6). SKN-1 is present at lower levels in all four P_1 descendants and undetectable in the four

AB descendants at the 8-cell stage, and SKN-1 is not detectable in any blastomeres by the 12-cell stage. SKN-1 is required to specify the fate of EMS, the parent of the two somatic founder cells, E and MS (Bowerman *et al.*, 1992).

The second putative transcription factor that specifies the fates of P_1-derived somatic founder cells, PAL-1, is a homeodomain protein (Hunter and Kenyon, 1996). The PAL-1 homoedomain is most similar in sequence to that of the *Drosophila* homeodomain protein Caudal, which, like PAL-1 in *C. elegans*, is required for patterning posterior cell fates in *Drosophila* (Hunter and Kenyon, 1996). PAL-1 is first detectable at the four-cell stage, in the nuclei of P_2 and EMS. PAL-1 remains present at high levels in all P_1 descendants until well after the 12-cell stage, when SKN-1 is undetectable. PAL-1 is required to specify the identities of the somatic founder cells C, a daughter of P_2, and D, a daughter of P_3.

Finally, PIE-1 is a Zn^{2+} finger protein that appears to separate the functions of SKN-1 and PAL-1, in part by virtue of its remarkable localization properties in the early embryo (Mello *et al.*, 1992, 1996). At the two-cell stage, PIE-1 is present cytoplasmically in P_1. During mitosis, PIE-1 localizes to the P_1 centrosomes, and as P_1 finishes dividing, PIE-1 leaves the centrosomes. PIE-1 disappears from the somatic daughter EMS but transits to the nucleus in the germline precursor, P_2. At each division of a germline precursor, PIE-1 localizes to the centrosomes during mitosis and then returns to the nucleus of the germline progenitor as mitosis ends. PIE-1 is thought to repress the activation of any somatic cell fate programs in germline precursors, maintaining the germline in a transcriptionally silent, inert, or undifferentiated state (Seydoux *et al.*, 1996).

The temporal and spatial regulation of SKN-1, PAL-1, and PIE-1 expression appears to play an important role in segregating the activities of SKN-1 and PAL-1, both of which are present at high levels in P_2 and EMS, even though each acts in only one of these two P_1 descendants to specify somatic founder cell fates (Mello *et al.*, 1992, 1996; Bowerman *et al.*, 1993; Hunter and Kenyon, 1996). By appearing at high levels before PAL-1, SKN-1 may predominate at the four-cell stage and specify EMS identity. Whereas SKN-1 specifies EMS identity, PIE-1 prevents SKN-1 from functioning in P_2 as part of its more general germline repressor function (Bowerman *et al.*, 1992; Mello *et al.*, 1992, 1996; Seydoux *et al.*, 1996). By the time P_2 divides to produce a C daughter free of PIE-1, SKN-1 is barely detectable and PAL-1 is present at high levels. Perhaps the higher levels of PAL-1 can override the fading levels of SKN-1 to specify C identity and, after P_3 divides, D identity (Hunter and Kenyon, 1996). Of course it is possible that as yet unidentified genes may provide additional means to regulate the time and place of SKN-1 and PAL-1 functions. For example, a factor localized to EMS could serve to block PAL-1 function in EMS much as PIE-1 blocks SKN-1 in P_2. Furthermore, because the maternal mRNAs for *pal-1*, *pie-1*, and *skn-1* are all distributed uniformly throughout early embryos, either translational regulation or differences in protein stability must account for their localized expression. Two

maternal genes, *mex-1* and *mex-3*, are required to prevent high levels of SKN-1 and PAL-1, respectively, from accumulating in anterior blastomeres and ectopically specifying EMS or P_2-like somatic cell fate patterns (see Section IV). Finally, as E, MS, C, and D all have very different fates, other genes must be necessary to distinguish E from MS and C from D (see the following).

Genetic studies of *skn-1*, *pal-1*, and *pie-1* indicate that they each play highly specific roles in specifying the fates of certain P_1 descendants without affecting the identities of other blastomeres in the early embryo. *skn-1* mutant embryos lack the endoderm normally made by E and the mesoderm normally made by MS. Instead, E and MS in *skn-1* mutant embryos each produce epidermal cells and body wall muscle, a fate similar to that of the C daughter of P_2 (Bowerman *et al.*, 1992). In the absence of SKN-1, the PAL-1 present in E and MS appears to respecify them to adopt more posterior C-like fates (Hunter and Kenyon, 1996). As mentioned earlier, in the absence of *skn-1* and *pal-1* function, very few or no differentiated somatic cell fates are produce by either P_2 or EMS. Thus, SKN-1 and PAL-1 appear to act very early to specify blastomere identities: In their absence, pattern formation appears absent even though cell division continues. Other blastomeres in *skn-1* mutant embryos appear to develop completely normally (Bowerman *et al.*, 1992). Both P_2 and ABp in *skn-1* mutant embryos produce normal patterns of cell division and cell fate. Although ABa fails to produce any anterior pharyngeal cells as *skn-1* mutant embryos completely lack a pharynx, the lack of ABa-derived pharyngeal cells is an indirect consequence of the requirement for *skn-1* function in EMS (Shelton and Bowerman, 1996). In the absence of *skn-1* function, MS is incapable of signaling wild-type ABa descendants to produce pharyngeal cells, but a wild-type MS can induce *skn-1* mutant ABa descendants to produce pharyngeal cells (Fig. 7). Thus, *skn-1* appears to specify EMS fate with respect to both the cell fate patterns it produces and the ability of MS to signal ABa.

The *pal-1* gene was first identified by the partial loss of function mutations that affect cell fate patterning postembryonically during larval development but are not lethal (Waring and Kenyon, 1990, 1991). A maternal role for *pal-1* was first suggested by the observation that the PAL-1 protein, in addition to being expressed zygotically in much of the posterior embryo, is also expressed maternally in P_1 descendants, beginning at the four-cell stage in P_2 and EMS (Hunter and Kenyon, 1996) (Fig. 6). The maternal requirements for *pal-1* were tested in two ways. First, for homozygous *pal-1* mutant mothers rescued by an extrachromosomal array containing the wild-type *pal-1* gene produce embryos, about 1 in 200 will lose the array specifically in the germline during mitotic division of early blastomeres. These animals grow up to produce broods of *pal-1* mutant embryos. Phenotypic analysis of these embryos indicates that P_2 usually fails to produce epidermal cells or body wall muscle cells, fates characteristic of C and D. Instead, P_2 in *pal-1* mutant embryos produces two germline-like cells resembling Z2 and Z3, the two germline precursors normally made by P_4, whereas the

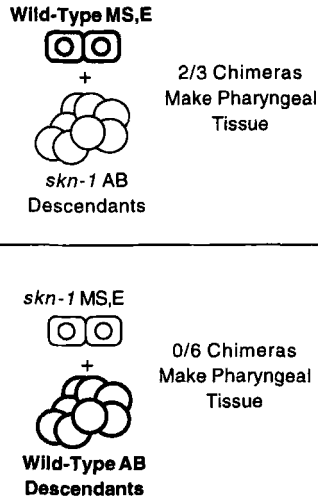

Fig. 7 SKN-1 is required for MS to signal AB descendants to produce pharyngeal cells at the 12-cell stage, but not for AB descendants to respond to MS signaling. By recombining in culture medium blastomeres isolated from wild-type (bold type) and skn-1 mutant embryos (light type), it is possible to reproduce early embryonic inductions and determine which blastomeres require particular gene functions.

remaining P_2 descendants appear small and undifferentiated (Hunter and Kenyon, 1996). Thus, in the absence of PAL-1, the low levels of SKN-1 present in C at the eight-cell stage are not sufficient to specify the production of any of the cell types normally made by E or MS. Similar results are observed when antisense *pal-1* RNA is microinjected into the ovaries of wild-type mothers to inactivate the endogenous maternal *pal-1* mRNA (Hunter and Kenyon, 1996).

Mutational inactivation of *pie-1* results in P_2 adopting a fate nearly identical to that of EMS, producing excess pharynx and intestine (Mello *et al.*, 1992). Thus, in the absence of the P_2-localized nuclear PIE-1 protein, the SKN-1 in P_2 specifies EMS fate ectopically. The smaller P_3 daughter of P_2 develops like E, and the larger daughter, C, usually develops like MS. Consistent with *pie-1* being required to block *skn-1* function in P_2, *pie-1;skn-1* double mutant embryos look like *skn-1* mutant embryos, failing to produce any pharyngeal or intestinal cells and instead making numerous epidermal cells and body wall muscle cells (Mello *et al.*, 1992). However, P_2 still fails to produce germline in *pie-1;skn-1* double mutant embryos, indicating that *pie-1* is required in P_2 for more than just blocking *skn-1* function; it is also required to specify germline. Studies of *pie-1* indicate that it functions as a general repressor of transcription in germline progenitors (Seydoux *et al.*, 1996). Several zygotic genes have been identified that begin transcription as early as the four-cell stage in somatic blastomeres. In all, 12 genes were studied that in wild type are transcribed in the somatic

daughter but not the germline daughter of a germline precursor. In all 12 cases, the gene was transcribed in both the somatic and the germline daughter in *pie-1* mutants. Mutations in the gene *mex-1* (see Section IV) have been shown to result in mislocalization of PIE-1 to somatic blastomeres, which in turn results in the ectopic repression of zygotic gene expression in the somatic blastomeres containing ectopic PIE-1 protein (Guedes and Priess, 1996). Thus, *pie-1* appears to function as a general repressor in germline precursors of somatic transcription programs. The observation that P_2 adopts an EMS-like fate in *pie-1* mutants indicates that SKN-1 may be the first embryonic determinant capable of activating pathways that specify somatic cell fates in P_1 descendants. Perhaps the absence of PIE-1 from the nucleus of P_1 indicates that zygotic gene expression in *C. elegans* does not begin until the four-cell stage. To summarize, during wild-type embryogenesis *pie-1* prevents *skn-1* function in P_2, limiting to EMS the production of *skn-1*-dependent endodermal and mesodermal cell fates and perhaps delaying the specification of P_2-derived somatic founder cells until a time when SKN-1 is nearly absent and PAL-1 can therefore predominate and specify C and D fates.

In addition to *pie-1*, six *mes* genes, *mes-1* through *mes-6*, have been identified that are maternally expressed and required for germline development. However, with the exception of *mes-1*, mutations in the *mes* genes do not affect the fates of embryonic blastomeres but instead result in the apparent degeneration, nonapoptotically, of the germline during larval development (Capowski *et al.*, 1991). Thus, *mes* mutations are maternal-effect sterile, or grandchildless, and *mes-2* through *mes-6* appear to act downstream of *pie-1* to either maintain germline or specify later stages of germline development in late embryos or larvae. Finally, other maternal genes that appear to be required specifically for P_1 and not AB fate have been identified. These include *mes-1*, already discussed, and *pos-1*, which will be discussed in Section IV.

2. The *mom* and *pop* Story: Wnt-Mediated Induction of Endoderm

The simple fates of most P_1 descendants (discussed earlier), the inability of P_2 and EMS to replace each other after having their positions interchanged, and the ability of P_1 to develop roughly normally after the removal of AB all seem to suggest that P_1 develops in a largely autonomous fashion. However, none of the preceding observations rule out important roles for cell interactions among P_1 descendants during development. In another simple and extremely informative set of experiments, in 1985, Schierenberg found that only germline precursors are able to maintain asymmetric divisions when allowed to develop in isolation after being extruded from the early embryos. He concluded that only germline precursors have an intrinsic polarity and predicted that somatic founder cells would require cell interactions with germline precursors in order to be properly polarized (Schierenberg and William, 1985). Seven years later, Goldstein showed

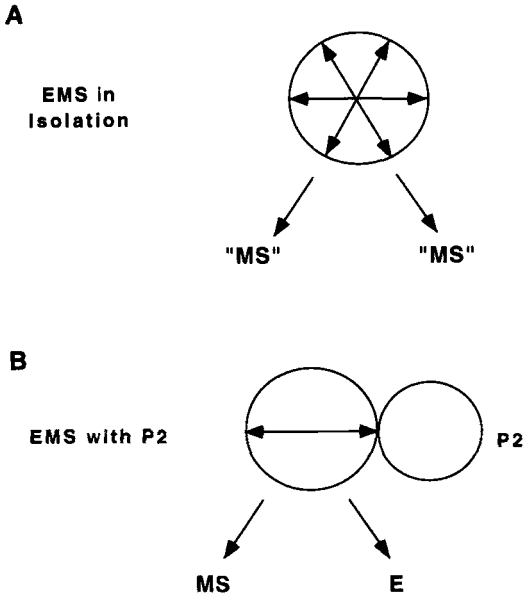

Fig. 8 A polarizing induction from P_2 signals EMS to orient its mitotic spindle axis and to induce the production of endoderm from the daughter of EMS born next to P_2. (A) In the absence of P_2 contact, EMS blastomeres cultured *in vitro* divide with randomly oriented mitotic spindles to produce two mesodermal precursors similar in fate to a wild-type MS blastomere. (B) When placed in contact with P_2, the EMS mitotic spindle rotates to lie along an axis defined by points at the center of P_2 and EMS, and EMS divides to produce one mesodermal precursor, MS, away from the site of contact with P_2 and one endodermal precursor, E, adjacent to P_2.

that the proper development of the somatic P_1 daughter EMS requires a polarizing signal from the neighbor and sister of EMS, the germline precursor P_2 (Goldstein, 1992). Normally, EMS divides to make one daughter, MS, that produces mesoderm and another daughter, E, that produces all of the endoderm in *C. elegans* (Sulston *et al.*, 1983). The signal from P_2 polarizes EMS such that the daughter of EMS born next to P_2 produces endoderm (Fig. 8). In the absence of the P_2 signal, EMS instead divides to make two MS-like daughters (Goldstein, 1992, 1993). Thus, the specification of E fate, the earliest founder cell born that makes a single cell type, requires an inductive signal from P_2 at the four-cell stage. Signals from P_2 not only polarize gut potential to one side of EMS but also orient the EMS mitotic spindle axis: Move P_2 to a different position on EMS, and the EMS spindle will rotate to "point" toward P_2, producing an E daughter close to P_2 and an MS daughter away from P_2 (Fig. 8). In an elegant series of experiments using isolated blastomeres placed in contact with each other at specific times after their birth, Goldstein (1995) found that the timing of the gut polariza-

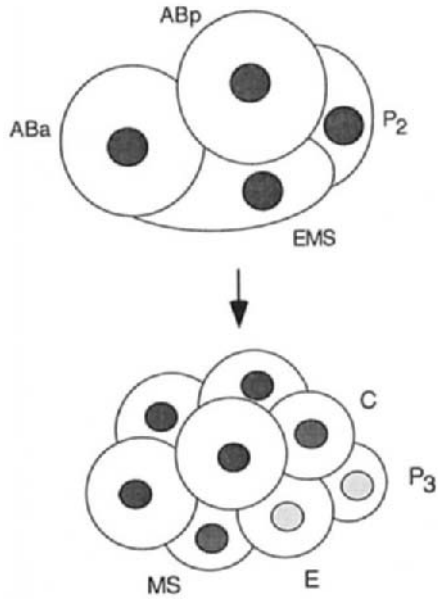

Fig. 9 POP-1 protein distribution in four-cell-stage and eight-cell-stage wild-type embryos. POP-1 contains a single HMG domain and is present in all nuclei at the four-cell stage. At the eight-cell stage, EMS divides to produce one daughter, MS, with high levels of nuclear POP-1 and one daughter, E, with low or undetectable levels of nuclear POP-1.

tion signal and the timing of the mitotic spindle orientation signal appear to be different, suggesting that two different signals from P_2 influence EMS fate.

pop-1 was the first maternal gene identified that is specifically required for distinguishing the fates of E and MS (Lin *et al.*, 1995). In *pop-1* mutant embryos, MS adopts an E-like fate, resulting in a 2-fold excess of intestinal cells at the expense of MS mesoderm. Thus, *pop-1* mutants have a phenotype opposite that caused by the elimination of P_2 signaling, producing two E-like daughters instead of the two MS-like daughters made in the absence of signaling. POP-1 is a putative transcription factor that contains a single HMG domain and is present in all nuclei at the four-cell stage. However, when EMS divides, nuclear POP-1 levels remain high in MS but drop in E (Fig. 9). Thus, down-regulation of nuclear POP-1 correlates with endoderm fate, and elimination of POP-1 from both E and MS in *pop-1* mutant embryos results in both EMS daughters adopting endodermal fates (Lin *et al.*, 1995). These results, together with those of Goldstein, suggest that polarization of EMS by P_2 signaling results in the differential segregation of gut potential to the daughters of EMS, such that nuclear POP-1 is present only in MS. If so, mutations in genes required for P_2 signaling should result in EMS producing two daughters that have high levels of POP-1 and adopt MS fates. Mutations in five such genes have been identified.

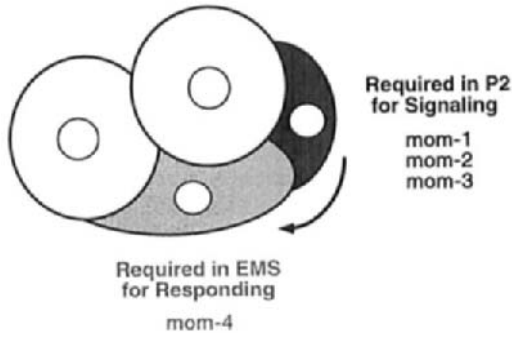

Fig. 10 Summary of which blastomeres require which *mom* gene functions based on an analysis of gut induction using genetically chimeric partial embryos made *in vitro* by recombining blastomeres isolated from mutant and wild-type embryos.

The mutationally identified genes required for the polarizing induction of gut potential in EMS are named *mom-1* through *mom-5*. In *mom* mutant embryos, EMS usually produces two MS-like daughters, resulting in excess mesoderm at the expense of all endoderm (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). By reassociating in culture medium the early blastomeres isolated from wild-type and *mom* mutant embryos, genetically mosaic partial embryos can be reconstituted to examine the requirements of different blastomeres for the different *mom* gene activities (Thorpe *et al.*, 1997). Three *mom* genes, *mom-1*, *mom-2*, and *mom-3*, are required in P_2 for signaling but not in EMS for responding, while *mom-4* is required in EMS for responding but not in P_2 for signaling (Fig. 10). All mutant alleles of the *mom* genes show incomplete penetrance for the gut defect. For example, even strong loss of function mutations in *mom-2* result in only about 75% of the mutant embryos lacking endoderm (Thorpe *et al.*, 1997). In almost all cases, mutations in the *mom* genes result in a completely penetrant defect in morphogenesis, a largely undefined and likely complex process that converts the round ball of cells made by embryonic cleavages into a long, thin worm. Thus, the *mom* genes probably participate in other processes in addition to the P_2 signaling that polarize gut potential in EMS.

Three of the five *mom* genes have been cloned, and all three are predicted to encode components of the widely conserved Wnt signal transduction pathway (Fig. 11). *mom-1* is a homologue of *Drosophila porcupine*, *mom-2* is a homologue of *Drosophila wingless/Wnt*, and *mom-5* encodes a *frizzled* homologue (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). Porcupine is a multipass transmembrane protein localized to the endoplasmic reticulum and required in signaling cells for proper processing and secretion of wingless (Perrimon, 1995). Wingless is a secreted glycoprotein that is required for many different cell signaling processes that pattern the bodies of both invertebrates and vertebrates

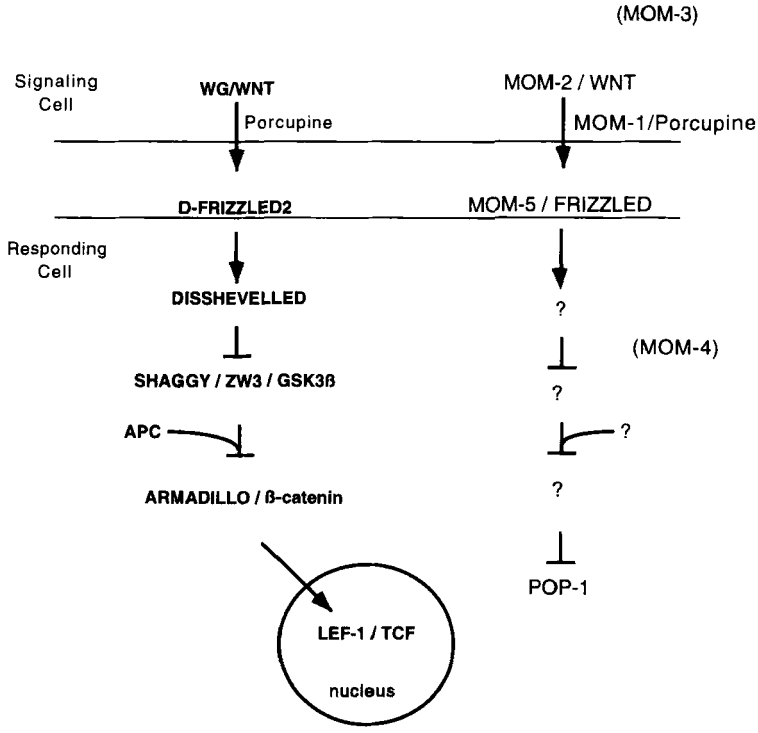

Fig. 11 A summary of the Wnt signal transduction pathway and the corresponding identities of *mom-1*, *mom-2*, and *mom-5*. The *mom-3* gene has not been cloned but is required in P_2 for signaling; *mom-4* has not been cloned but is required in EMS for responding to the polarizing signal from P_2.

(Moon, 1995). Thus, the requirements for *mom-1* and *mom-2* in P_2 are in accord with known functions of their *Drosophila* relatives. However, *mom-3* is also required in P_2 for signaling, and no other genes besides *porcupine* and *wingless* have been identified that are required in signaling cells for Wnt function. *mom-4* remains to be identified and thus could be any one of several components of the Wnt pathway known to act downstream of the signal (Moon, 1995). *mom-5* encodes a frizzled homologue and therefore is likely to be the receptor for MOM-2/WNT based on findings in *Drosophila* (Nusse, 1996).

A detailed discussion of the role of Wnt signaling in gut polarization is beyond the scope of this chapter. Instead, I focus on how the current analysis of Wnt signaling in *C. elegans* sheds new light on Wnt signaling mechanisms. As mentioned earlier, *mom-3* is a third gene required in signaling cells, along with *mom-1/porcupine* and *mom-2/wingless*, and therefore might represent a novel Wnt pathway component. Furthermore, the final step of gut polarization identified thus far is the down-regulation of POP-1 in E to permit endoderm fate. In mom mutant embryos, this down-regulation of POP-1 is lost, and POP-1 levels

remain high in both EMS daugzahters, consistent with POP-1 preventing either from adopting an endodermal fate (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). We conclude that to polarize gut potential, Wnt signaling down-regulates an HMG domain protein, POP-1, in E. By contrast, in all other studies of the Wnt pathway, signaling results in the activation of an HMG domain protein, such as LEF-1 or TCF, via an interaction with *armadillo*/β-catenin (Moon, 1995). Finally, intriguing defects in mitotic spindle orientation occur in the early blastomeres of *mom-1*, *mom-3*, and *mom-5* single mutant embryos and in *mom-1;mom-2* double mutant embryos (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). These results suggest that Wnt signaling affects both gut polarization and the P_2-induced orientation of the EMS mitotic spindle (discussed earlier), which were initially proposed to be two different processes (Goldstein, 1995). However, mutations in other Wnt pathway genes do not affect spindle orientation in EMS, and the relationship between these two process remains unclear. Nevertheless, these results provide evidence that Wnt signaling might regulate the polarity of the cytoskeleton in EMS, an intriguing possibility given the association of *armadillo*/β-catenin with the cytoskeleton at adhesion plaques, in addition to its cytoplasmic and nuclear locations during Wnt signaling to the nucleus (Moon, 1995). Finally, mutations in some *mom* genes affect spindle orientation not only in EMS but also in other somatic blastomeres, indicating that Wnt signaling may influence blastomere polarity throughout most of the early embryo (Rocheleau *et al.*, 1997; Thorpe *et al.*, 1997). But as Schierenberg first predicted, the embryonic germline precursors may rely more on autonomous polarization mechanisms (Schierenberg and William, 1985), and all germline precursors appear to have normal mitotic spindle orientations and asymmetric divisions in all *mom* mutant embryos examined thus far (Thorpe *et al.*, 1997).

In addition to Wnt signaling, other signals have been identified that influence the fates of MS and D. However, only two other molecules have been identified that, by genetic and molecular criteria, participate in cell interactions that influence blastomere identity. These two genes, *apx-1* and *glp-1*, are the two remaining blastomere identity group genes to be discussed in this review. Rather than influencing P_1 development, these two genes participate in a sequence of early inductions that specify the different identities of AB descendants. Other genes influencing P_1 development are described later in Section IV.

C. Cell Interactions That Specify the Fates of Anterior Blastomeres

Shortly after the finding that ABa and ABp are born with equivalent developmental potential (discussed earlier), the maternal gene *glp-1* was identified and shown to be required for a cell interaction that contributes to making ABa and ABp different (Priess *et al.*, 1987). In wild-type embryos, the MS blastomere signals the two granddaughters of ABa that touch MS to adopt fates that include the production of the cells forming the anterior part of the pharynx (Priess *et al.*,

1987; Hutter and Schnabel, 1994; Mango *et al.*, 1994b). If MS is killed with a laser microbeam at the end of the 8-cell stage, no pharyngeal cells are induced, but if MS is killed later in the 12-cell stage, the induction occurs and pharyngeal cells are made by the two ABa granddaughters. In *glp-1* mutant embryos, the 12-cell-stage induction of pharyngeal cells does not occur, and genetic mosaic studies of *glp-1* during larval development indicated that *glp-1* is required in responding cells (Austin and Kimble, 1987; Priess *et al.*, 1987). *glp-1* encodes a member of the Notch family of transmembrane receptors, which, like the Wnt pathway, participates in many cell interactions that pattern cell fates during vertebrate and invertebrate development (Austin and Kimble, 1989; Yochem and Greenwald, 1989). Antibodies to GLP-1 show that it is present at the cell surfaces of ABa and ABp in the 4-cell stage and persists at high levels in AB descendants until the 28-cell stage (Evans *et al.*, 1994). Thus, GLP-1 is present on the responding cells at the appropriate time to act as the receptor for the 12-cell stage signal from MS that induces the production of pharyngeal cells (Fig. 12). Lower

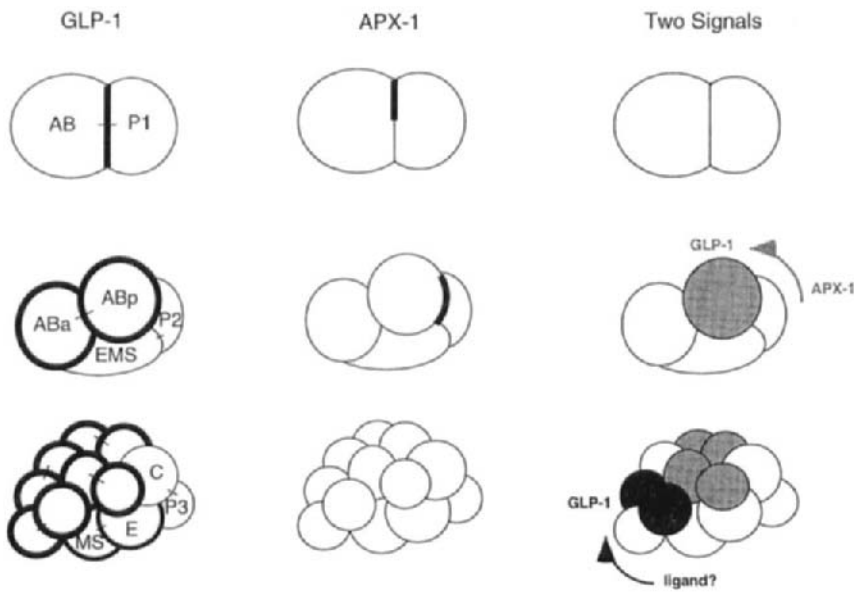

Fig. 12 Summary of GLP-1 and APX-1, Notch and Delta homologues, respectively, in two-, four-, and eight-cell-stage embryos. GLP-1 is first detected at the P_1/AB boundary at the 2-cell stage and on the surfaces of AB descendants at the 4-cell and 12-cell stages. APX-1 is first detected at the interface of P_1 and AB, but only in about one-half of the boundary; mechanisms controlling this early asymmetry and its functional significance remain unknown. APX-1 is present at the interface of P_2 and ABp at the 4-cell stage, but fades to undetectable levels by the 12-cell stage. APX-1 and GLP-1 are required for a cell–cell interaction at the four-cell stage that specifies ABp identity and breaks the initial equivalence of ABa and ABp (light shading). GLP-1 and an unknown ligand produced by MS are required for a second cell–cell interaction that specifies the fates of two granddaughters of ABa at the 12-cell stage (dark shading).

levels of GLP-1 are present in E and MS, and studies indicate that GLP-1 is required to receive a signal required for MS to produce body wall muscle (Schnabel, 1994).

The finding that MS induces ABa to produce pharyngeal cells raises an important question: If ABa and ABp are born equivalent, why does the MS signal influence only ABa descendants and not ABp descendants that touch MS? Because P_2 is the only four-cell-stage blastomere that touches only ABp and not ABa, P_2 was a logical candidate for sending a signal to break the equivalence of ABa and ABp at the 4-cell stage, and an earlier signal from P_2 might render ABp descendants resistant to the inductive signal from MS at the 12-cell stage. A variety of blastomere manipulation experiments provided evidence for such an interaction in wild-type embryos. If P_2 is killed with a laser microbeam just as it is born, ABp often fails to produce intestinal–rectal valve cells, an ABp-specific cell type (Bowerman *et al.*, 1992). Removal of P_2 within 5 min of its birth results in the absence of intestinal–rectal valve cells and the production of excess pharyngeal cells (Mango *et al.*, 1994a). If P_1 is prevented from dividing such that a large undivided P_1 touches both ABa and ABp, extra intestinal–rectal valve cells are made and no pharyngeal cells are induced (Mello *et al.*, 1994). Finally, if P_1 and AB are allowed to divide without an intact eggshell forcing their spindles to skew obliquely, P_2 is born out of contact with both AB daughters, in which case no intestinal valve cells and large numbers of pharyngeal cells are produced (Mello *et al.*, 1994). In sum, these experiments indicate that a signal from P_2 at the 4-cell stage is required for the production of ABp-specific cell types and for prevention of the production of pharyngeal cells by ABp in response to a signal from MS at the 12-cell stage.

Genetic evidence for a P_2 signal specifying ABp fate came both from the identification of the maternal gene *apx-1* and from an elegant analysis of temperature-sensitive alleles of *glp-1* (Hutter and Schnabel, 1994; Mango *et al.*, 1994a; Mello *et al.*, 1994). Mutations in *apx-1* result in ABp adopting a fate much like that of ABa: ABp in *apx-1* mutant embryos fails to produce ABp-specific cell types, and the ABp granddaughters that touch MS produce extra anterior pharyngeal cells in response to MS signaling at the 12-cell stage. Consistent with *apx-1* being required for P_2 signaling, it encodes a transmembrane protein related to the Delta family of Notch ligands, and the APX-1 protein is produced in P_2 at the four-cell stage localized to the interface of P_2 and ABp (Mickey *et al.*, 1996). The localization of APX-1 to the interface of P_2 and ABp could be simply due to clustering of APX-1 by its receptor on ABp. However, APX-1 is also present at the two-cell stage at the interface of P_1 and AB, but on only one side of the embryo (Fig. 12). Thus, APX-1 localization may be polarized in some manner that cannot be explained by contacts with neighboring blastomeres.

The identity of APX-1 as a Delta family member suggests that GLP-1, a Notch family member present on the surface of ABa and ABp at the four-cell stage, might act as the receptor for the APX-1 ligand made by P_2. Temperature shift

experiments using a conditional allele of *glp-1* showed that GLP-1 functions as the receptor both for the 4-cell-stage signal involving *apx-1*, which specifies ABp identity, and for the 12-cell-stage signal from MS, which induces pharyngeal cell production by ABa (Hutter and Schnabel, 1994; Mello *et al.*, 1994). If *glp-1* function is blocked by raising embryos at the restrictive temperature but then restored shortly after the four-cell stage by shifting to permissive temperature, the P_2 signal is blocked and ABp develops just as it does in *apx-1* mutant embryos, failing to produce ABp-specific cell types and insteading making excess anterior pharyngeal cells in response to the MS inductive signal. If *glp-1* function is blocked at both the 4-cell and 12-cell stages, neither ABp-specific cell types nor anterior pharyngeal cells are produced; instead all AB descendants adopt one of two "uninduced" ABa fates, that of a wild-type ABala or ABarp blastomere. Therefore, GLP-1 appears to function as the receptor for both the 4-cell-stage signal from P_2 and the 12-cell-stage signal from MS, which induces pharyngeal cell production by the two granddaughters of ABa that touch MS. Whereas the ligand for the MS signal remains unknown, MS requires SKN-1 function to express signaling activity, and presumably the MS ligand is also a Delta family member that activates the GLP-1/Notch receptor (Shelton and Bowerman, 1996).

The specific responses by AB descendants to the P_2 and MS signals—ABp fate vs induced ABa granddaughter fates—appear to depend not on the specific nature of the ligand or signaling blastomere but rather on time-dependent differences in the response of AB descendants to activation of the GLP-1 receptor. By using isolated blastomeres, it is possible to induce 12-cell-stage AB descendants to produce pharyngeal cells, either by placing them in contact with MS, the normal signaling blastomere from a 12-cell-stage embryo, or by placing them in contact with P_2 to form a "heterochronically" chimeric partial embryo *in vitro* (Shelton and Bowerman, 1996). Pharyngeal induction by P_2, but not by MS, requires *apx-1* function, indicating that APX-1 in P_2 can substitute for the MS signal to induce 12-cell-stage AB descendants to produce pharyngeal cells. Factors that presumably change with time in AB descendants to mediate the different responses to GLP-1 signaling at the 4-cell and 12-cell stages have yet to be identified, as do any autonomously acting factors responsible for specifying the uninduced fates of 12-cell-stage AB descendants.

A third maternal gene required for proper regulation of GLP-1 signaling in the early embryo, called *aph-2*, has been identified (C. Goutte and J. Priess, personal communication). Mutations in *aph-2* frequently result in mutant embryos having a phenotype similar to that of *glp-1* mutant embryos in which only the MS signal is defective: *aph-2* mutant embryos usually lack anterior pharyngeal cells and instead produce extra neurons and epidermis. However, about 10% of *aph-2* mutant embryos resemble *apx-1* mutants, with both ABa and ABp producing anterior pharyngeal cells. Thus, mutations in *aph-2* appear in some cases to result in a loss of *glp-1* function only at the 12-cell stage and in other cases to result in a loss of *glp-1* function only at the 4-cell stage. The APH-2 protein is novel but has a signal sequence and is predicted to be secreted and bound to the outer surface of

the plasma membrane via a GPI linkage. By making chimeric partial embryos in culture medium using isolated blastomeres from wild-type and *aph-2* mutant embryos, it has been shown that either signaling or responding blastomeres can supply *aph-2* function. Thus, APH-2 appears to be a membrane-bound extracellular protein that can be produced by either responding or signaling cells and somehow influences *glp-1* function during early embryonic inductions. It will be interesting to learn whether similar factors modulate Notch function during embryogenesis in other animals.

Finally, other signals have been identified that serve to specify the eight different fates of the AB descendants present in a 12-cell-stage embryo (Hutter and Schnabel, 1995). However, the gene products that mediate these other interactions remain to be identified, and thus these other interactions are not covered further in this chapter.

IV. The Intermediate Group Genes: *mex-1, mex-3,* and *pos-1*

This third and final group of mutants exhibits specific but relatively widespread defects in blastomere fates compared to the blastomere identity group mutants described earlier. Intermediate group mutant embryos usually have normal cleavages up until the division of P_3, which in wild-type embryos divides asymmetrically to produce a smaller germline progenitor, P_4, and a larger somatic daughter, D (Fig. 2). In *mex-1*, *mex-3*, and *pos-1* mutant embryos, P_3 divides equally and produces two daughters with similar fates (Mello *et al.*, 1992; Draper *et al.*, 1996; R. Hill and J. Priess, personal communication). However, the changes seen in blastomere identity are distinct for each intermediate group mutant, and in each mutant other blastomere identities are also affected (see the following). In addition to the P_3 defect, some *mex-1* mutant embryos show more global, *par*-like defects, including a small fraction that have an equal first cleavage (Schnabel *et al.*, 1996). P-granule localization defects parallel the cleavage defects: Normal or roughly normal segregation occurs until the division of P_3, at which time no P-granule segregation occurs. Again, *mex-1* mutants appear to be more severely defective: P-granules usually are segregated to P_1, P_2, and P_3, but they fail to associate with the posterior cortex in germline precursors, and many are lost to somatic daughters during cell division (Mello *et al.*, 1992; Schnabel *et al.*, 1996). Thus, *mex-1* mutants may be defective not in moving P-granules but in anchoring them to the posterior cortex.

A. Intermediate Group Gene Products

Molecular analysis has shown that two of the genes in this group, *mex-1* and *pos-1*, are related and both encode proteins with a Zn^{2+} finger domain (Guedes

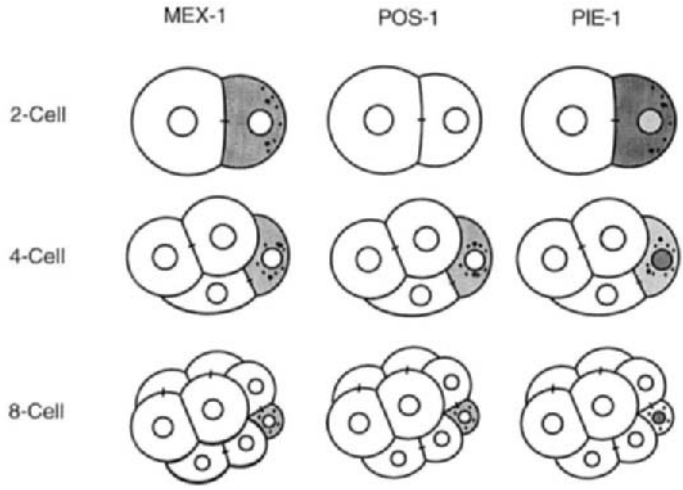

Fig. 13 Summary of the distributions of three TIS-11-like Zn^{2+} finger proteins in the early embryo. MEX-1, POS-1, and PIE-1 are all present in germline precursors and are associated with P-granules. All three proteins are first detectable at the two-cell stage in P_1. MEX-1 and POS-1 are cytoplasmic and associated with P-granules; PIE-1 at first is cytoplasmic and associated with P-granules in P_1 but becomes nuclear, localized, and associated with P-granules in later stages. No P-granule association is detectable for any of these proteins in oocytes.

and Priess, 1996; R. Hill and J. Priess, personal communication). The spacing of cysteine and histidine residues found in both *mex-1* and *pos-1* is also found in vertebrate TIS-11 genes, which are expressed early in response to treatment of fibroblasts with triphorbol esters but are of unknown function (Guedes and Priess, 1996). The MEX-1 and POS-1 proteins also show nearly identical distributions in the early embryo (Fig. 13). They are present in the cytoplasm of germline precursors, and after each division of a germline precursor they rapidly fade from the somatic daughter. Intriguingly, both proteins associate with P-granules in the early embryo. Like *mex-1* and *pos-1*, the maternal gene *pie-1* also encodes a TIS-11-like Zn^{2+} finger protein (Mello *et al.*, 1996). *pie-1* mutant embryos resemble *mex-1* and *pos-1* mutants in that P_3 divides equally, and the PIE-1 protein also is progressively localized to germline precursors and associates with P-granules at the two-cell stage (Mello *et al.*, 1992, 1996). PIE-1 is different from MEX-1 and POS-1 in that it localizes to centrosomes during mitosis and to nuclei during interphase beginning at the four-cell stage (Fig. 13). For all three TIS-11-like proteins, no association with P-granules is detectable in the maternal germline, due either to their absence or perhaps to masking by other proteins. In either case, these results indicate that P-granules are dynamic structures, raising the interesting possibility that P-granules load and perhaps unload different factors at different times during the early cleavages that generate found-

er cells. Finally, *mex-1* is required to restrict PIE-1 to the germline when P_3 divides: In *mex-1* mutant embryos, PIE-1 is mislocalized to and represses the transcription of zygotic genes in both P_3 daughters, indicating that in addition to being related these genes also have some regulatory interactions (Guedes and Priess, 1996). To summarize, three maternal genes, *mex-1*, *pos-1*, and *pie-1*, are all related by sequence and show similar localization to the germline and an association with P-granules. The functions of TIS-11-like Zn^{2+} finger domains are not known, but this group of genes, and how they interact, is likely to become an important focus in future studies of embryonic patterning in *C. elegans*.

B. Intermediate Group Mutant Phenotypes

Aside from the equal division of P_3, the phenotypes that result from inactivation of intermediate group genes are different from one another (see the following). However, in all three mutants, most affected blastomeres are transformed into more posterior fates (Draper *et al.*, 1996; Guedes and Priess, 1996; R. Hill and J. Priess, personal communication). On the basis of these observations, it seems reasonable to consider the possibility that intermediate group genes mediate assignments of blastomere identity based on interpretations of the AP polarity generated more directly by the par group gene products (discussed earlier). Nevertheless, the phenotypes of these three mutants are all quite distinct. As summarized in the following, *mex-1* and *mex-3* mutants have extensive defects in AB development in addition to P_3 defects, whereas *pos-1* mutants have extensive defects in P_1 development.

mex-1 mutant embryos are the most *par*-like of the intermediate group mutants. In some embryos, the first cleavage is equal, and P-granule partitioning is defective throughout the early cleavages of all mutant embryos (Mello *et al.*, 1992; Schnabel *et al.*, 1996). Most obvious in *mex-1* mutant embryos, the four granddaughters of AB often adopt fates similar to that of MS, a descendant of the posterior blastomere, P_1. Consistent with this finding, SKN-1 protein is mislocalized in *mex-1* mutant embryos, accumulating to high levels in ABa and ABp at the four-cell stage and in their daughters at the eight-cell stage (Bowerman *et al.*, 1993). Thus, *mex-1*, like *par-1* and *par-3*, is required for restricting the accumulation of high levels of SKN-1 to P_1 descendants (Bowerman *et al.*, 1997). Although many AB descendants develop like MS, in most cases they appear to adopt fates that are mosaics of AB and MS fates, as determined by cell lineage studies (Schnabel *et al.*, 1996). Thus, transformations in blastomere identities in the early *C. elegans* are by no means absolute and can instead be partial in nature. In some mutant embryos, E fails to produce gut and instead adopts a C-like fate, which is a more posterior fate in the sense that C is a daughter of P_2 and the posterior sister of EMS, the parent of E. The equal cleavage of P_3 in *mex-1* mutants results in a loss of germline and the production

of two D-like daughters that produce body wall muscle cells, as does a wild-type D blastomere. Finally, although some *mex-1* mutant embryos exhibit extensive defects in the development of both P_1 and AB descendants, in many *mex-1* mutant embryos, P_2 and EMS development, aside from the abnormal development of P_3, appears largely normal. Thus, whereas *mex-1* mutant embryos resemble *par* mutant embryos in many respects, *mex-1* mutant embryos may have less extensive losses of AP polarity.

mex-3 mutant embryos are like *mex-1* mutant embryos in that their most obvious defect is the abnormal development of AB granddaughters (Draper *et al.*, 1996). However, instead of adopting MS-like fates, they adopt C-like fates. As described earlier, *pal-1* is required for specifying C fate, and PAL-1 is present at high levels only in the nuclei of P_2 and EMS at the four-cell stage. In *mex-3* mutant embryos, PAL-1 is evenly distributed in all four-cell-stage blastomeres, suggesting that PAL-1 acts ectopically to specify C-like fates in the granddaughters of AB (Hunter and Kenyon, 1996). Consistent with this hypothesis, AB descendants no longer develop like C in *mex-1;pal-1* double mutant embryos, in that they no longer produce body wall muscle cells (Hunter and Kenyon, 1996). The MEX-3 protein contains two KH domains, found in RNA-binding proteins, and is present at higher levels in the cytoplasm of AB than in P_1 at the beginning of the two-cell stage and through the four-cell stage (Draper *et al.*, 1996). Furthermore, *pal-1* mRNA is present throughout the early embryo, and the 3′-UTR of *pal-1* mRNA is necessary and sufficient to localize the translation of a lacZ reporter RNA to P_1 descendants (Hunter and Kenyon, 1996). Thus, it has been proposed that MEX-3 directly regulates PAL-1 expression by acting as a translational repressor in AB, perhaps binding 3′-UTR sequences in *pal-1* mRNA (Draper *et al.*, 1996; Hunter and Kenyon, 1996). In later stage embryos, MEX-3 is present at very low levels only in P_2 descendants and is undetectable in other blastomeres.

How the complex expression pattern of MEX-3 is regulated remains largely unknown, but it appears to occur in part by localization of the *mex-3* maternal mRNA (Fig. 14). *mex-3* is unique among the maternal genes discussed thus far in showing an enrichment of mRNA in AB (Draper *et al.*, 1996). At the beginning of the one-cell stage, *mex-3* mRNA is distributed evenly but it fades to lower levels posteriorly by the time the zygote divides. During the two-cell and four-cell stages, higher levels of *mex-3* mRNA are present in AB and its daughters than in P_1 and its daughters. Subsequently, *mex-3* mRNA levels fade to below detectable levels except in the germline, an observation true of many maternal mRNAs in *C. elegans* (Seydoux and Fire, 1994). The stability of maternal mRNAs in the germline may simply reflect the apparently inert status of the germline, maintained at least in part by *pie-1* function (discussed earlier).

Finally, *mex-3* is unique among mutants with equal P_3 cleavages (*pie-1*, *mex-1*, *mes-1*, *mex-3*, and *pos-1*) in that both P_3 daughters adopt a germline fate (Draper *et al.*, 1996). In all the other mutants, both P_3 daughters adopt a D-like

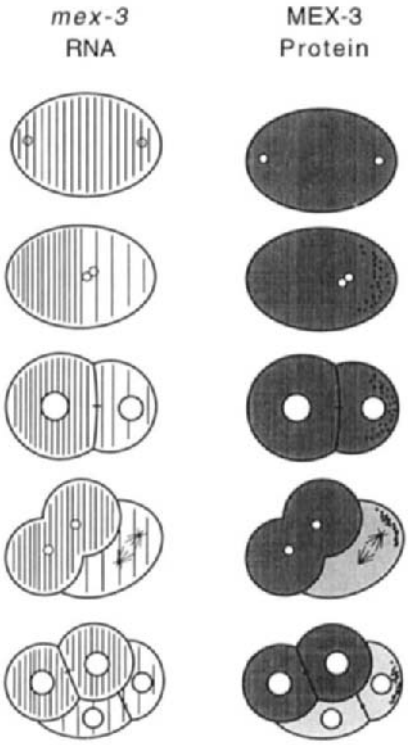

Fig. 14 Distribution of *mex-3* RNA and protein in one-, two-, and four-cell-stage wild-type embryos. The maternally expressed *mex-3* mRNA is evenly distributed in oocytes and early one-cell-stage embryos, but becomes enriched in the anterior part of the zygote shortly before mitosis and remains enriched in AB and its daughters at the two-cell and four-cell stages. Similarly, MEX-3 is first detected throughout oocytes and the one-cell-stage embryo, but begins to fade to lower levels in P_1 as it divides and remains enriched in ABa and ABp at the four-cell stage. MEX-3 is also associated with P-granules beginning late in the one-cell stage. The only other maternal gene that produces a localized mRNA is *pos-1*, which is present in P_1 but not in AB at the two-cell stage (not shown).

fate and produce body wall muscle cells (discussed earlier). Thus, not all blastomere transformations in the intermediate group mutants are to more posterior fates, as the transformation occurs in both directions for P_3 daughters depending on the mutant background. Also, as P-granules are mislocalized in all mutants when P_3 cleaves equally, their mislocalization is not sufficient to specify germline. However, as mentioned earlier, mutations in the maternal gene *pgl-1* result in a loss of detectable P-granules and sterility, indicating that P-granules are necessary but not sufficient for specifying germline fate and that somatic blastomeres may be resistant to any germline specification activities that P-granules may possess (S. Strome, personal communication).

As described earlier, *pos-1* also encodes a TIS-11-like Zn finger protein present in the cytoplasm of germline precursors and in P-granules beginning at the two-cell stage. Mutations in *pos-1* cause widespread defects in the development of P_1 descendants (R. Hill and J. Priess, personal communication). MS fails to produce body wall muscle, while E develops like C as in some *mex-1* mutant embryos. C development appears normal, but P_3 divides evenly as in *mes-1*, *mex-1*, *mex-3*, and *pie-1*. As in *mex-1* and *mes-1* mutant embryos, both P_3 daughters adopt a D-like fate, producing excess body wall muscle at the expense of germline. Finally, in both *pos-1* and *pie-1* mutant embryos, ABa and ABp produce anterior pharyngeal cells due to a lack of APX-1 expression (Mango *et al.*, 1994a; R. Hill and J. Priess, personal communication). Thus, mutations in *pie-1* and *pos-1* alter P_2 identity not only in terms of the cell fate patterns produced by P_2 but also in terms of P_2's ability to signal ABp via APX-1 to break the initial equivalence of ABa and ABp (discussed earlier). In contrast, P_2's ability to induce gut is normal, at least in *pie-1* mutants. Thus, mutations in genes that pattern the identities of P_1 descendants uncouple different temporal aspects of P_2 identity, even when comparing activities that both depend on maternally expressed regulatory factors.

V. Pathways of Blastomere Development

Thus far I have summarized a substantial body of work devoted to identifying maternal genes in *C. elegans* that regulate the early steps in pattern formation. This effort continues, but an even more challenging problem now is to understand how these gene functions are related. What are the genetic pathways that specify the eventual fates of individual blastomeres, and how do these pathways interact to coordinate their activities? Presumably the coordinated function of a limited set of such pathways results in the proper temporal and spatial regulation of transcription factors that regulate undefined sets of zygotic genes to specify blastomere identities. One example of such a genetic pathway involves the genes *par-1* and *mex-3* temporally and spatially regulating the expression of the putative transcription factor PAL-1 (Fig. 15).

A *par-1*, *mex-3*, and *pal-1* pathway can be understood most easily by referring first to the distributions of MEX-3 and PAL-1 in *par-1* mutant embryos (Fig. 15). MEX-3, normally present at higher levels in ABa and ABp than in P_2 and EMS, is present at high levels in all four-cell-stage blastomeres in *par-1* mutant embryos (Draper *et al.*, 1996). Consistent with the proposed role of MEX-3 as a translational repressor of *pal-1* in ABa and ABp in wild-type embryos, high levels of MEX-3 throughout *par-1* embryos correlate with the complete loss of PAL-1 protein (Hunter and Kenyon, 1996). Thus, mislocalization of MEX-3 to P_1 descendants causes ectopic repression of *pal-1* translation and hence a complete absence of PAL-1 from early *par-1* mutant embryos. Moreover, elimination

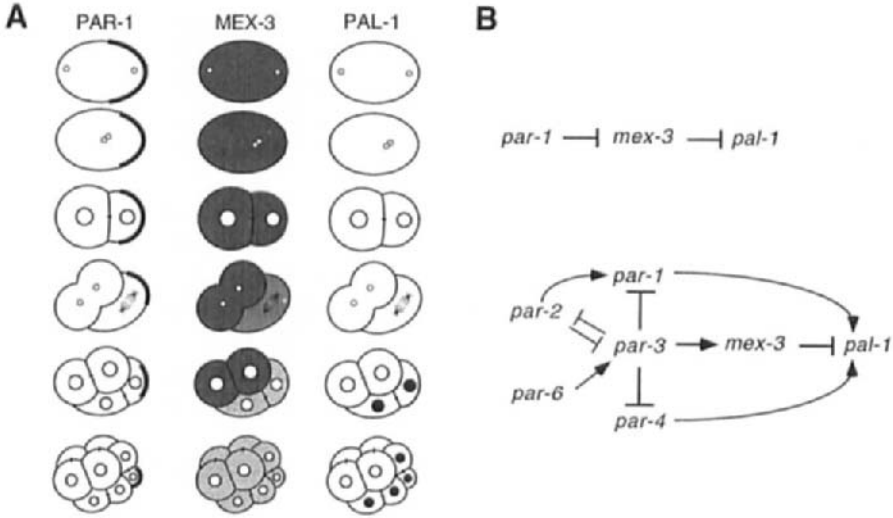

Fig. 15 Summary of PAR-1, MEX-3, and PAL-1 protein localization and models for the genetic interactions. See previous figure legends for a description of the protein localizations, illustrated in (A) for one-, two-, four, and eight-cell-stage wild-type embryos. (B, top) Simple pathway proposed for the regulation of PAL-1 expression by PAR-1 and MEX-3. (B, bottom) More complex network of gene function that attempts to account for all known functional interactions and protein localization data for the genes shown. *par-2* and *par-3* interact based on genetic studies and on analysis of their respective protein localization patterns in wild-type, *par-2*, and *par-3* mutant embryos. *par-2* is required for cortical localization of PAR-1, and *par-6* is required for cortical localization of PAR-6. *par-1* and *par-4* are both required for PAL-1 expression in posterior blastomeres, whereas *par-3* is partially required and *mex-3* is absolutely required to prevent PAL-1 expression in AB descendants. Therefore, *par-3* might indirectly limit PAL-1 expression to posterior blastomeres by restricting the *par-1*- and *par-4*-dependent activation of PAL-1 expression to posterior blastomeres. In this latter model, *par-3* is only partially required to limit PAL-1 expression to posterior blastomeres, and *mex-3* always functions to repress PAL-1 expression unless other factors, including *par-1* and *par-4*, overcome that repression to activate PAL-1 expression. By proposing such genetic pathways that connect the functions of the par group genes, through the intermediate group genes, to the localized function of blastomere identity group genes, it is now possible to begin to account more fully for the linkage between the establishment of AP polarity and the activation of blastomere identity programs in early founder cells. Bruce Draper generously provided materials for Figures 14 and 15.

of *mex-3* function in *par-1* mutant embryos restores PAL-1 expression and function in all four-cell-stage blastomeres (Draper *et al.*, 1996; Hunter and Kenyon, 1996). Finally, the localization of PAR-1, a putative Ser–Thr kinase, to the posterior cortex of P_1 in wild-type embryos correlates with the loss of *mex-3* mRNA and protein in P_1 and its daughters. Thus, PAR-1 may block *mex-3* function in the posterior part of the zygote, perhaps even directly by phosphorylating MEX-3, permitting the translation of *pal-1* in posterior blastomeres and eventually leading to the specification of the somatic founder cell fates derived from P_2 (Fig. 15).

However, studies of *par-3* mutant embryos indicate that the regulation of PAL-1 expression by *par-1* and *mex-3* may be more complex (Bowerman *et al.*, 1997). Like *par-1* mutant embryos, *par-3* mutant embryos mislocalize MEX-3 such that it is expressed at high levels in all four-cell-stage blastomeres. However, unlike *par-1* embryos, PAL-1 is expressed at normal levels and sometimes even mislocalized in *par-3* mutant embryos. One interpretation of this result is that *par-1* might act independently of *mex-3* to derepress the translation of *pal-1* mRNA (Fig. 15). While the linearity of a *par-1*, *mex-3*, and *pal-1* pathway in the early embryo requires additional testing, these studies provide an example of how genetic analyses in *C. elegans* are beginning to identify pathways that link the establishment of polarity in the zygote to the specification of individual blastomere identities in early stage embryos.

VI. Concluding Remarks

One purpose of this review has simply been to summarize progress in identifying maternal genes that regulate early steps in pattern formation. A more ambitious goal is to integrate this information into the construction of genetic pathways that can begin to explain at a molecular level how a cellularized embryo develops. Toward this latter end, I have tried to group the known maternal genes into categories that correspond not to close similarities in phenotype but rather to how early or late a particular gene might fit into a pathway of blastomere development. From what we know thus far, these pathways must link the specification of AP polarity initiated by sperm entry and PAR protein polarization into a reproducible pattern of founder cell identities specified by localized transcription factors such as PAL-1 and SKN-1. Genes within each of the three groups that I have defined in some cases appear to regulate each other, in addition to regulating a gene(s) in the group downstream of them. However, only one such pathway can be constructed that links members of all three groups: the possible *par-1*, *mex-3*, and *pal-1* pathway just described (Fig. 15). Almost certainly, these three groupings will prove inadequate to either describe all maternal genes or even integrate our understanding of those it can describe. Nevertheless, similar and perhaps more elaborate efforts should aid attempts to understand how a presumably complex network of gene regulation functions to specify the *C. elegans* body plan.

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4

Eye Development in *Drosophila*: Formation of the Eye Field and Control of Differentiation

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I. Introduction

The value of vision as a way for organisms to obtain long-range information about their environment is clear from the wide variety of species that have evolved systems for detecting light or processing more complex visual information. Research results have suggested that the basic mechanisms regulating the development of these systems may have been conserved throughout evolution. The insect compound eye, although very different in structure from the vertebrate eye, is also a complex and sophisticated visual organ. Insect eyes accomplish spatial resolution by using multiple copies of a simple unit eye precisely aligned on a curved surface, such that each has a slightly different orientation. In *Drosophila melanogaster*, each eye consists of approximately 800 of these unit eyes, called ommatidia. Each ommatidium contains eight photoreceptor cells, four lens-secreting cone cells, seven pigment cells, and a mechanosensory bristle [for a description of the structure of the adult retina, see Wolff and Ready (1993)]. The photoreceptors project to two optic ganglia of the brain, the lamina and medulla; two additional optic lobes, the lobula and lobula plate, are involved in higher order visual processing.

Most adult *Drosophila* structures, including the eye, develop from imaginal disks. These are groups of cells set aside in the embryo that grow and differentiate inside the larva and evert to become functional during metamorphosis. Differentiation in the eye disk is progressive, moving across the disk in a wavelike manner from posterior to anterior. The front of the wave is marked by an indentation in the disk known as the morphogenetic furrow (MF). Most cell division occurs in the unpatterned cells anterior to the furrow, while on the posterior side of the furrow the cells are organized into clusters that develop into the ommatidia [for a review, see Wolff and Ready (1993); Fig. 1]. Within each cluster, the photoreceptors differentiate in a defined sequence; signals produced by cells that differentiate early recruit the cells that differentiate later. The best example of this is the induction of the R7 cell, the last photoreceptor to differentiate, by an interaction between the sevenless receptor on its surface and the bride of sevenless ligand produced by the R8 cell, which is the first cell to differentiate [reviewed by Zipursky and Rubin (1994)]. There is no lineage relationship between the cells of an ommatidium (Ready *et al.*, 1976; Lawrence and Green, 1979); thus, cell fate and position are determined after cells complete their last division by local cell–cell interactions.

In this chapter, we will concentrate on the early stages of eye development, focusing on the mechanisms that determine the global pattern rather than those involved in the establishment of specific cell fates. We will discuss the specification of the eye disk to form an eye, the control of morphogenetic furrow initiation and propagation, and the coordination of growth with differentiation.

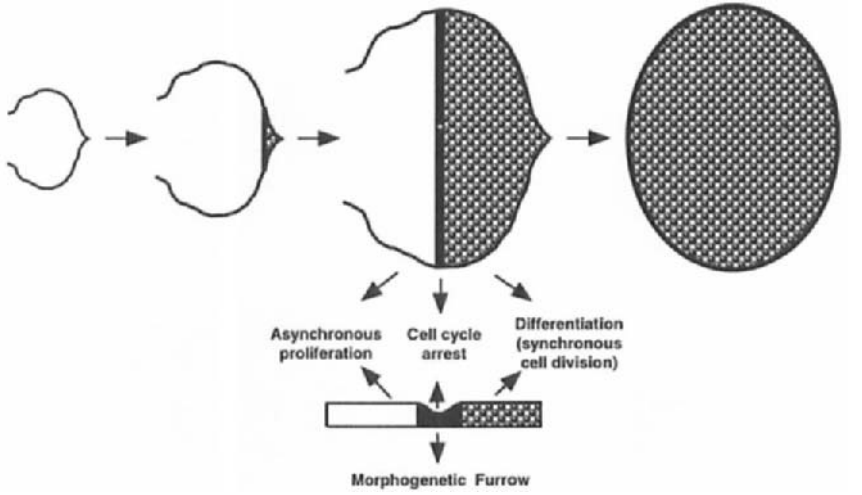


Fig. 1 Schematic representation of the retina at different stages of development. Shown from left to right are cartoons of eye disks of progressively mature stages, from early to late third instar larvae and an adult retina viewed from the top. Bottom: Lateral view of a portion of an eye disk in which the morphogenetic furrow has partially progressed across the disk. Posterior is to the right.

II. Determination of the Eye Primordium

The adult eye develops from an epithelium monolayer, the eye imaginal disk, which is part of the eye–antenna disk complex from which most of the head is formed (Haynie and Bryant, 1986). This eye–antenna disk has a complex embryonic origin, containing cells derived from at least three of the head segments, the acron, antennal, and maxillary segments. These cells are initially arranged in elongated groups in the epidermis; they are compressed late in embryogenesis and then invaginate into the embryo to give the disk its characteristic shape (Green *et al.*, 1993; Jurgens and Hartenstein, 1993). The region of the disk that forms the eye is derived primarily from the acron, the anterior-most region of the embryo (Jurgens and Hartenstein, 1993). A group of genes that distinguishes the eye disk from the other imaginal disks, giving it the potential to form an eye, is described in the following sections.

A. *eyeless*

Mutations in several genes result in the partial or complete loss of the eye. Of those that have been analyzed, *eyeless* (*ey*) seems to act earliest in eye determina-

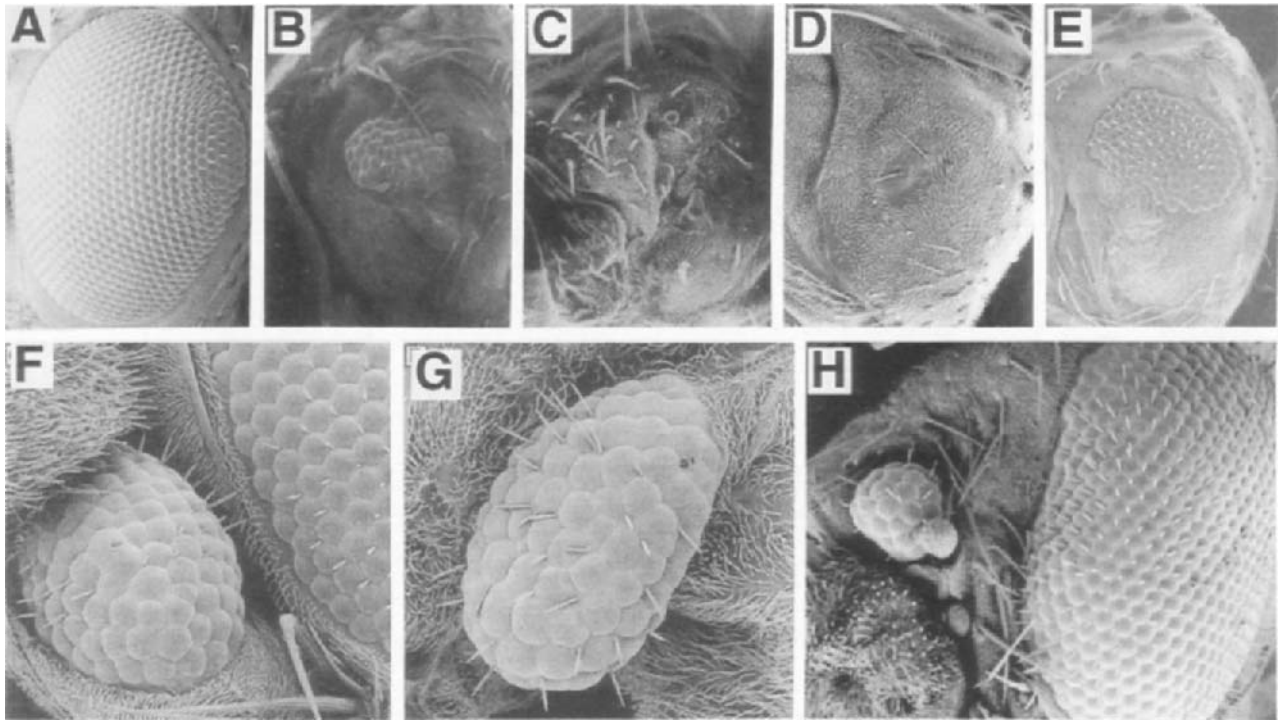


Fig. 2 Scanning electron micrographs of wild-type, mutant, and ectopic eyes. (A) Wild type. (B) *eyes absent*^{E1} (*eya*^{E1}), a strong *eya* allele. (C) *sine oculis*^{1} (*so*^{1}). (D) *dachshund*^{4} (*dac*^{4}). (E) *decapentaplegic*^{blk} (*dpp*^{blk}). (F and G) Ectopic eyes induced by misexpression of *eyeless* located on the antenna and under the wing, respectively. (H) Ectopic eye on the antenna induced by misexpression of *dachshund*. Magnification in F–H is approximately 4–5 times that in A–E. B was reproduced with permission from Bonini *et al.* (1993). C was reproduced with permission from Cheyette *et al.* (1994). F and G were reproduced with permission from Halder *et al.* (1995a). D and H were reproduced with permission from Shen and Mardon (1997). Posterior is to the right.

tion. *ey* is expressed in the eye primordium during embryogenesis. Later, in the third larval instar, *ey* expression is seen in the anterior, undifferentiated region of the eye disk (Quiring *et al.*, 1994). The identified alleles of *ey* cause only partial loss of the eye; they are transposable element insertions into an eye-specific enhancer (Quiring *et al.*, 1994) and do not completely remove the function of the gene (Renfranz and Benzer, 1989; Lindsley, 1992). *ey* has been called the "master control gene for eye morphogenesis" because of its striking ability to induce eye formation when ectopically expressed in other imaginal disks, such as wing and leg disks (Halder *et al.*, 1995a) (Fig. 2F,G). These ectopic eyes are surprisingly well-formed, containing large numbers of normally constructed ommatidia (Halder *et al.*, 1995a). It is not known what other factors must be present to allow *ey* to induce an eye; however, *ey* is also expressed in the embryonic central nervous system (CNS) and does not transform that into an eye.

Intriguingly, *ey* is homologous to the mouse *small eye* gene and the human *aniridia* gene; all three encode Pax-6 proteins, which contain DNA-binding paired domains and homeodomains (Hill *et al.*, 1991; Ton *et al.*, 1991; Quiring *et al.*, 1994). Both the mouse and human counterparts of *ey* are required for normal eye formation (Glaser *et al.*, 1992, 1994; Jordan *et al.*, 1992; Grindley *et al.*, 1995; Quinn *et al.*, 1996), as well as for the development of the nose, forebrain, and midbrain (Matsuo *et al.*, 1993; Grindley *et al.*, 1995; Stoykova *et al.*, 1996). Mouse Pax-6 is sufficient to induce ectopic eyes in *Drosophila*, demonstrating that the regulatory properties of the protein have been conserved (Halder *et al.*, 1995a). This result is surprising because the fly compound eye and the mammalian simple eye are structurally so different that they were thought to have evolved independently (Halder *et al.*, 1995b). A likely explanation is that Pax-6 was employed for regulating the light-responsive properties of the first photoreceptor cell and was subsequently co-opted for building the variety of structures that contain these cells in different species (Zuker, 1994). Support for this idea is provided by the observation that *ey* is expressed again late in eye development, during the pupal period, at the time of rhodopsin gene activation, and that the promoters of *Drosophila* and mammalian rhodopsin genes contain essential binding sites recognized by Pax-6 (Sheng *et al.*, 1997). Rhodopsin genes may have been the primary targets of Pax-6 early in evolution. Vertebrate lens crystallin genes also contain Pax-6-binding sites (Cvekl *et al.*, 1994, 1995a,b; J. Richardson *et al.*, 1995), and *small eye* is intrinsically required for lens formation (Fujiwara *et al.*, 1994; Grindley *et al.*, 1995); a corresponding function has not been demonstrated in *Drosophila*. Pax-6 appears to predate the appearance of photoreceptors as a homologue, is present in *Caenorhabditis elegans*, which has no photoreceptors; this homologue, *vab-3*, is used to determine the anterior region of the head (Chisholm and Horvitz, 1995).

A second *Drosophila Pax-6* homologue, *twin of eyeless* (*toy*), is more closely related to vertebrate *Pax-6* genes than is *ey*. *toy* is expressed earlier in embryogenesis than *ey*, and its ectopic expression induces both ectopic eyes and ectopic

ey expression. This suggests that *toy* normally acts upstream of *ey* to initiate eye development (T. Czerny, personal communication).

B. eyes absent

As the name *eyes absent* (*eya*) suggests, the eyes are missing in some *eya* mutants (Sved, 1986) (Fig. 2B). However, stronger *eya* alleles cause lethality or sterility, and expression of the gene is not restricted to the eye, showing that *eya* has functions in addition to eye determination (Bonini *et al.*, 1993; Boyle *et al.*, 1997). Larvae carrying *eya* alleles that cause complete loss of the adult eye have eye disks of reduced size in which no photoreceptors differentiate, and the level of cell death is greatly increased (Renfranz and Benzer, 1989; Bonini *et al.*, 1993). *ey* is still expressed in these disks, indicating that *eya* does not act upstream of *ey* (Halder *et al.*, 1995a). The cell death observed does not seem to be the primary consequence of loss of *eya* function, as clones of cells mutant for *eya*, analyzed in genetic mosaics, survive and proliferate (Fig. 3A,B; J. E. Treisman, unpublished data). However, the mutant cells fail to differentiate as photoreceptors and instead form head cuticle. Perhaps *eya* mutant cells in mosaic disks are rescued from death by an *eya*-regulated survival factor emanating from neighboring wild-type cells; this putative factor would be missing when the entire disk is mutant for *eya*. *eya* encodes a novel nuclear protein that is first detected in eye disks from second instar larvae; it forms a gradient with its highest levels at the posterior and lateral margins prior to the initiation of differentiation. Later, *eya* is expressed just ahead of the wave of differentiation and in differentiating photoreceptors (Bonini *et al.*, 1993). Interestingly, three mouse homologues of *eya* have been shown to be expressed during eye development. Two of them are regulated in the lens and nasal placode by Pax-6 (Xu *et al.*, 1997), suggesting that other conserved factors besides Pax-6 drive the early stages of eye development in vertebrates. Haplo insufficiency of a human homologue of *eya* leads to defects in the ear, kidney, and branchial arches (Abdelhak *et al.*, 1997); the homozygous mutant phenotype has not been described in vertebrates.

C. sine oculis

The eye is also completely or partially missing in *sine oculis* (*so*) mutants (Heitzler *et al.*, 1993) (Fig. 2C); as for eya^1, the eyeless so^1 mutation appears to disrupt an enhancer driving expression in the eye disk, while complete loss of *so* function is lethal (Heitzler *et al.*, 1993; Cheyette *et al.*, 1994). The so^1 phenotype is very similar to the eya^1 phenotype: the eye disk is reduced in size, no photoreceptors form, and large numbers of cells undergo apoptosis (Cheyette *et al.*,

1994). Unlike *eya*, clones of cells mutant for *so* do not appear to survive to the adult stage (Cheyette *et al.*, 1994). *so* is also required for development of the ocelli and larval photoreceptor organ and for invagination of the optic lobes, making it an essential gene throughout the visual system (Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994). *so* encodes a homeodomain protein expressed first in the eye disk in the early third larval instar, which is slightly later than the eya protein but is in a similarly graded pattern (Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994). Both *eya* and *so*, unlike *ey*, continue to be expressed in differentiating photoreceptors posterior to the morphogenetic furrow (Bonini *et al.*, 1993; Cheyette *et al.*, 1994; Quiring *et al.*, 1994; Serikaku and O'Tousa, 1994). Of three mouse *sine oculis* homologues, only one, *Six3*, is expressed in the developing eye; it is also present in anterior neural structures (Oliver *et al.*, 1995). Although *Six3* expression in the brain is not altered in *small eye* mutants (Oliver *et al.*, 1995), regulation of its expression in the eye cannot be addressed as the eyes are not formed. Again, this indicates conservation of the mechanisms of eye determination.

D. *dachshund*

Another mutation that has been shown to prevent eye formation affects the *dachshund* (*dac*) gene (Mardon *et al.*, 1994). In flies carrying strong *dac* alleles, few or no ommatidia are produced (Fig. 2D), although the eye disk grows to its normal size. *dac* encodes a novel nuclear protein expressed first in the eye disks of second instar larvae, in a gradient with its high point at the posterior margin (Mardon *et al.*, 1994). Targeted misexpression of *dac* in the ventral region of the antennal disk is able to induce eye development there (Shen and Mardon, 1997), leading to adult flies with ectopic eyes (Fig. 2H); however, this effect is considerably less robust than the eyes induced in multiple disks by ectopic *ey* expression. By several criteria, *dac* appears to act downstream of *ey*: (1) *dac* is induced by *ey*; (2) *dac* is required for the function of ectopic *ey*; and (3) *ey* is still expressed in *dac* mutant disks (Shen and Mardon, 1997). However, *dac* is also able to induce *ey* expression in the antennal disk (Shen and Mardon, 1997), suggesting that there may be a positive feedback loop through which *dac* maintains the expression of *ey*. It is not clear why the antennal disk is particularly sensitive to the effects of ectopic *dac*; perhaps dac cofactors are present in both the eye and antenna disks due to their close embryonic origin (Jurgens and Hartenstein, 1993). *eya* and *so* have been overexpressed in flies only using the heat–shock-inducible *hsp70* promoter, which allows the rescue of their respective mutant phenotypes but does not induce ectopic eye formation (Bonini *et al.*, 1993; Cheyette *et al.*, 1994; Serikaku and O'Tousa, 1994). Whether stronger and/or localized ectopic expression of these genes would be sufficient to induce *ey* expression and/or the formation of ectopic eyes remains to be seen.

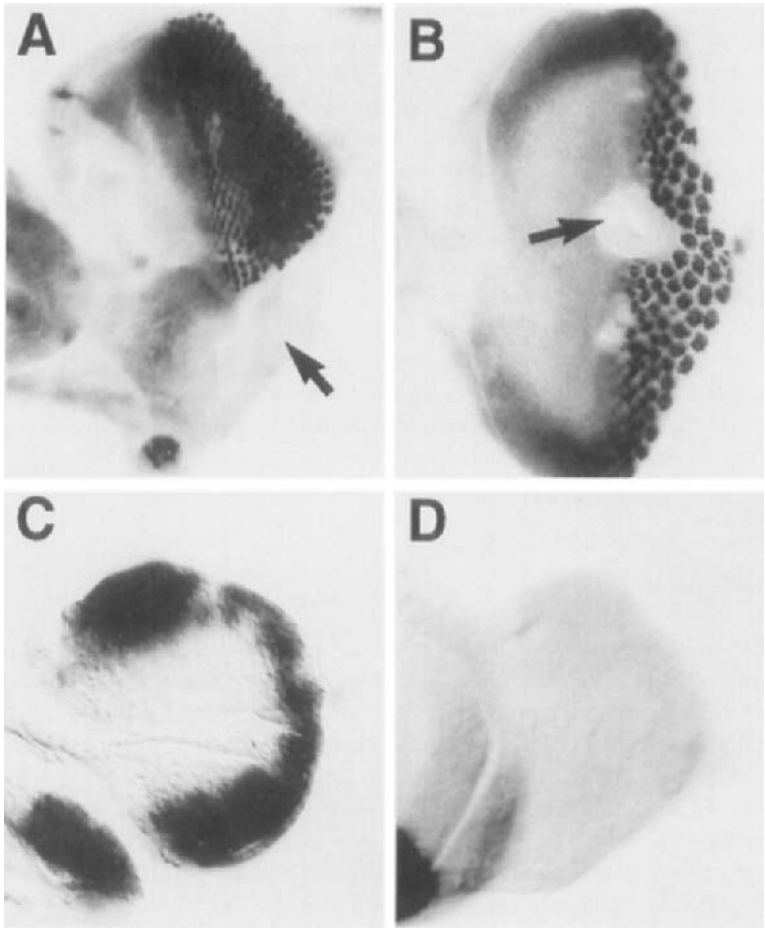


Fig. 3 Photomicrographs of eye disks displaying phenotypes associated with loss of the *eyes absent* (*eya*) function. (A and B) Eye disks carrying clones of cells mutant for *eya* located at the posterior margin and middle of the disk (arrows), respectively. Clones are marked by the absence of β-galactosidase expression (gray); differentiating photoreceptors are marked with an anti-Elav antibody (black staining), which recognizes a neuronal nuclear protein (Robinow and White, 1991). (C and D) Eye disks from early- and mid-third instar larvae carrying a *dpp–lacZ* reporter (Blackman *et al.*, 1991). (C) A wild-type eye disk displays *dpp* expression along the posterior and lateral margins. (D) Eye disk from an *eya* mutant larva; expression of *dpp* is abolished. Magnification in C and D is approximately 4 times that in A and B. Posterior is to the right.

E. Regulation and Other Genes

The regulatory relationships between the four genes just discussed have not been explicitly defined, except that *dac* acts downstream of *ey* (Shen and Mardon,

1997). On the basis of their times of expression, *ey* would be expected to act first, *eya* second, and *dac* and *so* third. Consistent with this, *ey* expression is not affected in *eya* or *so* mutants (Halder *et al.*, 1995a), although the converse has not yet been demonstrated. The purpose of this cascade of potential transcription factors is not clear; it may amplify or stabilize the initial decision to form eye tissue, or each factor may have functions not mediated by the others. Several additional genes, including *eyegone (eyg)*, *eyelisch*, *eye missing*, and *Lobe*, can mutate to eyeless phenotypes (Lindsley, 1992). The eye disk is reduced in size in *eyg* and *Lobe* mutants, placing them early in the process of eye determination. However, *decapentaplegic (dpp)* (see Section III.A) is still expressed in these mutants (Heberlein *et al.*, 1993; J. E. Treisman, unpublished data), suggesting that they may act farther downstream than *eya* and *so*, which are required for *dpp* expression in third instar disks (Fig. 3C,D; J. E. Treisman, unpublished data). *dpp* is also expressed in *dac* mutant disks (Mardon *et al.*, 1994), suggesting that *dac* acts relatively late in eye determination and thus rendering its ability to induce ectopic eyes all the more surprising. Because most of these genes are required for viability and were found to act in eye formation due to the fortuitous isolation of eye-specific alleles, many other essential genes may also contribute to the determination of the eye.

III. Initiation of Differentiation

During the third larval instar, cells in the eye disk begin to differentiate and assemble into photoreceptor clusters. This process initiates at the posterior tip of the disk and advances anteriorly row by row (Ready *et al.*, 1976). The basis for the temporal regulation of initiation is completely unknown, although it has been speculated that the levels of hormones such as ecdysone may play a role (Mardon *et al.*, 1994). The spatial regulation of initiation appears to depend on the interaction between a positive regulator encoded by the *dpp* gene and a negative regulator encoded by the *wingless* (*wg*) gene. The evidence for this is discussed in the following sections.

A. Function of *dpp*

The *dpp* gene encodes a member of the TGF-β family of secreted proteins (Padgett *et al.*, 1987) and has been shown to act at a distance to activate the expression of target genes in the wing disk (Lecuit *et al.*, 1996; Nellen *et al.*, 1996). *dpp* is expressed in the eye disk prior to any differentiation around the posterior and lateral margins of the disk; subsequently, expression becomes restricted to a narrow strip in the morphogenetic furrow (Masucci *et al.*, 1990; Fig. 4A, please see color plate). The first indication that *dpp* is required for

normal retinal morphogenesis came from the observation that the viable allele $dpp^{d\text{-}blk}$ has a specific effect on the eye: $dpp^{d\text{-}blk}$ flies have severely reduced eyes (Masucci *et al.*, 1990; Fig. 2E). In $dpp^{d\text{-}blk}$ eye disks, differentiation is restricted to a central dorsal region; although differentiation in this region normally advances in the anterior direction, it fails to spread laterally (Wiersdorff *et al.*, 1996; Chanut and Heberlein, 1997b; Fig. 4B). This mutation is a deletion of an eye-specific enhancer element (St. Johnston *et al.*, 1990; Blackman *et al.*, 1991), resulting in a lack of detectable *dpp* expression in the third instar eye disk (Masucci *et al.*, 1990). The mutant phenotype can be rescued by expressing *dpp* along the margins of the eye disk (Masucci *et al.*, 1990; Staehling-Hampton *et al.*, 1994). Interestingly, the mouse BMP-7 protein, a dpp homologue, is essential for development of the eye (Dudley *et al.*, 1995; Luo *et al.*, 1995). Another dpp homologue, BMP-4, is expressed in retinal progenitor cells at the dorsal ciliary margin of the eye (Papalopulu and Kintner, 1996).

Examination of the effect of complete loss of *dpp* function has been difficult, as the mutation is lethal and the gene acts highly nonautonomously so that most clones of homozygous mutant tissue are rescued, presumably by dpp protein diffusing from the surrounding wild-type cells. However, large mutant clones including the posterior margin were shown to prevent retinal differentiation (Heberlein *et al.*, 1993). A specific heteroallelic combination of embryonic lethal *dpp* mutations, dpp^{hr4}/dpp^{hr56}, which is temperature-sensitive for viability (dpp^{ts}; Wharton *et al.*, 1996), has been used to show that early loss of *dpp* results in the complete failure of differentiation whereas slightly later loss prevents differentiation from spreading laterally, in a manner that is similar to $dpp^{d\text{-}blk}$ (Chanut and Heberlein, 1997a; Fig. 4C,D). Finally, the analysis of clones of cells mutant for autonomously acting downstream components of the *dpp* signaling pathway (Fig. 5A) has confirmed that *dpp* signaling is essential for the initiation of differentiation; cells mutant for the type II receptor *punt* (Letsou *et al.*, 1995; Ruberte *et al.*, 1995), the type I receptor *thick veins* (*tkv*) (Affolter *et al.*, 1994; Brummel *et al.*, 1994; Penton *et al.*, 1994; Ruberte *et al.*, 1995), or the cytoplasmic component *Mothers against dpp* (*Mad*) (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995) are unable to initiate photoreceptor development (Burke and Basler, 1996; Wiersdorff *et al.*, 1996).

Experiments in which *dpp* was expressed ectopically support the idea that it is involved in patterning at the margins of the disk. Randomly positioned clones of cells that express *dpp* ectopically have an effect only on the anterior and lateral margins of the disk, where they induce ectopic differentiation and the generation of a second morphogenetic furrow that moves posteriorly (Pignoni and Zipursky, 1997). In some cases this leads to duplication of the entire eye disk (Pignoni and Zipursky, 1997). This effect can be induced nonautonomously by the expression of *dpp* in cells close to but not including the anterior margin (Chanut and Heberlein, 1997a; Pignoni and Zipursky, 1997), showing that this region of the margin responds to low levels of *dpp* whereas other regions of the disk are

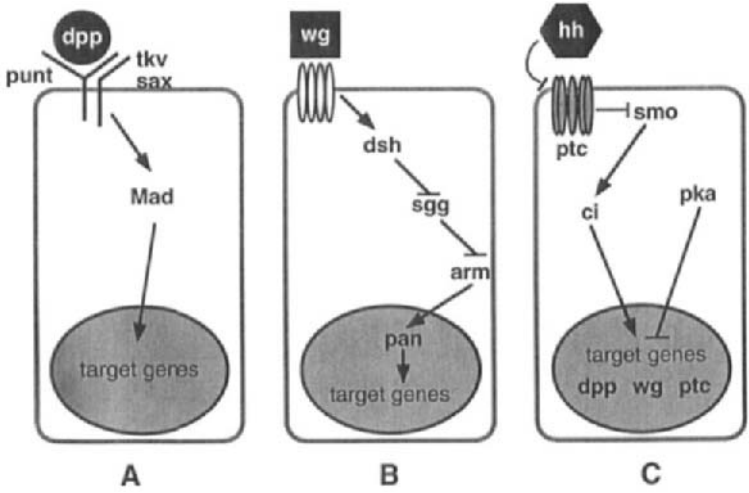


Fig. 5 Diagrams of the signal transduction pathways activated by *decapentaplegic* (*dpp*), *wingless* (*wg*), and *hedgehog* (*hh*). Positive genetic interactions are shown as arrows, and negative interactions are shown as two intersecting lines. See the text for details.

insensitive even to higher levels. It is not known which factors differentiate the margins from internal tissue and give them their unique sensitivity to *dpp*. However, some cells at the margin of the disk develop into head cuticle (Haynie and Bryant, 1986) so that a choice between the two cell fates must be made in this region. Both loss of function and ectopic expression conditions have also shown that *dpp* positively autoregulates its own expression: *dpp* expression is lost from *Mad* clones at the posterior margin (Wiersdorff *et al.*, 1996) and from dpp^{ts} disks grown at restrictive temperature (Chanut and Heberlein, 1997a). The endogenous *dpp* gene is activated at the anterior margin by nearby cells expressing *dpp* (Chanut and Heberlein, 1997a; Pignoni and Zipursky, 1997).

Clones of cells mutant for *dac* fail to initiate photoreceptor differentiation when located at the posterior margin, whereas internal clones have a much less severe effect (Mardon *et al.*, 1994). This is very similar to the phenotype of *punt*, *tkv*, or *Mad* clones, suggesting that *dac* could also act downstream of *dpp*. One difference is that *dpp* expression is not lost from *dac* clones (Mardon *et al.*, 1994), indicating that *dac* is not required for *dpp* autoregulation. However, ectopic *wg* is observed at the posterior margin of *dac* mutant disks (Treisman and Rubin, 1995); thus, *dac* is required for the ability of *dpp* to repress *wg* expression (see Section III.C). Because *dac* has only been ectopically expressed in regions where *dpp* is normally expressed (Shen and Mardon, 1997), its ability to induce ectopic eyes may require the simultaneous presence of *dpp*.

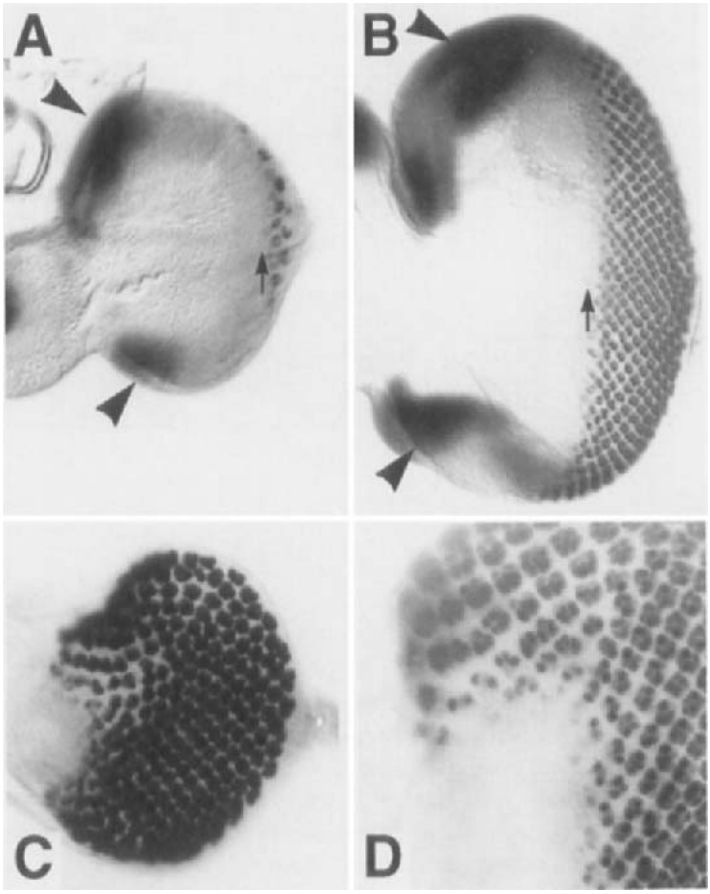


Fig. 6 Photomicrographs of eye disks showing the normal *wg* expression domains (A and B) and the consequences of loss of *wg* function (C and D). (A and B) Eye disks from wild-type mid- and late-third instar larvae, respectively, carrying a *wg–lacZ* reporter. Disks are stained with anti-Elav to localize differentiating neurons and X-GAL to identify *wg–lacZ* expression domains. *wg* is expressed along the dorsal and ventral margins of the disk (arrowheads) ahead of the morphogenetic furrow (arrows). (C and D) Eye disks from wg^{ts} larvae dissected approximately 48 hr after having been shifted to the restrictive temperature are stained with anti-Elav. Differentiation initiates precociously from the dorsal and ventral margins. Magnification in D is 3-fold higher than that in the other panels. Posterior is to the right; dorsal is up.

B. Function of *wg*

Although *dpp* is initially expressed at the lateral as well as the posterior margin, differentiation initiates only at the posterior tip of the disk. One reason for this seems to be that the *wg* gene is expressed at the lateral margins (Fig. 6A,B),

where it acts as an inhibitor of photoreceptor differentiation (Ma and Moses, 1995; Treisman and Rubin, 1995). *wg* is a member of the *Wnt* gene family (Nusse and Varmus, 1992), and its protein product appears to act as a secreted morphogen (Zecca *et al.*, 1996; Neumann and Cohen, 1997). Removal of *wg* function using a temperature-sensitive allele results in precocious differentiation starting from the dorsal and, more weakly, the ventral margins (Ma and Moses, 1995; Treisman and Rubin, 1995; Fig. 6C,D). The cells that normally express *wg* go on to form regions of the head capsule (Royet and Finkelstein, 1996); it is not clear whether the primary effect of *wg* is to prevent photoreceptor differentiation or to promote head capsule fate. Clones of cells mutant for *dishevelled* (*dsh*), a downstream component of the *wg* pathway (Fig. 5B), can form ectopic eye tissue on the top of the head (Heslip *et al.*, 1997), showing that those regions of the disk normally fated to become head capsule are competent to form eye in the absence of *wg* signaling. The weaker effect of loss of *wg* on the ventral margin of the disk suggests that an additional inhibitor, perhaps another *Wnt* gene, could be present in this region.

Ectopic expression of *wg* in randomly positioned clones of cells in the eye disk interferes with both the initiation and progression of differentiation (Treisman and Rubin, 1995). Loss of function of *shaggy/zeste-white3* (*sgg*), which encodes a kinase normally inhibited by *wg* signaling (Fig. 5B), has a similar effect, although ectopic *wg* appears to cause overproliferation and subsequent cell death while loss of *sgg* transforms the tissue to head cuticle (Treisman and Rubin, 1995; Heslip *et al.*, 1997). Expression of the transcription factor encoded by *optomotor-blind* (*omb*) is activated in response to *wg* pathway activity in the eye disk and may mediate its effects (Zecca *et al.*, 1996).

C. Interaction between *dpp* and *wg*

wg and *dpp* are expressed along the margins of the eye disk in apparently nonoverlapping domains (Figs. 3C, 6A,B, and 7). As mentioned previously, loss of *wg* function or ectopic expression of *dpp* along the disk margins leads to inappropriate initiation of differentiation, implying that *wg* and *dpp* have antagonistic roles in the eye. Some insight into the mechanisms underlying this functional antagonism comes from experiments where the effect of each gene on the other's expression and function was studied. Loss of *wg* function in wg^{ts} eye disks leads to ectopic expression of *dpp* along the anterior margin prior to the start of ectopic differentiation (Ma and Moses, 1995). On the other hand, ectopic expression of *wg* can inhibit differentiation at the posterior margin without obvious reduction in the levels of *dpp* expression (Treisman and Rubin, 1995), suggesting that *wg* signaling may inhibit the function of the dpp protein rather than its expression (Fig. 7). However, ectopic expression of high levels of *wg* along the margins causes a loss of *dpp* expression (U. Heberlein, unpublished). It

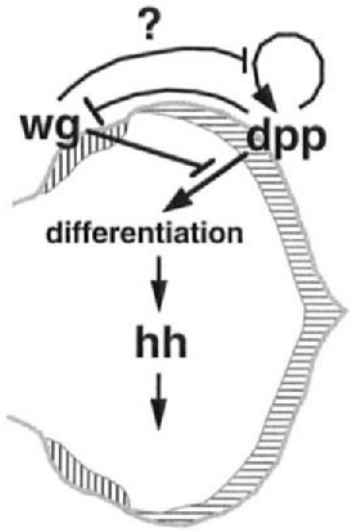


Fig. 7 Diagram summarizing the interactions between *wingless* (*wg*) and *decapentaplegic* (*dpp*). Expression of *dpp* is shown as horizontally hatched areas and expression of *wg* as vertically hatched areas. Positive interactions are shown with arrows, and negative interactions are indicated with two intersecting lines. Posterior is to the right.

is possible that low levels of wg inhibit dpp function, whereas high levels inhibit *dpp* transcription; as the function of *dpp* is required for its own expression, these effects may be related. Activation of the *wg* pathway by loss of *sgg* function is sufficient to prevent *dpp* expression (Heslip *et al.*, 1997), suggesting that complete inhibition of sgg kinase activity may require high levels of wg signaling. Thus, *wg* is necessary and sufficient to block the expression of *dpp*. Conversely, *dpp* is necessary and sufficient to repress inappropriate expression of *wg*. Clones of cells mutant for *punt* or *Mad*, which presumably cannot respond to the *dpp* signal, display ectopic *wg* expression when the clones are located at the posterior margin (Wiersdorff *et al.*, 1996; J. E. Treisman, unpublished data). In addition, ectopic *wg* expression is observed in dpp^{blk} eye disks (Wiersdorff *et al.*, 1996; Chanut and Heberlein, 1997a). Finally, overexpression of *dpp* can also repress *wg* in its normal expression domain (Chanut and Heberlein, 1997a; Pignoni and Zipursky, 1997). Thus, as has been demonstrated for the leg disk (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen *et al.*, 1996), *wg* and *dpp* display antagonistic functions during the development of the eye. This mutual antagonism may regulate the proper balance between their activating and inhibiting functions, which is crucial for normal retinal differentiation.

Whether *dpp* plays a positive role in photoreceptor differentiation or merely acts to prevent *wg* from inhibiting this differentiation is uncertain. However, the

analysis of double-mutant eye disks revealed that precocious differentiation initiating from the dorsal and ventral margins in wg^{ts} mutants is prevented by the simultaneous reduction of *dpp* expression using the dpp^{blk} mutation (Treisman and Rubin, 1995). This suggests that *dpp* does have a function in promoting differentiation, in addition to inhibiting *wg* expression. Another unanswered question is how the initial expression domains of *wg* and *dpp* are established. Perhaps these domains are established by the function of genes such as *ey*, *eya*, and *so* or by as yet unidentified factors. The target genes activated by the *dpp* pathway have not been identified in the eye, and how dpp activity ultimately leads to neural differentiation remains unclear.

IV. Progression of Differentiation

Once neuronal differentiation begins at the posterior tip of the eye disk, it progresses across the epithelium one row at a time, reaching the anterior margin approximately 2 days later. The anterior edge of this differentiation wave is marked by an indentation in the apical surface of the epithelium, the morphogenetic furrow (MF), which spans the disk along its dorsoventral axis (Ready *et al.*, 1976). The MF is the consequence of transient and localized changes in cell shape; cells have reduced apical–basal dimensions and greatly diminished apical surfaces (Wolff and Ready, 1991; Fig. 8). The mechanism and purpose of these cell shape changes are not known.

The MF temporally and spatially coincides with several important events in retinal morphogenesis. Ahead of the furrow, cells are unpatterned and undifferentiated, and they divide asynchronously. In the furrow, cells become synchronized in the G1 phase of their cell cycle as they begin to associate into evenly spaced clusters that will eventually form the individual ommatidia (Ready *et al.*, 1976; Tomlinson and Ready, 1987; Wolff and Ready, 1991). Some cells exit the cell cycle and begin differentiating as neurons immediately posterior to the MF (Fig. 4A). Photoreceptor R8 differentiates first and is followed by the pairwise addition of photoreceptors R2/5 and R3/4. The remaining undifferentiated cells undergo another synchronous round of mitosis, after which they sequentially differentiate into photoreceptors R1/6 and R7 and the cone cells. The pigment cells and the interommatidial bristle group also develop from this set of cells during the pupal stage [for a review, see Wolff and Ready (1993)]. The temporal sequence of eye development is laid out spatially in a single eye disk, with rows becoming successively older from the furrow to the posterior margin.

A. Mutations That Disrupt the Progression of Differentiation

Several mutations have been identified that specifically arrest the progression of the differentiation wave. In these so-called "furrow-stop" mutants, the adult flies

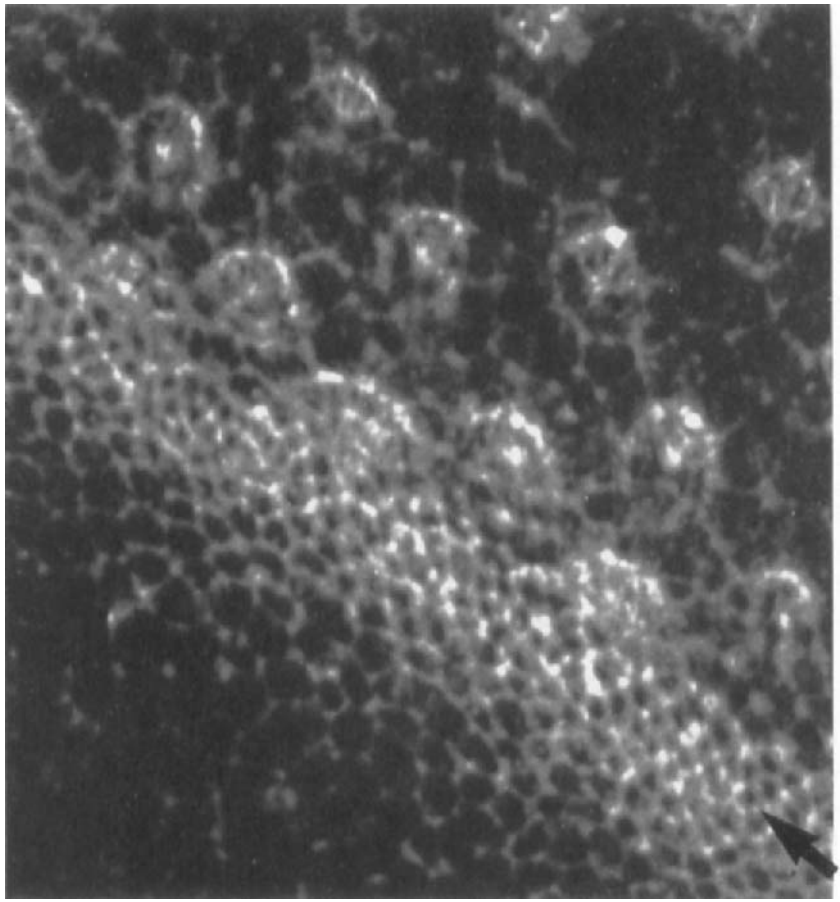


Fig. 8 Confocal photomicrograph of a portion of an eye disk stained with phalloidin to highlight the shape of cells. Cells in the furrow (arrow) have reduced apical surfaces. Cells posterior to the furrow begin to associate into evenly spaced clusters. Posterior is toward the top right corner. Reproduced with permission from Heberlein *et al.* (1993).

lack the anterior portion of the retina (Heberlein *et al.*, 1993; Fig. 9). The furrow-stop group includes several dominant mutations, such as *Bar* (*B*) and $rough^{DOM}$ (ro^{DOM}), and one recessive mutation, $hedgehog^{1}$ (hh^{1}), which is an eye-specific mutation in the segment polarity gene *hedgehog* (*hh*) (Mohler, 1988; see Section IV.C). The analysis of eye disks from furrow-stop mutants reveals that differentiation of new ommatidial rows stops after the furrow has moved partially across the disk. Ommatidia that had begun differentiating prior to this block continue to develop normally, causing the typical furrow-stop phenotype in which the most anterior row of ommatidia is formed by mature clusters containing a full comple-

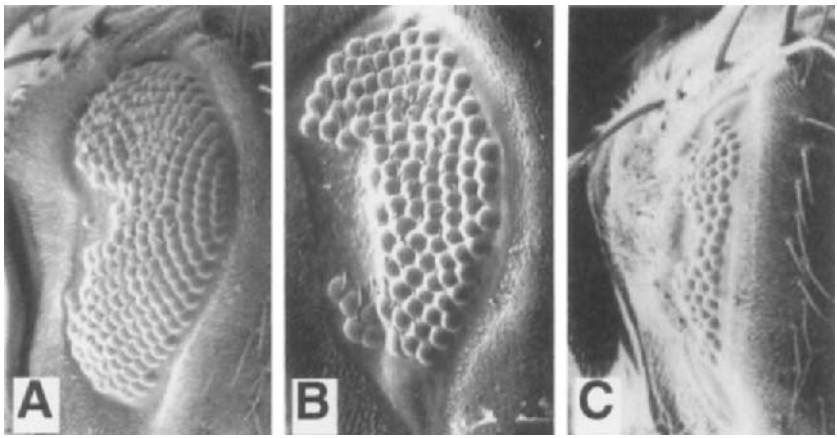


Fig. 9 Scanning electron micrographs of the eyes of furrow-stop mutants. Genotypes shown are (A) hh^1/hh^1, (B) $ro^{DOM}/+$, and (C) *Bar/Bar*. The anterior portion of the retina is missing from these mutants. Posterior is to the right. Reproduced with permission from Heberlein *et al.* (1993).

ment of photoreceptor neurons (Heberlein *et al.*, 1993; Chanut and Heberlein, 1997a; Fig. 10, please see color plate). Thus, the process by which the wave of differentiation moves across the eye disk is blocked in these mutants, without an effect on ommatidial maturation or neuronal differentiation per se. An indistinguishable furrow-stop phenotype is obtained when eye disks from larvae carrying a temperature-sensitive allele of *hh*, hh^{ts2}, are shifted to the nonpermissive temperature (Ma *et al.*, 1993). While a reduction in *hh* function across the whole eye disk, as achieved in hh^1 and hh^{ts2} larvae, leads to MF arrest, clones of cells homozygous mutant for complete loss-of-function *hh* alleles display only mild defects (Heberlein *et al.*, 1993; Ma *et al.*, 1993; Heberlein and Moses, 1995; Fig. 11). This nonautonomous function of *hh* could be explained if *hh* directly acts as a long-range-diffusible molecule, if *hh* induces a secondary diffusible signal, or both.

Both *Bar* and *rough* encode homeobox proteins normally expressed posterior to the MF (Tomlinson, 1988; Kimmel *et al.*, 1990; Higashijima *et al.*, 1992); the eye phenotype of the dominant alleles is caused by increased or inappropriate expression of the respective genes (Kojima *et al.*, 1991; Heberlein *et al.*, 1993). It has been shown that ectopic expression of *rough* inhibits the expression of *atonal* (*ato*) (Dokucu *et al.*, 1996), a gene required for neuronal differentiation in the retina (see Section IV.E), providing a likely explanation for the furrow-stop phenotype of ro^{DOM}.

B. *hedgehog* Function and Furrow Progression

hh, originally identified as a mutation that disrupts embryonic segmentation in *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), encodes a secreted protein

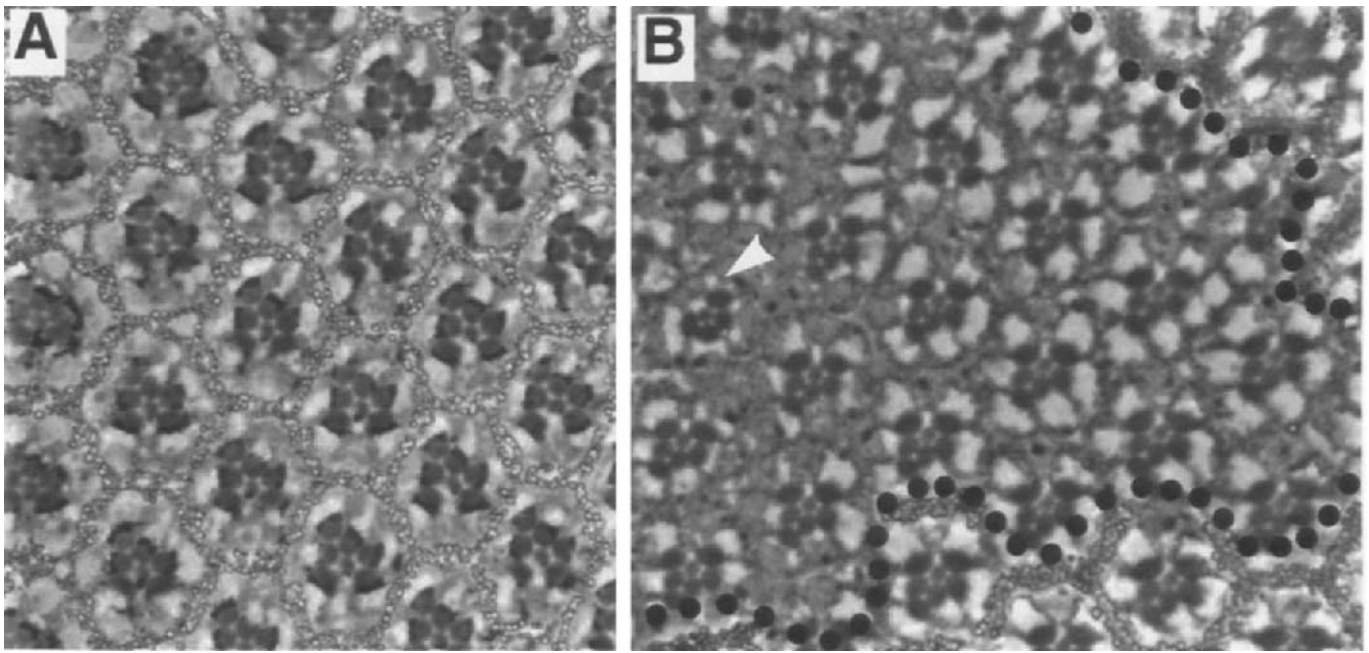


Fig. 11 Phase contrast photomicrograph of tangential sections through adult retinae. (A) Wild-type retina. (B) Retina containing a clone of cells with a homozygous mutant for a null *hh* allele. The mutant tissue is identified by the lack of the *white*$^+$ marker, which leads to the absence of pigment granules. The approximate location of the posterior border of the clone is highlighted with a dotted line. Most ommatidia within the mutant clone are normally structured; one exception is highlighted with the arrowhead. Posterior is to the right.

(Lee *et al.*, 1992; Mohler and Vani, 1992; Tabata *et al.*, 1992; Tashiro *et al.*, 1993) that also plays a crucial role in patterning various imaginal structures, such as the wing and leg (Basler and Struhl, 1994; Kojima *et al.*, 1994; Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995; Ingham and Fietz, 1995). Several vertebrate *hh* homologues have been identified and shown to function in multiple developmental events [for reviews, see Fietz *et al.* (1994), Ingham (1994, 1995), Pownall (1994), and Johnson and Tabin (1995)].

In the developing fly eye, *hh* is expressed in cells posterior to the MF (Lee *et al.*, 1992; Fig. 12). It has been assumed that these cells are the differentiating photoreceptors on the basis of two observations: First, an enhancer-trap insertion in the *hh* gene, in which a *lacZ* reporter (encoding bacterial β-galactosidase) is transcribed under the control of *hh* regulatory sequences, is expressed in photoreceptors as they differentiate (Ma *et al.*, 1993). Second, this expression requires that cells differentiate as neurons (Heberlein *et al.*, 1993). However, the possibility that additional cells located immediately posterior to the MF also express *hh* cannot be excluded.

A possible clue about how *hh*, expressed posterior to the MF, might act in furrow progression came from the observation that expression of *dpp* in the MF requires normal *hh* function; *dpp* expression is absent from the MF of hh^1 and

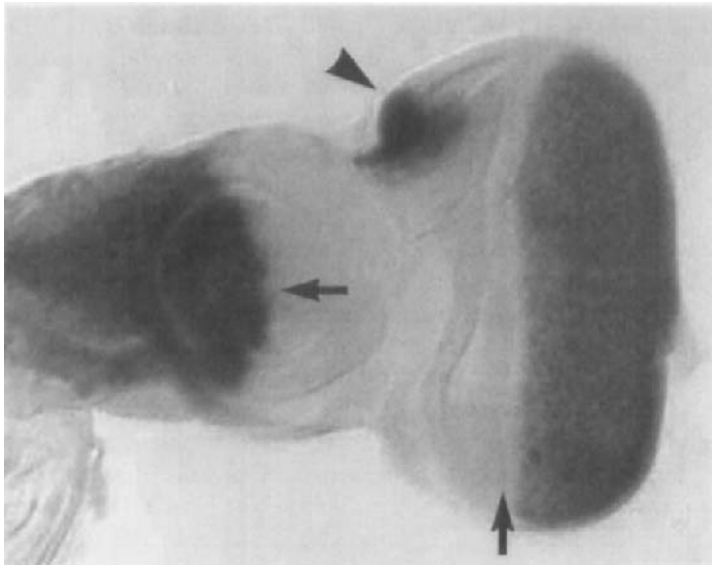


Fig. 12 Photomicrograph of an eye disk from a third instar larva carrying a *lacZ* enhancer–trap insertion in the *hh* locus stained with X-GAL. Line P30 (Lee *et al.*, 1992) faithfully reflects the expression pattern of the *hh* gene. Expression of β-galactosidase is seen posterior to the furrow (vertical arrow), in the presumptive ocellar region (arrowhead), and in the antennal disk (horizontal arrow). Posterior is to the right.

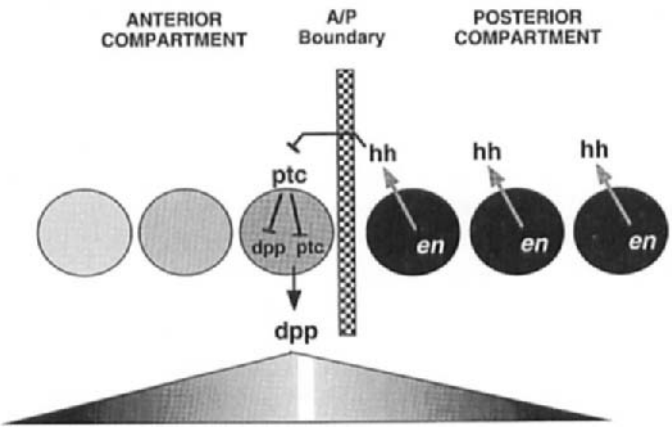


Fig. 14 Diagram of a "stable boundary." The signaling process across the "stable" anterior–posterior (A–P) compartment boundary in a wing disk is summarized. hh, expressed under the control of *en* in the posterior compartment, diffuses across the A–P boundary where it antagonizes the *ptc* function; this leads to the derepression of *dpp* and *ptc* expression. dpp protein acts as a morphogen patterning for both compartments. For details, see the text. Posterior is to the right.

hh^{ts2} mutant eye disks (Heberlein *et al.*, 1993; Ma *et al.*, 1993; Fig. 10). This, together with the fact that a loss of *dpp* function disrupts retinal morphogenesis (Spencer *et al.*, 1982; Masucci *et al.*, 1990; Heberlein *et al.*, 1993), led to the proposal that *hh* functions by inducing *dpp* as a secondary signal (Heberlein *et al.*, 1993). Ectopic expression of *hh* in clones of cells located anterior to the MF leads to ectopic induction of *dpp*, generation of ectopic furrows, and precocious neuronal differentiation (Heberlein *et al.*, 1995). These ectopic neurons begin expressing their endogenous *hh* gene, thus setting in motion waves of differentiation that can propagate in any direction across the disk (Fig. 13, please see color plate). Thus, *hh* is both necessary and sufficient to induce the expression of *dpp* and to propagate the furrow across the eye disk (Heberlein and Moses, 1995). Evidence suggesting that *dpp* does not execute all of the functions of *hh* will be discussed next.

hh signaling across the embryonic parasegmental boundary and the anterior–posterior compartment boundary of wing and leg disks has been studied extensively [for reviews, see Ingham (1995), Kalderon (1995), and Perrimon (1995); Fig. 14]. hh protein, expressed by cells lying posterior to both of these boundaries, diffuses anteriorly across the boundary where it induces the expression of target genes such as *dpp*, *wg*, and *ptc*. In addition to being a target of *hh* signaling, *ptc* encodes a multiple-pass membrane protein (Hooper and Scott, 1989; Nakano *et al.*, 1989) which, on the basis of genetic arguments, was postulated to be the *hh* receptor (Ingham *et al.*, 1991). The reported observation of a physical interaction

between ptc and hh proteins lends strong support to this hypothesis (Stone *et al.*, 1996). *ptc* acts through a series of additional components to constitutively repress the expression of *hh* target genes (Fig. 5C). These target genes are also repressed by *DCO*, which encodes the major catalytic subunit of protein kinase A (*pka*) (Lane and Kalderon, 1993). *hh* relieves this inhibition by antagonizing *ptc* function (Fig. 5C). Groups of cells that lack either *ptc* or *pka* function in wing, leg, or eye disks act as if they have received the *hh* signal, although they are not located near a source of hh (Chanut and Heberlein, 1995; Jiang and Struhl, 1995; Johnson *et al.*, 1995; Li *et al.*, 1995; Ma and Moses, 1995; Pan and Rubin, 1995; Sanicola *et al.*, 1995; Strutt and Mlodzik, 1995; Strutt *et al.*, 1995; Wehrli and Tomlinson, 1995). Thus, the molecules and signaling cascades that are activated (or prevented from inhibiting) by *hh* are very similar whether the signaling occurs across the parasegmental boundary in the embryo, the compartment boundary in the wing and leg disks, or the MF in the eye disk. Some striking differences among these systems, however, are that the boundary in the eye, the MF, is transient, moves, and does not involve stably inherited cell fates as do the other boundaries. Consistent with this, the engrailed transcription factor, which is required to activate *hh* transcription in the posterior compartment of wing and leg disks (Tabata *et al.*, 1992, 1995; Zecca *et al.*, 1995), does not control *hh* expression in the eye disk (Strutt and Mlodzik, 1996). Implicit in this "moving boundary" concept is the fact that cells that receive the *hh* signal must become cells that send the *hh* signal (Fig. 15). Because this transition from a recipient to a sender of the *hh* signal requires the receiving cell to differentiate as a photoreceptor neuron, the process of neuronal differentiation is central to furrow progression. Conversely, *hh* signaling is essential for progression of the neuronal differentiation wave. This tight linkage between differentiation and furrow progression, together with the repetitive nature of the process, often hinders the establishment of clear causal relationships.

C. *dpp* Function and Furrow Progression

While the available data support the notion that *hh* is necessary and sufficient to induce the expression of *dpp* and propagate the furrow across the eye disk, the role of *dpp* in this process is less clear. Mutations in the genes that act cell autonomously to receive and transduce the *dpp* signal (Fig. 5A) have been used to address the role of *dpp* signaling in MF progression. These include mutations in the type I receptors *tkv* (Burke and Basler, 1996; Penton *et al.*, 1997), type II receptor *punt* (Burke and Basler, 1996; J. E. Treisman, unpublished data), and the cytoplasmic signal transducer *Mad* (Wiersdorff *et al.*, 1996). This analysis, however, was complicated by the fact that these genes are required not only for the viability of the animal but also for cell proliferation and/or survival in clones of homozygous mutant tissue in mosaic animals. Therefore, the experiments have

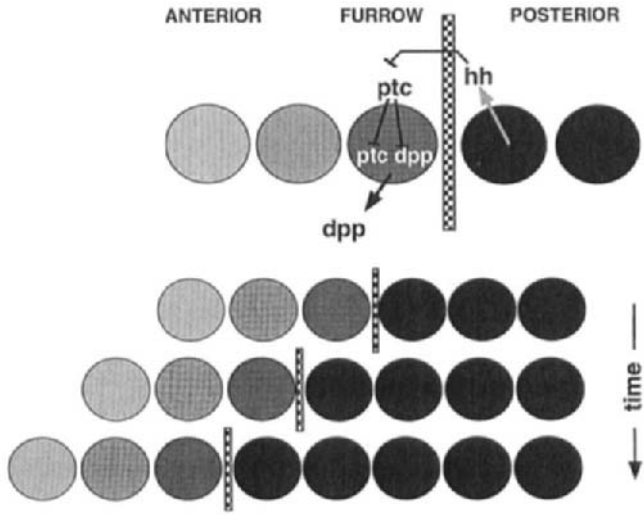


Fig. 15 Diagram of a "moving boundary." Signaling in the morphogenetic furrow is very similar to signaling across the A–P compartment boundary (see Fig. 14). However, in contrast to the "stable" compartment boundary, the furrow is a transient boundary that moves across the disk. Thus, cells that receive the *hh* signal become, in time, cells that send the *hh* signal. See the text for details. Posterior is to the right.

been limited to the analysis of small clones (for null alleles of *tkv*) or hypomorphic alleles (for *punt* and *Mad*). Adult eyes containing internal *tkv*, *punt*, or *Mad* clones display only minor irregularities (Burke and Basler, 1996; Wiersdorff *et al.*, 1996; Penton *et al.*, 1997). In third instar eye disks, progression of the MF is retarded slightly while crossing mutant tissue. These data suggest that only low levels of *dpp* signaling are needed for normal MF progression or that *dpp* acts through additional, as yet unidentified downstream components in this process. The latter possibility was addressed by generating very large clones of cells completely devoid of dpp production (Burke and Basler, 1996) using the *Minute* technique to give the mutant cells a growth advantage relative to the neighboring wild-type cells (Morata and Ripoll, 1975). Adult eyes carrying such large *dpp* clones are patterned normally. It was argued that the large size of these clones effectively rules out the possibility of rescue from surrounding wild-type tissue. However, the fact that these clones are able to proliferate suggests that they are indeed rescued, at least with regard to growth, by dpp presumably secreted by their wild-type neighbors. Finally, it has been shown that the removal of *dpp* by shifting a temperature-sensitive (ts) allelic combination to the nonpermissive temperature arrests, or at least slows, furrow progression (Chanut and Heberlein, 1997a). This effect, however, is observed only after the furrow has progressed

approximately halfway across the disk. Curiously, eye disks from larvae carrying a ts allele of *punt* show ectopic expression of *wg* ahead of the MF when grown at the restrictive temperature (Theisen *et al.*, 1996). Although it has not been determined whether the MF is arrested in these disks, ectopic expression of *wg* has been shown to block furrow progression (Treisman and Rubin, 1995). This raises the interesting possibility that one of the functions of *dpp* in the furrow is to block inappropriate expression of *wg*; whether this ectopic *wg* expression is induced by *hh* remains untested.

While it is difficult at this time to reconcile all of these data, it seems quite clear that the function of *hh* in the eye is not mediated solely by *dpp*. This is consistent with the observation that whereas ectopic expression of *dpp* along the disk margin can induce ectopic differentiation (Chanut and Heberlein, 1997a; Pignoni and Zipursky, 1997), ectopic expression of *dpp* or activation of the *dpp* signaling pathway (by ectopic expression of an activated form of the *tkv* receptor) in internal clones of cells ahead of the MF does not lead to precocious differentiation or the generation of ectopic furrows (Burke and Basler, 1996; Pignoni and Zipursky, 1997). Thus, *dpp* may play a permissive rather than an instructive role in MF progression by ensuring that conditions necessary for normal furrow progression, such as proper cell cycle control (see Section V.A), occur in synchrony.

D. Coordination of Initiation and Progression

As already discussed, *dpp* and *wg* have antagonistic effects on differentiation; *dpp* promotes the initiation of differentiation from the disk margins whereas *wg* inhibits it. Differentiation starts at a point near the optic stalk. As the furrow moves anteriorly across the disk and the disk continues to grow, differentiation must continuously reinitiate along the margins, a process that requires *dpp* function and signaling. In conditions of reduced *dpp* function, differentiation fails to spread laterally, and this is correlated with the ectopic expression of *wg* at the margins (Wiersdorff *et al.*, 1996; Chanut and Heberlein, 1997a). Because *wg* is expressed ahead of the MF along the dorsal and ventral margins, the advancing furrow must continuously counteract the inhibitory effect of *wg*. Normally, the lateral margins do not support differentiation until the MF reaches them. It is therefore possible that *dpp* expressed in a region of the MF close to the margin is able to inhibit *wg* expression at the margin, allowing differentiation to initiate de novo at each step of MF progression. This inhibition could be achieved perhaps through a nonautonomously induced increase in *dpp* expression at the margin. Thus, the role of *dpp*, through positive autoregulation and inhibition of *wg*, would be to coordinate the progression of differentiation at the center and edges of the retinal field.

E. Proneural Genes: *atonal* and *daughterless*

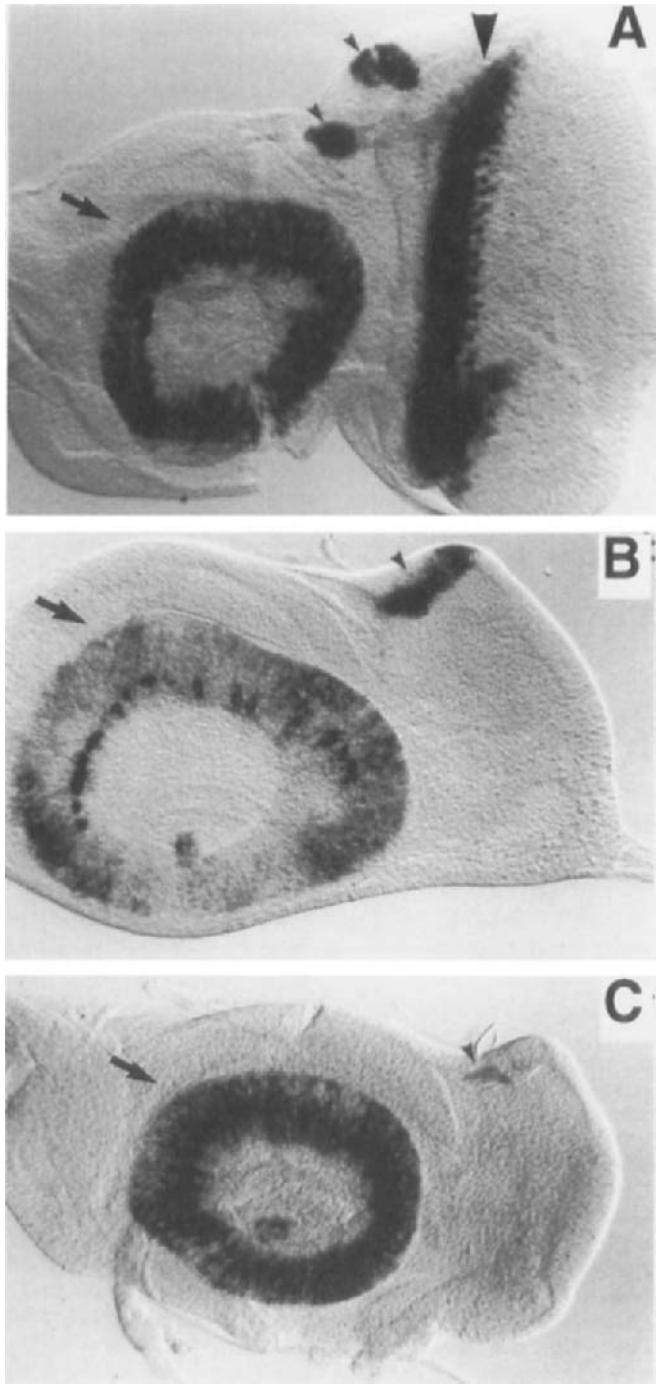
Neuronal differentiation in the retina requires the function of the proneural gene *atonal* (*ato*). Flies carrying the loss-of-function ato^1 mutation occasionally survive; these survivors lack chordotonal organs (stretch receptors) and are nearly eyeless (Jarman *et al.*, 1993, 1994). The remaining eye is very narrow and contains no photoreceptor neurons; only some pigment cells and bristles remain. The failure of photoreceptor differentiation in *ato* mutants is due to a defect in the specification of photoreceptor R8, the so-called ommatidial founder cell (Jarman *et al.*, 1994). It is believed that in the absence of an R8 the remaining photoreceptors fail to be recruited and induced to differentiate as neurons.

Eye disks from young ato^1 larvae appear indistinguishable from the wild type: the disks are normal in size and express *dpp* along the posterior and lateral margins, and the MF forms and even begins to move away from the posterior margin. However, neuronal differentiation is absent, *dpp* expression decays, and MF progression aborts (Jarman *et al.*, 1995). While this possibility has not been tested directly, it is likely that *hh* expression is missing from *ato* eye disks, leading to the observed failure in MF progression. Thus, the analysis of *ato* mutants has revealed that initiation and progression of the MF can be dissociated and that neuronal differentiation is crucial for continued furrow progression.

ato is expressed in a dynamic pattern prior to and during MF progression. Before initiation, *ato* expression is seen along the posterior margin in a pattern similar to that of *dpp*. During MF progression, *ato* is expressed in a strip of cells immediately anterior to the furrow. In the furrow, expression is restricted to evenly spaced groups of cells (called intermediate groups) and shortly thereafter to single R8 precursors (Jarman *et al.*, 1994; Fig. 16A). *ato* expression is completely dependent on normal *eya* and *so* function (Fig. 16B,C), suggesting that *ato* acts downstream of the early functions of these genes. In addition, normal *ato* function is required for *ato* expression in the intermediate groups and in R8, revealing a positive autoregulatory loop (Jarman *et al.*, 1995). The restriction of *ato* expression from the intermediate groups to single R8's requires signaling by the *Notch* (*N*) pathway; in eye disks from larvae carrying a temperature-sensitive allele of *N*, all cells of the intermediate group differentiate as R8's at the restrictive temperature (Baker and Zitron, 1995; Baker *et al.*, 1996).

ato encodes a basic helix–loop–helix (bHLH) protein and is thus a potential transcriptional regulator (Jarman *et al.*, 1993). *In vitro*, ato protein forms hetero-

Fig. 16 Photomicrographs of wild-type and mutant eye disks displaying the expression of *ato*, assayed by *in situ* hybridization. (A) Eye disk from a wild-type larva, *ato* is expressed in and near the furrow (large arrowhead), in the presumptive ocellar region (small arrowheads), and in the antennal disk (arrow). (B and C) Eye disks from *eya* and *so* mutant larvae, respectively, stained as in A. Posterior is to the right. Reproduced with permission from Jarman *et al.* (1995).


A
B
C

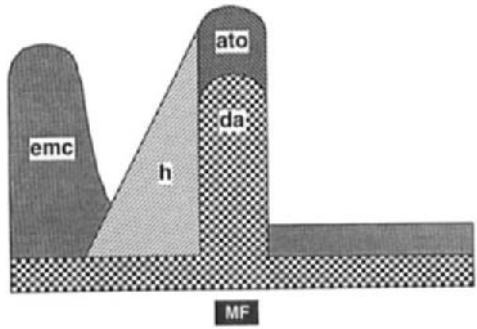


Fig. 17 Diagram of the expression patterns of HLH proteins in the eye disk relative to the position of the morphogenetic furrow (MF). See the text for details. Posterior is to the right. Adapted with permission from Brown *et al.* (1995).

multimers with another bHLH protein encoded by the *daughterless* (*da*) gene; these ato–da multimers act as sequence-specific DNA-binding complexes (Jarman *et al.*, 1993). *da* is required for numerous processes throughout development and is thought to function as a partner for *ato* and other spatiotemporally restricted bHLH proteins such as *achaetae* (*ac*) (Murre *et al.*, 1989). Loss of *da* function during eye development, analyzed in mosaic flies due to the embryonic lethality of *da* mutants, results in a failure of neuronal differentiation similar to that observed with a loss of *ato* function (Brown *et al.*, 1996). However, whereas *ato* is required only in R8, *da* function is required in R8 as well as in photoreceptors R2/5 and R3/4, which are the cells that differentiate immediately after R8. This requirement, while not absolute, suggests that *da* may act alone or together with an as yet unidentified partner in specifying R2/5 and R3/4.

While the expression of *da* is low throughout most of the eye disk, higher levels of expression are observed just ahead of the MF (Fig. 17). The cells that express high levels of *da* exactly correspond to the cells expressing *ato* (Brown *et al.*, 1996). In addition, *da* and *ato* regulate each other's expression patterns in a complex manner. Taken together, these data support the proposal that *ato* and *da* act as partners during proneural specification in the eye. However, *da* also appears to have functions that are independent of *ato*.

F. Antineural Genes: *hairy* and *extramacrochaetae*

hairy (*h*), like *ato* and *da*, encodes a bHLH protein; *extramacrochaetae* (*emc*) encodes an HLH protein lacking the basic DNA-binding domain. *h* is expressed in a dorsoventral strip located immediately anterior to the domain of *ato* expression (Brown *et al.*, 1991). *emc* expression, while fairly ubiquitous, is highest near the anterior margin of the disk (Brown *et al.*, 1995; Fig. 17). Loss of *h* or *emc*

function in the eye, analyzed in mosaic retinae (as both genes are required for viability), has little effect on normal development. However, loss of function of both *h* and *emc* causes the MF and the front of neuronal differentiation to accelerate by as much as eight ommatidial rows while traversing clones of doubly mutant tissue (Brown *et al.*, 1995).

While the exact mechanisms of *h* and *emc* function during eye development are not known, their roles as negative regulators of proneural function elsewhere in the fly's peripheral nervous system are better understood. *h* and *emc* use different strategies to block proneural gene function. *h* binds to promoter sequences in the proneural gene *achaetae* (*ac*) that are required for the proper repression of *ac* transcription, suggesting that *h* acts directly as a transcriptional repressor (Ohsako *et al.*, 1994; Van Doren *et al.*, 1994). *emc*, on the other hand, binds *in vitro* to several bHLH proteins, including ac and da, forming heterodimers (Van Doren *et al.*, 1991, 1992) that do not bind to DNA and are thus nonfunctional. It is unclear whether *h* and *emc* have redundant actions on the same target in the retina, presumably *ato*, or whether they act on different targets and have a synergistic effect.

G. Coordination of Proneural and Antineural Gene Function during Furrow Progression

Orderly progression of the MF requires the adoption of neuronal potential and subsequent differentiation to be tightly regulated both spatially and temporally, such that one row after another of ommatidia develops in exact synchrony. One logical way to achieve this synchrony is to couple differentiation to *hh* signaling. Indeed, *hh* has been shown to induce the expression of *ato* when expressed ectopically ahead of the MF (Heberlein *et al.*, 1995). These *ato*-expressing cells then differentiate neuronally and begin expressing their endogenous *hh* gene, which in turn leads to the induction of *dpp* and *ato* in neighboring cells. The repetition of this cycle allows the furrow to move across the disk. Curiously, ectopic expression of *hh* or loss of *pka* function ahead of the MF also leads to ectopic expression of *h* in nearby cells (Heberlein *et al.*, 1995; Pan and Rubin, 1995). This suggests that the strip of *h* expression ahead of the MF is induced normally, either directly or indirectly, by *hh*. Thus, *hh* seems to carry out two apparently paradoxical functions: it induces the expression of not only an activator of neuronal competence, *ato*, but also an inhibitor, *h*. It is likely that this mechanism ensures that *ato* expression and the adoption of neuronal competence are not achieved prematurely, allowing only one new row of ommatidia to be induced at a time. This in turn may be important for the orderly patterning of each row of ommatidia and correct projections of photoreceptor axons to the optic ganglia. A curious observation is that expression of *h* is expanded anteriorly in eye disks from ato^1 (Jarman *et al.*, 1995) and other furrow-stop mutants (U.

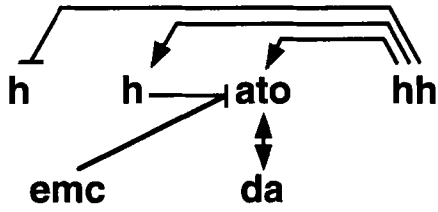


Fig. 18 Diagram summarizing the interactions between *hh* and the genes encoding HLH proteins as deduced from ectopic expression and loss-of-function experiments. *hh* induces the expression of *ato* and *h* in nonoverlapping domains ahead of the furrow. *hh* also appears to repress *h* expression in more anterior cells. *emc* and *da* block furrow progression and *ato* expression. *ato* and *da* regulate each other's expression. Positive interactions are shown with arrows and negative interactions with two intersecting lines. It is unclear whether the interactions shown are direct or indirect. The position of the furrow coincides with the region of *ato* and *da* expression. Posterior is to the right.

Heberlein, unpublished), implying that the anterior limit of *h* expression is also normally defined by signals emanating from the furrow. How *hh* regulates *ato* and *h* expression and how *ato* and *h* may regulate each other's expression remain unclear. However, what appears to be quite clear is that the advancing front of differentiation has long-range effects on cells located far ahead of the MF (Fig. 18).

H. A Missing Signal?

Loss-of-function and ectopic expression experiments lead to the inference that *hh* induces not only the expression of *dpp* but also the expression of the proneural gene *ato* (Heberlein *et al.*, 1995). Although it has not been tested directly, *ato* expression does not appear to be induced by *dpp*. First, *ato* expression is present in *tkv* mutant clones (Penton *et al.*, 1997). Second, neurons differentiate in cells that, in theory, cannot receive or transduce the *dpp* signal (Burke and Basler, 1996; Wiersdorff *et al.*, 1996; Penton *et al.*, 1997). This suggests that either *hh* induces *ato* directly or another *hh*-induced signal is responsible for *ato* induction. It has been shown that the *N* pathway is required not only to single out R8 cells from the intermediate groups (Baker *et al.*, 1996) but also to obtain high levels of *ato* expression in the intermediate groups and induce neuronal differentiation (Baker and Yu, 1997). The exact mechanisms by which *N* induces *ato* and neuronal differentiation are unknown. It is possible that *N* is required for cells to respond to *hh* or another *hh*-induced signal; conversely, *N* may play a more direct role in neuronal differentiation.

V. Cell Cycle Regulation

As the furrow progresses from the posterior tip to the middle of the eye disk, the epithelium grows extensively, increasing in size by approximately 6–8-fold. Thus, patterning, differentiation, and cell division must be tightly regulated, both temporally and spatially. This is particularly important in and near the MF, where the transition from asynchronous to synchronous cell division occurs (Ready *et al.*, 1976; Wolff and Ready, 1991; Thomas *et al.*, 1994). Cells divide asynchronously far ahead of the MF and are therefore found in either the M, G1, S, or G2 stage of the cell cycle. Immediately anterior to the MF, cells begin to become synchronous; there is an increase in mitosis and an arrest in the ensuing G1 (Thomas *et al.*, 1994). In the MF, cells are arrested in G1 (Thomas *et al.*, 1994). Behind the furrow, the precursors of R2–5 and R8 exit the cell cycle and begin differentiation while all other cells undergo one additional mitosis before differentiating (Wolff and Ready, 1991, 1993). The role of the latter cell division is to generate enough precursor cells to complete the assembly of ommatidia (Wolff and Ready, 1991, 1993; de Nooij and Hariharan, 1995).

Progression through the different stages of the cell cycle requires the activity of different cyclin-dependent kinases (Cdk) and their associated positive regulatory subunits, the cyclins (Cyc) (Norbury and Nurse, 1992; Morgan, 1995). Both positive and negative regulation ensure that this activation is specific for the appropriate stage of the cell cycle. Several Cyc and Cdk have been identified in *Drosophila* [for reviews, see Edgar (1994), Edgar and Lehner (1996), and Follette and O'Farrell (1997)]. Although their domains of expression in the developing retina are well-established, the assessment of their functions in the eye is incomplete and is an ongoing area of research. The expression patterns of several genes that play a role in cell cycle regulation near the MF (discussed in the following) are shown in Fig. 19.

A. Regulation of G2–M Transition

Synchronization of cells leading to G1 arrest in the MF is thought to occur in two steps: cells in G2 are driven into mitosis, and cells in G1 are prevented from reentering S phase. It has been proposed that one possible role of *dpp* in the MF is to promote the G2–M transition in cells located just ahead of the MF (Penton *et al.*, 1997). Clones of cells mutant for the *dpp* receptors *tkv* or *saxophone* (*sax*), and which encompass the MF, display persistent expression of the G2 cyclin, CycB, in the anterior region of the MF, suggesting that cells are arrested in G2. This arrest is transient as mutant cells enter delayed mitosis in the posterior region of the MF. Mutations in the *division abnormally delayed* (*dally*) gene display a similar phenotype; the domain of CycB expression and the domain

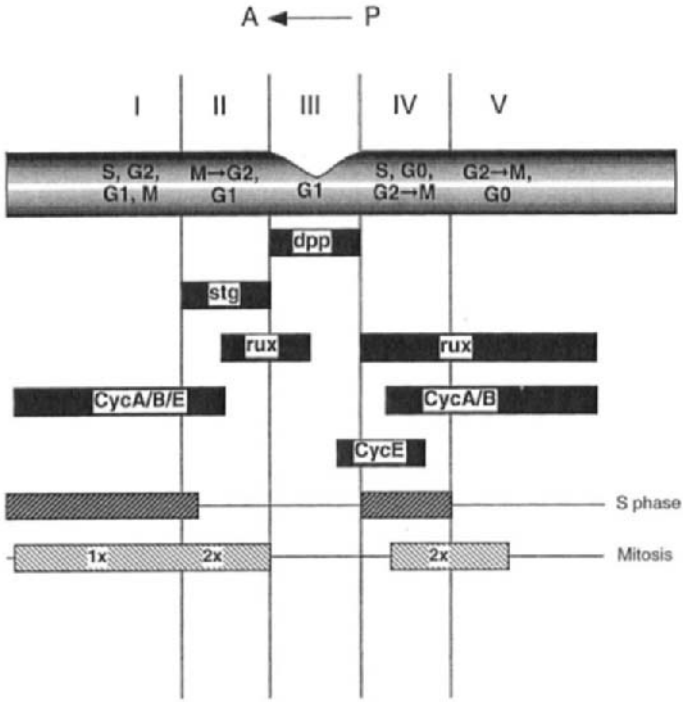
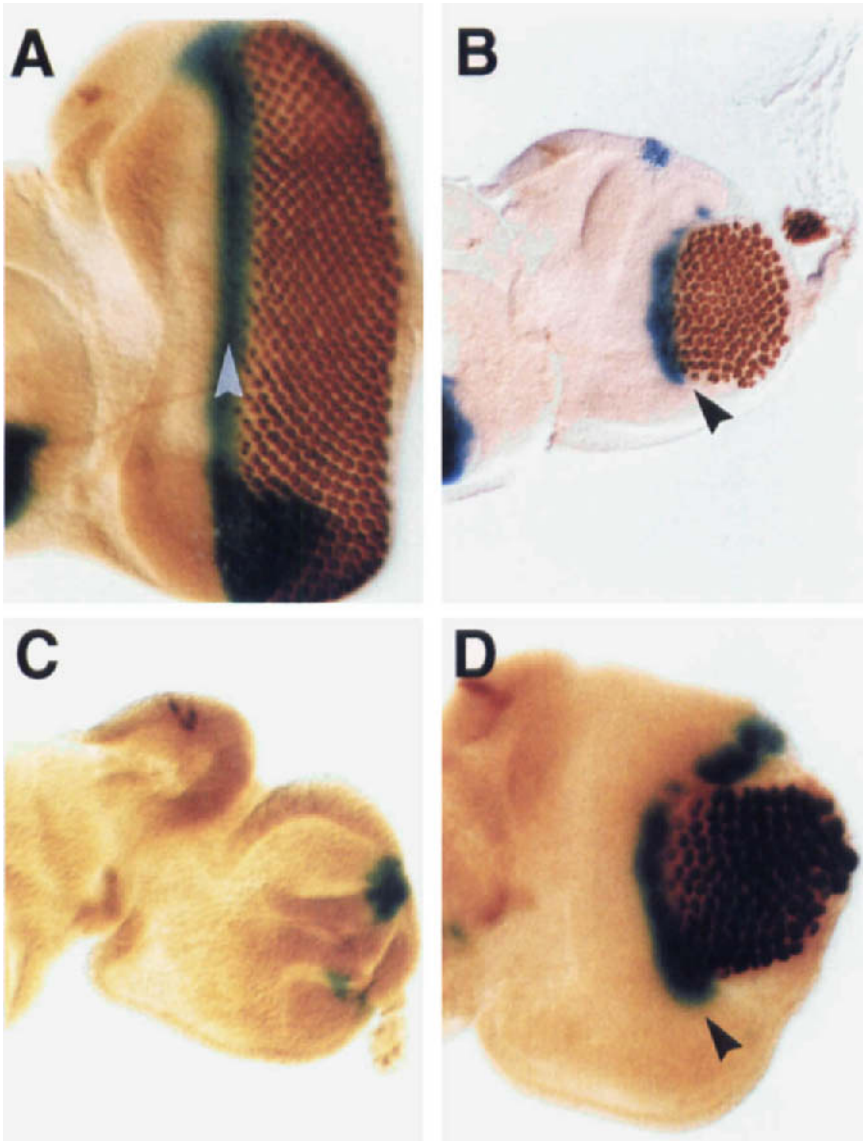


Fig. 19 Diagram summarizing the expression patterns of cell-cycle-regulated genes in the eye disk. The disk can be divided into five domains (I–V), each containing cells in different stages of the cell cycle. Adapted with permission from Thomas *et al.* (1994, 1997).

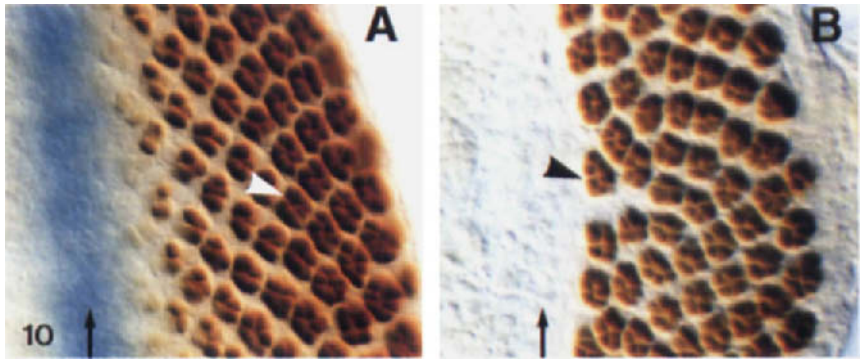
containing mitotic cells are shifted posteriorly in the MF (Nakato *et al.*, 1995). *dally* encodes an integral membrane proteoglycan that may act as a coreceptor for *dpp* in the eye (Nakato *et al.*, 1995).

The product of the *string* (*stg*) gene, a phosphatase homologous and functionally equivalent to the mitotic inducer Cdc25 (Edgar and O'Farrell, 1989; Kumagai and Dunphy, 1991), is also believed to drive the transition from G2 to M in cells located just anterior to the MF. *stg* is expressed in a band of cells just ahead of the MF (Alphey *et al.*, 1992), which coincides with a domain of increased mitosis (Thomas *et al.*, 1994). However, the effect of loss of *stg* function in the developing eye has not been established.

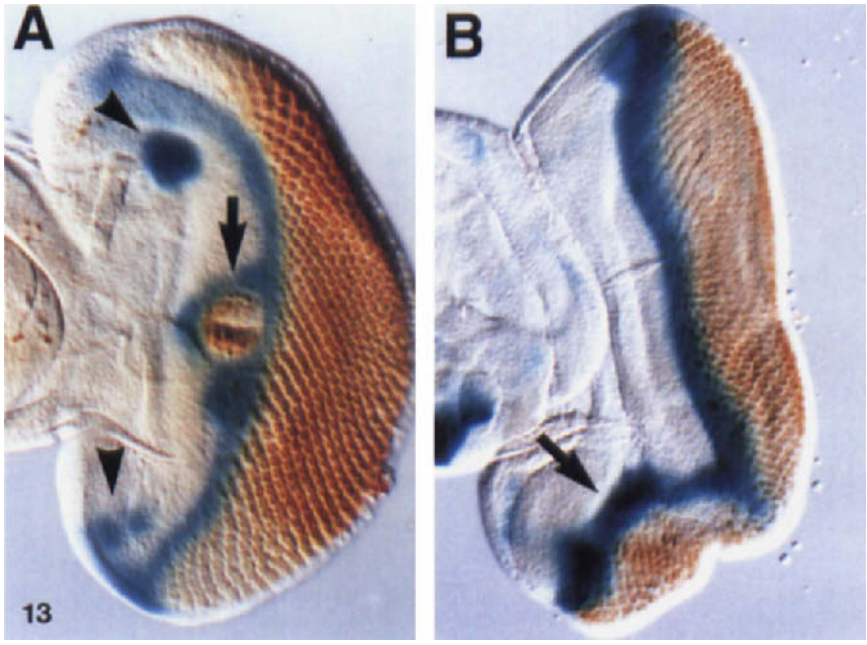
The three genes just described are believed to help drive cells located just ahead of the MF into mitosis as a first step toward G1 synchronization in the MF. One is tempted to speculate that the role of *dpp* signaling in the MF is to regulate the expression of *stg*, which in turn would activate a CycB–Cdk complex, thus driving cells into mitosis.



Chapter 4, Fig. 4 Photomicrographs of eye disks displaying phenotypes associated with the loss of *dpp* function. All disks are derived from late-third instar larvae and are stained with anti-Elav to mark differentiating photoreceptors posterior to the furrow (arrowheads) and with X-GAL to show the expression of the *dpp–lacZ* reporter in the furrow. (A) Wild type. (B) dpp^{blk} (C and D) dpp^{hr4}/dpp^{hr56} dissected 48 hr after a shift to the restrictive temperature. Differentiation is completely blocked in C and is restricted to the medial portion of the disk in D. Posterior is to the right. Reproduced with permission from Chanut and Heberlein (1997a,b).



Chapter 4, Fig. 10 Photomicrographs of third instar eye disks carrying the *dpp–lacZ* reporter stained with anti-Elav (to identify differentiating neuron posterior to the furrow) and X-GAL (to identify *dpp–lacZ*-expressing cells). (A) In a wild-type disk Elav-positive cells are behind the furrow (arrow), where *dpp* is expressed. (B) In an hh^1 eye disk expression of *dpp* in the furrow (arrow) is absent, and ommatidia in the first row (black arrowhead) contain a full complement of photoreceptors; in wild type this would only be seen several rows posterior to the furrow (white arrowhead). Posterior is to the right. Reproduced with permission from Chanut and Heberlein (1997a).



Chapter 4, Fig. 13 Photomicrographs of eye disks carrying clones of cells that ectopically express *hh* under the control of the *Tubulin α2* promoter generated using the "flp-out" technique (Struhl and Basler, 1993). Expression of the *dpp–lacZ* reporter is visualized by X-GAL staining (blue), and neuronal differentiation is marked by staining with the anti-Elav antibody (brown). (A) Disk carrying a group of precociously differentiating neurons (arrow) and groups of cells showing ectopic expression of *dpp* (arrowheads). (B) Disk in which an ectopic furrow (arrow) progressed from the ventral margin. Posterior is to the right. Reproduced with permission from Heberlein *et al.* (1995).

B. Control of G1

Cells in the MF are arrested in G1, an arrest thought to be necessary for proper cell–cell communication. In *roughex* (*rux*) mutants, cells in the MF fail to arrest in G1 and proceed prematurely into S phase and mitosis, leading to patterning defects (Thomas *et al.*, 1994). *rux* encodes a novel protein believed to suppress entry into S phase by preventing the accumulation of CycA: loss of *rux* function causes increased accumulation of CycA, whereas ectopic expression of *rux* leads to CycA degradation (Thomas *et al.*, 1997). As cells exit the furrow, some cells need relief from G1 arrest to enter their final mitosis. Cyclin E, which is expressed in these cells (H. Richardson *et al.*, 1995), has been shown to bind to rux protein *in vitro* and in a yeast two-hybrid assay (Thomas *et al.*, 1997). The binding of CycE is believed to destabilize rux; loss of rux leads to stabilization of CycA, which in turn promotes cells to complete S phase and enter the ensuing final mitosis (Thomas *et al.*, 1997).

The progression into S phase is also regulated by the product of the *regulator of cyclinA* (*rca1*) gene (Dong *et al.*, 1997). *rca1* mutants, which were isolated as dominant suppressors of *rux*, have an embryonic phenotype very similar to that of mutations in *cycA*. Ectopic expression of *rca1* promotes CycA accumulation and drives cells into S phase. This effect is reversed by reducing the *cycA* dosage, suggesting that *rca1* functions as a positive upstream activator of *cycA*. Thus, the antagonistic functions of *rca1* and *rux* may limit CycA activity. In the furrow, a critical balance between *rux*, *rca1*, and *stg* activities might lead to G1 arrest by preventing the activation of a CycA–Cdk complex during G1.

C. Coordination of Cell Cycle Regulation and Furrow Progression

The tight spatial and temporal linkage between patterning and cell cycle synchronization in the eye disk suggests that both events are under the same genetic control. Indeed, ectopic expression of *hh* ahead of the MF leads to ectopic expression of *stg* (Heberlein *et al.*, 1995). This is similar to the situation in the embryo, where *stg* expression is controlled by patterning genes that regulate the location of mitotic domains (Edgar *et al.*, 1994). High levels of expression of a *rux–lacZ* reporter is also seen in a band of cells just anterior to the MF (Thomas *et al.*, 1997). It will be interesting to determine whether *rux* expression is also regulated directly or indirectly by *hh*.

Another gene, *yan*, is required for cells to execute the choice between cell division and differentiation. In the eye disk, clones of cells mutant for *yan* overproliferate and fail to begin neuronal differentiation (Rogge *et al.*, 1995). It was postulated that in wild-type development, inactivation of *yan* in or near the MF is required to release certain cells from G1 arrest, driving them into their final

mitosis (Rogge *et al.*, 1995). Inactivation of *yan* is thought to occur via signaling through the *Drosophila* EGF receptor (Egfr). This is consistent with the observation that in *Ellipse*, a gain-of-function mutation in *Egfr*, too many cells behind the MF reenter S phase rather than differentiate neuronally (Baker and Rubin, 1992) [for a review on Egfr signaling in the eye, see Freeman (1997)]. How activation of Egfr and inactivation of *yan* regulate the choice between cell division and differentiation and how this regulation is coordinated with progression of the MF are not known.

VI. Concluding Remarks

The *Drosophila* eye disk develops in a remarkably coordinated manner; its differentiation begins at a precisely defined point and expands at a steady rate, laying down rows of evenly spaced ommatidial founder cells. Their further development results in the highly ordered structure of the adult compound eye. In this chapter we have discussed only the first phases of development, from the specification of the eye field to the establishment of ommatidial preclusters. Much progress has been made in this area of research, and we now understand some of the signals used to impose pattern on an apparently uniform tissue. Many unanswered questions remain, however. Although a number of apparent transcription factors encoded by *ey*, *eya*, *so*, and *dac* are required to allow the eye to form, the interactions between them and the target genes they regulate have not been well-defined. The disk is then spatially organized by the interaction between *dpp* and *wg*, but the molecular bases for this interaction, the initial establishment of the patterns of *dpp* and *wg* expression and the temporal control of differentiation, are not understood. The progressive pattern of development is controlled by *hh* and its target proneural, antineural, and cell cycle genes, but a mechanistic understanding of this process remains beyond reach. Also remaining unclear is the transition from *dpp*-controlled initiation to *hh*-controlled progression. We thus anticipate many more insights into these processes. As the mechanism of eye development appears to be conserved to an unexpected degree between flies and vertebrates, some of these insights will probably be applicable to other organisms as well.

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5

The Development of Voltage-Gated Ion Channels and Its Relation to Activity-Dependent Developmental Events

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Spontaneous activity is an essential feature in the development of the nervous system. The patterns of activity and the waveform and ionic dependence of the action potentials that occur during such activity are fine-tuned to carry out certain developmental functions, and are therefore generally not compatible with the mature physiological function of the cell. For this reason, the patterns of ion channel development that create spontaneous activity early in the development of a given cell type are complex and not easily predicted from the mature properties of that same cell. Ion channels are often found that are specific to early stages of development, and that either are not retained in the mature cell or whose properties are greatly changed during later differentiation. The exact significance of such patterns of channel development is just now becoming clear, as we understand more about the mechanisms linking spontaneous activity to later developmental events.

I. Introduction

The function of the mature nervous system depends on the electrical properties of individual cells and the synaptic connections among them. The correct develop-

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ment of both requires electrical activity. This was recognized many years ago as use dependence, where activity triggered by normal sensory input or motor output pathways early in development serves to refine either the synaptic connections in a particular circuit or the properties of individual cells within that circuit. It is now clear, however, that many activity-dependent developmental processes are independent of sensory or motor activity and that individual cells or small circuits must be spontaneously active at early stages to develop properly. Such spontaneous activity is a widespread, and perhaps ubiquitous phenomenon in nervous system development.

This is not surprising. The development of synaptic connections requires communication between the pre- and postsynaptic partners, and the normal activity-based synaptic transmission may be the most parsimonious way to accomplish this. Similarly, refinement of the mature populations of ion channels in the neuronal membrane by a preset developmental program that does not register the activity resulting from those channels would be difficult, given the sensitivity of activity to subtle changes in channel density and kinetics.

The properties that allow cells or small circuits to generate spontaneous activity of the type appropriate to drive developmental processes are likely to be incompatible with the functions of those cells in the mature nervous system. One therefore would expect the ion channel populations present early in development, especially during stages in which activity plays a critical role in development, to be qualitatively different from those in the mature cells and the patterns of channel development therefore to be more complex than can be easily predicted from the mature properties of a given cell. In this chapter, I will concentrate on how the development of ion channels is tuned to allow activity to serve its very different purposes in mature and developing cells.

II. Patterns of Ion Channel Development in Early Embryos

Ion channel development in excitable cells does not begin at the onset of terminal differentiation, but extends back to before fertilization. Although most work on the activity dependence of development has concentrated on the terminal differentiation of nerve and muscle, many significant events of channel development occur very early in embryogenesis. Some of these events are of known, profound biological significance, but many are poorly understood. The perspective they provide is crucial to a full understanding of the principles of channel development and activity dependence.

A. Ion Channels Are Present at the Earliest Stages of Development

Voltage-gated ion channels and the ability to generate action potentials are present in the unfertilized eggs of animals in most phyla (see Hagiwara and Jaffe,

1979; Moody, 1995). The significance of the variety of channel types present in oocytes of different species is sometimes clear, but in other cases obscure. For example, most egg cells need to generate a rapidly rising action potential when fertilized to prevent polyspermic fertilization (Jaffe, 1976). But it is not clear why in some oocytes the action potential is mediated by multiple Ca^{2+} channel types, whereas in other, often closely related species both Na^+ and Ca^{2+} channels are used (Block and Moody, 1987; Moody and Bosma, 1990). Another mystery is why mammalian eggs, which do not generate a voltage-dependent polyspermy block (Jaffe *et al.*, 1983) and which, indeed, do not depolarize at all during fertilization (Miyazaki and Igusa, 1981), also have these channels (Okamoto *et al.*, 1977). This may be an evolutionary relic of lower vertebrates in which the biology of fertilization dictates a higher risk of polyspermy than in mammals, or the channels may reflect an as yet unappreciated role for electrical activity in the prefertilization development of the egg or in the early cleavage stage development of the embryo. In most egg cells, depolarization-activated outward currents are small or absent, reflecting the requirement for sustained depolarization following fertilization to prevent polyspermy (Moody, 1995). In most egg cells, the primary resting conductance is due to an inwardly rectifying K^+ channel. Because it deactivates with small depolarizations, this channel serves the function (as it does in other, mature cells) of allowing large action potentials with minimal ion flux. This is likely to be critical for oocytes, where too much Ca^{2+} influx during the action potential could activate certain developmental programs inappropriately.

Complex forms of developmental modulation of voltage-gated channels occur in many species at very early stages of development, including oogenesis, meiotic maturation, fertilization, and the first few embryonic cell cycles. In the fully grown oocyte of the starfish, *Leptasterias hexactis*, the action potential is mediated by an inward Ca^{2+} current and a transient outward, or A type, potassium current. Oogenesis in this species requires 2 years. During oogenesis, the A current increases in amplitude monotonically, closely tracking plasma membrane surface area so that constant density is maintained during a period of growth that increases the surface area by 10-fold. The Ca^{2+} current, on the other hand, remains small during growth and then, near the end of oogenesis when growth is complete, abruptly increases in density. This increase closely coincides with the period when the nucleus migrates to the animal pole (Moody, 1985). After 2 years of oogenesis, oocytes mature (reinitiate meiosis in preparation for fertilization) in about 1 hr under the influence of the hormone 1-methyladenine (1-MA) (Kanatani, 1973). Application of 1-MA to *Leptasterias* oocytes causes a rapid loss of surface area (50% in 45 min) due to the removal of microvilli. During this hour, the A current decreases in precise proportion to the area whereas the Ca^{2+} current remains unchanged, implying a selective protection of Ca^{2+} channels from removal during membrane loss (Moody and Bosma, 1985). The resulting increase in the ratio of inward to outward currents increases the amplitude and

rate of rise of the action potential, which allows the eggs to depolarize rapidly at fertilization and thus initiate an effective polyspermy block (Miyazaki and Hirai, 1979). This developmental modulation of the ratio of inward to outward currents is reminiscent of similar events in the later development of the action potential (see the following). Ion channels also play various roles in the prefertilization development of sperm, and in the physiological responses of sperm to contact with the egg [see, for example, Babcock *et al.* (1992); Arnoult *et al.*, 1996].

After fertilization, the early embryo faces some potential problems with the ion channels present in the oocyte. On the one hand, activity necessary to prevent polyspermy might generate ion fluxes disruptive to later development and cleavage were it to occur at inappropriate times; on the other hand, channels that serve crucial functions after fertilization, such as those that set the resting potential, must continue to function while facing dilution by the large addition of new plasma membrane that accompanies the cleavage cycles. The response to these problems can be seen in the developmental events that follow immediately after fertilization. In the ascidian *Boltenia villosa*, for example, the Na^+ current that mediates the rising phase of the action potential at fertilization disappears completely during the 2 hr between fertilization and the first mitosis and does not reappear in any blastomere through gastrulation, 12 hr later (Block and Moody, 1987; Simoncini *et al.*, 1988). The inwardly rectifying K^+ current, which sets the resting potential, is maintained at constant *density* in all blastomeres through gastrulation, despite an approximately 8-fold increase in the total surface area of the embryo. A similar down-regulation of oocyte channels after fertilization is seen in other ascidians (Takahashi and Yoshii, 1981) and in mammals (Mitani, 1985). Presumably, the high membrane turnover during early embryogenesis includes the selective removal of some ion channels and the insertion of others.

The rapid selective loss of certain channel types after fertilization is strong circumstantial evidence for their function at earlier stages, especially when that loss occurs after a period of active up-regulation of channel numbers (Simoncini *et al.*, 1988; Greaves *et al.*, 1996). Loss of specific channels occurs at many later stages of development in many cell types and sometimes results in the permanent loss of an entire class of channels from the cell, even the permanent loss of excitability (Barres *et al.*, 1990). These findings similarly point to a developmental function for these channels during the window of time between their appearance and loss.

B. The Cell Cycle Clock as a Modulator of Channel Function in Embryonic and Mature Cells

Little is known about the physiological development of these cells in cleavage stage embryos. One generalization that can be made about this period, however,

is that the cell cycle clock is a powerful modulator of channel function. Cell-cycle-dependent channel modulation appears to occur by a combination of mechanical strain and protein kinase-based phosphorylation. In ascidian embryos, for example, inwardly rectifying Cl^- current density cycles with each cell cycle, increasing by more than 10-fold in 10–20 min at the exit from M phase and then falling again after cytokinesis (Block and Moody, 1990). The cycling does not, however, require actual cleavage, but proceeds normally in cleavage-arrested embryos. This current can also be up-regulated by more than 100-fold by cell swelling, but its response to an instantaneous increase in cell diameter (<5 sec) is slow (>20 min), suggesting that it is not responding directly to mechanical strain but rather to a strain-induced second messenger system. Blockers of cell-cycle-dependent protein kinase activity increase the response of the channel to swelling and, in some instances, directly up-regulate the current (Villaz *et al.*, 1995). Similar Cl^- channel modulation is seen in chromaffin, epithelial, and osteoblast cells (Doroshenko, 1991; Solc and Wine, 1991; Chesnoy-Marchais and Fritsch, 1989). These data suggest that mechanical strain and dephosphorylation interact in some way to up-regulate this current. This Cl^- channel clearly is not a typical "stretch-activated" channel: Nonselective cation channels that can be activated by suction applied to a patch pipet also exist in these embryos, but are not activated during cell division (Moody and Bosma, 1989). In embryos in which stretch-activated channels are activated during cleavage, they are activated by a combination of the mechanical strain of cleavage and an increase in the actual sensitivity of the channel to strain, caused by cAMP-dependent phosphorylation (Bregestovski *et al.*, 1992). Cell-cycle-dependent channel modulation has also been reported in mouse embryos where, again, cAMP-dependent phosphorylation may play a role in either modulating channel activity or triggering rapid channel insertion into the plasma membrane (Day *et al.*, 1993).

Although the biological significance of cell-cycle-dependent channel modulation in early embryos is not known, studies in mature cells indicate a close relationship between ion channel activity and proliferation. Ion channel activity changes with the proliferative state of cells (DeCoursey *et al.*, 1984; Partiseti *et al.*, 1993; Soliven *et al.*, 1989; and Wilson and Chiu, 1993), or in actively proliferating cells it changes with the phase of the cell cycle (Takahashi *et al.*, 1993; Bubien *et al.*, 1990). Ion channel blockers can prevent the proliferative response in lymphocytes (Chandy *et al.*, 1984), or in proliferating cells they can arrest the cell cycle at specific phases (Amigorena *et al.*, 1990; Ramsdell, 1991). These effects appear to involve Ca^{2+} fluxes across the plasma membrane, mediated directly by Ca^{2+} channels or, in the case of K^+ and Cl^- channels, indirectly by changes in membrane potential and, hence, the driving force for Ca^{2+} entry (Nilius and Wohlrab, 1992). The likely involvement of ion-channel-mediated effects on the cell cycle in tumor cells makes this work particularly relevant (Lepple-Wienhues *et al.*, 1996; Woodfork *et al.*, 1995; Wegman *et al.*, 1991; Chou *et al.*, 1995).

The particular sensitivity of Cl^- channels to cell-cycle-dependent modulation in both embryonic and mature cells (Block and Moody, 1990; Bubien *et al.*, 1990; Chou *et al.*, 1995) raises the additional possibility that they serve to regulate volume during the cell cycle. Embryonic and mature cells face different volume regulatory problems during proliferation. Embryonic cells cleave with minimal growth phase, and cells get progressively smaller while total embryo volume remains constant; mature cells, on the other hand, must grow between cleavages to maintain cell size. Expression of the water channel aquaporin I changes with the cell cycle (Delporte *et al.*, 1996), and misexpression of CFTR (Cystic Fibrosis Transmembrane Regulator) is associated with disruptions in the cell cycle (Schiavi *et al.*, 1996). In addition, the multidrug resistance protein, whose drug-exporting function causes drug resistance in tumor cells (Higgins, 1992), may be closely associated with volume regulatory chloride channel expression (Gill *et al.*, 1992; but see Viana *et al.*, 1995). In embryonic cells, it is possible that ion channels that are activated at certain phases of each cell cycle may serve as volume sensors involved in the transition between embryonic (volume-reducing) and mature (volume-maintaining) divisions.

III. Patterns of Ion Channel Expression during Terminal Differentiation

It is in comparing the physiological properties of cells at the onset of terminal differentiation with those in the mature state that we see the most profound and complex changes in ion channel properties that occur during development in an individual cell. It is also during this period that the developmental roles of activity can be seen in such processes as migration, target selection, and the maturation of ligand- and voltage-gated channels. Although information regarding ion channel development and the role of activity in development has been drawn from many systems, I have chosen five particularly instructive ones to review here. Not only have these been studied extensively but they each have elucidated important concepts that will define future studies in other cell types.

A. *Xenopus* Spinal Neurons

Presumptive neurons derived from the neural plate of *Xenopus* larvae differentiate morphologically and physiologically in culture in a way that approximates their *in vivo* development (Spitzer and Baccaglini, 1976; Spitzer and Lamborghini, 1976; Desarmenien *et al.*, 1993). About 6 hr after dissociation of the neural plate (stage 15), neurons can be morphologically identified. At this time they are capable of generating long-duration, Ca^{2+}-dependent action potentials and already possess high- and low-voltage-activated Ca^{2+} currents, a slowly

activating voltage-gated delayed K^+ current, and a Na^+ current (Barish, 1986; O'Dowd *et al.*, 1988; Gu and Spitzer, 1993). This indicates that ion channel development has proceeded considerably before overt morphological differentiation begins, which makes the initial events of electrophysiological differentiation difficult to study. During the next 24 hr in culture, the action potential duration shortens by more than 10-fold, driven by the 3-fold increase in K^+ current density and the 2-fold increase in its rate of activation (Barish, 1985, 1986; O'Dowd *et al.*, 1988; Lockery and Spitzer, 1992). Na^+ and Ca^{2+} currents change little during this period. Between 5 and 10 hr in culture (and *in vivo*), spontaneous elevations in intracellular Ca^{2+} are seen in the neurons at very low frequencies (3–4/hr) (Holliday and Spitzer, 1990). These transients can be blocked by preventing Ca^{2+} entry, and doing so prevents the acquisition of mature transmitter phenotype, reduces the speeding (but not the density increase) of the delayed K^+ current, and increases dendritic arborization [Holliday and Spitzer, 1990; Spitzer *et al.*, 1993; see Spitzer *et al.* (1994) for a review].

Several important principles have emerged from this work

1. Developmental processes can decode very low-frequency activity. Reproduction of the Ca^{2+} transients artificially in these neurons, by exposing them in zero Ca^{2+} solution to brief pulses of Ca^{2+}-containing high-K^+ solution, can rescue them from the zero Ca^{2+} effect. The sensitivity of developmental events to the frequency of Ca^{2+} transients in these neurons is remarkable: Artificial Ca^{2+} transients at 1/hr produced no rescue of K^+ current kinetics, whereas 2/hr produced a complete rescue (Gu and Spitzer, 1995). Given the relatively short duration of the transients (about 10 sec) in relation to their overall frequency of occurrence, this implies that the Ca^{2+}-triggered signal transduction pathway becomes Ca^{2+}-independent during the long intertransient intervals. Both Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) and protein kinase C (PKC) display properties of this sort. Autophosphorylation of CaM kinase can trap calmodulin and convert the kinase to a partly Ca^{2+}-independent form (Hanson *et al.*, 1994). This property can create a very steep dependence of kinase activity on the frequency of Ca^{2+} transients of exactly the sort required for low-frequency stimulus decoding. Similarly, Ca^{2+}-dependent protease activity can cleave PKC into a long-lived, constitutively active, Ca^{2+}-independent form (Suzuki *et al.*, 1992; Sessoms *et al.*, 1992). Rapid Ca^{2+} transients can also induce long-lasting responsiveness of neurons to neurotrophic factors (Schmidt *et al.*, 1996). Therefore, although in many cell types spontaneous activity occurs at fairly high frequencies (see the following), extremely low-frequency activity can be a developmentally potent event. One also should not lose sight of the fact that activity at frequencies of 3–6/hr is difficult to detect experimentally, and any

experiments searching for spontaneous activity in the developing nervous system must account for this low end of the frequency spectrum.

2. Outward K^+ currents are critical in controlling action potential waveform and the developmental function of activity. The amount of Ca^{2+} entry during spontaneous activity is critically dependent on spike duration, which is controlled in part by the amplitude and kinetics of the delayed K^+ currents. In *Xenopus* neurons, the increase in amplitude and speeding of activation of the delayed K^+ current are instrumental in shortening action potential duration during development to a duration no longer compatible with its developmental function (Barish, 1986; O'Dowd *et al.*, 1988; Lockery and Spitzer, 1992). If a rapidly activating K^+ current is misexpressed in these cells at early stages, prematurely truncating action potential duration, later development is disrupted although not in ways that are obviously consistent with the effects of directly blocking activity (Jones and Ribera, 1994; Jones *et al.*, 1995). Interestingly, the developmental changes in the delayed K^+ current are uniform and synchronous in a diverse population of spinal neurons, even though different families of delayed K^+ channels mediate the current in different neurons (Ribera, 1996). Although it is clear that action potential duration is a critical parameter in development, it is not entirely understood why. A long-duration action potential may not necessarily admit more Ca^{2+} to the cell than a short-duration one, and the details of Ca^{2+} channel kinetics are important to understand in order to predict the exact effect of spike duration. This question may be best solved experimentally by using voltage-clamp command waveforms that mimic naturally occurring action potentials (McCobb and Beam, 1991).

B. Ascidian Larval Muscle

One problem that has plagued studies of ion channel development is that overt morphological differentiation of many cells occurs after physiological development has begun. Thus, as in the case of *Xenopus* spinal neurons, by the time voltage-clamp recordings can be made on identified cells, substantial channel development has already occurred. Our group has circumvented this problem by exploiting an unusual property found in the embryonic muscle of a class of marine chordates, the ascidians. The muscle lineage in ascidian embryos develops autonomously, without inductive events for the most part (Satoh, 1994), and in certain species it is pigmented throughout development. We have used this pigment to identify muscle lineage cells and voltage-clamp them at 19 identified stages of development, beginning before fertilization and proceeding to the fully mature, contractile larval muscle (Greaves *et al.*, 1996). Our findings in these cells illustrate how channel populations early in terminal differentiation can profoundly differ from those in the mature cell and how coordination in the

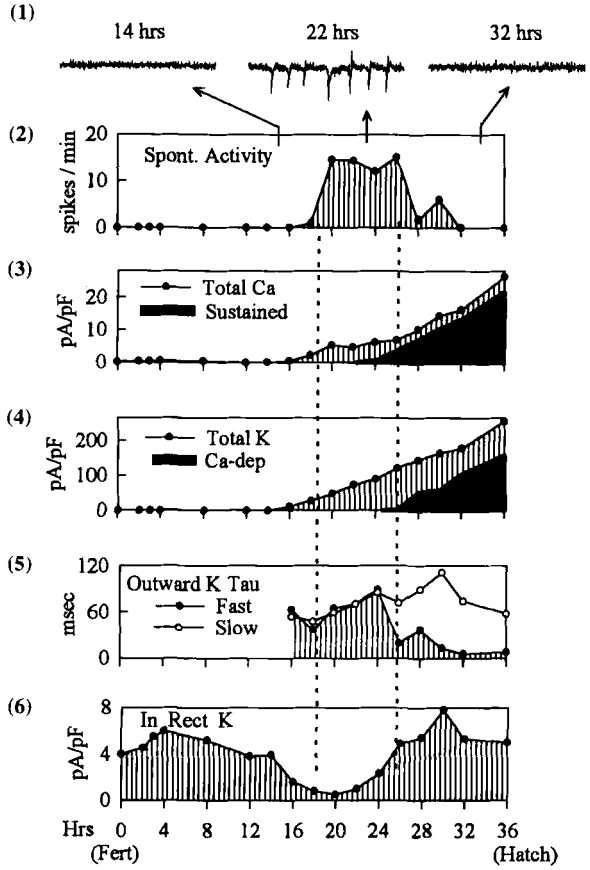

Fig. 1 Summary of the development of voltage-gated ionic currents and spontaneous action potentials in ascidian muscle. Development of the ascidian larva occupies 36 hr from fertilization to hatching of the tadpole. Each data point represents the mean value derived from between 4 and 36 cells at that stage. Staging was done by visible morphological features and then converted to standard times based on development at 12°C. Several developmental events for reference are the following: fertilization, 0 hr; gastrulation, 12 hr; neurulation, 16 hr; extension of the tail begins at about 18 hr; spontaneous movements and innervation of muscles occur at about 26–28 hr; hatching of the tadpole from the chorion, 36 hr. From top to bottom: (1) Cell-attached patch recordings of spontaneous action potentials at the three times indicated. (2) Mean spontaneous action potential frequency derived from such recordings. (3) Total calcium current density (filled circles) and its two components: high-threshold transient (vertical hatching) and high-threshold sustained (black). (4) Total outward K^+ current density (filled circles) and its two components: high-threshold, slowly activating, voltage-gated (vertical hatching) and low-threshold, rapidly activating, Ca^{2+}-activated (black). (5) Activation time constants for the total outward K^+ current. The fast time constant (filled circles) speeds markedly with the appearance of the Ca^{2+}-activated K^+ current; the slow time constant is retained throughout development because the voltage-gated, delayed K^+ current is retained after the Ca^{2+}-activated K^+ current appears. (6) Inwardly rectifying K^+ current density, showing its transient decline between 16 and 24 hr. The vertical dashed lines mark the period of spontaneous activity, showing how closely coordinated it is with the decline in the inward rectifier and the period during which the "embryonic" forms of the Ca^{2+} and outward K^+ currents predominate. Adapted from Figure 10 in Greaves *et al.* (1996).

development of different channels can direct early critical periods of spontaneous activity (see Fig. 1).

As the embryo undergoes neurulation, three synchronous events occur that define the "immature" electrophysiological state. The inwardly rectifying K^+ current, present since fertilization as the major (and perhaps only) resting conductance, virtually disappears. A high-threshold, inactivating Ca^{2+} current and a slowly activating delayed K^+ current appear, defining an action potential that is 10 times longer in duration than its mature counterpart. I_{Ca} and I_K densities rapidly increase to their final values, and then throughout the rest of development these currents do not change further in density or character. This immature state lasts approximately 6 hr, and during this period the cells generate spontaneous action potentials at a mean frequency of about 15/min. [Note that even this relatively low frequency is more than 100 times higher than that in *Xenopus* spinal neurons (Gu and Spitzer, 1995).]

This period of activity is terminated as the cells make the transition to the mature populations of ion channels. This transition, like the preceding one, is characterized by three simultaneous events. The inward rectifier reappears, rapidly attaining its previous density. A new Ca^{2+} current, high threshold but sustained, appears. And a new outward K^+ current, low-threshold, rapidly activating, and Ca^{2+}-dependent, also appears. These events terminate spontaneous activity and shorten the duration of the action potential by 10-fold.

When the spontaneous action potentials during the "immature" critical period of activity are blocked, the mature cells show two discrete deficits: The Ca^{2+}-activated K^+ current is absent, although all other currents are normal, and excitation contraction coupling is disrupted at some point downstream of Ca^{2+} entry (Dallman *et al.*, 1994).

Several important principles have emerged from this work

1. Tight coordination between the development of channels that determine action potential waveform and those that set the resting potential can drive spontaneous activity. There are two aspects to controlling spontaneous activity during critical developmental periods: Action potential waveform and ionic dependence must be appropriate to activate activity-dependent developmental programs, and something must trigger the action potentials in the appropriate patterns. Hence, coordination between resting and active channels is likely to be very important during development. The large sample size and detailed time line (550 cells over 19 stages) in these experiments were critical in revealing the absence of the inward rectifier at neurulation as a transient event, coordinated with the appearance of Ca^{2+} currents, and thus identifying its potential importance in controlling spontaneous activity.

2. The development of voltage-gated channels is itself activity-dependent, and this can lead to self-limiting spontaneous activity. Expression of the Ca^{2+}-activated K^+ current, the event that causes the increased density and activation rate of the outward K^+ current, is itself activity-dependent. This is similar to the situation in *Xenopus* neurons, where activity is required to speed outward K^+ current activation, even though in this case it occurs by speeding the voltage-gated K^+ current (O'Dowd *et al.*, 1988; Harris *et al.*, 1988). Outward K^+ current expression is also activity-dependent in *Xenopus* muscle (Linsdell and Moody, 1995). It may be a general phenomenon that spontaneous activity early in development is self-terminating, because the mature channels whose expression is activity-dependent often act to lessen activity. So the mature pattern of ion channel expression, which both terminates spontaneous activity and sets the adult signaling properties of the cell, is partly dependent on the correct expression of immature channel types.

3. The detailed biophysical properties of embryonic channels may be finely tuned to serve the developmental functions of activity. For example, the primary function of the speeding and density increase of the outward K^+ currents during development is usually thought to be to shorten the action potential, but examination of the composite currents under voltage clamp suggests another role. The duration of the net inward current, that is, the time from the onset of a stimulus to the point at which the K^+ current sufficiently activates to reverse the net inward current to outward, becomes progressively shorter with development as the outward K^+ currents become larger and faster [see Greaves *et al.* (1996), Fig. 7]). With long net inward currents, a cell can be stimulated by slowly depolarizing stimuli, whereas with a brief net inward current that cell will accommodate and not generate an action potential. So whatever is driving low-frequency spontaneous activity is likely to become progressively less effective as a stimulus as the outward K^+ current grows and speeds. A second point concerns embryonic Ca^{2+} currents. It is not immediately obvious why early in ascidian muscle development, when the action potential is long, the Ca^{2+} current is an inactivating type but later, when the spike is brief, the Ca^{2+} current is sustained. There are several interesting hypotheses to explain this. The inactivating Ca^{2+} channels could actually mediate greater Ca^{2+} entry than the sustained ones, if they obligatorily reopen on spike termination in making the transition from the inactivated to the closed states (Slesinger and Lansman, 1991). Inactivating channels could limit spike frequency in early development by taking a long time to recover from inactivation. But it is also possible that Ca^{2+} entry is not the effective event that transduces spontaneous activity into its developmental sequalae. The immature form of the Ca^{2+} channel might be directly coupled to internal Ca^{2+} release mechanisms, much like the voltage-sensing dihydropyridine (DHP) receptor of vertebrate skeletal muscle (Chavis *et al.*, 1996), and the current waveform might simply reflect the kinetic properties of the Ca^{2+}

channel type that carries out this coupling function. It can be difficult experimentally to distinguish between a small amount of Ca^{2+} entry that triggers subsequent internal Ca^{2+} release from a mechanism based solely on directly triggered Ca^{2+} release [see, for example, Holliday *et al.*, (1991)].

C. *Xenopus* Embryonic Skeletal Muscle

Muscle cells dissociated from the somites of neural plate stage *Xenopus* embryos begin to differentiate morphologically within about 30 min, roughly 6 hr earlier than neurons. Recordings from muscle at this time reveal currents that are small or absent, indicating that muscle develops physiologically later in relation to overt morphological differentiation than do spinal neurons in the same animal. Both inwardly rectifying and delayed K^+ currents appear within 4 hr, followed by Na^+ and Ca^{2+} currents at 6–8 hr (Spruce and Moody, 1992; Linsdell and Moody, 1995). This sequence is different from that in *Xenopus* neurons and ascidian muscle, where Ca^{2+} and/or Na^+ current predominates at early stages. Both K^+ currents in *Xenopus* muscle develop in two phases: an initial rise in density, a 6–8-hr plateau, and finally a second rise. During this plateau, Na^+ and Ca^{2+} current densities are increasing rapidly. This creates a situation analogous to that in ascidian muscle, where the ratio of inward to outward and resting currents increases during a window of developmental time, raising the possibility of a period of spontaneous activity (Greaves *et al.*, 1996). The application of blockers of activity during this period prevents the second rise in both K^+ current densities (Linsdell and Moody, 1995), much the way that activity block in ascidian muscle prevents the appearance of the Ca^{2+}-activated K^+ current (Dallman *et al.*, 1994) or in *Xenopus* neurons prevents the speeding of the delayed K^+ current (Desarmenien and Spitzer, 1991).

The early phases of development of both K^+ currents were unaffected by blockers of activity, an unsurprising result given that the Na^+ and Ca^{2+} currents that might mediate activity are not yet present at those times. Does this mean that, in mechanistic terms, the early phases of K^+ current development do not respond to activity? It does not. Ectopic electrical activity in these cells at early stages created by misexpressing a mammalian Na^+ channel moves the initial appearance of both K^+ currents to earlier stages of development (Linsdell and Moody, 1994). [In *Xenopus* neurons, increasing the frequency of activity does not push developmental events beyond their normal levels, but in this case activity was increased during the normal period of its occurrence rather than being created at abnormal times (Gu and Spitzer, 1995).]

Some important principles that have emerged from this work

1. Creating ectopic electrical activity during development can reveal activity dependence that blocker studies miss. Activity dependence of the early

phases of K^+ current development was shown only by misexpression experiments, which created activity at developmental stages when it would not normally occur, not by applying blockers of activity, suggesting that many aspects of development are sensitive to activity over a much longer time than actual activity exists. This situation is analogous to competence for induction, where a tissue is likely to be receptive to induction over a much longer period of time than it is actually exposed to the inducer (Okado and Takahashi, 1990). Broad periods of competence may represent a safety factor in development to compensate for variations in the timing of events such as spontaneous activity, or they may reflect conservation in the basic signal transduction pathways that can alter developmental processes. A potentially important effect of this broad sensitivity to activity is that pathologies that create ectopic activity can be very disruptive to the nervous system, even at stages where blocker studies do not indicate the presence of activity-dependent developmental events.

2. Ion channel development in vertebrate muscle is very complex, even in the absence of innervation. Many events in the normal development of vertebrate skeletal muscle are driven by electrical activity that results from innervation. These include expression of the mature form, spatial distribution of the acetylcholine (ACh) receptor, and expression of the resting Cl^- conductance (e.g., Heathcote, 1989; Dutton *et al.*, 1993). Our results show that muscle is not simply the passive recipient of nerve-induced activity. Rather, innervation imposes its influence on an already complex endogenous pattern of cell-autonomous ion channel development, which includes periods of spontaneous activity that precede nerve contact. It will be very interesting to examine the details of the physiological effects of innervation in light of this information. In some ways, this is analogous to the situation in the mammalian visual system, discussed in the following section, in which experience-driven activity participates in development, but is superimposed on a complex series of non-experience-dependent developmental events, some of which are themselves driven by spontaneous activity.

D. Mammalian Visual System

The visual system of higher mammals is perhaps the classic example of a complex set of neural circuits that require experience-driven activity to develop properly. Much of the functional architecture of the visual cortex is established without sensory experience, but requires visual input to be maintained after birth [see Shatz (1990) for a review]. It has become clear that spontaneous activity plays an important role in the establishment of visual circuitry at earlier stages.

Connections between retinal ganglion cells and the lateral geniculate nucleus (LGN) form a complex topographic map in which input from the two eyes is segregated into distinct layers in the LGN, and, within one eye-specific layer,

retinal input forms a coherent spatial map of the retina. Eye-specific layering and, possibly, spatial mapping of input to the LGN require spontaneous action potentials in the retinal ganglion cells, action potentials generated before visual input is possible. The ganglion cells initially grow into the LGN and project to all layers. Later, they prune connections to inappropriate layers and elaborate many new connections in the appropriate layers. The pruning and elaboration are eliminated when spontaneous activity in the ganglion cells (Maffei and Galli-Resta, 1990) is blocked with tetrodotoxin (Shatz and Stryker, 1988). Direct measurement with extracellular electrode arrays confirms the existence of spontaneous activity in the retina and shows that its characteristics are consistent with its function in identifying ganglion cells to their LGN targets, both by eye and by spatial location within an eye (Meister *et al.*, 1990). Bursts of action potentials form waves of activity that cross the retina, and waves are separated by relatively long periods of silence. Because the periods of activity are brief compared to the intervals between them, it is unlikely that the two eyes would be synchronously active, and thus all ganglion cell terminals that are active within a given interval could be assumed by the LGN cells to come from the same eye. Because the activity spreads across the retina, terminals that are synchronously active within a short interval could be assumed by the LGN to be adjacent in the retina, thus providing cues to establish a spatial map on the LGN. [During the waves of activity, on and off cells of the retina show different patterns of firing, which may be involved in their segregation into sublaminae in the LGN (Wong and Oakley, 1996).]

Dependence on spontaneous activity is not confined to the LGN connections. The initial branching of LGN axons when they grow into layer four of the visual cortex also requires activity that does not depend on visual input (Herrmann and Shatz, 1995). Although these data have been cited as an exception to the rule that the initial processes of axon pathfinding and targeting occur independently of activity (Goodman and Shatz, 1993), there was never much evidence for this generalization in the first place, given substantial data for the role of activity in neuron responsiveness to neurotrophic factors (Schmidt *et al.*, 1996; McAllister *et al.*, 1996), in dendritic elaboration (Gu and Spitzer, 1995), and in neuronal migration (Komuro and Rakic, 1992).

What causes the spontaneous activity in the retinal ganglion cells? Voltage-clamp studies indicate that three changes in Na^+ current properties occur in the cells during development, all of which would tend to increase excitability: density increases, the steady-state activation curve shifts to more negative potentials, and the steady-state inactivation curve shifts to more positive potentials (Skaliora *et al.*, 1993). All of these changes, however, are essentially complete before the period of spontaneous activity begins, indicating that although they may be permissive for activity they probably are not the immediate cause. The delayed K^+ current inactivates progressively more slowly with development, a change that spans the period of spontaneous activity, and could be a limiting factor in the development of repetitive firing ability (Skaliora *et al.*, 1995). Some caution

should be exercised in the interpretation of this timing, however, because it is possible that effects of physical dissociation [see, for example, Spruce and Moody (1992)] or removal of the cells from a retinal environment containing diffusible factors controlling channel properties [see, for example, Kofuji *et al.* (1996)] could affect the apparent developmental time course seen.

Several Important Principles Have Emerged from This Work

1. Neurons can identify themselves to their targets using activity. In this system, we see how, once the gross connectivity between retina and geniculate is established, activity can be used to communicate information about the identity of the neuron to its potential targets. By generating waves of action potential activity that propagate across the retina in a time much shorter than the intervals between activity, a retinal ganglion cell can identify itself to the geniculate neurons as being from the same eye or a different eye from another ganglion cell and as being located adjacent to or distant from another ganglion cell on that retina. Clearly, some kind of competition is involved in layering the geniculate, and it remains unclear how in one layer terminals from the ipsilateral retina "win," whereas in another layer terminals from the same two ganglion cells might compete with the opposite outcome. A mechanism based on the sequential formation of geniculate layers, in which pruning of terminals from a ganglion cell in one layer up-regulates its activity so that is has the competitive advantage in the next layer, could, in principle, provide the answer.

2. Systems that use experience-driven, activity-dependent development can also use non-experience-driven, activity-dependent development. The visual system as a whole, and possibly individual connections within it (thalamocortical connection to establish ocular dominance columns), uses both types of activity-dependent developmental mechanisms. Although "spontaneous" activity in this system clearly is not driven by the normal sensory route, its origin is not entirely clear. Cholinergic synaptic transmission from amacrine cells is necessary to propagate the waves (Feller *et al.*, 1996), but the initial excitation may be intrinsic to individual ganglion cells (or amacrine cells).

D. Weaver Mouse Mutation

In the mouse neurological mutant *weaver*, the granule cell precursors of the external germinal layer of the cerebellum proliferate normally, but subsequently fail to migrate and differentiate. The *weaver* mutation results in a single amino acid substitution in the pore region of the G protein-gated inward rectifier, GIRK2 (K_{IR} = 3.2). This mutation renders the channel constitutively active (rather than requiring $G_{\beta\gamma}$), nonselectively permeable to cations, including Ca^{2+}

(rather than K^+ selective), and sensitive to block by certain cation channel blockers (Slesinger *et al.*, 1996; Kofuji *et al.*, 1996; Liao *et al.*, 1996). The *wv*GIRK2 channel also shows activation by intracellular Na^+, making a component of its activation regenerative (Silverman *et al.*, 1996). The defect in cultured *wv* granule neurons can be rescued by cation channel blockers that block the mutated GIRK2 or by transplant of the neurons into wild-type cerebellum (Kofuji *et al.*, 1996; Gao *et al.*, 1992).

How does the defective *wv*GIRK2 cause the defects in granule cell development? The *in vitro* rescue of the *weaver* defect by blockers of *wv*GIRK2, which do not restore normal GIRK2 channel function, suggests that it is the defective channel and not the absence of the normal channel that causes cell death (Kofuji *et al.*, 1996). This most likely results from the constitutively active inward Ca^{2+} and Na^+ fluxes through *wv*GIRK2. The rescue of the defect in the presence of wild-type granule neurons suggests that some local factor in the external germinal layer of normal cerebellum suppresses *wv*GIRK2 function and that normal GIRK2 function is required for release or production of this factor. These data suggest the possibility that the role of this inward rectifier in granule cell development is analogous to the role of the voltage-gated inward rectifier in ascidian muscle development detailed earlier. Perhaps it is not the presence of normal GIRK2 that is required for the differentiation of granule cells but its transient absence that, as in ascidian muscle, might control a period of activity-dependent development. That absence might be controlled by local factors in the external germinal layer, and this critical period of activity is disrupted by the presence of *wv*GIRK2 and restored by blocking *wv*GIRK2.

Why does the mutation have such different effects on neurons in different regions of the brain? The motor defects in the *weaver* mutation result from disruptions of neuronal development in both the cerebellum and substantia nigra, but the modes of cell death caused by the *weaver* mutation in these two areas are very different [see Slesinger *et al.* (1996) for a review]. These pleiotropic effects may result from the different effects of *wv*GIRK2 in homo- vs heteromultimeric GIRK channels. At high levels of expression, *wv*GIRK2 channels show the defects summarized earlier, which lead to a constitutive influx of Na^+ and Ca^{2+} ions. But low levels of *wv*GIRK2 expression, at least in the *Xenopus* oocyte expression system, can suppress wild-type GIRK1 function (Slesinger *et al.*, 1996). This indicates that the effect of the *weaver* mutation on a given cell depends not only on the levels and timing of GIRK2 expression in that cell but also on the expression of other normal channels in the GIRK family.

The *weaver* mutation exerts its effects not during target selection or later refinement of connections and not during axon outgrowth and pathfinding, but during the early phases of neuronal migration. Of course, the *weaver* data themselves do not indicate a role for activity at these stages, because the phenotype results from aberrant ion conduction through the mutated channel and not from the loss of normal channel activity. Other data do, however, indicate a role for

activity driven by specific Ca^{2+} channel types in granule cell migration in the cerebellum (Komuro and Rakic, 1992), and the finding that mammalian central neurons express a variety of voltage- and transmitter-gated ion channels even before they exit the cell cycle (Mienville *et al.*, 1994; LoTurco *et al.*, 1990) is suggestive of a general role for activity very early in mammalian neurogenesis.

IV. Conclusion

Ion channel development is a complex phenomenon designed to create two distinct types of electrical activity in cells. At early stages, the types and properties of the channels present in the cell drive periods of spontaneous activity that play a critical role in development. At later stages, channels mediate the mature signaling properties of the cell. Because later ion channel development itself is among the developmental processes that are dependent on preceding periods of activity, the correct early phases of channel development are required to produce the correct later phases, even though the actual channels at the two times may be very different. The developmental roles of activity include processes occurring very early in neurogenesis, at least before neurons have reached their final destinations in the nervous system and probably even earlier, at the time before final cell division. The fact that voltage-gated ion channels are present in embryos even before fertilization suggests that activity-independent and -dependent processes interact throughout development. This contrasts with the often proposed model in which activity-dependent events in neural development follow and refine the results of activity-independent events.

Because embryonic channel properties are finely tuned to serve the developmental functions of activity, they are likely to be incompatible with the mature signaling properties of the same cell. That this is the case is clear in at least two of the systems discussed earlier, ascidian muscle and mammalian retinal ganglion cells: the patterns of spontaneous action potentials that are essential early in development would be disruptive to the function of the mature cell. In the retina, synchronous bursts of activity that cross the retina every few minutes independent of visual input would send aberrant visual information to the cortex. In ascidian muscle, spontaneous action potentials in bursts of several Hertz independent of synaptic input would, in the mature larva, result in ectopic muscle contraction and disrupt the pattern of swimming necessary for larva dispersal. Similarly, the long duration of the embryonic action potential itself, even if it did not occur spontaneously, clearly would be incompatible with the rates of evoked firing necessary in the mature cell. In *Xenopus* spinal neurons, for example, the action potential just after neurulation has a duration of about 35 ms, compared to 1.5 ms 24 hr later. *In vivo* in the tadpole, spinal neurons fire at frequencies of 50–100 Hz and occasionally higher (Clarke *et al.*, 1984). Although synaptic input can shorten spike duration, it is very unlikely that immature spike durations could

support the normal patterns of swimming on the motor side or the normal sensory activity of mature Rohon–Beard cells. Indeed, modeling studies indicate that a reduction in outward current amplitude toward immature values precludes sustained rhythmic activity and disrupts the swimming pattern output in *Xenopus* (Dale, 1995). For these reasons, cells requiring a period of spontaneous activity during development must terminate that activity during maturation, and the action potential waveform and ionic dependence must be brought to their mature states for successful development of the cell to proceed. The termination of spontaneous activity at characteristic times of development is seen clearly in ascidian muscle, *Xenopus* spinal neurons, and mammalian retina (Greaves *et al.*, 1996; Gu and Spitzer, 1995; Meister *et al.*, 1990). The mechanism by which this is achieved is partly understood in ascidian muscle, where both the reappearance of the inwardly rectifying K^+ current and the speeding of the outward currents caused by the appearance of a Ca^{2+}-activated K^+ current participated in terminating activity (Greaves *et al.*, 1996).

The fine-tuning of channel biophysical properties in embryonic cells serves both to control the pattern of spontaneous activity and to optimize ion fluxes during activity. The long open time of the embryonic ACh receptor channel in vertebrate skeletal muscle, for example, helps match the duration of current flow to the long time constant of embryonic muscle (Jaramillo *et al.*, 1988). This impedance matching helps drive muscle activity at early stages, which controls both the switch to the mature ACh channel type and distribution and the later appearance of resting Cl^- permeability (Heathcote, 1989), which in turn shortens the membrane time constant. In this case, one can readily see the high degree of optimization of embryonic channel isoforms to their function and the self-limiting nature of many aspects of embryonic channel expression.

One feature of early channel development in many cells is the prominence of low-threshold transient (T type) Ca^{2+} currents (Gonoi and Hasegawa, 1988; Kubo, 1989). These could serve to lower spike threshold and promote activity (Gu and Spitzer, 1993). They could also mediate a large fraction of Ca^{2+} entry during slow depolarizations, because the overlap of the inactivation and activation vs voltage curves creates a steady "window current" at voltages near the resting potential (Barish, 1991), or during action potentials, because they are open when the driving force for Ca^{2+} entry is large (McCobb and Beam, 1991). The properties of the high-threshold Ca^{2+} currents found early in development are also likely to be optimized to allow spontaneous activity to result in the appropriate Ca^{2+} entry. For example, high-threshold inactivating Ca^{2+} currents, such as those found early in ascidian muscle development (Greaves *et al.*, 1996), could, in principle, maximize Ca^{2+} entry during a single spike while limiting the frequency of the activity. If the channels obligatorily reopened in making the transition from open to closed states at spike termination (Slesinger and Lansman, 1991), a burst of Ca^{2+} entry upon spike repolarization could occur that is greater than that achieved by a sustained Ca^{2+} current during the action poten-

tial. At the same time, the subsequent recovery from inactivation could result in a long postspike refractory period, thus limiting firing frequency. Finally, the significance of this embryonic Ca^{2+} current could be in its direct coupling to intracellular Ca^{2+} stores (Chavis *et al.*, 1996) rather than its current waveform per se, so that activity is simply creating voltage excursions that activate the Ca^{2+} channel voltage sensor and the Ca^{2+} entry itself is not critical.

Clearly, a great deal of information is needed about the signal transduction pathways coupling activity to developmental events in a given cell before the exact significance of embryonic channel expression can be appreciated. For example, *Xenopus* spinal neurons require a certain frequency of activity during a critical period to develop properly (discussed earlier). But although Ca^{2+} entry during the action potentials is required, most of the increase in intracellular Ca^{2+} with activity results from a release from intracellular stores (Holiday *et al.*, 1991). Therefore, the Ca^{2+} current during the action potential can be reduced substantially without affecting the resulting intracellular transient, and the exact spike waveform can hardly be very critical in these cells given that activity-blocked cells can be rescued by repetitive pulses of Ca^{2+}-containing high-K^+ solution (Gu and Spitzer, 1995). Spike duration, however, must be above a minimum level, because premature truncation of the action potential with a misexpressed K^+ channel can disrupt development (Jones and Ribera, 1994). Thus, we need to know the "safety factor" in the coupling of activity and development in each cell in order to interpret the significance of patterns of channel development.

The development of almost every class of ion channel has been found to be activity-dependent. These include muscle ACh and NMDA receptors (Carmignoto and Vicini, 1992; Goldman *et al.*, 1988), muscle Cl^- channels (Heathcote, 1989), and Na^+, delayed K^+, and Ca^{2+}-activated K^+ channels in several cell types (Linsdell and Moody, 1995; Greaves *et al.*, 1996; Dallman *et al.*, 1994; Mori *et al.*, 1993). In many cases, these changes represent a form of self-regulation of spontaneous activity. For example, activity-dependent up-regulation of outward K^+ current density or speeding of outward K^+ current kinetics serves to terminate developmentally critical periods of spontaneous activity (discussed earlier). This probably reflects a specific case of the general phenomenon that at least some part of the suite of ion channel properties that underlie the mature physiological functioning of nerve and muscle cells is activity-dependent in its development. Activity dependence of mature channel development makes a great deal of sense, especially for neurons in complex circuits. Subtle changes in the patterns of activity in a mature neuron can be very disruptive to its functioning in a complex circuit, and quite small changes in the properties of the ion channels it expresses can have profound effects on those patterns of activity. Therefore, the most secure method of ensuring appropriate channel development is to put activity itself in the feedback loop that controls the development of activity. This can lead to cases in which the function of one

channel is dependent, both chronically and acutely, on other channels. In ascidian muscle, for example, developmental expression of the Ca^{2+}-activated K^+ current requires activity driven by the embryonic form of the Ca^{2+} current, whereas in the mature cell the same Ca^{2+}-activated K^+ current requires Ca^{2+} entry through the mature form of the Ca^{2+} channel for its activation.

However, it is equally clear that all channel development is not activity-dependent. Even in a single cell type, closely related aspects of channel development differ greatly in their activity dependence. For example, in *Xenopus* spinal neurons the developmental increase in delayed K^+ current density is not activity-dependent, whereas the increase in activation rate is (Desarmenien and Spitzer, 1991; see Spitzer, 1991). Similarly, in ascidian muscle the developmental appearance of Ca^{2+}-activated K^+ currents requires preceding spontaneous activity, whereas the appearance of the mature form of the Ca^{2+} current, which is responsible for activating these K^+ currents, does not (Greaves *et al.*, 1996; Dallman *et al.*, 1994). In *Xenopus* embryonic muscle, early phases of delayed K^+ current development do not require activity (although they can be influenced by artificially imposed activity) whereas later phases do (Linsdell and Moody, 1995). Of course, the development of channels that are responsible for the spontaneous activity itself is unlikely to be activity-dependent. Furthermore, if a subset of later channel development is activity-independent, the cell may be able to better control situations in which the mature density of a channel serves more than one critical function. For example, the correct regional distribution of Ca^{2+} channel density in a neuron serves both to control Ca^{2+} entry and to set the ratio of inward to outward currents that determines the patterns of activity. The insertion of Ca^{2+} channels during development could be determined independently of activity to set the appropriate regionalized Ca^{2+} entry, and the development of outward K^+ currents could be targeted, by being activity-dependent, to make that absolute Ca^{2+} channel density compatible with a certain level of excitability in the mature cell.

The dependence of ion channel development on preceding ion channel function has important implications in the interpretation of transgenic channel knockout experiments. Elimination of the function of a single channel type throughout development is likely to disrupt the development of other channels, so that the physiological effects of the knockout at all stages after that may be due to disruptions in channels that were not directly targeted in the experimental design. This problem can be circumvented to some extent by confining the knockout to particular stages and cell types by, for example, linking a dominant negative K^+ channel subunit to a tissue- and stage-specific promoter.

Activity-dependent developmental events can occur in single, isolated cells in culture or in cultured populations at densities that make paracrine effects highly unlikely [see, for example, Henderson and Spitzer (1986); Greaves *et al.*, 1996]. Although such experiments do not rule out autocrine effects, it seems most likely that such activity-dependent events are triggered by Ca^{2+} entry into single cells

followed by Ca^{2+}-activated second messenger cascades within those same cells [see, for example, Mendelzon *et al.* (1994); Klarsfeld *et al.*, 1989; Desarmenien and Spitzer, 1991; Mori *et al.*, 1993]. Because Ca^{2+} is such a promiscuous player in second messenger systems, it has been difficult to sort out the downstream effects of activity in any satisfactorily complete way. Even knowledge of the regulatory sequences conferring activity dependence of a given gene (e.g., Dutton *et al.*, 1993) may leave some ambiguity, given that such single elements can be activated by more than one second messenger (Sheng *et al.*, 1991; Dash *et al.*, 1991).

Activity-dependent developmental events can also require paracrine signaling between cells, often cells of different types (Barres and Raff, 1993; Kaiser and Lipton, 1990). This type of signaling often involves the secretion of neurotrophic or other survival factors that promote the growth and survival of adjacent cells. The fact that neurotrophin action, production, and secretion are all activity-dependent (McAllister *et al.*, 1996; Lindholm *et al.*, 1994; Blochl and Thoenen, 1996; Schmidt *et al.*, 1996) has the potential to create complex feedback loops in neural development, particularly ones involving activity-dependent competition for synaptic strength or survival.

Perhaps the more important idea, however, is the general one that activity-dependent developmental events and activity-dependent plasticity in the mature nervous system are likely to use the same basic mechanisms [see, for example, Kandel and O'Dell (1992)]. Thus, in some sense, development and learning in the nervous system are two ends of a continuum of activity-dependent processes, with development dependent on spontaneous activity at one end, activity-dependent plasticity in the mature nervous system at the other end, and development dependent on experience-driven activity in the middle.

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6

Molecular Regulation of Neuronal Apoptosis

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Apoptosis is a fundamental biological process used by all muticellular organisms to eliminate unwanted or superfluous cells, and is a prominent feature of normal neural development. Developmentally occurring neuronal apoptosis serves to match the number of neurons to the requirements of their synaptic targets and to rid the nervous system of inappropriate connections. While it is generally accepted that apoptosis is a "suicide program" inherent in all cells, the molecular basis of this program is just beginning to be unraveled. Evidence from numerous recent studies indicate that a variety of proteins are involved in the transmission of external signals to the cell-death machinery within the cell. This review describes many of the recent findings of the regulatory pathways and genes that have been implicated in the induction or suppression of apoptosis in neurons. Copyright © 1998 by Academic Press.

I. Introduction

The development of the vertebrate nervous system is accompanied by a massive loss of neurons from virtually every area [for a review, see Oppenheim (1991)]. Although culminating in the death of about one-half of all neurons that are produced, the process occurs in a highly systematic manner that is spatially and

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temporally reproducible. Developmentally regulated neuronal death therefore has been considered a prime example of programmed cell death (PCD), a term originally coined to describe the spatially and temporally predictable cell loss that occurs during normal vertebrate ontogeny (Glucksmann, 1951; Kerr *et al.*, 1972). Research in the past decade has firmly established that PCD does not occur solely during development but is a fundamental biological process that occurs at all stages of animal life, serving to eliminate unwanted, superfluous, infected, and potentially harmful cells [for reviews, see Steller (1995) and Jacobson *et al.* (1997)]. The physiological relevance of PCD is underscored by its occurrence in virtually all higher eukaryotes, including plants, slime molds, nematodes, insects, and vertebrates (Jacobson *et al.*, 1997). In the developing nervous system, PCD primarily serves to match neuronal number with target size and to rid the nervous system of neurons making improper or no connections (Oppenheim, 1991). Competition for limiting amounts of trophic factor secreted by neuronal targets determines which and how many neurons ultimately survive. Besides trophic factors, synaptic transmission and electrical activity are also important regulators of survival in some neuronal populations during development (Oppenheim, 1991). In addition to these target-dependent stimuli, target-independent regulation of neuronal survival has also been described (Yaginuma *et al.*, 1996).

One of the best-characterized forms of PCD is apoptosis (Kerr *et al.*, 1972). Cells undergoing apoptosis display characteristic morphological features. These include membrane blebbing and nuclear and cytoplasmic condensation, followed by fragmentation of the cell into membrane-bound apoptotic bodies that are rapidly phagocytosed by macrophages or neighboring cells without leakage of cellular contents. Fragmentation of genomic DNA into oligonucleosomal fragments as a result of nuclease activation is almost always observed in apoptosis and widely accepted as a biochemical hallmark of apoptotic death. In contrast to apoptosis, necrosis, which is often a result of toxicity or trauma, is typified by cell swelling and lysis, random degradation of macromolecules, and spillage of cytoplasmic contents, which triggers an inflammatory response.

Whereas apoptosis clearly is of great benefit when undertaken in a controlled fashion, failure to regulate this death process is believed to be involved in a variety of diseases [for a review, see Thompson (1995)]. In the nervous system, increased or ectopic apoptosis has been implicated in several neurodegenerative diseases, including Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis, ataxia telangiectasia, and retinitis pigmentosa [Cotman and Anderson, 1995; Thomas, 1995; Hartley, 1994; Portera-Cailliau, 1995; for reviews, see Bredesen (1995) and Thompson (1995)]. Although the loss of neurons that immediately follows stroke or ischemia is largely necrotic, the ensuring slower and more widespread degeneration that is responsible for much of the damage in these conditions has apoptotic characteristics (Y. Li *et al.*, 1995; Linnik *et al.*, 1993). Outside the nervous system, increased apoptosis has been shown to occur in T-cells of patients with acquired immunodeficiency

syndrome (AIDS), as well as in some hematologic diseases. On the other hand, inhibition of normal apoptosis contributes to cancer, some autoimmune disorders, and persistence of certain viral infections (Thompson, 1995).

Although likely to represent the major form of PCD in the nervous system, apoptosis may not be the only mode by which neurons die. Other forms of neuronal death with different morphological features have also been described (Chu-Wang and Oppenheim, 1978; Clarke, 1990; Pilar and Landmesser, 1976). The cellular and biochemical changes associated with these morphologically variant forms of degeneration have yet to be characterized. It is possible that the molecular mechanisms regulating these nonapoptotic forms of neuronal PCD are distinct from those operating during apoptosis.

The objective of this chapter is not to provide a comprehensive description of cell death in the nervous system, but rather to summarize discoveries on the molecular and biochemical mechanisms regulating neuronal apoptosis with a focus on the components of the cell death machinery. Initially, a brief description of cell culture paradigms commonly used to study apoptosis in neurons will be presented. Next, genes that have been implicated in the induction or suppression of apoptosis will be described. Finally, cytoplasmic molecules and pathways that regulate neuronal apoptosis will be outlined. As will become apparent to the reader, a unifying mechanism for apoptosis has yet to be established in neurons (or other cell types), although common themes clearly are emerging. Much of the existing information points to a rather complicated picture in which a given cell type may utilize distinct molecules for the same final result: death.

II. *In Vitro* Paradigms of Neuronal Apoptosis

Although apoptosis is widespread and ultimately leads to the death of large numbers of neurons, the study of the mechanisms regulating this process *in vivo* has been hampered by the fact that apoptotic death is not synchronous. This means that at any given moment only a very small proportion of cells is dying in a specific area of the nervous system. Moreover, apoptotic cells are rapidly degraded and cleared, adding to the difficulty of detecting apoptosis *in vivo*. It is for this reason that the true magnitude of cell loss during normal development was not appreciated for many years. Most studies of the biochemical and molecular mechanisms of neuronal death therefore have been performed using *in vitro* systems. Although several different paradigms of neuronal apoptosis have been established over the past few years and yielded important information, three commonly used cell culture systems are described here.

A. Sympathetic Neurons Deprived of Nerve Growth Factor (NGF)

Cultured sympathetic neurons from embryonic rat superior cervical ganglion (like their *in vivo* counterparts) depend on nerve growth factor (NGF) for survival

(Levi-Montalcini and Booker, 1960; Martin *et al.*, 1988). NGF deprivation induces apoptotic death, which can be inhibited by macromolecule synthesis inhibitors as well as other agents including cyclic AMP and elevated potassium (K^+; Rydel, 1988; Martin *et al.*, 1988; Edwards and Tolkovsky, 1994). Metabolic changes such as a drop in glucose uptake, macromolecule synthesis, and mitochondrial function occur within 12 hr of NGF deprivation, and DNA fragmentation as well as apoptosis-associated morphological changes can be seen soon after (Deckwerth and Johnson, 1993). By about 24 hr, neither macromolecule synthesis inhibitors nor other neuroprotective agents such as cyclic AMP analogues or re-added NGF can rescue most neurons from death, suggesting that the neurons are committed to death by this time (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994; Martin *et al.*, 1988). Cell death begins at around this time and is completed by 72 hr of NGF deprivation.

B. Pheochromocytoma PC12 Cell Line Deprived of Serum or NGF

Rat PC12 cells (Greene and Tischler, 1982) are normally maintained in culture medium containing serum. Serum withdrawal leads to apoptosis (Batistatou *et al.*, 1993; Rukenstein *et al.*, 1991), which can be prevented by NGF. In this paradigm, apoptosis cannot be prevented by RNA and protein synthesis inhibitors, suggesting that the components necessary for the induction of death are normally present in this cell line. PC12 cells can be induced to differentiate irreversibly into a sympathetic neuronlike phenotype by prolonged treatment with NGF and therefore have been used frequently as a system to study neuronal cell processes (Greene and Tischler, 1982). NGF deprivation induces apoptosis in differentiated PC12 cells which, in contrast to undifferentiated cells, can be prevented by macromolecule synthesis inhibitors (Mesner *et al.*, 1992; Pittman *et al.*, 1993). Similar to sympathetic neurons, metabolic and energetic changes are significant by about 24 hr after deprivation in PC12 cells (Mills *et al.*, 1995). Cell death also begins around this time.

C. Cerebellar Granule Neurons Deprived of Potassium

Granule neurons from postnatal rat cerebellum cultured in medium containing serum and depolarizing levels of K^+ survive and mature *in vitro* (Gallo *et al.*, 1987). The simultaneous lowering of serum and K^+ levels induces apoptosis (D'Mello *et al.*, 1993). Death begins at about 16 hr after the switch to low-K^+ medium, killing over 50% of the neurons within 24 hr (D'Mello *et al.*, 1993). Neuron death is accompanied by DNA fragmentation and can be inhibited by RNA and protein synthesis inhibitors, insulin-like growth factor (IGF-1), and cyclic AMP (D'Mello *et al.*, 1993). Several different laboratories have examined

the time point of commitment to death, which varies (depending on the study) from 2 to 6 hr after the decrease in K^+ (Galli *et al.*, 1995; Miller and Johnson, 1996; Nardi *et al.*, 1997; Schulz *et al.*, 1996; Yan *et al.*, 1994). The survival effects of depolarizing levels of K^+ may serve as an *in vitro* correlate of the positive effects of electrical activity on neuron survival [for a review, see Harris (1981)]. Cerebellar granule neurons also die following treatment with glutamate or specific glutamate receptor agonists. In contrast to death by K^+ deprivation, the death induced by glutamate is mainly due to necrosis (Dessi *et al.*, 1993; Ignatowicz *et al.*, 1991). Because granule neurons can be induced to die by either apoptosis or necrosis, they serve as an excellent model to study the molecular mechanisms underlying the differences between these two modes of neuronal degeneration.

It is noteworthy that common agents such cyclic AMP and elevated K^+ can support the survival of a number of different neuron types. In contrast, the survival of nonneuronal cells generally cannot be maintained by these agents. Treatment of neurons with elevated K^+ causes membrane depolarization, leading to the influx of calcium (Ca^{2+}) through voltage-gated L-type Ca^{2+} channels (Collins and Lile, 1989; Gallo *et al.*, 1987; Koike *et al.*, 1989; Scott and Fisher, 1970; Wakade and Thoenen, 1984). The increased cytosolic Ca^{2+} can substitute for neurotrophic factors in a variety of central and peripheral neurons *in vitro* [for a review, see Franklin and Johnson (1992)]. One of the events downstream of increased cytosolic Ca^{2+}, at least in some neurons, is the activation of Ca^{2+}/calmodulin-dependent kinase (Hack *et al.*, 1993). In comparison to the pathway activated by elevated K^+, an increase in cytosolic Ca^{2+} is not necessary for the survival effects of either cyclic AMP or growth factors such as NGF or IGF-1 (Franklin and Johnson, 1992; Galli *et al.*, 1995). Although it is not known whether increased cytosolic cyclic AMP is associated with neuronal survival *in vivo*, one biological factor that does increase cyclic AMP and promote survival of cultured neurons is the neuropeptide pituitary adenylate cyclase-activating poypeptide (PACAP) (Cavallaro *et al.*, 1996; Chang and Korolev, 1997; Villalba *et al.*, 1997). PACAP is found in the brain and its receptors are expressed by neurons (Basille *et al.*, 1993; Cavallaro *et al.*, 1996; Masuo *et al.*, 1994; Villalba *et al.*, 1997). Neuron survival by NGF and IGF-1 occurs via activation of their tyrosine kinase receptors [for a review, see Meakin (1992)]. In the case of IGF-1, another protein, viz., the insulin receptor substrate (IRS) protein, is also involved in signal transmission following IGF-1 receptor activation (D'Mello *et al.*, 1997). Although these agents initially activate distinct signaling mechanisms, it is likely that the pathways utilized by elevated K^+, cyclic AMP, and IGF-1/NGF ultimately converge to affect common molecular targets. Supporting this notion is the finding that rescue by cyclic AMP or KCl occurs with a time-course that is identical to that elicited by IGF-1/NGF (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994; Galli *et al.*, 1995).

III. Genes and Apoptosis

Apoptosis is often referred to as a "suicide" process primarily because of the belief that the dying cell synthesizes molecules necessary to kill itself. This idea has been based almost entirely on the observation that RNA and protein synthesis inhibitors can prevent cell death that occurs *in vivo* during development as well as in most (but not all) cell culture systems (Oppenheim *et al.*, 1990; Schwartz *et al.*, 1990; Tata, 1966). However, that new gene expression is essential for apoptotic death has not yet been conclusively demonstrated, and apoptosis by a transcription-independent mechanism has been described in some paradigms. One line of evidence against the need for new gene expression is that virtually all nucleated mammalian cells can be induced to undergo apoptosis by treatment with high doses of staurosporine, an inhibitor of several protein kinases, even in the presence of the translational inhibitor, cycloheximide (Weil *et al.*, 1996). Although these experiments indicate that the synthesis of new proteins is not required, the production of very small amounts of one or more highly active cell-death-inducing proteins cannot be completely ruled out, even in the presence of cycloheximide. It is noteworthy, that, although capable of inducing apoptosis in all cells and tissues that have been examined, the molecular target(s) of staurosporine within the cell is not know. Unlike staurosporine, which is a broad-spectrum inhibitor, other protein kinase inhibitors are not universal inducers of apoptosis. Much stronger evidence against the need for gene activation comes from experiments showing that apoptotic death can be induced even in mammalian cytoplasts, which lack a nucleus entirely (Jacobson *et al.*, 1994).

A majority of experiments performed on differentiated neurons both *in vivo* as well as in culture have indicated that new gene expression is necessary for apoptosis induced by virtually every death-inducing stimuli. For instance, naturally occurring as well as axotomy-induced neuronal death in the chick embryo can be inhibited by inhibitors of RNA and protein synthesis (Oppenheim, 1991). Apoptosis of NGF-deprived sympathetic neurons is also prevented by macromolecule synthesis inhibitors (Edwards and Tolkovsky, 1994; Martin *et al.*, 1988). As described in the following, expression of at least one of the induced genes (the *c-jun* gene) has been found to be necessary for apoptosis in these neurons. Similarly, in K^+-deprived cerebellar granule cells, as well as in trophic-factor-deprived sensory and central neurons, apoptosis is inhibited by the blockade of macromolecule synthesis (D'Mello *et al.*, 1993; Milligan *et al.*, 1994; Scott and Davies, 1990; Yan *et al.*, 1994). It is quite possible that the molecules synthesized in response to apoptotic stimuli in neurons (and nonneuronal cells) serve only to activate or suppress the cell death machinery, which otherwise exists entirely in the cytoplasm. Once triggered, the progression of events leading to cell death is totally independent of the nucleus. If this is the case, how high concentrations of staurosporine can bypass the need for gene expression will have to be resolved. In cerebellar granule neurons for example, lowering of K^+

induces transcription-dependent cell death, whereas staurosporine treatment can induce apoptosis even if translation is inhibited (Taylor *et al.*, 1997). This result clearly shows that whether apoptosis is transcription-dependent or -independent depends more on the stimulus used than the specific cell type. It is possible that at high concentrations staurosporine treatment can induce modifications, which under physiological or near-physiological conditions is absolutely dependent on the synthesis of new gene products. Therefore, apoptosis induced during development or *in vitro* by trophic factor/K^+ withdrawal may involve a programmed sequence of events that is triggered by new gene expression and leads to alterations such as the inhibition of a survival-promoting kinase or the activation of a pro-apoptotic molecule that is normally inactivated by phosphorylation—actions that staurosporine is capable of mimicking. Regardless of whether staurosporine-mediated apoptosis activates physiological pathways, identification of the target of its death-inducing action could have clinical benefits.

Strong support for the existence of apoptosis-inducing genes under natural conditions also comes from the nematode *Caenorhabditis elegans*, in which a genetic pathway of apoptosis has been established [for a review, see Ellis (1986)]. Similarly, in the fruit fly, *Drosophila melanogaster*, genetic analysis has led to the identification of three death-inducing genes: *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*. The expression of the *reaper* gene in particular is both necessary and sufficient for apoptosis during development in all cell types examined (P. Chen *et al.*, 1996; Grether *et al.*, 1995; Steller, 1995). Besides the expression of pro-apoptotic or "death genes," whose increased expression leads to apoptosis, the survival status of a cell can be profoundly affected by the activity of anti-apoptotic genes or "survival genes," which function to inhibit apoptosis. Decreased expression of this class of genes may increase the vulnerability of cells to death. The stoichiometric balance between pro-apoptotic and anti-apoptotic gene products therefore could determine whether a cell lives or dies. Described in this section are some genes that have been implicated in the regulation of apoptosis in neurons.

A. AP-1 Transcription Factors

In NGF-deprived sympathetic neurons, expression of several genes is induced just before the time these cells become committed to death. Among these are the genes encoding AP-1 transcription factors such as c-fos, fos B, c-jun, and jun B (Estus *et al.*, 1994). Induction of c-fos and c-jun is also seen in cultured cerebellar granule neurons switched to low-K^+ medium, as well as in other neuronal cell types undergoing apoptosis (Anderson *et al.*, 1995; Miller and Johnson, 1996). In addition to *in vitro* systems, induction of these genes and several other immediate-early genes (IEGs) is seen in vulnerable regions of the brain following ischemia (Gubits *et al.*, 1993; Newmann-Haefelin *et al.*, 1994; Wessel *et al.*,

1991). In sympathetic neurons, induction of c-jun is believed to be necessary for apoptosis (Estus *et al.*, 1994; Ham *et al.*, 1995). This conclusion is based on the finding that a blockade of c-jun activity by microinjection of neutralizing antibodies or overexpression of a dominant negative form of this transcription factor protects sympathetic neurons from NGF-deprivation-induced death (Estus *et al.*, 1994; Ham *et al.*, 1995). Whether the increased expression of other transcription factors may also be necessary for neuron death has not been established. Induction of IEGs is, however, not restricted to neurons undergoing apoptosis but also occurs in response to other types of stimuli such as neurotrophic factor treatment (Wu *et al.*, 1989) and membrane depolarization (Bartel *et al.*, 1989; Monnier *et al.*, 1994), which do not cause cell death. Furthermore, c-jun and other IEGs are implicated in a variety of other biological actions, including cell proliferation (Kovary and Bravo, 1991), neuron differentiation (Bartel *et al.*, 1989; Wu *et al.*, 1989), and nerve regeneration (Jenkins *et al.*, 1993), and in learning and memory (Dragunow, 1996). A role for c-jun in neuron survival has also been suggested (Haas *et al.*, 1996). The importance of c-jun is underscored by the fact that mice lacking this transcription factor die prenatally. Surprisingly, an examination of several areas of the developing nervous system as well as other tissues in c-jun null embryos shows no abnormality in the pattern of cell death that usually occurs during early embryogenesis (Johnson *et al.*, 1993; Roffler-Tarlov *et al.*, 1996). Whether cell death occurring at later stages is affected in these mutant mice could not be evaluated because of their short life span. It is possible that c-jun may primarily serve to connect external stimuli to different signaling pathways, one of which is responsible for cell death. Whether c-jun induces apoptosis rather than some other biological effect might depend on the expression of additional interacting proteins within the cell, as well as on other factors inside and outside the cell that regulate its survival.

Several studies have implicated c-fos in the apoptotic death of various neuronal and nonneuronal tissues and in certain pathological conditions (Colotta *et al.*, 1992; Gillardon *et al.*, 1994; Smeyne *et al.*, 1993). In NGF-deprived sympathetic neurons however, only a small fraction of cells undergoing apoptosis display c-fos induction, and microinjection of neutralizing c-fos antibodies does not prevent cell death in this paradigm (Estus *et al.*, 1994; Ham *et al.*, 1995). As observed with c-jun null mutants, mice lacking c-fos display no alterations in the pattern or extent of developmental or injury-induced neuron death (Roffler-Tarlov *et al.*, 1996). Similarly, the normal pattern of death is retained in co-fos-deficient mice in other nonneural tissues that display extensive cell loss, such as the palate or the interdigital zone of the developing limb (Roffler-Tarlov *et al.*, 1996). These findings along with the observation that transgenic mice overexpressing the c-fos or c-jun genes are viable and suffer no obvious increase in cell death during development argues against a simple causal relationship between the expression of either of these genes and the apoptotic pathway.

B. Cell-Cycle-Associated Genes

The *c-myc* oncogene, which is a potent activator of proliferation, can induce apoptosis under conditions of restrained cell proliferation such as serum deprivation (Evan *et al.*, 1992), suggesting that apoptosis could result in a situation in which cell proliferation is initiated but cannot be successfully completed. Cells undergoing apoptosis also display some characteristics of mitotic cells, such as chromatin condensation, breakdown of the nuclear envelope, rounding up of the cell, and some cytoskeletal changes. On the basis of such observations, it has been proposed that in postmitotic cells such as neurons in particular, apoptosis might represent an abortive attempt by cells to traverse the cell cycle (Batistatou *et al.*, 1993; Heintz, 1993; Rubin *et al.*, 1993). Supporting this concept is the demonstration that blockade of DNA synthesis by induction of a dominant inhibitory form of the ras protein protects undifferentiated as well a differentiated PC12 cells from apoptosis (Ferrari and Greene, 1994). Furthermore, trophic-factor-induced apoptosis of PC12 cells, as well as sympathetic neurons, can be inhibited by treatment with pharmacological blockers of the G1/S phase of the cell cycle (Farinelli and Greene, 1996), strengthening the possibility that abortive cell cycle progression underlies apoptosis in neurons. Cyclin D1, which is involved in the progression of the cell cycle, is also induced in NGF-deprived sympathetic neurons (Freeman *et al.*, 1994). However, the inability of antisense cyclin D1 oligonucleotides to induce apoptosis in the presence of NGF or to decrease the susceptibility of sympathetic neurons to NGF deprivation (Greenlund *et al.*, 1995a) raises the possibility that cyclin D1 induction may not be necessary for apoptosis. In cerebellar granule neurons undergoing low-K^+-induced apoptosis, cyclin D1 gene expression is not increased (Miller and Johnson, 1996), implying that even if it contributes to the apoptosis of sympathetic neurons, cyclin D1 is not necessary for the death of some CNS neurons.

Other cell-cycle-associated molecules have also been implicated in the induction of apoptosis. For instance, the expression of p53, which arrests the cell cycle at the G1/S border in response to DNA damage, is increased during apoptosis in some paradigms. Neurons from mice lacking p53 are protected against apoptosis induced by some stimuli, but die as readily as cells from normal mice in response to other types of stimuli (Enokido *et al.*, 1996; Eves *et al.*, 1996; Wood and Youle, 1995). For example, while cerebellar granule neurons from p53-deficient mice are resistant to irradiation-induced apoptosis, the developmental death of these neurons *in vivo* as well as the low-K^+-mediated apoptosis of these neurons in culture is p53-independent (Enokido *et al.*, 1996; Wood and Youle, 1995). Apoptosis by a p53-independent mechanism has also been demonstrated in differentiated neuronal cell lines (Eves *et al.*, 1996). Finally, p53 null mice develop to adulthood without any obvious abnormalities in neurodevelopment (Donehower *et al.*, 1992). These results show that although necessary for apoptosis in

response to certain stimuli, p53 expression is not obligatory for neuronal apoptosis. It has been suggested that p53 may not "induce" apoptosis per se, but rather makes cells more susceptible to cell death especially in response to stimuli that damage DNA or affect cell cycle progression, such as irradiation and anticancer drugs (Miyashita and Reed, 1995). Another gene known to be involved in the control of cell cycle progression is the *retinoblastoma* (*Rb*) gene. Mice lacking *Rb* display abnormal and massive neuron loss during development and die prenatally (Lee *et al.*, 1992). In this case, increased and ectopic mitosis is observed in the brain of mutant mice (Lee *et al.*, 1992), consistent with a link between the cell cycle and neuronal apoptosis.

C. *bcl-2* and Related Genes

The *bcl-2* gene was first discovered as a result of its location at the break point of translocations between chromosomes 14 and 18, which is found in most human follicular B-cell lymphomas [for reviews on this topic, see Gajewski (1996), Reed (1996), and Yang (1996)]. Subsequent studies showed that, in contrast to most other protoncogenes, overexpression of *bcl-2* promotes tumorigenesis by preventing apoptosis rather than increasing cell proliferation. *bcl-2* protects a wide variety of cell types from undergoing apoptosis, in response to stimuli ranging from ionizing radiation and oxidative stress to viral infection and growth factor deprivation. It is now known that *bcl-2* is only one member of a family of structurally related genes, including *bax, bcl-X, mcl-1, Al, bad, bak*, and *bag-1*, all of which participate in the regulation of apoptosis. Interestingly, whereas some members of the *bcl-2* gene family inhibit apoptosis, other members actually promote cell death. In addition to the redundancy in the number of *bcl-2*-related genes, alternative splicing generates additional forms of these proteins, and these isoforms are expressed at different levels in different tissues. In the case of *bcl-X*, the long form, $bcl\text{-}X_L$, inhibits apoptosis, whereas the short form, $bcl\text{-}X_S$, promotes death. Further complicating the picture is the finding that some members of the family can both promote and inhibit apoptosis, depending on the cell type and stimulus used (Cortazzo and Schor, 1996; Kiefer *et al.*, 1995; Middleton *et al.*, 1996).

Although it is known that down-regulation of *bcl-2* gene expression is associated with susceptibility to apoptosis, the molecular mechanisms regulating the expression of *bcl-2* or related genes are not well-understood. One factor believed to repress transcription of the *bcl-2* gene is p53 (Miyashita *et al.*, 1994). Binding sites for p53 are also found in the *bax* gene promoter, although in the case of *bax* p53 is an activator of transcription (Miyashita and Reed, 1995). Lower *bax* expression is also seen in tumors with loss of p53 function (Krajewski *et al.*, 1995a), which is consistent with a role for p53 in maintaining elevated levels of *bax*. In addition to the level of expression posttranslational modification of *bcl-2*

family members is also important. Indeed, phosphorylation of *bcl-2* lowers its effectiveness against apoptosis (Haldar *et al.*, 1995). Deletion of a region of *bcl-2* that contains potential phosphorylation sites transforms *bcl-2* into an active form under conditions where it is normally in an inactive form (Chang *et al.*, 1996). As observed with *bcl-2*, phosphorylation of *bad* leads to blockade of its function (Wang *et al.*, 1996; Zha *et al.*, 1996). In contrast to *bcl-2* however, *bad* is a death-stimulating member of the family. It is known that *bcl-2* proteins can form homodimers or heterodimerize with other family members and that this feature can increase or antagonize the effectiveness of specific *bcl-2* proteins to regulate cell survival (Oltvai and Korsmeyer, 1994; Yang *et al.*, 1995). For instance, dimerization of the death-inducing members *bad* or *bax* with either *bcl-2* or *bcl-*X_L lowers the ability of these latter members to promote survival (Oltvai and Korsmeyer, 1994; Yang *et al.*, 1995). Furthermore, phosphorylation of *bad* or mutations that prevent its heteromerization with *bcl-*X_L permit *bcl-*X_L to exert its survival-promoting effect even in the presence of *bax* (Zha *et al.*, 1996). The widespread distribution of some of the death-inducing members of the family, such as *bak* and *bax*, raises the possibility that cell death activity is controlled primarily by inhibiting the activities of apoptosis-inducing molecules. Supporting this possibility is the finding that susceptibility to death correlates with the level of *bax* in homodimers versus that in heterodimers with *bcl-2* or *bcl-*X_L (Yang *et al.*, 1995). *bad* may exert its death-promoting action not directly as an effector molecule but rather by heterodimerizing with *bcl-2* or *bcl-*X_L, hence sequestering them and preventing their interaction with *bax* (Yang *et al.*, 1995). However, the ability of mutant *bcl-*X_L proteins that are unable to bind *bax* to still inhibit apoptosis (Chang *et al.*, 1996) argues in favor of an active role for *bcl-*X_L in preventing death.

Exactly how *bcl-2* and related family members act to regulate cell death is unclear and the subject of intense investigation. Several modes by which *bcl-2* may influence apoptosis have been suggested, and these include inhibition of antioxidant pathways, regulation of calcium homeostasis, participation in protein transport across membranes, regulating cytochrome c release from mitochondria, modulation of signal transduction pathways via interaction with signaling molecules such as r-Ras and Raf-1 (for review: Reed, 1997). Binding of *bcl-2* with other proteins including the phosphatase calcineurin, the p53 binding protein p53-BP2, the prion protein Pr-1, as well as several proteins of unknown function such as Nip-1, Nip-2, and Nip-3 has also been demonstrated although the significance of these findings has yet to be fully understood. Direct interaction of *bcl-2* with effectors of the cell death machinery such as some caspases has also been demonstrated (for reviews: Reed, 1997; Reed *et al.*, 1996). Recent studies also indicate that *bcl-X, bcl-2*, and *bax* are capable of forming channels in synthetic lipid membranes (Antonsson *et al.*, 1997; Minn *et al.*, 1997; Schendel *et al.*, 1997). It is possible that membrane insertion and pore formation by *bax* might promote apoptosis by permitting the flow of ions or small molecules across

specific intracellular membranes. For example, insertion of *bax* in mitochondrial membranes could alter membrane permeability leading to alterations in the transmembrane potential, a change often observed to precede apoptotic death. Interestingly, the channel-forming activity of *bax* in lipid membranes can be inhibited by *bcl-2* (Antonsson *et al.*, 1997). Whether *bcl-2* and related proteins actually form channels *in vivo* has yet to be demonstrated, however.

In the nervous system, *bcl-2* is highly expressed during development. While the expression of *bcl-2* is retained in the peripheral nervous system, it declines to low levels in the postnatal and adult brain (Abe-Dohmae *et al.*, 1993; Castren *et al.*, 1994; Ferrer *et al.*, 1994; Merry *et al.*, 1994). A correlation between the expression of *bcl-2* and neuron survival has been observed. In sympathetic neurons for example, *bcl-2* expression decreases upon NGF (Allsopp *et al.*, 1993; Greenlund *et al.*, 1995b). Overexpression of *bcl-2* protects these neurons against NGF-deprivation-induced apoptosis (Allsopp *et al.*, 1993; Garcia *et al.*, 1992; Greenlund *et al.*, 1995b). Similar protection has been observed in sensory neurons deprived of NGF or BDNF (Allsopp *et al.*, 1993). In addition to trophic factor deprivation, overexpression of *bcl-2* in PC12 cells inhibits death in response to apoptosis-inducing stimuli such as serum deprivation and Ca^{2+} ionophore treatment (Mah *et al.*, 1993). In sympathetic neurons, *bcl-2* overexpression does not prevent the decline in cellular functions that occurs after NGF deprivation, such as the drop in protein synthesis (Greenlund *et al.*, 1995a). This suggests that *bcl-2* acts downstream of these changes and possibly in the terminal stages of the apoptotic pathway. The neuroprotective effects of *bcl-2* overexpression are recapitulated in *bcl-2*-overexpressing transgenic mice, which show a reduction in neuron death during development as well as following axotomy, ischemia, and induced retinal degeneration (Dubois-Dauphin *et al.*, 1994; Farlie *et al.*, 1995; Martinou *et al.*, 1994). However, not all neuronal populations are rescued by *bcl-2* overexpression. For example, the death of certain sensory neurons induced by ciliary neurotrophic factor (CNTF) deprivation occurs even if *bcl-2* is overexpressed (Allsopp *et al.*, 1993). Mice lacking *bcl-2* have also been generated and analyzed (Nakayama *et al.*, 1994; Veis *et al.*, 1993). Surprisingly, developmentally regulated neuron death, which is largely completed by birth, occurs to the same extent in these mice as in wild-type mice (Michaelidis *et al.*, 1996). However, these mice display the degeneration of several neuronal populations following birth, indicating that although *bcl-2* is not absolutely necessary during prenatal development, it is critical for neuron survival thereafter (Michaelidis *et al.*, 1996).

Interestingly, axotomized motoneurons from *bcl-2*-deficient mice can be rescued from degeneration by the local application of neurotrophic factors with efficiency similar to that in wild-type animals (Michaelidis *et al.*, 1996), suggesting that *bcl-2* is not a necessary component of the survival pathway utilized by neurotrophic factors. The absence of gross abnormalities in *bcl-2*-deficient mice in comparison with the rather severe neural defects seen in mice lacking neuro-

trophins [for a review, see Snider (1994)] supports the conclusion that additional cellular components are required for the survival effect of neurotrophic factors. In addition to its effect in the maintenance of neuron survival, *bcl-2* has been shown to promote the regeneration of severed retinal axons of all ages both *in vitro* and *in vivo* (Chen *et al.*, 1997). Neurons from mice deficient in *bcl-2* do not have the ability to grow axons, indicating the *bcl-2* expression is essential for the regeneration process (Chen *et al.*, 1997). The effect of *bcl-2* on axon growth occurs in the absence of any neurotrophic factor and may be independent of its anti-apoptotic activity process (Chen *et al.*, 1997).

In comparison to *bcl-2*, other members of this family, such as *bcl-*X_L, *bcl-*X_S (products of the *bcl-X* gene), *bax*, and *mcl-1*, are expressed at relatively high levels in the central nervous system both during development and in the adult (Frankowski *et al.*, 1995; Garcia *et al.*, 1992; Gonzalez-Garcia *et al.*, 1995; Krajewski *et al.*, 1994). It is therefore likely that these members may be more important than *bcl-2* in regulating neuron death in the brain. In cultured cerebellar granule neurons, for example, apoptosis induced by K^+ deprivation is preceded by decreased expression of the anti-apoptotic *bcl-*X_L transcript of the *bcl-X* gene with a concomitant increase in the pro-apoptotic spliced form, *bcl-*X_S (D'Mello *et al.*, 1997). An essential role for the *bcl-X* gene in the survival of central neurons is the finding that mice lacking *bcl-X* die *in utero* around embryonic day 13 with extensive neuronal apoptosis in the brain and spinal cord (Motoyama *et al.*, 1995). Telencephalic cells from *bcl-X*-deficient embryos have been analyzed in culture (Roth *et al.*, 1996) and have been found to be significantly more susceptible to apoptosis when challenged with stimuli such as serum deprivation (Roth *et al.*, 1996). This increased apoptosis of *bcl-X*-deficient telencephalic cells is exacerbated rather than reduced by inhibitors of protein synthesis (Roth *et al.*, 1996), suggesting that the survival of *bcl-X*-deficient neurons requires ongoing protein synthesis. Besides promoting survival of central neurons, expression of *bcl-*X_L also inhibits death of peripheral neurons caused by NGF deprivation (Frankowski *et al.*, 1995; Gonzalez-Garcia *et al.*, 1995).

In contrast to the neuroprotective actions of *bcl-X*, expression of the bax gene is associated with apoptosis. During cortical and cerebellar development *bax* expression is high during the period of naturally occurring neuronal death and falls to virtually undetectable levels in the adult (Vekrellis *et al.*, 1997). Overexpression of *bax* in sympathetic neurons induces apoptosis which is blocked by coexpression of *bcl-*X_L (Vekrellis *et al.*, 1997) supporting a role for elevated *bax* expression in neuronal death as well as the hypothesis that the relative levels of proapoptotic and antiapoptotic members of the *bcl-2* family is a critical determinant of a cells viability status. *bax* mRNA and protein levels are upregulated in vulnerable regions of the brain after injury (Chen *et al.*, 1996a; Krajewski *et al.*, 1995b), suggesting that in addition to developmentally regulated death, *bax* may be involved in injury-induced neuronal degeneration in the brain. Like *bcl-2*-

lacking mice, however, *bax*-deficient mice are viable and do not display a dramatically aberrant phenotype (Knudson *et al.*, 1995), indicating that *bax* expression is not solely responsible for the cell death that occurs in the brain or other parts of the nervous system during development. An examination of the superior cervical ganglia and facial motoneurons in *bax*-deficient mice does show increased cell numbers, indicating that although not generally required for neuron death, *bax* is necessary for the developmentally occurring cell loss in at least some neuronal populations (Deckwerth *et al.*, 1996). Moreover, sympathetic neurons and motoneurons are more resistant to apoptosis induced by NGF deprivation and axotomy, respectively, confirming the importance of *bax* in the promotion of apoptosis (Deckwerth *et al.*, 1996) in these neuron types. *bcl-X*-deficient mice have been interbred with *bax*-deficient mice to generate mice deficient in both *bcl-X* and *bax* (Shindler *et al.*, 1997). Although incapable of preventing the embryonic lethality of *bcl-X*-deficient mice, *bax* deficiency significantly reduces the cell death that occurs in the brain and spinal cord of *bcl-X*-deficient embryos (Shindler *et al.*, 1997). Similarly, *bax* deficiency reduces the increased level of apoptosis of *bcl-X*-deficient telencephalic cells *in vitro* (Shindler *et al.*, 1997).

IV. Cytoplasmic Regulators of Neuronal Survival or Death

A. Caspases

In *C. elegans*, two genes, *ced-3* and *ced-4*, are critical for developmentally occurring cell death [for a review, see Ellis (1986)]. Expression of another gene, *ced-9* (the mammalian homologue of which is *bcl-2*), inhibits the death-inducing activities of *ced-3* and *ced-4*, thereby permitting cell survival. *ced-3* was found to encode a protein related to the mammalian interleukin-1β-converting enzyme (ICE), which subsequently led to the discovery of several other structurally and functionally related proteases now collectively referred to as caspases [for reviews on this subject, see Chinnaiyan and Dixit (1996) and Alnemri (1997)]. As observed with *ced-3*, the activation of one or more caspases has been found to occur during apoptosis of mammalian cells, and overexpression of these proteins induces apoptosis in cells that are normally viable. Conversely, caspase inhibitors such as the cowpox virus CrmA protein, the bacteriophage p35 protein, and certain peptide methyl ketones and peptide aldehydes, which bind irreversibly to the active-site cysteine, can inhibit apoptosis [for reviews, see Alnemri (1997), Chinnaiyan and Dixit (1996), and Livingston (1997)]. All caspases are synthesized as inactive enzymes and require proteolytic processing for activation. The mechanisms regulating the cleavage and activation of caspases is unclear, although both autoproteolysis and cleavage of one caspase by another have been shown to occur. On the basis of structural similarities, caspases can be classified

into three subfamilies: (1) the ICE subfamily, which includes ICE, ICEII/TX, ICE-reIII, and ICE-re1III; (2) the CPP32 subfamily, which includes CPP32/Yama/apopain, ICE-LAP3, Mch2, Mch3, Mch4, Mch5, and Mch6; and (3) the Nedd-2/Ich-1 subfamily.

As seen in other cell types, caspase activation induces apoptosis in neurons [reviewed in Schwartz (1996)]. Initial evidence came from experiments showing that transfection of the baculovirus p35 gene (which inhibits caspases) into a neuronal cell line protected them from apoptosis induced by a variety of death-inducing stimuli (Rabizadeh *et al.*, 1995). Other evidence implicating caspases in neuronal apoptosis comes from experiments in which overexpression of the ICE gene in cultured sensory neurons induced apoptosis even in the presence of NGF (Gagliardini *et al.*, 1994). Death induced by ICE overexpression can be blocked by coexpression of *bcl-2* or the cowpox virus gene, *crmA* (Gagliardini *et al.*, 1994). Surprisingly, however, mice deficient in ICE develop normally and are viable and fertile (Kuida *et al.*, 1996; P. Li *et al.*, 1995). No abnormalities within the nervous system have been reported in these mice. Whereas these results suggest that ICE itself is not required for programmed cell death during development, there is likely to be functional redundancy among caspases. In contrast to the virtually normal phenotype of mice lacking ICE, CPP32 null mutants show profound abnormalities in the developing brain and these mice die within a few weeks after birth (Kuida *et al.*, 1996). The brains of CPP32-deficient mouse embryos display ectopic cell masses containing fully differentiated neurons, indicating decreased apoptosis in the developing nervous system (Kuida *et al.*, 1996).

Peptidic methyl ketone inhibitors have been used to examine the role of various caspases in neuronal apoptosis. Among the most commonly used inhibitors are YVAD.cmk (which is selective for the ICE subfamily), DEVD.fmk (selective for the ced-3 subfamily), and ZVAD.fmk, which is a broad-spectrum inhibitor capable of inhibiting the actions of ICE, CPP32, and other members (Livingston, 1997). Experiments utilizing such selective inhibitors have revealed that, depending on the neuron type, different caspase subfamilies may be activated during apoptosis. Even in a single neuron type, distinct caspases may be activated in response to different death-inducing stimuli. For example, while the ICE subfamily inhibitor, YVAD, blocks trophic factor deprivation-induced death of motoneurons, it does not prevent motoneuron death induced by limb-bud removal (Milligan *et al.*, 1995). Caspase inhibitors have been used in a number of studies using cerebellar granule neurons. In these neurons, YVAD.cmk is only weakly effective in preventing apoptosis induced by the lowering of K^+ (Aglieco *et al.*, 1997; Armstrong *et al.*, 1997). This observation, along with the lack of ICE cleavage in apoptotic granule neurons (Schulz *et al.*, 1996), suggests the ICE itself is not involved in low-K^+-mediated death. Other inhibitors such as the CPP32-selective inhibitor, DEVD.fmk, and the broad-spectrum caspase inhibitor, ZVAD.fmk, are partially effective in preventing the death of K^+-deprived gran-

ule neurons (Aglieco *et al.*, 1997; Armstrong *et al.*, 1997), raising the possibility that members of the CPP32 subfamily are involved. A CPP32-immunoreactive protein has been reported to be activated (cleaved) in apoptotic granule neurons, raising the possibility that CPP32 mediates death in these neurons (Armstrong *et al.*, 1997). However, another study utilizing other CPP32 antibodies found no evidence of CPP32 processing after K^+ deprivation (Aglieco *et al.*, 1997). Poly(ADP)–ribose polymerase (PARP), a protein that is normally proteolyzed by activated CPP32 and often used as an indicator of CPP32 activation, is not cleaved in K^+-deprived cerebellar granule neurons (Aglieco *et al.*, 1997; Taylor *et al.*, 1997). This, along with partial inhibition by the potent CPP32 inhibitor DVED.fmk, argues against the need for CPP32 in low-K^+-mediated death of granule neurons. A caspase that is very closely related to CPP32 (designated as IRP) has recently been cloned and found to be expressed in cultured granule neurons and neuron-enriched regions of the brain (Ni *et al.*, 1997). Expression of IRP is increased in K^+-deprived granule neurons as well as *in vivo* during development when naturally occurring neuron death takes place (Ni *et al.*, 1997). In contrast to K^+-deprivation-mediated death, apoptosis of granule neurons induced by staurosporine is accompanied by PARP cleavage (Taylor *et al.*, 1997), indicating that a caspase distinct from the one activated by low K^+ is utilized in staurosporine-mediated neuron death. Although peptidic inhibitors such as YVAD.cmk and DEVD.fmk point broadly to a particular subfamily, caspase families, they do not, unfortunately, reveal the identities of the specific members that are activated.

As observed with ICE and CPP32 *in vitro*, overexpression of Nedd-2 can also induce apoptosis in neuronal cell lines (Kumar *et al.*, 1994). Furthermore, inhibition of endogenous Nedd-2 expression by antisense olionucleotide treatment inhibits apoptosis in PC12 cells induced by growth factor deprivation, but not death induced by other stimuli (Troy *et al.*, 1997). Cleavage of proNedd-2/ICH-I has also been reported to occur in NGF deprived sympathetic neurons (Deshmukh *et al.*, 1996). Taken together, it seems likely that members of all three caspase subfamilies are involved in neuron death although, depending on the type of neuron and the stimulus used, the caspase that is responsible may differ.

B. Reactive Oxygen Species and Oxidative Stress

Excessive production of reactive oxygen species (ROS) causes cell damage. Several lines of evidence support a causal role for ROS in apoptosis in neurons as well as in other cell types [for a review, see Slater *et al.* (1995)]. For example, exogenously provided oxidants and agents that increase the intracellular production of ROS induce apoptosis in neuronal cell lines (Enokido and Hatanaka, 1993; Estevez *et al.*, 1995). Apoptotic death of neurons induced by a variety of stimuli, including serum–trophic factor deprivation (Ferrari *et al.*, 1995; Green-

lund *et al.*, 1995a; Satoh *et al.*, 1996), K^+ deprivation (Schulz *et al.*, 1996), exposure to high oxygen levels (Enokido and Hatanaka, 1993), exposure to β-amyloid (Behl *et al.*, 1994; Forloni *et al.*, 1993), glutamate-mediated excitotoxicity (Bonfocco *et al.*, 1995), and ischemia (Linnik *et al.*, 1993), is accompanied by increased production of ROS. Treatment of these neurons with ROS scavengers, antioxidants, and ROS-metabolizing enzymes can protect against apoptosis in many cases (Bonfocco *et al.*, 1995; Ferrari *et al.*, 1995; Ratan *et al.*, 1994; Satoh *et al.*, 1996). Elevated levels of ROS are seen in cortical neurons from cultured fetal Down's syndrome brains (Busiglio and Yankner, 1995). The premature death of these neurons in culture can also be prevented by ROS scavengers and metabolizing enzymes (Busiglio and Yankner, 1995), implicating ROS in the neuronal degeneration in this neuropathological disorder. Events downstream of ROS accumulation in a dying cell are not clear, but may include modulation of gene expression. It is known that ROS formation activates transcription factors such as NF-κB (Schreck *et al.*, 1991). Activation of NF-κB has been implicated in apoptosis in some paradigms (Grimm *et al.*, 1996; Slater *et al.*, 1995), although other studies have shown that this transcription factor actively inhibits apoptosis (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996; Wang *et al.*, 1996). Besides the fact that antioxidants cannot always protect against apoptosis, however, one piece of evidence that strongly argues against an obligatory role for ROS is the finding that apoptosis occurs in the absence of mitochondrial respiration and in cells grown essentially without oxygen, conditions that should virtually eliminate the production of ROS (Jacobson *et al.*, 1994; Shimuzu *et al.*, 1995).

A key enzyme in the metabolism of ROS is copper–zinc superoxide dismutase (SOD1). In humans, mutations in this gene are often the cause of familial amyotrophic lateral sclerosis (FALS), a degenerative disease characterized by motor neuron loss. Although it has not been convincingly demonstrated that the degeneration in FALS is due to increased ROS production caused by loss of SOD1 activity, this hypothesis has been tested in several neuronal systems with mixed results. In PC12 cells, antisense oligonucleotides against SOD1 inhibit cell survival, supporting a neuroprotective role for this enzyme (Troy and Shelanski, 1994). In cultured sympathetic neurons, however, microinjection of SOD1 delays but cannot not substantially prevent apoptosis induced by NGF deprivation (Greenlund *et al.*, 1995a). Moreover, the re-addition of NGF rescues sympathetic neurons from death even after the full-blown increase in ROS production has taken place (Greenlund *et al.*, 1995a). Similarly, in serum-deprived PC12 cells and embryonic cortical neurons, survival factors prevent apoptosis without affecting the increase in ROS levels (Enokido *et al.*, 1992; Hinshaw *et al.*, 1993). Taken together, these results suggest that elevated ROS levels are in themselves not fatal. Convincing data from several laboratories now indicate that the pathological changes in familial ALS are not due to the loss of SOD1 enzyme activity, but result from a new toxic property of the mutated SOD1 enzyme (Gurney *et al.*, 1994; Rabizadeh *et al.*, 1995; Wong *et al.*, 1995). Therefore, transgenic mice expressing mutant

forms of the *SOD1* gene develop the pathological symptoms of FALS, including motoneuron degeneration, whereas mice lacking or overexpressing the wild-type enzyme are normal (Gurney *et al.*, 1994; Wong *et al.*, 1995). Taken together, it seems likely that ROS production lowers a cell's resistance to apoptosis, and overexpression of ROS-metabolizing genes such as *SOD1* therefore can be protective. However, ROS generation does not appear to be obligatory for apoptosis.

C. The Phosphoinositide 3-Kinase (PI 3-Kinase)–Akt Pathway

PI 3-kinase is the enzyme that phosphorylates phosphoinositides (PIs) on the D-3 position of the inositol ring, leading to the generation of the phospholipids PI-3-P, PI-3,4-P, and PI-3,4,5-P [for a review, see Kapeller and Cantley (1994)]. This enzyme, composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, is activated by several growth factors as well as by some hormones and has been implicated in a variety of responses, including metabolic processes (such as glucose uptake and superoxide production), mitogenic signaling, membrane ruffling, and vesicle trafficking (Kapeller and Cantley, 1994). In addition to these actions, activation of PI 3-kinase is required for the prevention of apoptosis by NGF in serum-deprived PC12 cells (Yao and Cooper, 1995). The anti-apoptotic and, conversely, survival-promoting action of PI 3-kinase is not restricted to PC12 cells and NGF. Indeed, PI 3-kinase activation is necessary for the survival of cultured cerebellar granule neurons by IGF-1 (D'Mello *et al.*, 1997; Dudek *et al.*, 1997). In contrast, however, PI 3-kinase is not required for the survival of these neurons by elevated K^+ (D'Mello *et al.*, 1997; Dudek *et al.*, 1997), suggesting that neuron survival can be mediated by PI 3-kinase-dependent (growth-factor-activated) and -independent (high-K^+-activated) pathways.

A downstream effector of PI 3-kinases in several systems is the serine–threonine kinase, Akt/PKB (Franke *et al.*, 1995). In cerebellar granule neurons, as well as in nonneuronal cells in which PI 3-kinase has been found to be required for survival, activation of PI 3-kinase leads to the activation of Akt/PKB (Dudek *et al.*, 1997; Franke *et al.*, 1997b). Activation of Akt/PKB is mediated by its binding to membrane-associated PI-3,4-P2 which is produced as a result of PI 3-kinase activation (Franke *et al.*, 1997a). Supporting the idea that Akt/PKB activation occurs downstream of PI 3-kinase is the finding that overexpression of Akt/PKB prevents neuronal apoptosis in the absence of PI 3-kinase activity (Dudek *et al.*, 1997). Furthermore, dominant negative forms of Akt/PKB interfere with IGF-1-mediated cell survival in spite of PI 3-kinase activation (Dudek *et al.*, 1997). Besides Akt/PKB, downstream effector of PI 3-kinase in some systems is the ribosomal protein kinase $p70^{S6kinase}$. However $p70^{S6kinase}$ is not required for IGF-1-mediated survival in neurons or nonneuronal cells (Dudek *et al.*, 1997; Franke *et al.*, 1997b). The molecular target of Akt/PKB in the anti-apoptotic pathway is yet to be identified. Members of the *bcl-2* family could conceivably be affected. For example, phosphorylation of the death-promoting

member *bad* in response to treatment of hemopoietic cells with *IL-3* has been shown to induce its binding to 14-3-3, rather than to *bcl-X_L*, thereby permitting *bcl-X_L* to exert its survival promoting activity (Zha *et al.*, 1996).

D. p75 Neurotrophin Receptor

NGF can bind independently to two different receptors—with high affinity to the trkA tyrosine kinase receptor and with lower affinity to the p75 neurotrophin receptor [for a review, see Meakin (1992)]. The p75 receptor is thought to increase binding of NGF to trkA, enhance the specificity of trk receptors for neurotrophins, and mediate retrograde transport of some neurotrophins [for a review, see Chao (1995)]. However, it is now known that the p75 receptor also promotes cell death under some conditions. In neuronal cell lines, the pro-apoptotic action of p75 is activated by the absence of NGF (Rabizadeh *et al.*, 1993). This lethal action of p75 is also displayed in late prenatal and postnatal cultures of sensory neurons in the absence of NGF, although at earlier stages the same neurons require p75 expression for their development and survival (Barrett and Bartlett, 1994). Transgenic mice expressing the intracellular domain of p75 display significant cell loss in certain peripheral and central neuronal populations, confirming the ability of p75 to mediate cell death (Majdan *et al.*, 1997). That the absence of p75 is neuroprotective under some circumstances has been demonstrated using p75, which show increased survival of cholinergic forebrain and neostriatal neurons (Van der Zee *et al.*, 1996). The p75 receptor contains a "death domain" motif, which is also found in other apoptosis-inducing proteins such as the p55 tumor necrosis factor (TNF) and the Fas receptors as well as in molecules that associate with these receptors such as the TRADD, FADD, and RIP proteins [for a review, see Cleveland (1995)]. On the basis of a number of studies, it seems likely that it is via the death domain that surface molecules such as the p75 and TNF receptors are connected to intracellular components of the cell death machinery. The functional significance of this motif in promoting cell death is underscored by the finding that its deletion abrogates the ability of these molecules to mediate apoptosis (Stanger *et al.*, 1995).

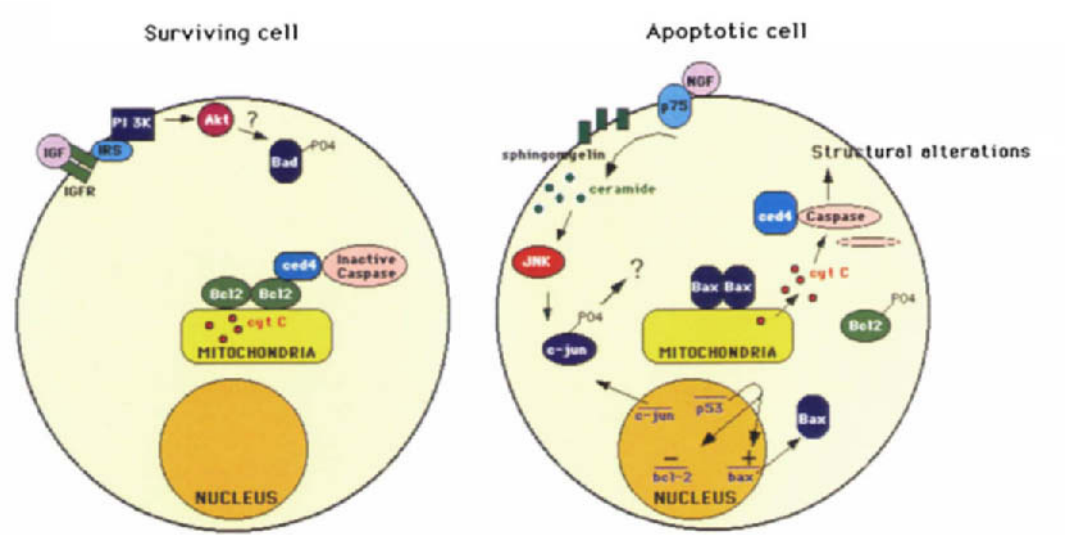
In contrast to the mechanism seen in these neuronal systems where p75-mediated apoptosis is triggered by the absence of NGF, in chick retinal neurons and in cultured oligodendrocytes expressing p75 but not trkA it is the binding of NGF to p75 that induces apoptosis (Casaccia-Bonnefil *et al.*, 1996; Frade *et al.*, 1996). Intriguingly, treatment with other neurotrophins such as BDNF and NT3, which also bind p75, does not affect the survival of oligodendrocyes, suggesting that this pathway of apoptosis is restricted to NGF. Also noteworthy is the finding that NGF treatment of other nonneuronal cell types that express p75 but not trkA does not induce apoptosis. Although the mechanism by which apoptosis is triggered upon the binding of NGF to p75 is not known, sustained elevation of intracellular ceramide levels by activation of the sphingomyelin pathway is believed to be involved (Casaccia-Bonnefil *et al.*, 1996). Neurotrophins that do not

affect cell survival do not increase ceramide levels in oligodendrocytes. Likewise, a sustained increase in ceramide is not observed by p75-expressing cells that are not killed by NGF treatment (Casaccia-Bonnefil *et al.*, 1996). Ceramide has been associated with apoptosis in other neuron types (Brugg *et al.*, 1996; Weisner and Dawson, 1996). For example, apoptosis of embryonic chick cortical neurons induced by staurosporine treatment is accompanied by the hydrolysis of sphingomyelin to ceramide (Weisner and Dawson, 1996). Exogenous addition of sphingomyelinase, which increases sphingomyelin hydrolysis, or inhibitors of ceramidase, which maintain high ceramide levels, also leads to apoptosis in these neurons (Weisner and Dawson, 1996).

How does elevated ceramide cause apoptosis? Ceramide can activate c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), a member of the mitogen-activated protein kinase (MAPK) family, and does so in oligodendrocytes induced to die by NGF treatment (Casaccia-Bonnefil *et al.*, 1996). JNK/SAPK activation is also associated with apoptosis in serum-deprived PC12 cells (Xia *et al.*, 1995) and in some nonneuronal systems (Liu *et al.*, 1996; Seimiya *et al.*, 1997). In lymphoid cell lines, for example, ceramide accumulation activates JNK, leading to apoptosis (Cuvillier *et al.*, 1996), while treatment with sphingosine 1-phosphate, the metabolite of ceramide, inhibits cell death. Sphingosine 1-phosphate stimulates the extracellular signal-regulated kinase (ERK) pathway as well as inhibits the ceramide-induced activation of JNK (Cuvillier *et al.*, 1996). This is consistent with results obtained from PC12 cells indicating that apoptosis following serum deprivation depends on the inhibition of ERK signaling, in addition to the activation of JNK (Xia *et al.*, 1995).l The target of JNK is c-jun, and phosphorylation of c-jun increases its transactivating action as well as reduces its degradation (Musti *et al.*, 1997; Smeal *et al.*, 1991). Contrary to what is seen in PC12 cells however, in NGF deprived sympathetic neurons JNK activation does not always cause apoptosis and a suppression of JNK activity does not necessarily prevent cell death, suggesting that at least in some neurons, signaling mechanisms in addition to or other than JNK are critical in the commitment to death (Virdee *et al.*, 1997). Also in contrast to PC12 cells, ERK signaling does not appear to be important for the survival of cultured sympathetic neurons (Virdee and Tolkovsky, 1996) or cerebellar granule cells (Gunn-Moore *et al.*, 1997).

E. Interactions between Cellular Components in the Regulation of Apoptosis

Although research has led to the identification of several molecules that affect the survival status of a cell, how these various death-inducing and death-inhibiting components interact at the molecular level remains to be elucidated. Participating in the apoptosis of most, if not all, cell types (neuronal and nonneuronal) and in response to virtually every stimuli examined are one or more members of the caspase family (related to the *C. elegans* ced-3 protein) and members of the *bcl-2*



Chapter 6, Fig. 1 General models of apoptosis regulation in neurons. In neurons such as the cerebellar granule neuron, treatment with trophic factors such as IGF-1 (left-hand side) leads to the sequential activation of IRS, PI 3-kinase, and Akt/PKB. Events downstream of Akt/PKB have yet to be elucidated, although phosphorylation of *bcl-2* family members such as *bad* is conceivable. *bad* phosphorylation would inhibit its ability to induce apoptosis. *bcl-2* expression prevents the activation of caspases. Release of cytochrome c from the mitochondria, which is involved in the activation process, is inhibited by *bcl-2* and other family members. Also affecting the activation of caspases is *ced-4* or its mammalian homologue(s). *ced-4* is dragged to intracellular membrane locations by *bcl-2* and modified, which prevents caspase activation. The panel on the right shows a model of a neuron, such as the chick retinal neuron, in which NGF activates apoptosis by binding to the p75 receptor in the absence of the high-affinity trkA receptor. This stimulates the conversion of sphingomyelin to ceramide. Sustained elevation of intracellular ceramide activates JNK, which can phosphorylate the transcription factor c-jun. Increased expression of c-jun is necessary for the induction of apoptosis in some neurons. The target(s) of c-jun in the apoptotic pathway is not known. Another molecule whose expression is associated with apoptosis is p53. One mode by which increased p53 expression might promote apoptosis is by influencing the transcription of the *bcl-2* and *bax* genes. Transcription of the *bcl-2* gene is suppressed while transcription of the apoptosis-inducing gene, *bax*, is stimulated by p53. The expression of *bax* along with the absence of *bcl-2* permits the release of cytochrome c from the mitochondria. Along with the interaction with unmodified *ced-4*, the release of mitochondrial cytochrome c causes the activation of caspases, leading to structural alteration in the cell and culminating its demise.

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family of proteins (related to the *C. elegans* ced-9 protein). Besides *ced-3,* the product of the *ced-4* gene is necessary for programmed cell death in *C. elegans.* A mammalian homologue of *ced-4* has not yet been identified. Analysis of the interactions between the various *C. elegans* proteins has been studied by their expression in nonneuronal mammalian cells. These studies reveal that *ced-4* is the molecular link between the death-inducing proteins, ced-3/caspases, and the death-inhibiting proteins, ced-9/bcl-2 (Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997). In the absence of ced-9/bcl-2, *ced-4* interacts with ced-3/caspase, leading to *ced-3* activation (Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997). In the presence of ced-9/bcl-2, however, *ced-4* is bound to ced-9/bcl-2 and translocated from its diffused cytoplasmic location to specific intracellular membrane sites (possibly mitochondrial membrane) where *bcl-2* resides (Wu *et al.*, 1997). Either because of its removal from the cytosol or because of a ced9/bcl-2-induced modification, *ced-4* is unable to activate ced-3/caspase (Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997). *bax* and other death-promoting members of the bcl-2 family may act by disrupting the interaction between *bcl-2* or *bcl-X_L* and *ced-4.* Another event important in caspase activation is translocation of cytochrome c from mitochondria to the cytosol (Kluck *et al.*, 1997; Yang *et al.*, 1997). Overexpression of bcl-2/ced-9 prevents cytochrome c release from the mitochondria, thereby inhibiting caspase activation and apoptosis (Kluck *et al.*, 1997; Yang *et al.*, 1997). Activation of caspases leads to the proteolysis of important enzymes, cytokines, as well as structural and other proteins (Alnemri, 1997; Chinnaiyan and Dixit, 1996), leading to DNA fragmentation and cell death. Although exactly how DNA fragmentation is activated remains unclear, a protein that induces fragmentation has been identified (Liu *et al.*, 1997). Interestingly, activation of this protein, designated the DNA fragmentation factor (DFF), occurs via proteolytic cleavage by CPP32 (Liu *et al.*, 1997).

As described in this chapter, in addition to caspases and bcl-2 proteins, other proteins such as AP-1 and p53, molecules including ceramide, ROS, and signaling pathways such as the PI 3-kinase–Akt/PKB pathway have been implicated in the regulation of apoptosis in neurons. Figure 1 (please see color plate) summarizes many of these findings and provides general models for how apoptosis might be suppressed or induced in neurons. Whereas some of the events shown in the figure represent findings from nonneuronal systems, it is likely that the same or related mechanisms operate in neurons. Verification and extension of these findings will certainly lead to therapeutic approaches for the prevention or treatment of neuropathological conditions such as neurodegenerative diseases and stroke.

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7

A Novel Protein for Ca^{2+} Signaling at Fertilization

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At fertilization in all species studied the sperm activates the egg by causing an increase in the level of cytoplasmic free Ca^{2+} concentration. It is still not established how the sperm causes the changes in Ca^{2+} in the egg, which in the majority of eggs is due to release from internal stores. Current hypotheses about the signaling molecules involved in fertilization are confounded by the fact that for many eggs the fertilization-associated Ca^{2+} increase is readily mimicked by parthenogenetic activating agents. One exception to this is found for mammalian eggs where there are a series of Ca^{2+} oscillations observed at fertilization that have distinct characteristics. In this context we discuss three different theories of how sperm trigger Ca^{2+} release in eggs. We present the case that the sperm mediates its Ca^{2+} mobilization effects after gamete membrane fusion by introducing a specific protein into the egg cytoplasm. Our argument is based upon the fact that only the mammalian sperm protein factor can trigger a pattern of Ca^{2+} oscillations that is similar to that induced by the sperm in mammalian eggs. The sperm factor activity is correlated with a novel signaling protein that we have called oscillin and which may mediate Ca^{2+} release via a novel mechanism.

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I. Intracellular Ca^{2+} and Egg Activation

A. Egg Activation

The union of a sperm and an egg at fertilization triggers the development of the egg into an embryo. There are many biochemical and morphological changes brought about in the egg by the fertilizing sperm. These changes are collectively known as egg activation (Whittingham, 1980; Whitaker and Steinhardt, 1982; Jaffe, 1983; Yanagimachi, 1994). In most vertebrate species, meiosis is arrested before fertilization, and sperm-induced activation of the egg leads to the completion of meiosis and the subsequent entry of the early embryo into the mitotic cell cycles (Yanagimachi, 1994). Egg activation also is usually associated with exocytosis of cortical granules, which are part of the mechanism that prevents polyspermy (Whitaker and Steinhardt, 1982; Kline, 1993; Yanagimachi, 1994). In the eggs of some species, such as sea urchins, activation includes an increase in the rates of respiration and protein synthesis (Whitaker and Steinhardt, 1982). In other eggs, such as those of mammals, there are changes in the pattern of proteins that are translated after fertilization (Howlett, 1986). The focus of this chapter is to discuss the signal transduction mechanisms that are involved in initiating sperm-induced egg activation. Most of our work has focused on the signaling mechanism in mammals because eggs (or more correctly oocytes) of this group have specific features that make them especially suited to resolving problems of signal transduction. These evident features in mammalian eggs concern changes in intracellular Ca^{2+}. However, because a change in intracellular Ca^{2+} appears to underlie signaling at fertilization in all species, our discussion also considers the signaling mechanisms in other groups of animals, such as echinoderms and amphibians.

A major problem arising in studies of fertilization is the question of whether those experimental stimuli that have been found to trigger egg activation have any relevance to the physiological mechanism operating at fertilization or whether their activating properties are coincidental and physiological egg activation operates by quite a different mechanism. This problem is not a new one. It significantly affected the conclusions of one of the earliest pioneers of fertilization research, Lazzaro Spallanzani, who studied fertilization in frogs (Magner, 1979). Spallanzani was the first person to clearly demonstrate that the addition of semen to eggs was sufficient to trigger them to develop into embryos. However, the extensive experiments he carried out on the nature of this phenomenon led him to incorrectly conclude that the spermatozoa in the semen were not involved in the process of fertilization. Instead he believed that a soluble portion of the semen harbored the ability to activate the development of the egg. A key reason for Spallanzani reaching this conclusion appears to have been his finding that the treatment of semen with certain reagents (e.g., vinegar) could kill the spermatozoa, but did not prevent the ability of semen to trigger early development

(Magner, 1979). It is now thought that Spallanzani was observing a form of artificial activation. Unfortunately, his assay, which looked for the initial cell divisions, was unable to distinguish the artificial stimulus from the real one. This example is a pertinent demonstration of how the criteria one adopts for assessing the ability of an agent to mimic the events at fertilization can dramatically affect the conclusions that are made about its physiological role. Hence, the degree of confidence that one can place in any putative activating factor's actual role at fertilization is critically dependent on the criterion of activation itself. We suggest that the specific pattern of Ca^{2+} changes at fertilization in mammalian eggs represents the most stringent criterion for activation. This leads us to make some novel proposals for the nature of the agent involved in egg activation in mammals.

B. The Central Role of Ca^{2+}

Multiple different treatments have been shown to cause egg activation in sea urchin, frog, and mammalian eggs (Whitaker and Steinhardt, 1982; Jaffe, 1983; Whittingham, 1980). We now know that the link between many of these different activating treatments is their capacity to artificially raise intracellular Ca^{2+} levels in the egg. The central role of elevated Ca^{2+} in the process of egg activation was hinted at in early parthenogenetic studies, which showed that the stimulation of sea urchin egg activation by some detergents only takes place in calcium-containing sea water (Moser, 1939). However, the first clear evidence of the critical role of Ca^{2+} was the demonstration that the Ca^{2+} ionophore, A23187, could induce the normal parameters of egg activation (cell cycle initiation and exocytosis) in sea urchin, frog, and hamster eggs (Steinhardt *et al.*, 1974). The suggestion that a Ca^{2+} increase played a key role in mammalian egg activation was also supported by the demonstration that activation and development to the blastocyst stage are triggered by iontophoretic injection of Ca^{2+} in the mouse (Fulton and Whittingham, 1978).

Because Ca^{2+} could trigger activation in all types of eggs examined, the next important step in demonstrating the important role of intracellular Ca^{2+} was to show that Ca^{2+} changes accompany normal fertilization. A variety of methods for detecting Ca^{2+} (e.g., Ca^{2+}-sensitive electrodes, aequorin, and Ca^{2+}-sensitive fluorescent dyes) were used to show that a rise in intracellular Ca^{2+} occurs during fertilization. This was observed first in fish eggs (Ridgeway *et al.*, 1977) and since then in the eggs of every deuterostome species studied so far, including sea urchins, frogs, mice, and humans (Whitaker and Steinhardt, 1982; Jaffe, 1983; Busa, 1990; Miyazaki *et al.*, 1993b; Taylor *et al.*, 1993; Swann and Ozil, 1994). Ca^{2+} increases have also been demonstrated in ascidians and several types of protostome egg at fertilization (Speksnijder *et al.*, 1989; Deguchi and Osanai, 1994; Stricker, 1996). The changes in Ca^{2+} concentration almost always

involve an increase of about an order of magnitude, which is the same kind of magnitude of Ca^{2+} change that accompanies striated muscle contraction or neurosecretion (Berridge, 1993). The conclusive demonstration of the central role of Ca^{2+} came from studies that showed that inhibition of the Ca^{2+} rise by loading the cytoplasm with Ca^{2+} chelators blocks all of the events of egg activation at fertilization in sea urchin, frog, and mouse eggs (Whitaker and Steinhardt, 1982; Kline, 1988; Kline and Kline, 1992).

Along with the discovery that Ca^{2+} is the physiological mediator of egg activation, it has become clear that there are substantial differences in the pattern of Ca^{2+} release in eggs from different species. A question of considerable interest is the source of these differences and understanding what functional purpose they serve. A general feature of Ca^{2+} signaling in eggs, as with somatic cells, is that it involves positive feedback mechanisms whereby a small Ca^{2+} increase stimulates an additional, large release of Ca^{2+} (Jaffe, 1983; Whitaker and Swann, 1993; Berridge, 1993). In sea urchin, frog and fish eggs, the increase in intracellular Ca^{2+} takes the form of a single explosive wave that crosses the egg, starting from the point of sperm entry (Jaffe, 1983; Busa, 1990; Whitaker and Swann, 1993). The duration of the single Ca^{2+} increase in these eggs lasts for 5–10 min. However, quite a different pattern of Ca^{2+} release is observed in mammalian eggs, where the sperm triggers a series of periodic increases in Ca^{2+}, or transients, that occur at regular intervals, continue for several hours, and are referred to as Ca^{2+} oscillations (Miyazaki and Igusa, 1981; Cuthbertson and Cobbold, 1985; Fissore and Robl, 1993; Swann and Ozil, 1994). In mammals these oscillations trigger cortical granule exocytosis, completion of meiotic division, and entry into the interphase as marked by the appearance of two pronuclei (Kline and Kline, 1992). Different patterns of Ca^{2+} oscillations have been reported in other species of eggs such as marine bivalves, nemertean worms, and ascidians (Speksnijder *et al.*, 1989; Deguchi and Osanai, 1994; Stricker, 1996). In marine bivalves and ascidians, the initial Ca^{2+} rise is large with additional smaller Ca^{2+} oscillations occurring on top of, or after, the initial rise. In nemertean worms, the oscillations are similar in form to those in mammalian eggs in that there are large, distinct Ca^{2+} spikes that occur on a fairly even baseline of Ca^{2+} (Stricker, 1996). In ascidian oocytes, after the initial Ca^{2+} rise, the smaller increases appear to be related to the timing of meiotic events (Speksnijder *et al.*, 1989). Understanding of the differences in the dynamics of Ca^{2+} release at fertilization in different species is likely to require a more precise knowledge of the sperm agent or agents that trigger Ca^{2+} release in the egg. This question remains a major unresolved problem.

C. The Criteria for Assessing Signaling Mechanisms at Fertilization

A major challenge for those seeking to understand the signaling mechanism involved in egg activation at fertilization is the identification of the physiological

messenger that triggers Ca^{2+} release in the egg. We consider that one can best uncover the factors involved in egg activation by monitoring the most important feature of signaling at fertilization, which is the Ca^{2+} increase itself. One concern with using eggs from sea urchin, fish, and frog for assessing putative signaling agents is that it is possible to mimic the sperm-induced, single, large transient increase in Ca^{2+} with a variety of agents (Whitaker and Swann, 1993). For example, in the sea urchin egg, injection of inositol 1,4,5-triphosphate ($InsP_3$), cyclic ADP ribose, GTP analogues, or cyclic GMP all cause a single Ca^{2+} increase somewhat similar to that seen at fertilization (Swann and Whitaker, 1986; Rakow and Shen, 1990; Whalley *et al.*, 1992; Galione *et al.*, 1993). However, the pattern of repetitive Ca^{2+} oscillations seen during mammalian fertilization represents a distinctly different response. Many parthenogenetic stimuli, such as the application of 7% ethanol or Ca^{2+} ionophore, cause a single, prolonged Ca^{2+} increase in hamster and mouse eggs (Cuthbertson *et al.*, 1981; Swann and Ozil, 1994). Figure 1D shows the Ca^{2+} increase in a mouse egg after the addition of Ca^{2+} ionophore. These kinds of responses are completely unlike that observed at fertilization (see Fig. 1A). Direct injection of Ca^{2+} or electroporation in the presence of Ca^{2+} also triggers a single, large Ca^{2+} transient in mammalian eggs (Igusa and Miyazaki, 1983; Ozil and Swann, 1995). These procedures can lead to some degree of egg activation; however, they do not lead to Ca^{2+} oscillations and, thus, do not match the response observed with sperm

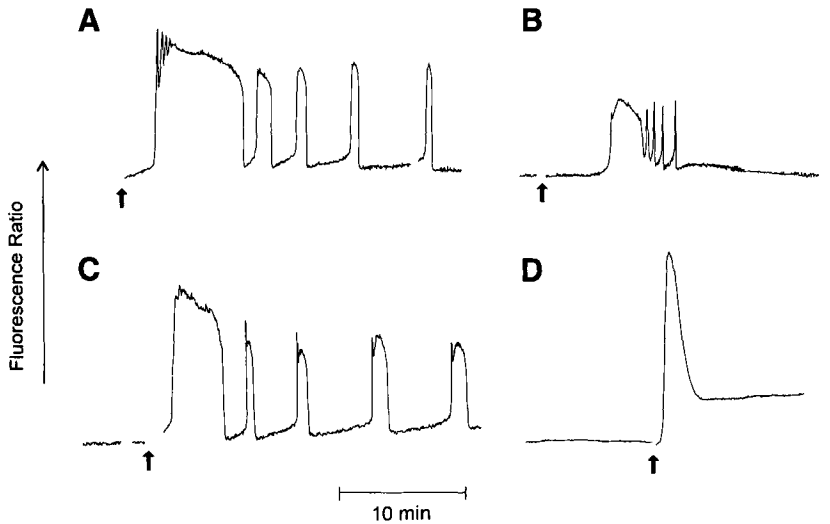

Fig. 1 Ca^{2+} changes in mouse eggs. Ca^{2+} levels were monitored with intracellular fura red [see Lawrence *et al.* (1997) for details]. Each trace is from a separate egg under identical conditions. In A the egg was fertilized; in B 250 μM carbachol was perfused onto the egg; in C the egg was injected with cytosolic sperm extract; and in D 5 μM ionomycin was added.

(Igusa and Miyazaki, 1983; Ozil and Swann, 1995). The sperm-dependent Ca^{2+} oscillations in mammalian eggs, therefore, provide a better "fingerprint" of the physiological signaling mechanism. It is important to examine the precise pattern of oscillations because some artificial agents can cause Ca^{2+} oscillations in mammalian eggs. However, even those artificial stimuli that do cause Ca^{2+} oscillations in mammalian eggs do not mimic the same pattern induced at fertilization (Igusa and Miyazaki, 1983; Swann *et al.*, 1989; Kline and Kline, 1992; Swann, 1992). Consequently, the criterion of causing the same pattern of Ca^{2+} oscillations as seen at fertilization in mammals shall form an important part of our discussion concerning the possible agents that the sperm uses to trigger Ca^{2+} release in eggs.

Figure 1A illustrates the start of the train of Ca^{2+} oscillations seen after fertilization in the mouse egg. These oscillations are of relatively low frequency. The Ca^{2+} oscillations can last for several hours and they generally maintain a constant amplitude during this period. Each of the Ca^{2+} increases in the mouse, and most other mammalian species, lasts for about 1 min (Miyazaki *et al.*, 1993b; Swann and Ozil, 1994), although the frequency and total number of Ca^{2+} increases, or spikes, seem to vary. Some of the variation in mammalian eggs is likely to be species-dependent. However, even in one species, such as the mouse, the range of frequencies varies from one spike every 2 min (Kline and Kline, 1992) to one spike every 20–40 min (Cuthbertson and Cobbold, 1985), depending on the technique for monitoring Ca^{2+} levels. Higher frequency oscillations in hamster eggs are clearly associated with higher degrees of polyspermy (Miyazaki and Igusa, 1981). Although the first few Ca^{2+} transients in hamster eggs are wavelike and similar to those in the sea urchin egg (Miyazaki *et al.*, 1986, 1993b), subsequent Ca^{2+} transients appear to be synchronous pulses. This latter characteristic is itself linked to the fact that the Ca^{2+} oscillations seen at fertilization are associated with a heightened sensitivity to Ca^{2+}-induced Ca^{2+} release (see the following). In hamster eggs, the spatial distribution of Ca^{2+} transients has been measured by imaging the light output from injected aecquorin or by using fura-2 fluorescence combined with a confocal scanning microscope (Miyazaki *et al.*, 1986, 1993b). After the fusion of a single sperm, the first Ca^{2+} increase travels as a wave moving away from the point of fusion, crossing the egg in about 6 sec. The subsequent Ca^{2+} increases start from a more diffuse area near the point of sperm entry and then spread into the remainder of the egg. After several responses have occurred, the Ca^{2+} increases are initiated over the entire egg volume, and no wave is detectable within the time resolution of the measuring system (Miyazaki *et al.*, 1986, 1993b). This suggests that an agent introduced, or induced, by the sperm irreversibly changes the egg so that Ca^{2+} increases are initiated from increasingly larger volumes of the egg cytoplasm until, after several minutes, the Ca^{2+} increase occurs more or less synchronously throughout the egg.

The changes in spatial Ca^{2+} dynamics are themselves linked to a very characteristic change in the egg after fertilization—its sensitivity to Ca^{2+}-induced

Ca^{2+} release (CICR). Injection of Ca^{2+} into unfertilized hamster eggs can cause Ca^{2+} release, but the response requires a relatively large injection of Ca^{2+} and generally can only be observed once in the hamster egg (Igusa and Miyazaki, 1983; Swann, 1991). In mouse eggs, there is no evidence for this kind of CICR in the unfertilized egg (Kline and Kline 1994; Ozil and Swann, 1995). However, after fertilization in mouse, hamster, and rabbit eggs there is an increase in the sensitivity of CICR as is evident in their responses to injections of Ca^{2+} (Igusa and Miyazaki, 1983; Fissore and Robl, 1994), voltage-gated Ca^{2+} (Swann, 1990), and Ca^{2+} influx after electrical field pulses (Ozil and Swann, 1995). This means that very small increases in Ca^{2+} in the egg can trigger regenerative Ca^{2+} release from intracellular stores. The postfertilization increase in sensitivity of this process of CICR in hamster eggs is at least 10-fold (Igusa and Miyazaki, 1983). The assays of sensitized CICR are made some minutes after Ca^{2+} oscillations have been initiated, which corresponds to the time at which the whole egg cytoplasm is the potential source for each Ca^{2+} increase.

In summary, to be considered for the role of a putative fertilization signaling agent, an agent ought to be capable of generating a pattern of Ca^{2+} oscillations similar to those seen at fertilization, with this being linked to a concomitant sensitization of the CICR mechanism in the egg. At least three hypotheses are being advocated for the signaling mechanism used to generate Ca^{2+} release at fertilization (Nuccitelli, 1991; Shen, 1992; Whitaker and Swann, 1993). These are the Ca^{2+} conduit hypothesis (Jaffe, 1991; Creton and Jaffe, 1995), the receptor linked to $InsP_3$ production hypothesis (Jaffe, 1990; Foltz and Shilling, 1993; Miyazaki *et al.*, 1993b; Williams *et al.*, 1992; Ohlendiek and Lennarz, 1995; Dupont *et al.*, 1996), and the soluble sperm factor hypothesis (Dale *et al.*, 1985; Dale, 1988; Stice and Robl, 1990; Swann, 1990; Wu *et al.*, 1997). Before we consider these three ideas, there is one more important factor in understanding the potential signals that are involved in triggering Ca^{2+} release in eggs.

At fertilization, we may not be dealing with a conventional transmembrane signaling problem. This is because the signal to the egg is provided by another cell, the sperm. If sperm–egg fusion precedes Ca^{2+} release, then it is reasonable to consider the problem as one in which one small part of the cell communicates to the greater part. The timing of sperm–egg fusion relative to Ca^{2+} release was first measured in sea urchin eggs by McCulloh and Chambers (1992), who measured fusion electrically by monitoring the increase in capacitance caused by the extra plasma membrane that the sperm contributes at fertilization. In voltage-clamped eggs they found that sperm–egg fusion coincided with the onset of the initial inward current. This inward current response always preceded the start of the Ca^{2+} wave by at least 7 sec (on average 15 sec) (Shen and Steinhardt, 1984). These data show that the sperm and egg are in intracellular contact for several seconds before the Ca^{2+} increase is initiated. In addition to this, it has also been shown that dextran-linked fluorescent dyes can diffuse from the egg into the sperm before the initial Ca^{2+} increase in mouse eggs (Lawrence *et al.*, 1997).

Moreover, these studies in mouse egg suggested that sperm–egg fusion invariably occurred 1–3 min before the first Ca^{2+} spike and that gamete fusion and the occurrence of Ca^{2+} release are so tightly coupled that they cannot be separated from one another (Lawrence *et al.*, 1997). In many other species, fusion has occurred at least by the time the sperm tail stops beating, which again supports the conclusion that gamete membrane fusion takes place prior to Ca^{2+} release (Nuccitelli, 1991; Whitaker and Swann, 1993).

Because sperm–egg fusion appears to precede Ca^{2+} release, we can interpret another notable feature of the fertilization response as the delay, or latent period, that occurs between sperm–egg fusion and the onset of regenerative Ca^{2+} release (Shen and Steinhardt, 1984; Nuccitelli, 1991; Whitaker and Swann, 1993). The latent period should represent the time between sperm–egg fusion and the onset of Ca^{2+} release. Very different latent periods have been found between different related species. For instance, in mammals the first Ca^{2+} transient in hamsters occurs after only 1–10 sec, whereas in mice the time delay is 5–10 min (Miyazaki and Igusa, 1981; Jaffe, 1983). Some of these differences appear to be due to the sperm, because if zone-free hamster eggs are fertilized with mouse sperm, there is also a delay of about 10 min before the onset of Ca^{2+} oscillations (Igusa *et al.*, 1983). Little is known about the events occurring during the latent period except they are very temperature-dependent, with a Q_{10} of $\sim$ 2.3, which suggests that they may reflect the time required for enzymatic reactions (Allen and Griffen, 1958). This is in contrast to the wave of Ca^{2+} release that occurs after the latent period with a Q_{10} of $\sim$ 1.4, which suggests a diffusion-limited reaction (Allen and Griffen, 1958). Ca^{2+} oscillations in mammalian eggs seem to require the continuous presence of an activating stimulus (triggering of a single Ca^{2+} transient does not trigger oscillations). Thus, if the sperm uses the same agent to cause a single Ca^{2+} wave in sea urchin eggs that it does to cause oscillations in mammalian eggs, we would have to assume that it was continuously present or induces the production of some other molecule in the egg cytoplasm for several hours after sperm–egg fusion. This would be compatible with a sperm-mediated mechanism of triggering Ca^{2+} oscillations that involves continuous enzymatic activity.

II. Hypotheses for Activating Ca^{2+} Release in Eggs at Fertilization

A. The Ca^{2+} Conduit Hypothesis

One of the most logical suggestions of how the sperm triggers Ca^{2+} release in eggs is that, after sperm–egg fusion, the sperm itself "injects" Ca^{2+} into the egg (Jaffe, 1983, 1991). Ca^{2+} introduced in this manner could then act as a trigger for further Ca^{2+} release by CICR (Fig. 2A). This hypothesis is consistent with

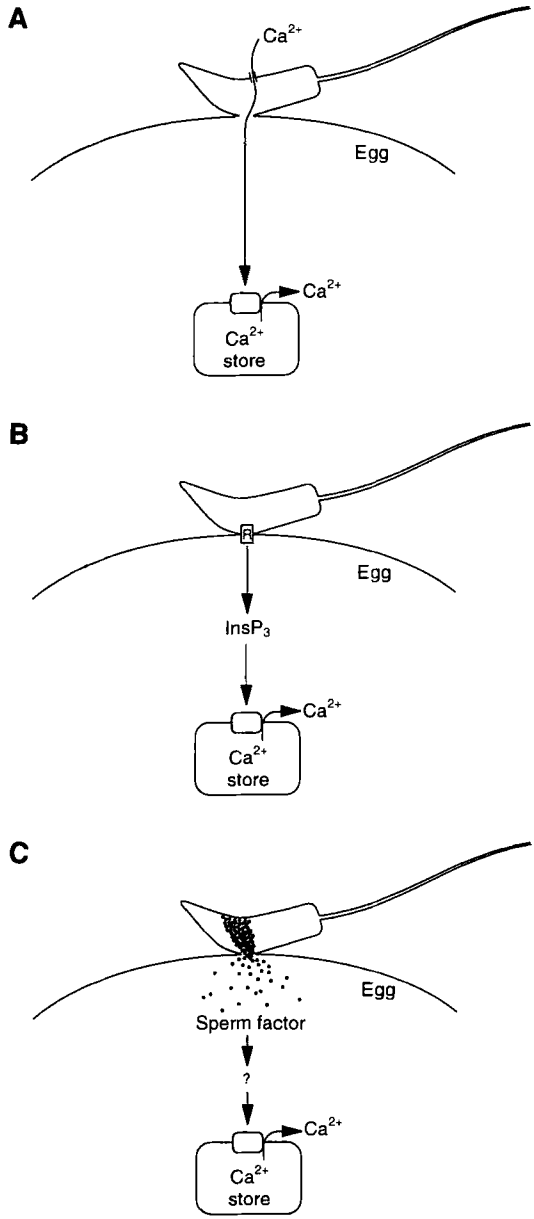

Fig. 2 Schematic diagram illustrating the three models for signaling that result in the triggering of Ca^{2+} oscillations in eggs at fertilization. (A) Sperm as a conduit for Ca^{2+} entry; (B) receptor-linked $InsP_3$ production; (C) soluble sperm factor. See text for discussion.

the finding that sperm–egg fusion takes place before Ca^{2+} release. Further support for this hypothesis is provided by the finding that sperm from many species take up Ca^{2+} prior to fusion with the egg (Jaffe, 1983; 1991). This hypothesis was originally proposed in the simplest form whereby a bolus or "bomb" of Ca^{2+} was introduced by the sperm upon sperm–egg fusion. Another version proposes that the sperm acts as a conduit to enable Ca^{2+} to flood into the egg through Ca^{2+} channels in the sperm membrane (Jaffe, 1991; Creton and Jaffe, 1995). The Ca^{2+} that enters the egg is taken up by the Ca^{2+}-ATPase of the intracellular stores, and this leads to an overload of Ca^{2+} release. Such a Ca^{2+}-overload-induced release has been observed in the sarcoplasmic reticulum of muscle (Endo, 1977) and may be the mechanism by which Ca^{2+} oscillations are generated in skinned cardiac muscle cells (Fabiato, 1983). The conduit model is more plausible than the previous explanation because it requires that there be some delay between sperm–egg fusion and the initial Ca^{2+} release. In this model, the temperature dependency of the latent period may also be explained by the enzymatic activity of the Ca^{2+}-ATPase. It is also supported by observations that the rapid removal of extracellular Ca^{2+} inhibits activation at fertilization in sea urchin eggs (Creton and Jaffe, 1995).

A number of findings suggest that Ca^{2+} alone is unlikely to be the signaling agent at fertilization. In mammals, it is possible to introduce Ca^{2+} into the mammalian egg experimentally in a number of ways: Ca^{2+} can be injected by iontophoresis (Igusa and Miyazaki, 1983; Swann, 1991), action potentials can be generated that cause Ca^{2+} influx across the plasma membrane (McNiven *et al.*, 1988), or electrical field pulses may be used to transiently permeabilize the plasma membrane in the presence of Ca^{2+} (Sun *et al.*, 1992; Ozil and Swann, 1995). However, in each of these cases only a single Ca^{2+} increase is observed, and even in hamster eggs, where there is evidence that CICR can be generated, this kind of response is only seen once in any one egg and oscillations are never produced (Swann and Ozil, 1994). Sustained injection of Ca^{2+} into unfertilized hamster or mouse eggs fails to mimic the Ca^{2+} transients observed at fertilization and at best causes a series of small and critically damped oscillations (Igusa and Miyazaki, 1983; Swann, 1990, 1992, 1994). Similarly, repetitive action potentials in high-Ca^{2+} media also fail to cause oscillations (McNiven *et al.*, 1988; Swann, 1990). In fact, all procedures that artificially increase the influx of Ca^{2+} into the egg lead to a decrease in the sensitivity of CICR (Igusa and Miyazaki, 1983; Swann, 1991). This is the opposite of what is seen at fertilization where there is a more than an order of magnitude increase in the sensitivity of the CICR mechanism (discussed earlier). Thus, it appears highly unlikely that Ca^{2+} influx alone can be the messenger that activates eggs at fertilization in mammals.

The Ca^{2+} conduit model has been proposed for sea urchins on the basis of experiments that show that egg activation by the sperm is inhibited by rapid replacement of the extracellular Ca^{2+} during fertilization (Creton and Jaffe, 1995). These results differ from previous data, which suggested that extracellular

Ca^{2+} was not needed for fertilization and egg activation in sea urchins (Schmidt *et al.*, 1982). However, it still is not clear how the Ca^{2+} influx mechanism works because in sea urchin eggs there is no evidence that CICR exists (Swann and Whitaker, 1986). In mammals, the simple experiment to remove extracellular Ca^{2+} and look at sperm-induced events cannot be carried out because sperm–egg fusion is itself dependent upon extracellular Ca^{2+}. However, the influx of Ca^{2+} at fertilization has been examined by using Mn^{2+} ions as a surrogate for Ca^{2+}. Mn^{2+} ions have the advantage that they can cause the quenching of fura-2 fluorescence, which is normally used to monitor intracellular Ca^{2+} (McGuiness *et al.*, 1996). Consequently, one can monitor divalent cation influx and Ca^{2+} release in a single egg during fertilization. In mouse eggs, it was shown that the measurable divalent cation influx occurs after the initial Ca^{2+} increase at fertilization (McGuiness *et al.*, 1996). This observation is inconsistent with a Ca^{2+} conduit model.

The Ca^{2+} conduit model may be more applicable to other eggs, such as those of the frog, because in these eggs the injection of Ca^{2+} can at least trigger a Ca^{2+} wave similar to that seen at fertilization (Busa and Nuccitelli, 1985). However, it is notable that, in these eggs, injection of other Ca^{2+}-mobilizing agents, such as $InsP_3$, can also mimic the sperm in this respect (Busa and Nuccitelli, 1985; Busa *et al.*, 1985). This observation reinforces the important point about the criterion used for activation, because it is clear that the dynamic pattern of the oscillations in mammalian eggs allows us to readily distinguish the poor mimic from the natural response at fertilization. In frog eggs, the ability to trigger a Ca^{2+} wave by itself does not help one distinguish between candidate messengers. If a common mechanism is involved in generating Ca^{2+} release in vertebrate eggs at fertilization, then we suggest that Ca^{2+} influx is not likely to play the key role.

B. The Receptor-Linked IP_3 Production Hypothesis

The second hypothesis that has been advanced to explain the mechanism of egg activation proposes that the induction of Ca^{2+} release is triggered by a surface-mediated interaction between egg and sperm (Jaffe, 1990; Foltz and Shilling, 1993). Sometimes referred to as the "honorary hormone hypothesis," it envisages the sperm as acting in a manner analogous to the way hormones act in somatic cells (Fig. 2B). Upon binding of the egg and sperm, a ligand on the surface of the sperm is believed to interact with an egg surface receptor, thus triggering a signaling cascade that leads to the elevation of inositol 1,4,5-triphosphate ($InsP_3$) (Jaffe, 1990; Foltz and Shilling, 1993; Miyazaki *et al.*, 1993a; Schultz and Kopf, 1995). The signaling cascade is most commonly believed to involve a G-protein-linked receptor, which upon activation stimulates a phospholipase Cβ; this in turn produces $InsP_3$, which releases stored Ca^{2+}, and diacylglycerol (DAG),

which activates protein kinase C (Foltz and Schilling, 1993; Miyazaki *et al.*, 1993a; Schultz and Kopf, 1995). An alternative proposal is that the cascade proceeds via the activation of a tyrosine-kinase-linked receptor, which can trigger polyphosphoinositide (PI) turnover via phospholipase Cγ (Dupont *et al.*, 1996) and so could produce $InsP_3$ by this pathway (Foltz and Schilling, 1993; Schultz and Kopf, 1995).

The receptor-linked production of $InsP_3$ model has been the most popular proposal for how the sperm triggers egg activation. The widespread involvement of the PI messenger system in calcium signaling in numerous cells and tissue types, together with the finding that the phospholipases needed for generating $InsP_3$ are present within eggs, would be compatible with the involvement of $InsP_3$ in Ca^{2+} release at fertilization (Dupont *et al.*, 1996). Further, the injection of $InsP_3$ has been shown to trigger Ca^{2+} release in eggs of the sea urchin, frog, hamster, mouse, cow, and rabbit (Whitaker and Irvine, 1984; Busa *et al.*, 1985; Swann and Whitaker, 1986; Miyazaki, 1988; Swann, 1992; Kline and Kline, 1994; Fissore and Robl, 1993; Fissore *et al.*, 1995). In addition, injection of nonhydrolyzable GTP analogues or sustained injection of $InsP_3$ can trigger a series of Ca^{2+} oscillations in various mammalian eggs (Miyazaki, 1988; Fissore, and Robl, 1994). Stimulation of cell surface receptors, either endogenously occurring or exogenously expressed, can also trigger Ca^{2+} release and egg activation in starfish, frogs, and mammals (Kline *et al.*, 1988; Miyazaki *et al.*, 1990; Williams *et al.*, 1992; Shilling *et al.*, 1994; Swann, 1992).

Further evidence for the role of $InsP_3$ production at fertilization has come from the use of inhibitors of the PI signaling pathway, although some of the inhibitors used may not be highly specific in their interactions. Heparin is a widely used competitive inhibitor of the $InsP_3$ receptor-release channel (Ghosh *et al.*, 1988). Injection of heparin into egg blocks Ca^{2+} release at fertilization in some species such as the frog, mouse, rabbit, and cow (Nuccitelli *et al.*, 1993; Kline and Kline, 1994; Fissore and Robl, 1993; Fissore *et al.*, 1995), whereas it is reported to have a less significant effect on Ca^{2+} release in sea urchin, hamster, and pig eggs (Rakow and Shen, 1990; Crossley *et al.*, 1991; Miyazaki *et al.*, 1993a; Sun *et al.*, 1994). The dose of heparin used in each set of experiments may be important because an increase in the concentration of heparin from ~200 μg/ml to ~1 mg/ml makes it effective at blocking fertilization in sea urchins (Mohri *et al.*, 1995). However, it is not known whether heparin is acting specifically to only block $InsP_3$ receptors in eggs particularly when used at concentrations >1 mg/ml, because heparin also binds tightly to many cytosolic proteins. Another inhibitor that has also been applied to eggs is the phospholipase C antagonist, U73122, which has been reported to inhibit Ca^{2+} oscillations in mouse eggs (Dupont *et al.*, 1996). However, U73122 can also inhibit strontium-induced Ca^{2+} oscillations in mouse eggs (K. Swann and Y. M. Lawrence, unpublished data), an unexpected observation because strontium is thought to stimulate Ca^{2+} release in cells via direct action on the Ca^{2+}-release channels (Lee, 1993). In addition,

U73122 has been shown to deplete Ca^{2+} stores and inhibit Ca^{2+} influx in some somatic cells (De Moel *et al.*, 1995; Willems *et al.*, 1994). This may be related to the fact that U73122 is a structural analogue of the sulfhydryl reagent *N*-ethylmaleimide and therefore may have multiple sulfhydryl-sensitive targets in the cell. It is also notable that the commonly used "inactive analogue," U73343, is identical to U73122 in all respects expect that it lacks the double bond in the maleimide ring, which is known to be essential for reaction with sulfhydryl groups (Jocelyn, 1972).

An apparent exception to the use of nonspecific inhibitors is the finding that injection of a mouse monoclonal antibody to the $InsP_3$ receptor blocks all forms of Ca^{2+} oscillations in mouse and hamster eggs (Miyazaki *et al.*, 1992, 1993b). The epitope for the $InsP_3$ receptor antibody is in the channel domain of the molecule and does not inhibit the $InsP_3$-binding site (Nakade *et al.*, 1991). This result, therefore, implicates the $InsP_3$ receptor channel in Ca^{2+} release at fertilization, but it does not show that $InsP_3$ is the molecule that activates Ca^{2+} release through the channel.

There is evidence that increased PI turnover occurs at fertilization in sea urchin and frog eggs (Turner *et al.*, 1984; Stith *et al.*, 1994), but the data do not provide much support for a simple theory in which $InsP_3$ production accounts for Ca^{2+} release. Mass assays based on studies of $InsP_3$ levels did not detect an increase at fertilization in sea urchin eggs, although the $InsP_3$ concentration was shown to oscillate during the cell cycle (Ciapa *et al.*, 1994). In frog eggs, a 2-fold increase in $InsP_3$ mass levels was detected at fertilization (Stith *et al.*, 1994). This increase was unaffected by injecting heparin or Ca^{2+} chelators. Because both of these agents block the Ca^{2+} wave at fertilization, we can conclude that the $InsP_3$ increase is caused not by the Ca^{2+} wave, but by the sperm. When polyspermy was induced by a variety of agents such as heparin (Stith *et al.*, 1994), the increase in $InsP_3$ was still about 2-fold. This result was surprising because similar studies that monitored Ca^{2+} changes in heparin-injected eggs suggested that each sperm that fuses after heparin injection causes a local Ca^{2+} increase at the site of fusion (Nuccitelli *et al.*, 1993). Consequently, these data imply that the amount of Ca^{2+} release in heparin-injected eggs is directly proportional to the number of sperm fusing, but this is not closely related to the $InsP_3$ concentration.

There are additional unresolved issues with the receptor linked to $InsP_3$ production model. For egg activation to be triggered via a surface-mediated interaction, a direct link to a Ca^{2+} release mechanism is required. Some candidate molecules involved in the binding and fusion steps of fertilization in sea urchin and mammals have been identified; however, there is no evidence that they are directly involved in Ca^{2+} release in the egg (Myles, 1993; Ohlendiek and Lennarz, 1995; Schultz and Kopf, 1995). In sea urchin, a sperm surface molecule named bindin has been identified as interacting with the egg surface membrane (Glabe, 1985). Bindin is a fusogenic protein and shows amino acid sequence homology to viral fusion proteins (Glabe, 1995; Glabe and Clark, 1991). The

bindin receptors in the egg membrane have been identified as single-pass transmembrane proteins and are not predicted to be directly linked to G-proteins (Foltz, 1995). Addition of binding to sea urchin eggs does not trigger cortical granule exocytosis (a simple assay for the existence of a Ca^{2+} increase) (Abassi and Foltz, 1994). The addition of soluble forms of bindin is also ineffective in causing egg activation, although it does mimic the sperm in causing increased tyrosine phosphorylation of the bindin receptor (Abassi and Foltz, 1994). The increase in tyrosine kinase activity itself does not result in Ca^{2+} release because tyrosine kinase inhibitors do not block cortical granule exocytosis at fertilization in sea urchins (Moore and Kinsey, 1995). These data suggest that *bona fide* receptor stimulation alone does not lead to Ca^{2+} release in sea urchin eggs. In mammals, the key molecules on the egg involved in mediating the egg–sperm interaction appear to be integrins, which are known to be involved in a variety of cell–cell interactions (Myles, 1993; Foltz, 1995; Snell and White, 1996). Integrins may act to bind the sperm to the egg membrane prior to the interaction of gamete membranes, which leads to fusion. One candidate is a protein identified on the surface of guinea pig sperm called fertilin, which shows some sequence homology to viral coat proteins also known to be involved in cell fusion events (Snell and White, 1996). There is no direct evidence that the stimulation of this pathway can lead to Ca^{2+} release in the egg.

The only direct evidence that receptor simulation can lead to Ca^{2+} increases in eggs comes from two sources. In *Urechis* eggs, addition of a species-specific sperm surface bindin can trigger a Ca^{2+} influx response similar to that seen at fertilization (Gould *et al.*, 1986; Gould and Stephano, 1987). It was found that RGD-containing peptides, shown to bind to integrin receptors in a wide variety of systems, stimulated Ca^{2+} increase in frog eggs (Iwao and Fujimura, 1996). Because such experiments result in a Ca^{2+} increase in these species and not in others (discussed earlier), it is possible that there are genuine species differences in the mechanism of egg activation at fertilization. However, it should be noted that Ca^{2+} influx responses indistinguishable from those at fertilization can also be stimulated in *Urechis* eggs by the addition of trypsin (Jaffe *et al.*, 1979). Frog eggs are also known to be able to generate a Ca^{2+} wave, similar to that at fertilization, simply by pricking of the egg with a needle or providing any stimulus that causes a local Ca^{2+} increase in the egg (Busa and Nuccitelli, 1985). Hence, the observation of a Ca^{2+} increase, and of egg activation, in frog and *Urechis* eggs may not be a reliable criterion for assessing potential sperm-borne activating agents. Our thesis proposes that it is only the specific pattern of Ca^{2+} oscillations in mammalian eggs that can provide the required stringent constraints on the selection of candidate activating molecules.

Taking the pattern of Ca^{2+} oscillations at fertilization in mammalian eggs as the benchmark for assessing potential mediators of fertilization would suggest that $InsP_3$ is not the sole mediator of the fertilization process. Although it is widely accepted that manipulations that increase $InsP_3$ in mammalian eggs can

trigger Ca^{2+} release, the pattern of release observed is significantly different from that seen at fertilization (Swann and Ozil, 1994). In hamster egg, the Ca^{2+} oscillations produced in response to injection of GTPγS, after treatment with serotonin, or following injection of $InsP_3$ (or the nonhydrolyzable analogue) are all high-frequency, critically damped responses (Swann *et al.*, 1989; Miyazaki *et al.*, 1990; Galione *et al.*, 1994). This pattern is in contrast to fertilization, where the Ca^{2+} transients show a large range of frequencies but very little damping or decrease in amplitude over 2 hr (Igusa and Miyazaki, 1983). The desensitization to GTP analogues and receptor stimulation in hamster eggs appear to include a negative feedback loop involving protein kinase C (Swann *et al.*, 1989). However, there is also an irreversible desensitization to $InsP_3$ injection itself in hamster eggs (Galione *et al.*, 1994). In mouse eggs, the response to sustained $InsP_3$ injection is not damped, but it is always of higher frequency than that seen at fertilization (Swann, 1994). Reduction of the injection dose of $InsP_3$ leads to lower amplitude Ca^{2+} oscillations, but not lower frequency oscillations. Receptor stimulation in mouse eggs also leads to small-amplitude, high-frequency of Ca^{2+} oscillations that are unlike those seen at fertilization (Swann, 1992). Figure 1B shows Ca^{2+} oscillations induced in mouse eggs after stimulation of acetylcholine receptors by the addition of carbachol. These oscillations are invariably smaller and of higher frequency than those seen at fertilization. It is not yet clear how a simple theory in which the sperm acts on a receptor to generate $InsP_3$ within the egg can explain these data. In addition, when mouse eggs were aged *in vivo*, a desensitization to $InsP_3$-induced Ca^{2+} oscillations was observed, although no desensitization was found in response to the sperm (Jones and Whittingham, 1996). The ability to generate Ca^{2+} oscillations in aged eggs by sperm, but not $InsP_3$, again suggests that the generation of $InsP_3$ is not the critical factor in Ca^{2+} release at fertilization.

C. The Cytosolic Sperm Factor Hypothesis

To accommodate the evidence that remains incompatible with the first two models of fertilization, a third distinct hypothesis has been advanced to explain egg activation by the sperm (Fig. 2C). This model proposes that, after fusion of the gamete membranes, a soluble, cytosolic sperm factor is introduced into the egg and triggers Ca^{2+} release and egg activation (Dale *et al.*, 1985; Stice and Robl, 1990; Swann, 1990; Wu *et al.*, 1997). As with the Ca^{2+} conduit hypothesis, this model requires that the sperm is fused together with the egg before Ca^{2+} release occurs, which has been shown (McCulloh and Chambers, 1992; Lawrence *et al.*, 1997).

Over 10 years ago it was demonstrated that a cytosolic extract made from sea urchin sperm could trigger exocytosis and, by implication, Ca^{2+} release when injected into sea urchin eggs (Dale *et al.*, 1985). Further experiments in ascidian

oocytes suggested that a soluble sperm factor could also trigger membrane current responses similar to those at fertilization (Dale, 1988). The nature of the sperm factor responsible for these effects was not characterized. Subsequent studies led to the suggestion that $InsP_3$ may be the sperm component responsible for egg activation in both sea urchins and humans (Iwasa *et al.*, 1990; Tosti *et al.*, 1993). This conclusion was based upon a mass assay of total $InsP_3$ content in sperm (Iwasa *et al.*, 1990; Tosti *et al.*, 1993). However, these mass assays of $InsP_3$ have also suggested that sea urchin eggs contain micromolar levels of $InsP_3$, which should be capable of causing Ca^{2+} release (Whitaker and Irvine, 1984; Galione *et al.*, 1993). The proposal that $InsP_3$ is the sperm factor in sea urchin eggs is also inconsistent with the observation that heparin injection, at concentrations that can block $InsP_3$-induced Ca^{2+} release, does not block the sperm-induced Ca^{2+} response (Rakow and Shen, 1990; Crossley *et al.*, 1991). In mammalian eggs, a single bolus injection of $InsP_3$ from the sperm would also be insufficient to trigger the sustained oscillations of Ca^{2+} that occur at fertilization (Miyazaki, 1988; Galione *et al.*, 1994).

Other potential sperm factors for sea urchins might include molecules such as cyclic ADP-ribose and cyclic GMP, which act via ryanodine-sensitive Ca^{2+} release channels in triggering Ca^{2+} release. The requirement in sea urchin eggs of both the ryanodine-sensitive and $InsP_3$-sensitive Ca^{2+} release mechanisms implies that a scheme involving small-molecular-weight sperm factors would comprise a cocktail of messengers (Galione *et al.*, 1993). However, if the sperm is envisaged as introducing both $InsP_3$ and cyclic ADP-ribose into the egg, this would not explain the temperature dependence of the latent period at fertilization in sea urchins, because these small molecules would rapidly diffuse and trigger Ca^{2+} release without any obvious requirement for intermediate enzymes (Galione *et al.*, 1993). A mechanism involving cyclic ADP-ribose alone would also have to be species-specific, because it does not trigger Ca^{2+} release in frog or most mammalian eggs (Galione *et al.*, 1993; Kline and Kline, 1994). The difficulty in distinguishing between the various candidate messengers at fertilization in eggs, where only a single Ca^{2+} transient is required for fertilization, further supports the case for studying mammalian eggs, where putative signaling molecules from sperm are required to produce Ca^{2+} oscillations.

Evidence for a mammalian sperm factor has been provided by the finding that injection of cytosolic sperm extracts into mouse, hamster, and human eggs causes Ca^{2+} oscillations similar to those seen at fertilization (Swann, 1990, 1992; Homa and Swann, 1994). Figure 1C shows an example of the Ca^{2+} response in a mouse egg injected with cytosolic sperm extract. The active sperm factor was shown to be of high molecular weight and was destroyed by heat or trypsin treatment, indicating that the factor was protein in nature (Swann, 1990). The sperm factor caused Ca^{2+} oscillations only when injected and was without effect when presented outside the egg. The oscillation-inducing activity, or oscillogen, was only found in extracts derived from sperm and was not detected in extracts from other

tissues (Swann, 1990). Calculations of the number of sperm equivalents injected into each egg suggest that, within an order of magnitude, there is enough activity in a single sperm to trigger the appropriate response at fertilization (Swann, 1990, 1993). The initial finding that sperm cytosol contains a protein-based oscillogen that can cause Ca^{2+} oscillations in mammalian eggs (mouse and cow) has been confirmed independently by others working on mammalian eggs (Wu *et al.*, 1997). Evidence has also been presented for the existence of a high-molecular-weight factor that can cause Ca^{2+} oscillations when injected into eggs of the nemertean worm, *Cerebratulus lacteus* (Stricker, 1997).

The sperm oscillogen mimics the Ca^{2+} oscillations seen at fertilization in a more convincing manner than any other agent, generating Ca^{2+} oscillations that remain of fixed amplitude but vary in frequency, depending on the amount injected (Swann, 1990, 1994). Moreover, the oscillogen can sensitize the Ca^{2+} release mechanisms in the egg in a manner reminiscent of the increase in sensitivity of the CICR mechanisms that occurs after fertilization (Swann, 1990, 1994). Injection of a protein-based sperm extract was also shown to lead to egg activation in mouse and rabbit eggs (Stice and Robl, 1990; Swann, 1990). However, although egg activation is observed after sperm extract injection, it is important to emphasize that activation alone is insufficient to support the claim for a sperm oscillogen that is relevant to fertilization. It is the stimulation of the striking pattern of Ca^{2+} oscillations that resemble those induced by the sperm that provides the essential basis for a sperm factor hypothesis. Indeed, it was noted that injection of the low-molecular-weight fraction from sperm cytosol could occasionally cause a single Ca^{2+} increase in hamster eggs (Swann, 1990). The nature of this low-molecular-weight factor was not investigated further as it does not appear to be required for the distinct oscillatory response at fertilization.

Further indirect support for the cytosolic sperm factor hypothesis in mammals has been provided by the clinical practice of intracytoplasmic sperm injection (ICSI). In this procedure, spermatozoa are injected directly into the egg cytoplasm, leading to fertilization and subsequent development to term (Van Steirtinghem, 1994). Hundreds of successful pregnancies have been made possible by this technique for otherwise infertile couples. The success of this procedure may not have been predicted by the Ca^{2+} conduit hypothesis nor the receptor-linked $InsP_3$ production hypothesis, but it is entirely compatible with the sperm factor hypothesis.

The activation of eggs during ICSI was initially thought to be a side effect of the injection of sperm culture medium (which contains Ca^{2+}; however, some reports suggest that this is not always the case. A moiety within the sperm is believed to be responsible for triggering egg activation, because some reports clearly suggest that the injection of permeabilized sperm is ineffective in causing activation (Dozortsev *et al.*, 1995). However, the evidence from ICSI has to be taken with some degree of caution, because in some species, including humans, there is evidence that the injection procedure alone can activate the egg (Markert,

1983; Gearon *et al.*, 1995). It is therefore of critical importance that there are indications that ICSI can lead to Ca^{2+} oscillations. One report has suggested that ICSI in human eggs is successful because it leads to the generation of Ca^{2+} oscillation several hours after injection (Tesarik *et al.*, 1994). It has also been shown that ICSI in nemertean worms leads to the generation of Ca^{2+} oscillations similar to those seen at fertilization in this species (Stricker, 1996). These experiments demonstrated that boiling the sperm (to denature proteins) prior to injection destroyed their ability to produce Ca^{2+} oscillations, suggesting that the active factor in the worm sperm may be related to the oscillogen in mammals (Stricker, 1996).

Further supportive evidence for the soluble sperm factor hypothesis has come from pronuclear transfer experiments. When the female or male pronucleus from a fertilized zygote is transferred into an unfertilized egg, the membrane of the transplanted pronucleus breaks down, Ca^{2+} oscillations are induced, and the recipient egg is activated (Kono *et al.*, 1995). The ability to trigger these oscillations is specific to the pronuclei of a fertilized zygote. In contrast, pronuclei from parthenogenetically activated eggs, including those from eggs activated by strontium, which also triggers Ca^{2+} oscillations, do not possess this capacity (Kono *et al.*, 1995). The assay of Ca^{2+} release after pronuclei transfer appears to provide an assay that distinguishes sperm-induced stimulation from any known parthenogenetic stimuli in mammals. It was of some interest, therefore, to find that pronuclei from eggs previously activated by the injection of sperm cytosolic extracts could trigger Ca^{2+} oscillations in unfertilized mouse eggs (Kono *et al.*, 1995). These studies support the argument for the existence of a protein oscillogen in sperm cytosolic extracts that, when injected into eggs, can act in the same way as the sperm at fertilization. These pronuclei transfer experiments also suggest that the sperm oscillogen may associate with the newly forming nucleus, either by interacting with the outer nuclear membrane, and hence the endoplasmic reticulum, or by entering into the nucleoplasm.

III. A Sperm Protein Involved in Ca^{2+} Release in Mammalian Eggs

A. The Identification of the 33-kDa Protein

Evidence for the protein nature of the oscillogen suggested that it would be possible to isolate by using conventional protein separation steps. A fractionation procedure with successive chromatography columns that utilized dye affinity interaction, ion exchange, and hydroxylapatite columns was employed with the injection of fractionated sperm extract into mouse eggs as an assay. By using this approach, the oscillogen activity from hamster sperm was found to correlate

specifically with a soluble protein that exhibited an apparent subunit mass of 33 kDa, which has been referred to as oscillin (Parrington *et al.*, 1996). In its native form, the 33-kDa protein exists as an oligomeric complex of ~200 kDa based on nondenaturing gel electrophoresis (Parrington *et al.*, 1996; J. Parrington and F. A. Lai, unpublished). The presence of the 33-kDa protein within an oligomer is consistent with the earlier observation that oscillogen activity is associated with a fraction of >100 kDa (Swann, 1990), although the components of the oligomeric complex are unknown. A high-molecular-weight sperm factor would also be consistent with the pattern of Ca^{2+} oscillations observed during fertilization in mammals (Miyazaki *et al.*, 1986). As described in Section I.A, the Ca^{2+} waves in hamster eggs originate from a trigger zone that slowly spreads across the egg after sperm–egg interaction. The spread of such a trigger zone is a much slower process than the spread of the rapid Ca^{2+} waves themselves. If the diffusion of a sperm protein oscillogen were responsible for this phenomenon, then it would have to be a high-molecular-weight protein (Swann, 1996).

Generation of a mouse monoclonal antibody specific to the hamster 33-kDa protein allowed a number of significant findings on the expression and localization of the hamster 33-kDa protein to emerge (Parrington *et al.*, 1996). First, it was shown by immunoblot analysis that the 33-kDa protein may be sperm-specific, as it was expressed at much higher levels in sperm than in other tissues. This finding is consistent with the lack of oscillogen activity in extracts from somatic cells (Swann, 1990). Second, immunofluorescence localization studies showed that the 33-kDa protein was localized to the equatorial region around the sperm head and confirmed that the 33-kDa protein is a soluble, cytosolic protein. This conclusion could be made because specific staining required sperm fixation, indicating the soluble nature of the 33-kDa protein, whereas nonpermeabilized sperm did not show localized staining, indicating an intracellular localization for the 33-kDa protein (Parrington *et al.*, 1996). The immunocytochemical localization of the sperm hamster 33-kDa protein to the equatorial region of the sperm head is unaffected by the acrosome reaction (K. Swann, J. Parrington, and F. A. Lai, unpublished). In contrast to these findings, immunocytochemistry with a distinct monoclonal antibody, C3, raised against a 29-kDa protein that was found in the final stages of purification of sperm extracts but that did not correlate with oscillogen activity showed a pattern very different from that of the 33-kDa protein, staining a caplike structure associated with the acrosome of hamster sperm (Parrington *et al.*, 1996). The specific equatorial localization of the 33-kDa protein is also of interest given its putative role in triggering Ca^{2+} oscillations at fertilization, as electron microscopy studies have shown that the equatorial region overlying the segment is the first region of the sperm to fuse with the egg (Yanagimachi, 1994; Bedford *et al.*, 1979; Arts *et al.*, 1993). Moreover, the plasma membrane overlying the equatorial segment appears to be the sole fusogenic domain of sperm (Arts *et al.*, 1993), because liposomes made from human spermatozoa only fuse with the equatorial segment.

B. A Novel Mechanism for Generating Ca^{2+} Oscillations

The precise mechanism that the 33-kDa protein uses to generate Ca^{2+} oscillations remains unclear. Cloning of the cDNA encoding the hamster 33-kDa protein suggested a novel mechanism of action due to the observed sequence homology to the bacterial enzyme, glucosamine-6-phosphate isomerase (Parrington *et al.*, 1996). This enzyme in bacteria is believed to function as a deaminase and reversibly catalyzes the metabolism of glucosamine 6-phosphate to fructose 6-phosphate and ammonia (Calcagno *et al.*, 1984). Although it has been studied for some years and its activity has been detected in both prokaryotic (Bates and Pasternak, 1965; White, 1968; Calcagno *et al.*, 1984) and eukaryotic (Comb and Roseman, 1958; Singh and Datta, 1979; Weidanz *et al.*, 1995) cells, knowledge about its exact role remains unclear. Because the bacterial deaminases form into a hexameric structure in solution (Altamirano *et al.*, 1993), the oscillogen may also form hexamers of 33-kDa subunits. Whether the similarity of the hamster 33-kDa protein to a bacterial deaminase as an enzyme has any relevance to the Ca^{2+}-mobilizing properties of the 33-kDa protein has yet to be shown. The idea that the oscillogen response involves an enzymatic process is in accord with the high Q_{10} of the latent period, which occurs between sperm–egg fusion and the onset of Ca^{2+} release (see Section I.C). However, it is not apparent that the 33-kDa protein can cause Ca^{2+} oscillations simply by input into a standard metabolic pathway, because the injection into mammalian eggs of the metabolites, fructose 6-phosphate and glucosamine 6-phosphate, or application of ammonia does not cause Ca^{2+} release or Ca^{2+} oscillations (K. Swann, unpublished). The presence of enzyme activity for the 33-kDa protein that is distinct, but related to that of glucosamine-6-phosphate isomerase activity, and that is linked to generating Ca^{2+} oscillations, still remains a possibility. There are no obvious indications that sugar metabolism is related to Ca^{2+} release at fertilization, although it is intriguing that, in the initial report of the temperature dependence of fertilization, Allen and Griffen (1958) also noted that the only substance that shortened the duration of the latent period was periodate, a compound used diagnostically to oxidize and identify sugars. It may also be relevant that many glycolytic enzymes, including phosphoglucomutase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase, have been shown to interact with intracellular Ca^{2+} release channels (Caswell and Corbett, 1985; Lee *et al.*, 1992).

Additional ideas relating to the signaling mechanism of the sperm oscillogen arise from studies of the effect on eggs of the sulfhydryl agent, thimerosal. Thimerosal has been shown to trigger Ca^{2+} oscillations in hamster, mouse, rabbit, and human eggs that are very similar to those seen at fertilization (Swann, 1991, 1992; Cheek *et al.*, 1993; Fissore and Robl, 1993; Homa and Swann, 1994). Application of thimerosal does not alter $InsP_3$ production (Hecker *et al.*, 1989) but appears to trigger Ca^{2+} release from both $InsP_3$ and ryanodine receptors by directly increasing their sensitivity to CICR (Swann, 1991; Galione *et al.*,

1994). Evidence for direct action of thimerosal on the channels has been obtained from reconstitution studies with purified $InsP_3$ and ryanodine receptors (Kaplin *et al.*, 1994; Abramson *et al.*, 1995). This is consistent with an involvement of both $InsP_3$ and ryanodine receptor Ca^{2+} release channels in the generation of Ca^{2+} oscillations at fertilization or after sperm extract injection (Miyazaki *et al.*, 1992, 1993b; Swann, 1992; Jones *et al.*, 1995; Ayabe *et al.*, 1995). However, unlike fertilization and sperm-extract-induced oscillations, the effects of thimerosal are reversed by adding the reducing agent dithiothreitol (Swann, 1991; Cheek *et al.*, 1993). This would require that the sperm oscillogen does not act solely as a sulfhydryl-oxidizing agent, but that it effects a novel modification of the Ca^{2+} release channels in the egg that could lead to the generation of the same pattern of Ca^{2+} oscillations that was seen at fertilization.

There is the possibility of a much wider role for the 33-kDa protein as a cell signaling molecule in somatic cells. Sperm cytosolic extracts have been shown to trigger Ca^{2+} oscillations when injected into rat dorsal root ganglion neurons (Currie *et al.*, 1992) and hepatocytes (Berrie *et al.*, 1996). The Ca^{2+} oscillation in rat hepatocytes are similar in character to those seen in mammalian eggs, persist in Ca^{2+}-free media, and, hence, appear to be due to the release of Ca^{2+} from intracellular stores (Berrie *et al.*, 1996). These findings suggest that the action of the 33-kDa protein to generate intracellular Ca^{2+} signals involves components common to many cells. Whether this implies that the 33-kDa protein also has an endogenous role in such somatic cell types remains to be demonstrated. Although immunoblot analysis indicated that the hamster 33-kDa protein was relatively abundant in sperm (Parrington *et al.*, 1996), the protein may be present but at low levels in other tissues. The apparent germ cell specificity or enrichment of the 33-kDa protein compared to other tissues, indicates that the control mechanisms governing expression in somatic cells may be very different from those operating during spermatogenesis.

C. Are There Multiple Signaling Mechanisms at Fertilization?

In our discussion of the way in which a sperm causes Ca^{2+} release, and in the schematic diagrams of Fig. 2, we have considered each potential mediator or mechanism in isolation. It is possible that more than one signaling mechanism could operate at fertilization. By analogy with receptor-mediated signaling in somatic cells, it has been suggested that multiple mechanisms may be operating in parallel when the sperm activates an egg (Foltz and Shilling, 1993; Belton and Foltz, 1995; Schultz and Kopf, 1995). If the sperm were to stimulate multiple pathways, this would imply that there is more than one sperm-derived activating agent. In mammals, it has been suggested that the receptor-linked production of $InsP_3$ mechanism operates to trigger the initial Ca^{2+} increase, with a sperm cytosolic factor being introduced into the egg that is responsible for the

later Ca^{2+} oscillations (Miyazaki *et al.*, 1993b). Further complexity would be added if there is also the possibility that species-specific differences exist in eggs, with each species using a different array of messengers (Whitaker and Swann, 1993).

A hypothesis that employs multiple messengers cannot be excluded at this time, although there is no compelling evidence supporting such an idea. The existence of just one protein factor, the sperm oscillogen, that initiates the Ca^{2+} changes and egg activation at fertilization is also entirely plausible for two principal reasons. First, all of the events necessary for activation at fertilization appear to be triggered by a single response, the increases in Ca^{2+}, whereas in somatic cells increases in Ca^{2+} alone appear to be insufficient to stimulate proliferation, a process that requires multiple pathways to be utilized (Berridge, 1993). Because egg activation and subsequent cell divisions can all be linked to the Ca^{2+} signal, it is possible for the sperm to use one signaling agent as there is only one target: the release of Ca^{2+} from intracellular stores (Whitaker and Steinhardt, 1982; Jaffe, 1983). Second, in mouse and hamster eggs, the entire pattern of oscillations at fertilization can be simulated by the injection of sperm extracts (Swann, 1990, 1994). Mimicking of the precise frequency of oscillations induced by the sperm is related to the dose, because the frequency of oscillations increases with the amount of sperm oscillogen injected into the egg. A mechanism that employed both the oscillogen and increased $InsP_3$ production, or an oscillogen and increased Ca^{2+} influx, would invariably lead to a much higher frequency of Ca^{2+} oscillations than that seen at fertilization in mouse eggs (Swann, 1994). Therefore, the sperm oscillogen alone may offer a sufficient and simple explanation of the pattern of Ca^{2+} oscillations and all the other events of egg activation in mammals.

We propose a simple scheme for signaling at fertilization, as illustrated in Fig. 2C. In this sperm oscillogen hypothesis, the soluble proteins diffuse from the sperm head into the egg at fertilization to trigger Ca^{2+} release from intracellular Ca^{2+} stores. The oscillogen mediates a process resulting in a decrease in the threshold for CICR that can occur via either of the known intracellular Ca^{2+} release channels (Berridge, 1993). The action of the sperm oscillogen may involve the modulation of Ca^{2+} release via specific sulfhydryl groups that are common to both types of Ca^{2+} release channel (Kaplin *et al.*, 1994; Abramson *et al.*, 1995). Once CICR is sensitized to a critical threshold, Ca^{2+} release occurs regeneratively from resting Ca^{2+} levels. Diffusion of the oscillogen throughout the egg cytoplasm would then facilitate Ca^{2+} release from different regions of the egg cytoplasm, a process that has been observed by using confocal scanning of mouse eggs after sperm extract injection (Carroll *et al.*, 1994). A sperm factor related to the mammalian sperm oscillogen may also be involved in the initial trigger for Ca^{2+} release in nonmammalian eggs, such as those of the frog and sea urchin. However, in these species the sperm factor is required to trigger only a single endogenous wave of Ca^{2+} release, which itself employs a mechanism

different from that of the sperm oscillogen because it has a different temperature dependence (Allen and Griffen, 1958).

Finally, we emphasize that the sperm oscillogen model can still incorporate a role for increased PI turnover at fertilization (Turner *et al.*, 1984; Nuccitelli, 1991) that entails the generation of diacylglycerol and stimulation of protein kinase C. In sea urchin eggs, increased turnover of PI phospholipids can occur as a result of the initial Ca^{2+} rise (Whitaker and Irvine, 1984), and indirect assays of protein kinase C stimulation at fertilization suggest that it is activated via the Ca^{2+} increase (Crossley *et al.*, 1991). In mammalian eggs, it is also evident that protein kinase C stimulation at fertilization follows a transient Ca^{2+} increase (Collona and Tatone, 1993; Gallicano *et al.*, 1993). Thus, a sperm protein that functions to release Ca^{2+} from stores could be the single messenger that activates the egg and stimulates multiple downstream signaling pathways, including an array of phospholipid messengers.

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8

The Development of the Kidney

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I. Introduction

This chapter sets out to describe what is known about the earlier stages of development of the vertebrate metanephric kidney [the development of the pro- and mesonephroi is reviewed in Vize (1997)]. We will concentrate mainly on the mouse and use descriptive morphology as a platform for considering both the molecular mechanisms underpinning kidney morphogenesis and differentiation and the ways in which these processes can go awry and lead to congenital kidney

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disorders, particularly in humans. As will soon become apparent, however, it is not possible to consider any one of these aspects in isolation: Such has been the speed of progress over the past decade that the study of congenital kidney disease and the search for key genes that regulate nephrogenesis are as the two wheels of a bicycle. Moreover, the study of the molecular biology of the developing kidney is illuminating aspects of developmental morphology that have received very little attention in the past. Indeed, kidney development is turning out to be much more complicated than was suspected even 5 years ago, and we are nowhere near the position where the basic molecular mechanisms controlling kidney development are even approximately understood. This survey thus is to be seen as the view of the traveller.

Those who are in the field and who set out to read this chapter will already know why the kidney is such a wonderful model system for developmental biologists, especially for those who want to maintain a foothold in medical research. For the casual browser of these pages whose knowledge of nephrogenesis may be limited, we start with a brief summary that covers what goes on as the kidney forms and how its development can go awry and lead to congenital abnormalities and then list the tools for investigating these events and the problems that are under investigation. We hope that, having tasted this *hors d'oeuvre*, browsers from other fields will then want to stay for the rest of the meal.

To pursue this metaphor a little further, the reader of this chapter might expect to find each course weighed down with a substantial helping of molecular data, and, indeed, the past decade has produced an enormous wealth of information on patterns of gene expression in the developing kidney. We have chosen to be as light with this ingredient as possible, mainly because the detail makes heavy reading and will certainly quickly become out-of-date, and because much of it does not illuminate the processes of nephrogenesis in any profound way. Therefore, we have chosen to concentrate more on the biology of the system, but an up-to-date summary of the very great majority of gene expression patterns can always be found at the web site of the Kidney Development Database (Davies and Brandli, 1997); the reader is encouraged to use this resource to illuminate more fully the issues discussed in this chapter.

A. A Simple Introduction to the Formation of the Mouse Kidney

The mature kidney is a fairly complex organ attached to an arterial input vessel and two output vessels, the vein and the ureter. Inside, the artery and vein are connected by a complex network of capillaries that invade a large number of glomeruli, the proximal entrance to nephrons, which are filtration units that link to an arborized collecting-duct system that drains into the ureter. In addition, there are nerves and the juxtaglomerular apparatus, a set of specialized cells that produce renin. It should be said at the beginning that almost all of the work on the

development of the kidney has focused on how the nephrons and collecting-duct systems form, particularly in their early stages, but, as is now becoming clear, the other components play a more substantial role in development than was previously expected.

The essentials of kidney development have been known for many years and this review stands on many others, with the reader being directed to those of Saxén (1987) and Ekblom (1992) in particular for coverage of the classic experimental work on the emergence of the developmental phenotype. It is more difficult to recommend other general reviews that cover work on the underlying genetic regulatory mechanisms, not because they are wrong or dull, but because such is the speed of progress that even the most recent have been overtaken by the flood of molecular data. This, together with other useful information, should always be obtainable from the Kidney Development Database (Davies and Brandli, 1997), as mentioned previously.

Although the adult kidney is complex, its early morphogenesis seems relatively simple. The mouse metanephros starts to form just before embryonic day 11 when, on each side of the embryo, an epithelial tube called the ureteric bud grows from the nephric duct and extends some 200–300 μm into a small dense mass of about 5000 cells, called the metanephrogenic mesenchyme, which is located within the intermediate mesoderm at about the level of the middle of the hindlimb. A few hours after the bud invades the metanephrogenic mesenchyme, a reciprocal interaction takes place with two main results. First, the bud starts to bifurcate, eventually forming the collecting-duct system that will drain through the nephric duct into the bladder. Second, the metanephrogenic mesenchyme starts to differentiate, first into stem cells (E11.5), which are seen at the kidney periphery, and later into their descendants, the nephrons of the cortex (>E13) and the mature stroma of the medulla (Fig. 1). At about this time, the neural and vascular components of the kidney start to differentiate.

Developing kidneys show an unusual geographical feature: Those nephrons that are at the cortex–medullary border are the oldest formed and most mature, those within the cortex are younger, and those at the periphery, where the tips of the growing collecting-duct system are containing metanephrogenic stem cells, are new nephrogenic condensations that are still forming. Given that the kidney blood vessels enter and exit with the ureter, the kidney thus can become functional at its center while its periphery is still differentiating.

This brief summary, most of which could have been written 40 years ago, highlights the diverse set of developmental mechanisms involved in kidney morphogenesis. The more obvious of these are the following: budding of the nephric duct and its directed extension into the metanephrogenic mesenchyme; reciprocal inductions between the bud and the metanephrogenic mesenchyme; branching morphogenesis; control of stem-cell growth and differentiation; formation of mesenchymal condensations; mesenchyme-to-epithelial transition; epithelial morphogenesis and fusion; and patterning of the filtration capabilities

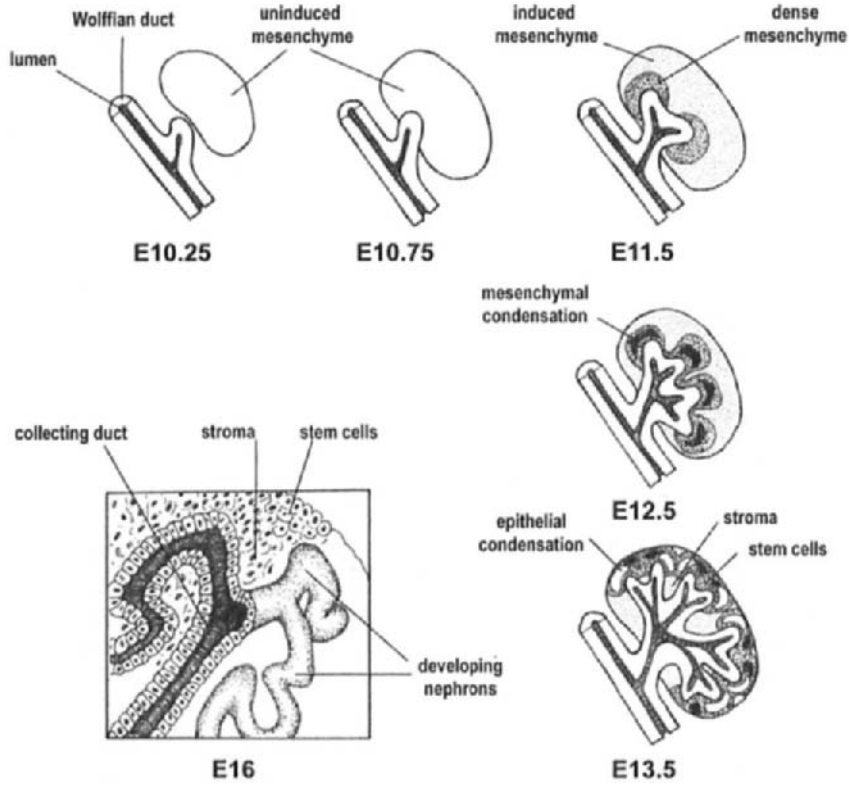

Fig. 1 The development of the mouse kidney. At E10.25 or so, the ureteric bud forms off the Wolffian duct and extends towards the uninduced mesenchyme of the metanephric blastema, reaching it some 12 h later. Over the next day, the bud invades the mesenchyme and starts to bifurcate while the mesenchyme is induced, with some of it condensing around the tips of the duct. A day later (E12.5), small dense (black) condensations form that will become nephrons. Over the next day, and as the bud continues to bifurcate to produce the collecting-duct system, a profound change takes pace in the mesenchyme lineage. Here, existing condensations epithelialize (white) as new condensations (black) continue to form from the dense mesenchyme. This is now at the periphery of the rudiment where it acts as a population of stem cells, while the less dense mesenchyme has moved toward the center of the rudiment where it will form stromal cells. By E16, the epithelialized nephrons have fused to the collecting-duct system. (Drawings are not to scale and were produced by Amy Carless.)

along the length of the nephron. Thus, there are direct analogies between the kidney and many other tissues, and aficionados of embryogenesis will realize that this list covers all major developmental mechanisms. As will become clear later, kidney formation also requires most of the minor ones too!

One reason for studying the kidney is the belief that solving problems in this tissue will be easier than in other organs and, hence, that work on the kidney has

a greater significance than it might appear at first sight (We are aware that the same claim has been made for many other tissues but tend to find such claims unconvincing, as will be discussed). Another is that some important congenital kidney disorders in humans can be approached through mouse models (the best known being Wilms' tumor, in which growth and differentiation of the stem cells go awry); indeed, such disorders and others that can be manufactured through transgenic technology (Table I) are providing valuable keys to understanding normal kidney development (see later discussion).

B. Strategies and Tools: *In Vitro*, Markers, Knockouts, Cells, and Cell Line, the Kidney Development Database

The main reason for making the claim that we will understand the morphogenesis of the kidney before that of other equally complicated tissues is that many of the events already outlined will take place in relatively simple organ culture under well-defined conditions (one unexplored exception seems to be stem-cell maintenance and growth). In such cultures, branching morphogenesis and nephron formation occur, with each nephron extending, differentiating, folding to form a glomerulus, and fusing to a growing duct (Grobstein, 1955).

Table I Developmental Kidney Diseases[a]

Disease	Pathology	Etiology (if known)
ADPKD	Cystic	Genetic: autosomal dominant mutation of polycystin
Alport syndrome	Glomerular defect	Collagen chain mutations
ARPKD	Cystic	Genetic: autosomal recessive, gene unknown (6p21 in human)
Beckwith–Wiedemann	Growth disorder	Genetic: autosomal, gene unknown (11p15 in human)
Meckel's syndrome	Cystic	Genetic: autosomal recessive, gene unknown (17q in human)
Medullary cystic disease	Cystic	Genetic: autosomal dominant
Nephronophthisis	Cystic	Genetic: autosomal recessive, gene unknown (2p in human)
Tuberous sclerosis	Cystic	Genetic: autosomal dominant mutation of tuberin
von Hippel–Lindau	Cystic	Genetic: autosomal dominant, gene unknown (3p in human, probably cloned)
Wilms' tumor	Tumor	Genetic: autosomal recessive, ~15% due to mutation in WT1 (11p13 in human)

[a]For additional details, see Section III of the text.

The ability of the kidney and, indeed, isolated metanephrogenic mesenchyme (MM), to develop in culture (Fig. 8) means that the developing tissues can be subjected to a wide variety of experimental procedures designed to investigate their molecular and cellular properties and to test hypotheses about developmental mechanisms. The accessibility of the kidney to this type of investigation is underlined by the fact that expression patterns of some 250 genes are described in the Kidney Development Database, and the availability of this resource is a second reason for believing that we will understand the molecular interactions that underpin kidney development before those of other tissues.

Nevertheless, the problem with gene expression data in the kidney, as in every other tissue, is that knowledge of expression does not necessarily lead to an understanding of function (except under the guilt-by-association hypothesis). Knockout data using either transgenic mice *in vivo* or antisense technology *in vitro* can help to determine the function of key molecules, although the redundancy question remains as difficult to deal with here as elsewhere.

C. What This Review Includes and What It Sets Out To Achieve

What we would *like* to include in this review is a brief summary of the key steps in the development of the kidney followed by a terse analysis of the cellular and molecular mechanisms that underpin them. This would then be followed by a section showing how they go awry in congenital kidney disorders. Such a review is, unfortunately a decade or two into the future. Instead, this review proper starts with a slightly more detailed description of the various stages of normal kidney differentiation (Section II.A), which is followed by a summary of the roles played by growth and death (Section II.B) and a discussion of the extent to which kidney cell lines will emulate normal developmental processes *in vitro* (Section II.C). The following section covers kidney disorders, either natural [Wilms' tumor (Section III.A) and polycystic kidney disorders (Section III.B)] or induced through homologous recombination in transgenic mice. In a sense, these sections pose the detailed questions currently being investigated. The next and longest section considers the progress that has been made toward finding these answers, particularly with respect to competence in the metanephrogenic mesenchyme (Section IV.A), lineage relationships for the various cell types in the kidney (Section IV.B), ureteric bud induction and branching morphogenesis (Section IV.C), and mesenchyme induction (Section IV.D) and its downstream effects (Section IV.E). Accompanying these sections are tables that detail some of the core data about kidney development. It will soon become apparent that none of the obvious problems of kidney development as yet has a completely satisfactory solution. The cynic might say that the past decade of work has merely shown that things are far more complicated than the early workers ever expected; the cynic would, as ever, be correct, but would not be telling the whole truth. As a result of much clever work in the past few years, we not only have a far deeper insight into

the phenomenology of kidney development but are beginning to correlate it with the ever-increasing amounts of molecular data that are now available.

The study of kidney development is thus passing through interesting times as it seeks to make the leap from the phenotype to the genotype. It is not easy to see where the journey will lead, but the chapter ends with a view of the foothills that are likely to be surmounted in the next few years (Section V.A) and the mountains over whose tops we can hope to see in a decade's time (Section V.C). The developing kidney is small but complicated, accessible but opaque, and interesting but challenging. There is much to be done and work for as many as want to solve its problems.

II. Growth and Differentiation

A. Growth and Development of the Kidney *in Vivo*

1. Normal Development

All metanephric ("permanent") kidneys that have been examined initially develop in a similar way. Later development in larger organisms differs from that in smaller ones in the production of lobed kidney not present in the smaller organisms (this variant increases the surface:volume ratio as the diameter increases, thus allowing more nephrons to form from peripherally located stem cells). Here, we consider the mouse kidney because it has been subjected to the closest analysis, but the reader should not expect there to be major differences from the kidneys of other organisms, other than in timing.

Once induced, the kidney rudiment grows approximately exponentially (Fig. 2) until birth, initially doubling in volume every 8 hr or so, but slowing down to double in about 12 hr for the last few days of gestation. A very wide range of activities does, however, take place behind this envelope of regularity, and it is perhaps surprising that the net result is such uniform growth. It is convenient to divide kidney development into three distinct phases: (i) establishment, (ii) steady-state growth, and (iii) the mature state.

In the first phase that immediately follows induction (~E10.75), the ureteric bud extends and bifurcates while the MM segregates (~E11.5) into a prenephrogenic and a prestromal component, with the former adjacent to the bud and the latter occupying the periphery of the rudiment. One day later (E12.5 for the mouse), these components seem to have reversed locations and the nephrogenic stem cells have taken up their characteristic location at the cortex of the rudiment. It is probably at this point that the first nephrogenic condensations form and start to differentiate.

The second phase, that of steady-state development, starts when the essential features of the developing kidney are in place (~E13.5) and involves the ureteric bud–collecting-duct tree continuing to extend and bifurcate while the cortical stem cells divide rapidly, maintaining themselves and continuing to produce

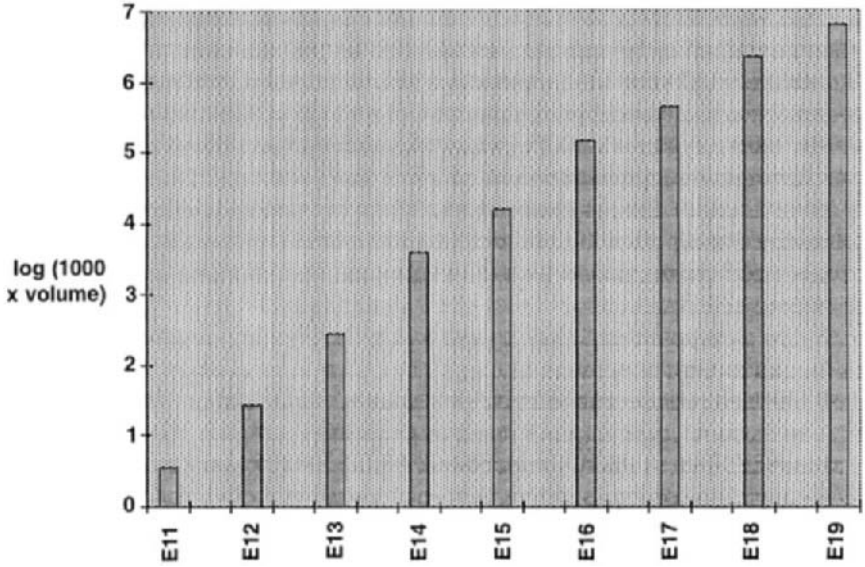

Fig. 2 The growth of the kidney. A log plot showing that the kidney volume (mm^3) grows exponentially, doubling in volume approximately every 9 h, up to about E16 when relative growth rates slightly decline (data courtesy of Anne Grattan).

small mesenchymal condensations adjacent to the tips of the arborizing collecting-duct system. The condensation and subsequent differentiation of the nephrons continue in the cortex until near birth, so that the farther a condensation is from the periphery, the older (and more differentiated) it is. At this stage, therefore, geography reflects history.

Each condensation undergoes a series of changes that leads to it forming a nephron: First, it becomes epithelial and forms a small cyst. The cyst then sprouts two tails. The first, on the side opposite the duct, gives it a comma shape (capillaries soon invade the space between the tail and the condensation to initiate the renal capsule), whereas the second (which will become the distal tubule) is near the collecting duct and gives the structure an S shape. The subsequent events are not entirely clear, but observations using markers specific for the duct system suggest that the domain of collecting duct adjacent to the distal rudiment extends and fuses with it (Davies, 1994). At the same time, the renal capsule differentiates and the proximal tubule forms. Differentiation to this stage probably takes about 2–3 days.

In this second phase, the medullary region of the developing kidney seems to include no more than the base of the collecting-duct tree, which is embedded in a very loose stroma of cells and a rich extracellular matrix and was, for many years, assumed to take a relatively passive role in the proceedings, merely providing space that the loops of Henle could invade. It is, however, worth mentioning for two reasons. First, the observations on the BFK−/− mouse (Hatini *et*

al., 1996), which are discussed later, have forced us to revise the role of the medullary cells in mediating nephrogenesis, and, second, several hundred neurons together with a renal ganglion differentiate within this stroma, even *in vitro* (Karavanov *et al.*, 1995).

The third phase of kidney development reflects the end of nephron morphogenesis and the beginning of kidney function. There is, of course, no sharp border between these second and third phases because mature nephrons located at the corticomedullary border can be functional while the more peripheral nephrons are still differentiating. Nevertheless, on the basis of the morphology and emergence of differentiation markers in the nephrons and the behavior of the embryo, it seems as if the metanephros starts to function at around E16 when the mature nephron structures start to form and their loops of Henle descend into the medulla. Indeed, it is at about this time that the collecting-duct system starts to expand at its base and form calyces, the enlarged lumens into which urine flows on its way to the ureter.

By now, the great majority of nephrons that will form are in place, and one can get some sense of the growth that has occurred by considering the number of nephrons that are present. Although exact counts have not been made, two independent lines of analysis suggest that there are 1000–2000 nephrons in the mature mouse kidney. On the basis of relative size, the mature mouse kidney (~8 mm across) has about 0.1% of the volume of a human kidney (~10 cm across) with its 1.5 million nephrons (Fawcett and Raviola, 1994) and so should have ~1000 nephrons. Absolute size calculations give a similar figure: In the mouse kidney, each nephron is about 4 mm in length and about 100 μm in diameter apart from its larger, spherical glomerulus. If we assume that 50% of the kidney's volume is composed of nephrons, then there is space for about 2000 nephrons with each being composed of several thousand cells. This is an impressive figure for it means that, as the initial mesenchymal blastema itself had several thousand cells, each on average probably gave rise to one or two nephrons.

As birth approaches, the ability of the stem cells to produce nephrogenic condensations declines and these stem cells are lost soon after birth (in the human, small groups may be found that are called "rests," and mutations in these can lead to Wilms' tumor). Meanwhile, the stromal cells seem to be lost mainly because their division rates are so slow compared with the net growth in organ volume (Sainio *et al.*, 1994). With the loss of the stem and stromal cells and the filling of the medulla with loops of Henle, the metanephros acquires its adult form [for details, see, for example, Fawcett and Raviola (1994)].

B. Growth and Death

1. Mitosis

As has already been noted, the metanephros grows remarkably fast, doubling in size every 8 hr or so over the period E11–E16, and, as might be expected, many

of the cells express the proliferating cell nuclear antigen (PCNA), a marker for being within the S phase (L. McLaren and J.B.L. Bard, unpublished). The most remarkable feature is the degree of PCNA expression within the stem-cell population in the cortical rind, where almost every cell seems to express the antigen during the period E12–E16. A similar result is obtained with the use of BrdU where, at E16, more than one-half of the cortex is in the S phase at any moment as compared to about 12% for the medulla (Sainio *et al.*, 1994).

It is not clear, at first sight, whether the rapid growth of the kidney requires locally synthesized growth factor or whether it reflects the intrinsic ability of all of the kidney cell types to divide in the presence of nonspecific factors found ubiquitously in serum. The fact that kidney rudiments will develop, to some degree at least, in serum-free medium supplemented only with transferrin suggests at first that all necessary growth factors are synthesized by the kidney itself. However, things are not quite that simple. The growth of the kidney in culture is actually rather slow, and even after 4 days in the presence of serum it has only doubled in size twice (Bard and Ross, 1991). After that, there seems to be further differentiation, but no more growth. As cultured kidneys flatten in culture, this observation is compatible with the need for replacement of the kidney-synthesized factors that diffuse away from the rudiment.

The observations on two types of knockout mice provide positive evidence that the growth of the metanephric mesenchyme needs such factors (see Fig. 9). Loss of BMP-7 has no effect on early nephrogenesis, but, in its absence, growth soon slows and the mice are born with small kidneys and few nephrons (Dudley *et al.*, 1995), a result suggesting that BMP-7, which is synthesized by the cells of the collecting duct, is needed for the growth of the stem cells. This view is confirmed by the observations of Vukicevic *et al.* (1996), who showed that the addition of BMP-7 to isolated but induced metanephric mesenchyme *in vitro* enabled the cells to divide and differentiate. The second knockout is that of BF-2, a transcription factor expressed in the stromal cells of the medulla (Hatini *et al.*, 1996). In the BF-2−/− mouse, however, it is the cortical cells that fail to grow or form proper nephrogenic aggregates (discussed earlier). It is thus clear that BF-2 controls the production, by the stroma, of factors that regulate growth and differentiation of the metanephrogenic mesenchyme. As the only growth factor known to be produced by the stroma is TGFβ, and as its knockout has no effect on kidney development (Boivin *et al.*, 1995), it seems as if there are additional growth factors regulating growth and development and synthesized within the stroma that are still to be discovered.

As for the other cell types, things are clear only for the growth of neurons for whom the growth factor neurotrophin-3 seems necessary for survival and differentiation (Karavanov *et al.*, 1995). This factor generally is needed for neural crest cell differentiation and thus points to a neural crest source for the neuronal precursors.

2. Apoptosis

Much of the mitogenic effort is, in a sense, wasted: A large proportion, estimated to be as much as 50%, of the cells in the developing kidney are born to die (Camp and Martin, 1996). This is a surprisingly high number given the fact that the kidney doubles in size approximately every 8 hr (McLaren and J.B.L. Bard, unpublished). In p53-overexpressing mice even more cell death takes place (Godley *et al.*, 1996), but a small kidney still forms. Reference is made later to the fact that uninduced metanephrogenic mesenchyme shows strong suicidal tendencies, which are laid aside only when it is induced into the stem-cell state. However, it has become apparent that apoptotic death plays a role in development even after induction has taken place.

Assays for apoptosis based on DNA degradation, nuclear staining, and electron microscopy (Koseki *et al.*, 1992; Coles *et al.*, 1993) have revealed prominent apoptosis throughout all stages of renal development. One major wave is associated with early nephron formation, with many of the cells adjacent to newly forming nephric epithelia showing pyknic nuclei (Koseki *et al.*, 1992); this may reflect a mechanism to eliminate cells that were involved in the first stages of condensation, but were not included in the set that became committed to epitheliogenesis. A second wave is seen in the S-shaped body, where many cells in the region of the glomerular cleft die (Coles *et al.*, 1993). A third wave, occurring later, is seen in the medullary epithelia, including the ureteric bud.

The extent of apoptosis in a developing system generally is controlled by specific trophic factors (Raff, 1992). Coles *et al.* (1993) have shown that renal apoptosis can be dramatically reduced by treatment with excess epidermal growth factor (EGF), and it has been suggested that is possible that this and other growth factors are used to match the number of cells available for nephrogenesis to the length of the ureteric bud available for connection to nephrons (Camp and Martin, 1996). In transgenic mice lacking *bcl-2* (see Fig. 9a,b, below), there is too much early apoptosis followed by hyperproliferation, perhaps in an attempt to compensate for the abnormally high apoptosis. The result is the formation of epithelial cysts (Veis *et al.*, 1993). Whereas the precise mechanism of cyst formation is not yet understood, the phenotype of these *bcl-2−/−* kidneys provides strong evidence for the importance of a proper balance between proliferation and death if normal anatomy is to develop.

C. Differentiated Cell Types That Develop in the Kidney

As morphogenesis takes place, differentiation markers are acquired and lost, and much of the work in the latter part of the 1980s was dedicated to documenting these changes [see, for example, Ekblom (1992)]. The development of the nephron can therefore be documented by changes in gene expression profiles, and it is

Table II Useful Markers for Different Tissue Types in the Developing Kidney

Tissue type	Useful marker
Ureteric bud–developing collecting duct	Calbindin-D-28K (unique to developing CD in normal culture conditions, but *in vivo* or in culture medium containing 1,21-dihydroxyvitamin D_3 mature distal tubules also express it; Davies, 1994).
	Cytokeratin 7 (Moll *et al.*, 1991)
	IGFBP3 (Matsell *et al.*, 1993)
	KDN-1 (Burrow, 1993)
Uninduced metanephrogenic mesenchyme	Pax-2 not yet induced (Rothenpieler and Dressler, 1993); this tissue will only be present at the very early stages of renal development
Stem cells	I1 (Svennilson *et al.*, 1995) with Pax-2 (Rothenpieler and Dressler, 1993)
Nephrogenic condensates	Strong NCAM (it is expressed weakly in MM and stem cells, but greatly up-regulated in condensates; Klein *et al.*, 1984)
	WT-1 also up-regulated (Armstrong *et al.*, 1992)
Early epithelium	No unique marker, but one could use Pax-8 (Poleev *et al.*, 1992) in the absence of glomerular, proximal tubule, and distal tibule markers
Developing glomerulus	α_3-Integrin (Korhonen *et al.*, 1990, 1992)
	Desmin (some cells only; Bachmann *et al.*, 1983)
Proximal tubule	CD15 (Bard and Ross, 1991)
	Brush border antigen (Miettinen, 1986)
Distal tubule	Uromucoid (Tamm–Horsfall antigen) (Hoyer *et al.*, 1974)
Stroma	Tenascin (Aufderheide, 1987)
	BF-2 (Hatini, *et al.*, 1996)
Endothelium	PECAM (Baldwin *et al.*, 1994)
Juxtaglomerular apparatus	Renin (but earliest expression is more widespread; Jones *et al.*, 1990)
Neurons	Neurofilament 200 (Sainio *et al.*, 1994)

possible to identify many cell types by using specific markers (Table II). It will be helpful to list here the range of cells, with a key gene or two that it expresses and that can be used as a marker(s).

D. Kidney-Derived Cell Lines as Models for Development

1. Classical Renal Cell Lines

Kidney-derived cell lines, such as Madin–Darby canine kidney (MDCK) collecting-duct epithelium, have long been used in cell culture work for investigating problems as diverse as virus replication, cell polarization, induced scattering and cell–cell junction formation (e.g., Pasdar and Krzeminski, 1992; Timbs and Spring, 1996; Schultze *et al.*, 1996). However, their use to investigate the

mechanisms of kidney development has been much less frequent. The most promising results are probably those that have been obtained from the MDCK cell-cyst model system.

MDCK cells grown on planar plastic substrates form polarized epithelial monolayers, but those grown in a three-dimensional suspension form small hollow cysts. If the three-dimensional matrix is an "inert" substrate such as agar, then the apicobasal polarity of the cells is opposite that in a normal kidney tubule—their basal surfaces are innermost and their apical ones outmost (like an early mammalian embryo). If, on the other hand, they are cultured in a matrix containing, for example, collagen, their polarity reverses so that they form the basal-out, apical-in structure typical of real kidney tubules (Wang *et al.*, 1994). This phenomenon underlines the importance of cell–matrix interactions for the establishment of epithelial anatomy, a subject that will be considered in more detail in Section IV.E.2.

MDCK cysts growing in these collagen gels show interesting morphogenetic responses that some researchers believe to reflect processes taking place in normal development (Fig. 3). For example, if they are treated with the growth factor HGF/SF, they extrude long cellular spikes in a process that has been likened to branching morphogenesis (Montesano *et al.*, 1991). It is, however, important to view these analogies critically as there is no strong evidence to suggest that the spikes formed from MDCK cysts are homologous to true branched epithelial tubules, and real ureteric bud cells do not behave in the same way (Sainio *et al.*, 1997).

2. The Search for Nephrogenic Cell Lines

While research into kidney development is made much easier by the facility with which organ rudiments will develop *in vitro*, it is hampered by one serious limitation of the system: So far it has proved impossible to establish a cell line that can be grown in bulk and then induced to undergo organotypic development. This is a nuisance to researchers who would like to use transfection techniques to generate specific mutants whose phenotypes could be characterized in culture [see Burrow and Wilson (1994) for a review]. Two strategies are being attempted to circumvent this: (a) immortalization of cells by transformation with temperature-sensitive SV40 large T antigen and (b) induction of multiplication of wild-type metanephrogenic mesenchyme cells without the induction of differentiation.

The immortalization strategy is based on the SV40 large $T^{ts\text{-}58}$ transgenic mouse of Jat *et al.* (1991). This animal carries a temperature-sensitive allele of SV40 large T antigen under the control of a γ-interferon-inducible promoter. At 37°C and in the absence of γ-interferon, the antigen is inactive and the animal's cells are normal. If the cells are taken from an animal of this strain and cultured at 33°C with γ-interferon, the T antigen becomes active and forces the cells to multiply—at least in theory. Subsequent transfer to the nonpermissive tempera-

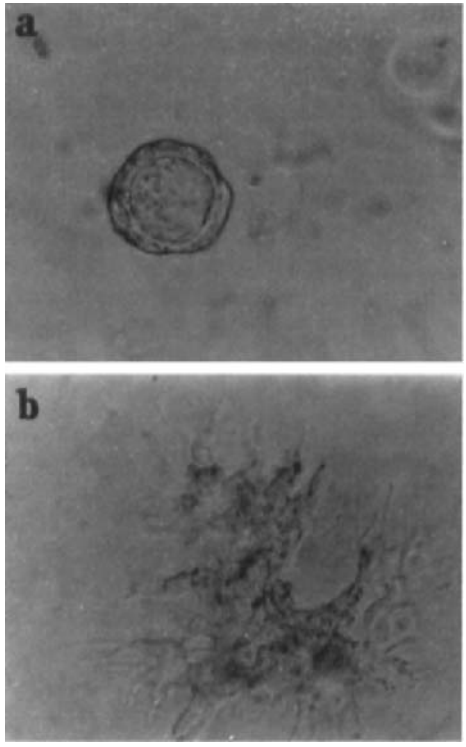

Fig. 3 HGF acts as a morphogen on MDCK cell cysts. These cells (derived from collecting duct) cultured in suitable extracellular matrix form simple cysts in the absence of HGF (a), but in the presence of HGF (b) the cysts push out extensions in a process that has been compared to the epithelial branching seen in normal organogenesis. Micrograph courtesy of Sarah Wallis.

ture will cause the cells, now far greater in number, to return to their previous behavior (again, in theory). Use of temperature-sensitive SV40 large T alleles has been effective with several types of cell, for example, neurons (Giordarno *et al.*, 1993, 1996) and myocytes (Miller *et al.*, 1994; Benito *et al.*, 1993), but so far has failed to yield a nephrogenic cell line. It has, however, been useful with already epithelial renal cell lines (Piedagnel *et al.*, 1994; Hosoyamaad *et al.*, 1996). Thus far, the cell lines that have been produced seem to have already progressed beyond the stem-cell stage as they express cytokeratins.

The second strategy arises from the conclusion, which will be explained later in Section IV.D.2, that the induction of nephron development has two phases, the first of which switches cells from quiescence and apoptosis to vigorous growth, and the second of which causes differentiation. The aim is to induce the first without the second and thus create a multiplying population of nephrogenic stem cells. This appears to have been achieved by Barasch *et al.* (1996), whose elegant

technique combines the use of SV40 $T^{ts\text{-}58}$ transgenic cells with exploitation of two-phase induction. Barasch *et al.* have created a cell line from the ureteric buds of SV40 $T^{ts\text{-}A58}$ transgenic mice and have shown that conditioned medium from this line switches wild-type metanephrogenic mesenchyme cells into vigorous growth without compromising their ability to be later induced into nephrogenesis. The technique may enable researchers to transfect nephrogenic cells in the near future and so provide them with a key tool for investigating the molecular basis of nephrogenesis.

III. Congenital Disorders of Nephrogenesis and Their Significance

A. Wilms' Tumor

Wilms' tumor is the most common solid-tissue cancer of childhood, affecting 1 in 10,000 in the population and occurring in both sporadic and familial forms (although only about 1% of patients have a family history of the disease). Like most cancers, it presents with a somewhat variable histology, but "classical" (triphasic) Wilms' tumors show large numbers of proliferative blastemal cells, in which small islands of epitheliogenic and stromogenic differentiation are scattered that parody normal kidney development. The blastemal cells do not express Pax-2 (Dressler and Douglas, 1992) and therefore are unlike normal nephrogenic stem cells (see Section II).

Genetic study of Wilms' tumor family trees has resulted in the identification of a tumor-suppressor gene called *WT1*, which maps to the 11p13 region of chromosome 11 [for a review, see Coppes and Williams (1994)]. *WT1* is mutated in approximately 15% of tumors; the etiology of the remaining 85% remains mysterious, although there is one other site, closely linked to the *IGFII* gene on chromosome 11 in the 11p15 region, in the region of the Beckwith–Wiedemann locus that may account for some of them. A third gene is likely to be on chromosome 16 [for a review, see Tay (1995); Ward, 1997]. The role of *WT1* and its possible role in Wilms' tumor are discussed later.

B. Congenital Cystic Kidney Diseases

This set of disorders leads to the formation of large cysts within the epithelial tubules (see Fig. 4a), both nephric and collecting duct, in which normal physiological processes are inhibited or reversed. Although the mature kidney is a relatively robust tissue and can fulfill its filtration functions in the presence of considerable insult (and abnormal morphogenesis, e.g., horseshoe kidneys, Fig. 4b), the size and number of the cysts and the associated renal hyperplasia of these diseases usually lead to kidney failure.

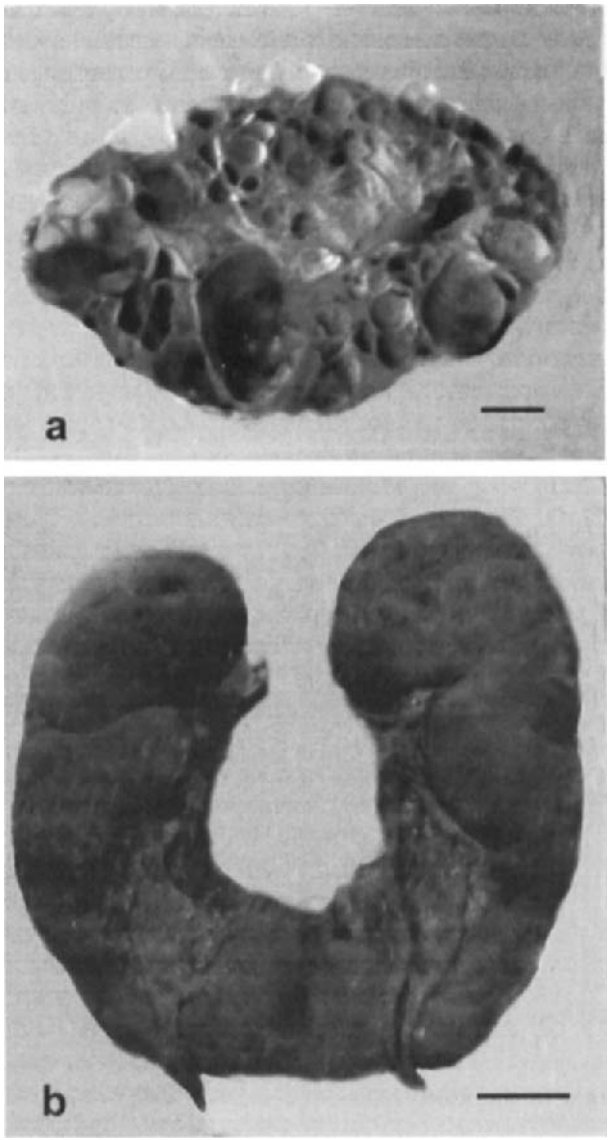

Fig. 4 Abnormal human kidneys. (a) a cystic kidney, with the cysts being up to several cm in diameter. (b) a horseshoe kidney. Bar = 2 cm. (Material from the Anatomy Museum, Edinburgh University.)

1. Autosomal Dominant Polycystic Kidney Disease

Mutations in three genes have been shown to lead to this phenotype. Polycystin (PKD1), the first of these, seems to be a membrane-traversing protein that may have a role in cell adhesion and is strongly expressed in fetal kidneys and less strongly expressed in adult kidneys (Palsson *et al.*, 1996). PKD2, the second, was identified by positional cloning and is similar to PKD1 in that it is a membrane protein, is related to the family of voltage-activated calcium channels, and contains a potential calcium-binding domain (Mochizuki *et al.*, 1996). There is also evidence for a third, ADPKD, gene (Daoust *et al.*, 1995), but it has not yet been cloned.

2. Autosomal Recessive Polycystic Kidney Disease

This version of polycystic kidney disease (ARPKD) is a rare but lethal inherited disorder resulting in the formation of cysts within the kidneys that are enlarged and dysfunctional. The gene responsible for ARPKD has been mapped to chromosome 6p21.1-cen (Zerres *et al.*, 1994).

3. Nephronophthisis (Medullary Cystic Disease)

This autosomal recessive disease is the most common form of early onset cystic disease that leads to renal failure. Although the underlying genetic defect has yet to be identified, the gene for juvenile nephronophthisis (*NPH1*) has been mapped by linkage analysis to chromosome 2q13. As a complete YAC contig of 7MB containing the region has now been constructed, it should not be too long before the gene is cloned and analyzed (Hildebrandt *et al.*, 1996).

4. Meckel's (or Meckel–Gruber) Syndrome

This monogenic, autosomal recessive disorder is recognized by three sets of abnormalities, neural tube closure defect, large polycystic kidneys, and polydactyly, and leads to the death of the fetus *in utero* or shortly after birth. Linkage analysis assigns the *MES* locus to chromosome 17q21-q24 (Paavola *et al.*, 1995).

C. Some Other Kidney Disorders with a Genetic Basis

Apart from Wilms' tumor and the polycystic kidney diseases, there are several rather obscure, but still serious congenital human kidney disorders that have been known for many years on the basis of their histology and symptoms. However, considerable effort has been put into elucidating their genetic and molecular bases, and the list that is briefly discussed here demonstrates the success of this strategy.

1. von Hippel-Lindau's Disease

This disorder, which may be familial (dominant) or sporadic, predisposes individuals to renal cell carcinoma, hemangioblastomas of the central nervous system, and pheochromocytoma. The disease now seems to be due to mutations in *VHL*, a tumor-suppressor gene on chromosome 3p that is expressed, *inter alia*, in the proximal tubular epithelium of the developing and adult kidney (Kessler *et al.*, 1995). It is thought to play a role in regulating the transcription of, as yet, unidentified downstream genes (Richards *et al.*, 1996).

2. Alport Syndrome

Alport syndrome (or hereditary nephritis) is an inherited kidney disease caused by irregularities and disruptions in the glomerular basement membrane and is associated with hematuria and sensorineural deafness. The common, *X*-linked form is associated with mutations in a gene encoding a novel basement membrane (type IV) collagen α_5-chain (Zhou *et al.*, 1991), while mutations in the α_3- and α_4-chains have been reported for the rarer autosomal forms of the disease (Mochizuki *et al.*, 1994).

3. Denys–Drash Syndrome

Patients with this syndrome show early onset nephropathy, a high risk of Wilms' tumor (WT), and pseudo-hermaphroditism. Careful analysis of *WT1*, the Wilms' tumor gene, shows that the disorder is associated with mutations in the zinc-finger regions. *In vitro* studies of mutated genes show that they fail to bind to *WT1* target sites (Little *et al.*, 1995), although it is not yet clear how this failure manifests itself as a lesion in the glomerulus.

4. Beckwith–Wiedemann's Syndrome

In the context of the kidney, Beckwith–Wiedemann's syndrome gives a predisposition toward Wilms' tumor, and genetic analysis has shown that maternally expressed imprinted genes may be involved that are in the chromosome 11p15 region [for a review, see Ward (1997)]. Three such genes whose expression is altered in the disease are *IGFII* (Ogawa *et al.*, 1993), the human cyclin-dependent kinase inhibitor, p57KIP2 (Hatada *et al.*, 1996; Matsuoka *et al.*, 1996), and KVLQT1, which spans much of the interval between p57KIP2 and *IGFII* (Lee *et al.*, 1997).

5. Tuberous Sclerosis

This dominantly inherited disease of the skin, retina, and heart is characterized by the presence of small benign fibrous tumors; it is often manifested in the kidney as angiomyolipomas (~50%) and cysts (~30%). The disease seems to be caused

by a mutation in one of two genes that act as tumor suppressors. The first is on chromosome 9 and has yet to be cloned, whereas the second, tuberin (the *TSC-2* gene is on chromosome 16), is a widely expressed 180-kDa polypeptide that exhibits specific GTPase-activating activity *in vitro* toward Rap1, with which it colocalizes, and that functions as a Rab5 GTPase-activating protein (GAP) in modulating endocytosis (Wienecke *et al.*, 1997; Xiao *et al.*, 1997).

D. Other Mutants Showing Defects in Renal Development

Of the 30 or more knockout mice with deletion of genes expressed during normal kidney development, 22 have no recognizable renal defect. Examples of those with particularly interesting renal defects are shown in Table III.

Table III Selected Mutants with Interesting Renal Phenotypes[a]

Mutant genotype	Phenotype	Reference
bcl-2−/−	Polycystic kidneys	Nagata *et al.* (1996)
BF-2−/−	Hypoplastic, underdeveloped collecting duct, few nephrons but many overlarge condensates	Hatini *et al.* (1996)
BMP-7−/−	1. Dudley and Luo mutants: severe hypoplasia; renal development seems to start properly but growth subsequently ceases	Dudley *et al.* (1995; Luo *et al.* (1995)
	2. Jena mutant: adequate glomeruli formed but poor development of distal tubules; polycystic	Jena *et al.* (1997)
GDNF−/−	Lack of ureteric bud; can be rescued *in vitro* by exogenous GDNF	Sanchez *et al.* (1996); Pichel *et al.* (1996); Moore *et al.* (1996)
p53 overexpression	Half-size kidneys	Godley *et al.* (1996)
Pax-2−/−	Failure of ureteric bud and nephron formation	Torres *et al.* (1995)
PDGFB−/− or PDGF-R−/−	Too few glomeruli	Soriano (1994); Leveen *et al.* (1994)
c-ret−/−	Variable failure of ureteric bud	Schuchardt *et al.* (1994)
Wnt-4−/−	MM condenses but does not epithelialize	Stark *et al.* (1994)
WT1−/−	Ureteric bud does not grow, metanephrogenic mesenchyme dies and cannot be induced by wild-type inducers	Kreidberg (1994)

[a]Additional mutants may be found in The Kidney Development Database (Davies and Brandli, 1997).

IV. Experimental and Genetic Analysis of Kidney Development

A. WT1 and the Early Stages

Renal development begins when a ureteric bud grows out from the Wolffian duct and toward the metanephrogenic mesenchyme. Without this early event taking place, all subsequent metanephric development will fail, so it is perhaps surprising that rather little is known about these critical early events. The first evidence of the metanephrogenic mesenchyme is the appearance of a domain of condensed mesenchyme (~250 μm in diameter) at the level of the midpoint of the hindlimb buds in each of the two bands of the intermediate mesoderm. It has recently become clear that these early stages of development absolutely depend on the activity of *WT1*, a gene cloned on the basis of its role in Wilms' tumor. WT1 is a zinc-finger protein, with transcription and splice-regulating activity that is expressed in normal metanephrogenic mesenchyme but not in the Wolffian duct or ureteric bud. It has 10 exons and 2 of these (5 and the KTS domain of 9, part of zinc-finger 3) are under independent control, so that 4 isoforms are coexpressed.

Expression analysis of WT1 shows that transcripts are present in the mouse in three distinct regions: Low-level expression is present in the uninduced metanephrogenic mesenchyme as soon as this tissue can be identified (Armstrong *et al.*, 1992). Following induction, the gene is expressed at noticeably higher levels in the metanephrogenic mesenchyme surrounding the ureteric bud and also in nephrogenic condensations. Its expression here is transitory, but the third and highest level of expression, in the podocyte layer of the differentiating renal capsule, is maintained until well after birth. The gene is also expressed elsewhere in the embryo (Armstrong *et al.*, 1992), particularly in regions where a mesenchyme-to-epithelial transition will take place (e.g., presumptive mesothelial cells and the gonad).

In WT1−/− animals, ureteric bud outgrowth fails, and the condensed mesenchyme taken from the regions of the embryos that should be occupied by metanephrogenic mesenchyme cannot be induced, even by wild-type inducers in culture. Instead, it rapidly undergoes apoptosis (Kreidberg *et al.*, 1993). This phenotype carries two implications: (1) WT1 function is required to establish a normal MM phenotype ("competence"); and (2) ureteric bud outgrowth is induced by the presence of normal MM.

As ureteric bud outgrowth takes place at a distance, WT1 almost certainly has to act through the release of a diffusible inducing molecule. The production of normal and ectopic ureteric buds from the Wolffian duct can certainly be triggered by the experimental application of a growth factor gradient; for example, placement of beads soaked in high concentrations of GDNF near the Wolffian duct can produce supernumerary ureteric buds that grow toward the bead (Sainio

et al., 1997). The natural inducing molecule might be GDNF, which is expressed by uninduced metanephrogenic mesenchyme (Trupp *et al.*, 1995), but the observation that not all GDNF−/− mice show complete absence of ureteric buds (Moore *et al.*, 1996) argues that there is some redundancy with other, so far unidentified, factors.

Sequence analysis of the *WT1* gene reveals it to be a transcription factor of the zinc-finger family with four such fingers; it also has a GC-rich region that is a potential target for trans-activating factors. Several laboratories have identified binding regions for *WT1* in the upstream regions of genes involved in nephrogenesis, such as *syndecan-1* (Cook *et al.*, 1996) and *EGR1* (see Maheswaran *et al.*, 1993). Transfection studies have pointed to possible stimulatory or inhibitory interactions between *WT1* and p53 (see Maheswaran *et al.*, 1993), the retinoic acid α-receptor (Goodyear *et al.*, 1995), *EGR1*, and *bcl-2* and *c-myc*, where loss of *WT1* led to the deregulation of these genes and contributed to tumor formation (Hewitt *et al.*, 1995). In the absence of *WT1*, normal changes in the expression of these genes fail to take place.

An additional function for *WT1* has emerged from expression analysis using antibodies to *WT1*. Larsson *et al.* (1995) showed that the presence or absence of an exon carrying the KTS amino acid sequence, between zinc-fingers 3 and 4, determines whether the WT1 protein is associated with nuclear DNA or with splicing factors in the spliceosomes, suggesting that the protein has two very distinct roles. In this context, it is intriguing that a search for *WT1* homologues across the vertebrates showed that the gene was present in chick, alligator, frog, and zebrafish as well as in mouse and humans (Kent *et al.*, 1995), with both the zinc fingers and the transregulatory domain exhibiting a high level of similarity. However, only one of the two alternatively spliced regions, the three-amino acid KTS insertion between zinc-fingers 3 and 4, is found in species other than mammals. The functional significance of this diversity is still obscure.

Although *WT1* was cloned almost a decade ago, only recently have there been substantial rewards for the large amount of effort that has been put into analyzing what controls its expression and how it exerts such a powerful effect on nephrogenesis. Although little is understood about the reasons for the WT1 expression pattern, Hofmann *et al.* (1993) have shown that its upstream region contains at least four start sites and a range of other intriguing features, the most important of which may be that the WT1 promoter region contains potential recognition sites for WT1/EGR, Pax-8, and GAGA-like transcription factors. The first of these points to a possible autoregulatory stimulation or inhibition of WT1 expression (Rupprecht *et al.*, 1994) and the second to a way in which WT1 is up-regulated in the podocyte layer soon after Pax-8 starts to be expressed; this view is strengthened by the fact that transfected Pax-8 isoforms can stimulate WT1 expression in cells (Dehbi and Pelletier, 1996; Pelletier, 1996b). A similar and earlier role also seems to be taken by Pax-2, which is expressed by MM very soon after induction (see the following), and this activity may account for the initial up-regulation of

WT1 in the induced MM and early condensates (Dehbi *et al.*, 1996; Pelletier, 1996a).

Another intriguing and unexpected observation has been that there is a second gene, *WIT-1* (Gessler and Bruns, 1993) in the 11p13 region (in humans) some 2 kb upstream from the *WT1* gene and coexpressed with it, but transcribed in the opposite direction, apparently from the *WT1* promoter (Eccles *et al.*, 1994), albeit at some 10% of the level of *WT1* transcription. Hewitt *et al.* (1996) suggest that *WIT-1* may be an antisense regulator of *WT1*.

As a whole, these studies show that *WT1* is a gene whose expression can be regulated by a range of activators and that can, in turn, activate or repress a wide variety of proteins. Progress in understanding the WT1 network has been rapid, and we can hope that we will soon have a solid genetic picture of how this gene is regulated and how it establishes the metanephric mesenchyme and guides its developmental responses as nephrogenesis proceeds.

B. Lineage in the Developing Kidney

Surprisingly little is known about lineage relationships in developing kidneys. It is clear that the comparatively few tissue types present in a very early kidney rudiment (ureteric bud, metanephrogenic mesenchyme, and perhaps some endothelial and neural crest cells) have to give rise to a much larger variety of cell types in the mature kidney (see Section II.B). Nonetheless, details of who gives rise to whom and in what order choices between developmental pathways are made remains, for the most part, uncertain. The classic story is that the ureteric bud develops into collecting duct and that metanephrogenic mesenchyme forms stroma and nephrons, but this is clearly inadequate as it provides no source for neurons (which grow in culture as well as *in vivo*) blood cells, and the juxtaglomerular apparatus.

1. The Collecting Ducts

The development of the ureteric bud has traditionally been thought to involve the least uncertainty over lineage: it develops as an outgrowth from the Wolffian duct. The bud, in turn, was believed to give rise to only the collecting-duct system, and the collecting-duct system was believed to arise solely from the ureteric bud. The work of Herzlinger *et al.* (1993) and Qiao *et al.* (1995) has, however, cast doubt on this nice, simple story. By using the techniques of retroviral cell marking and dil cell labeling, these researchers observed that the progeny of labeled ureteric bud cells could later be found in nephric epithelium. On this basis, they claimed that the cells of a labeled bud can leave it, undergo an epithelium-to-mesenchyme transition, and join the nephrogenic mesenchyme cells, where they then undergo a reverse mesenchyme-to-epithelial transition to become part of the nephrons.

Does this result mean that nephrons are made simply from cells that leave the top of the ureteric bud, migrate a short distance, and reepithelialize? Several observations suggest not, the most powerful of which is the well-established phenomenon of nephrogenesis in uninduced mesenchymes separated from ureteric bud and recombined with a completely different inducer (e.g., spinal cord) in culture (Grobstein, 1955). This works even for mesenchymes of mutant mice that have no ureteric buds (Schuchardt *et al.*, 1996). The potential contribution of nephrogenic cells by the ureteric bud observed by Herzlinger *et al.*, albeit intriguing, is therefore not necessary for nephrogenesis.

Similar work from the Herzlinger group has also provided evidence for cell traffic in the other direction; labeled mesenchyme cells appear to become incorporated into the growing ureteric bud (Qiao *et al.*, 1995). This two-way traffic between cell types, if a feature of normal kidney development, complicates cell lineage issues considerably and merits further investigation.

2. Mesenchyme Derivatives

The clump of cells grouped together as metanephrogenic mesenchyme gives rise to neuronal cells, vascular endothelium, renal stroma, cells of the juxtaglomerular complex, and excretory nephrons. Neuronal elements are widely assumed to develop from neural crest, a major source of migrating cells with neurogenic and other fates. Invasion of the metanephrogenic area by neural crest cells has been described in developing birds (Weston, 1963; LeDouarin, 1969; LeDouarin and Theillet, 1974), but their development at that site has not been followed in detail.

Vascular elements of the kidney have traditionally been thought to arise solely via angiogenic invasion of the organ rudiment by capillaries from elsewhere in the embryo [see Saxén (1987) for a review], a process that can be mimicked by culturing kidney rudiments on the chorioallantoic membranes of chick eggs (Sariola *et al.*, 1983). The idea that external vessels are the only source of renal capillaries is further supported by the observation that isolated kidney rudiments do not form capillaries in standard culture conditions. It has, however, become clear that isolated cells in the early (E10.5) metanephrogenic mesenchyme do express the endothelial markers VEGFR and Tiel, which are characteristic of endothelial cells (Landels *et al.*, 1994; Loughna *et al.*, 1996). If transgenically marked metanephrogenic mesenchymes of this age are transplanted into an unmarked host kidney, the capillary network that develops includes marked endothelial cells, confirming vasculogenesis from endothelial precursors endogenous to the metanephrogenic mesenchyme.

Early stromal cells are first seen soon after induction at the periphery of the kidney, where they express BF2 (Hatini *et al.*, 1996). Later, stromal cells expressing $BF2^+$ are seen, mainly in the medulla, with small groups of them also appearing in the cortex, where they may later form the cells of the juxtaglomerular complex.

The apparently homogeneous metanephrogenic mesenchyme thus give rise to

four very different cell types: nephric epithelium, vascular endothelium, juxtaglomerular cells, and renal stroma. This may be explained by three distinct models: (1) MM consists of one cell type, the pluripotency of which embraces stromogenic, vasculogenic, and epitheliogenic fates, which diverge only after induction. (2) MM consists of a mixture of two morphologically indistinguishable cell types, one of which is restricted to vasculogenic growth even before induction and the other is pluripotent for both epitheliogenic and stromogenic fates. (3) MM consists of three or more cell types, restricted even before induction to epitheliogenic, stromogenic, juxtaglomerular, and vasculogenic fates. Discrimination between the models requires that cell-marking experiments be performed before commitment to epitheliogenesis takes place. The mesenchyme-marking experiments reported so far result in clones that are either purely epithelial or purely mesenchymal (Herzlinger *et al.*, 1994), but labeling was undertaken too late in development to rule out models 1 and 2. This issue urgently needs to be addressed, because each model implies the existence of quite different cell-signaling mechanisms to control subsequent differentiation (see Section IV.D.4).

Cell-labeling studies have provided good evidence that, before their nephrogenic differentiation, mesenchyme cells remain pluripotent with respect to the part of the nephrons to which their daughters can give rise; the clonal progeny of labeled mesenchymal cells can appear in multiple segments of a maturing nephron (Herzlinger *et al.*, 1994). Once differentiation of specialized nephron segments begins, the pluripotency of cells presumably is lost, although the order in which choices between fates become irrevocable is completely unknown.

The last segment of what is conventionally regarded as the nephron, the very short segment that attaches to the collecting duct and is called the connecting tubule, appears by its expression of markers and its response to signaling molecules to share the properties of collecting-duct cells rather than those of the nephron (Davies, 1994). This and the fact that connecting tubules have never been observed to form in the absence of a developing collecting-duct system suggest that this segment is in fact derived as an outgrowth from the collecting duct. The issue will be difficult to prove, however, because the apparent interconversions of ureteric bud and mesenchymal cells reported by Herzlinger *et al.* make the interpretation of conventional labeling experiments very difficult.

C. Collecting-Duct Growth and Bifurcation

1. Signals That Control Collecting-Duct Morphogenesis

Like the epithelia of other glandular organs (e.g., lung, salivary gland, mammary gland), development of the collecting-duct system (Fig. 5) from the ureteric bud depends completely on signals emanating from the mesenchyme that surrounds it. Isolated in culture, the ureteric bud fails to develop, and it also fails to arborize when recombined with most types of embryonic mesenchyme tested (although

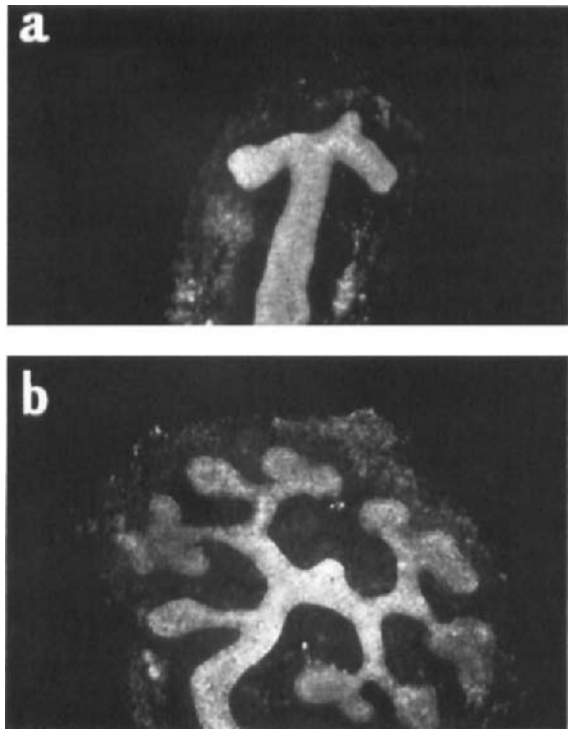

Fig. 5 Development of the collecting duct system; the initially unbranched ureteric bud bifurcates (a) then grows and undergoes more rounds of bifurcation to generate a tree-like epithelium (b) with, eventually, 250–500 tips (mouse).

some mesenchymes, such as lung, do support limited development; Sainio *et al.*, 1997).

The molecular identities of factors that renal mesenchyme uses to support and control ureteric bud development have been sought by three main techniques:

- Examination of the phenotypes of mutant mice showing abnormal ureteric bud development
- Specific inhibition of candidate molecules by antibody, antisense, transgenic, and biochemical techniques
- Testing the ability of candidate molecules to rescue the development of collecting-duct systems that has been experimentally inhibited in some way.

Four sporadic mouse and chick mutants show defective ureteric bud development: *Danforth's Short Tail* (*Sd*), *limb deformity* (*ld*), *fused* (*Fu*) and *Wingless* (*Wg*) (Gluecksohn-Schoenheimer, 1943; Maas *et al.*, 1994; Theiler and Gluecksohn-Waelsch, 1956; Ede, 1978). Three of these also cause defective limb develop-

ment, although the significance of this correlation is not understood: We therefore cannot exclude the possibility that the apparently linked effects are effectively byproducts of events elsewhere, and perhaps earlier, in the region. The molecules associated with *Fu*, *Sd*, and *Wg* have not yet been identified, although intensive mapping of the *Sd* region in the mouse (Alfred *et al.*, 1997) promises an early answer. *Limb deformity* (*ld*) arises from a defect in formins, which are nuclear proteins that are more likely to be concerned in the response to regulators than in the signaling system itself.

Specific inhibition of molecules in culture, or in transgenic mice, has yielded several signaling systems that are able to influence collecting-duct development (Table IV). The first of these is hepatocyte growth factor–scatter factor (HGF/SF), which is produced by the mesenchymal stem cells of developing kidneys (Sonnenberg *et al.*, 1993; Woolf *et al.*, 1995); its c-MET high-affinity receptor tyrosine kinase is expressed by the epithelium of the ureteric bud–developing collecting duct (Sonnenberg *et al.*, 1993; Woolf *et al.*, 1995). The HGF/SF–c-MET system was therefore an obvious candidate paracrine regulator, and its physiological function was tested by the addition of function-blocking antibodies to kidney rudiments growing *in vitro*; the antibodies strongly inhibited collecting-duct development (Woolf *et al.*, 1995). This result provided such firm evidence for HGF/SF having an essential paracrine rôle that the result of an HGF−/−

Table IV Candidate Regulators of Collecting-Duct Development

Candidate regulator of collecting-duct development	Evidence for	Evidence against
HGF	HGF expressed in mesenchyme and its receptor (c-met) expressed by ureteric bud	HGF−/− mice show no renal defects
	Acts as a branching morphogen in the MDCK cyst model system	
	Rescues growth (*not* branching) in S-GAG-deprived kidneys	
	Anti-HGF blocks renal development in culture	
GDNF	GDNF expressed by mesenchyme and its receptor (c-RET) by ureteric bud	GDNF also expressed by mesonephros (which does not support collecting-duct arborization)
	GDNF beads elicit supernumerary ureteric buds	
	GDNF rescues branching in S-GAG-deprived cultures	GDNF supports growth but not branching of isolated ureteric buds
	GDNF−/− mice show little or no collecting-duct development	
	GDNF receptor (c-RET) mutants show reduced collecting-duct development	

transgenic knockout experiment came as a surprise—the kidneys seemed to develop normally (Schmidt *et al.*, 1995; Uehara *et al.*, 1995). This paradox has yet to be resolved.

A morphogenetic function for the signaling system based on a second growth factor, GDNF, and its high-affinity receptor tyrosine kinase, c-RET, is supported by both culture and transgenic knockout data. GDNF is synthesized by uninduced metanephrogenic mesenchyme and nephrogenic stem cells, whereas c-RET is expressed by the epithelium of Wolffian duct and ureteric bud. Transgenic knockout mice of the GDNF−/− and c-RET−/− types show marked inhibition of ureteric bud–collecting–duct development, although both show some variation in phenotype severity from mouse to mouse. Treatment of kidneys growing *in vitro* with beads soaked in high concentrations of GDNF causes an increase in collecting-duct branching in the vicinity of the beads, whereas the growth factor maintains the morphology of isolated ureteric buds in hanging-drop culture (Sainio *et al.*, 1997).

Another growth factor whose absence from transgenic knockout mice results in the failure of collecting-duct development is BMP-7 (Dudley *et al.*, 1995; Luo *et al.*, 1995). The *BMP-7* gene is transcribed by the ureteric bud–collecting duct itself, as well as by nephrogenic stroma and developing nephrons, and loss of BMP-7 also inhibits the development of nephrogenic mesenchyme into nephrons (see the following). It is not therefore clear whether the collecting-duct defect in BMP-7-deficient mice results directly from the developing collecting duct requiring BMP-7 or from a secondary effect caused by abnormal development in the surrounding mesenchyme.

A slightly different approach to understanding duct morphogenesis has been the use of purified growth factors to "rescue" growth-inhibited kidney rudiments in culture. Many growth factors bind to sulfated glycosaminoglycan (S-GAG) coreceptors on the surface of cells that serve to concentrate these factors (and thus increase their chances of binding neighboring high-affinity receptors) and may facilitate binding to the high-affinity receptors by inducing conformational change (Rapraeger *et al.*, 1991; Lyon *et al.*, 1994). The depletion of cultured kidney rudiments of their S-GAGs results in complete and reversible inhibition of collecting-duct growth and branching [Fig. 6a of Davies *et al.* (1995)]. This inhibition can be relieved by simultaneous treatment with concentrations of certain growth factors at concentrations high enough to compensate for the lack of S-GAGs. Under these conditions, HGF/SF restores growth to the developing collecting-duct system without activating branching morphogenesis, so that the epithelium develops into an abnormally long unbranched tube [Fig. 6b of Davies *et al.* (1995)]. GDNF, on the other hand, activates the branching program well [Fig. 6d of Davies *et al.*, (1995)], so well indeed that it can even induce branching from the "wrong" end of the ureteric bid (Sainio *et al.*, 1997). The implication of these results, that the growth and branching aspects of arborization seem to be controlled separately, may have important implications for the development of

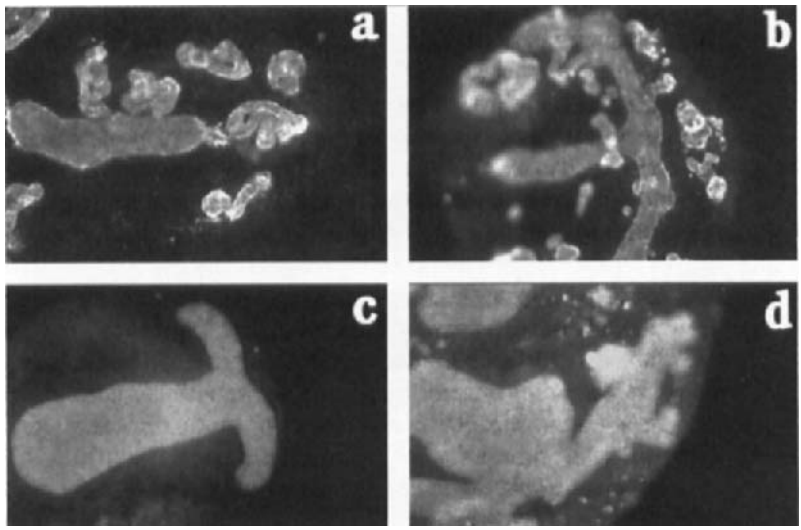

Fig. 6 Morphogenetic effects of HGF and GDNF in kidneys depleted of sulphated glycosaminoglycans. (a) Kidneys depleted of sulphated glycosaminoglycans (S-GAGs) show very limited collecting duct branching, compared with controls (e.g., Fig. 7, top panel), although they still form nephrons (panel (a)-nephrons are not visible in panels (c) and (d) because they are stained for a collecting-duct-specific marker). Treatment of the S-GAG-depleted kidneys with exogenous HGF causes their collecting ducts to extend but not to branch (b), while treatment with GDNF causes branching and swelling of the ducts without much extension (d—compare with the control culture (c)).

glandular organs in general. Each has a characteristic epithelial shape primarily governed by the surrounding stroma, so that, for example, salivary gland epithelium placed in lung stroma develops in the approximate shape of lung epithelium (Deucher, 1975). Different ratios of mitogenic and branch-generating signals might provide a simple explanation for the characteristic anatomies of glandular epithelia and the different degrees to which they fill three-dimensional space (their fractal dimensions).

2. Mechanisms of Collecting Duct Morphogenesis

Once it has received signals that induce it to grow and branch, the collecting-duct primordium must activate morphogenetic mechanisms that enable it to change its shape and mount a directed invasion of the surrounding stroma. The nature of most of these mechanisms remains mysterious, but some sketchy details are emerging from experiments.

Collecting-duct development traditionally has been described as involving terminal dichotomous branching. The situation may not, however, be that simple,

and two pieces of evidence, both obtained from culture work, suggest that internodal branching might also exist. A general property of trees that develop by terminal branching, first noticed by Leonardo da Vinci [see Long, (1994) for a review], is that the ratio of the diameter of a branch of generation n to that of a branch of generation $(n + 1)$ is a constant (da Vinci's number). This means that the ratio of the diameter of the trunk to that of the first main branches is the same as that of the first main branches to that of the secondary branches, and so on; in modern parlance, the tree shows self-similarity (fractal geometry). The arborization of many botanical trees, and also that of zoological "trees" such as canine airway epithelium, follows this general pattern (Nelson *et al.*, 1990). Attempts to calculate the da Vinci number (or the related fractal dimension) of the collecting ducts of kidneys developing in culture do not, however, work if one measures the branch generation number (primary, secondary, tertiary, etc.) by assuming that only terminal branching takes place. One can obtain a constant da Vinci number only by assuming that the internodes of branches of generation n can directly give rise to branches of generations $>(n + 1)$ (J. A. Davies, unpublished). Tantalizing as these results are, the question of whether internodal branching really takes place or whether branching is always terminal but can be very unequal will only be settled by the time-lapse studies underway. The second piece of evidence comes from restarting the arborization of ureteric buds whose branching development has been stopped; new branch tips arise along the lengths of old branches, not just at their termini (Davies *et al.*, 1995).

Whatever the choices of branch pattern available, investigation of their generation mechanisms is made simpler by the fact that branching seems to be independent of growth; ureteric buds of kidneys treated with concentrations of methotrexate sufficient to block DNA synthesis (and therefore cell cycling) still undergo branch initiation, although the branches fail to grow out (Davies *et al.*, 1995). This result is in accord with similar observations on the independence of branching from growth that have been obtained in the developing salivary gland (Nakanishi and Ishii, 1989).

Branching morphogenesis requires certain regions of a tubule wall to bend; in these regions, cells must become wedge-shaped, with a narrowed basal surface along the convex curve at the tip of the branch and a narrowed apical surface at concave surfaces where the branch leaves its parent tubule. One obvious potential mechanism for mediating this deformity of cell shape is cytoskeletal tension generated by actin and myosin; another is pressure of growth. Actin microfilaments are indeed concentrated at the sites of maximum cell deformation (J. A. Davies, unpublished, Fig. 7), but there is as yet no unambiguous evidence that the actin–myosin interaction is the main agent for branch initiation. Drugs such as cytochalasin D, which interfere with microfilament polymerization, do block branch initiation very effectively (B. Harron, L. Ramage, and J. A. Davies, unpublished), but far too many cellular processes are likely to be affected by this treatment to allow firm conclusions to be drawn.

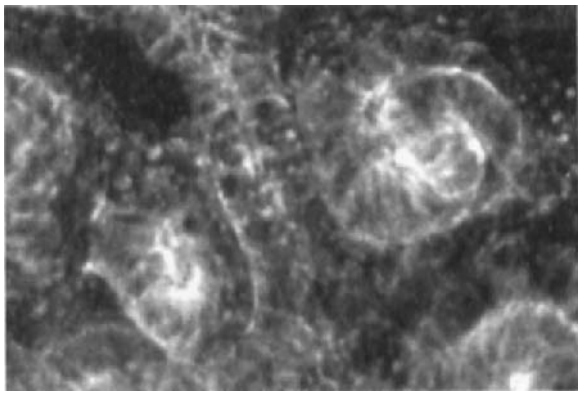

Fig. 7 Phalloidin staining of developing nephrons reveals a high concentration of filamentous actin where bending of the tubule is taking place, as predicted from a model in which localized "purse-string" contraction of apical microfilament networks drives bending morphogenesis.

In addition to changing its shape to initiate new branches, the growing collecting-duct epithelium has to invade the mesenchymal matrix that surrounds it. The mechanisms underlying this have not been studied in detail, but evidence implicates matrix metalloprotease 2, whose activity correlates with collecting-duct morphogenesis *in vitro* (McCormack and J. A. Davies, unpublished).

D. Mesenchyme Induction

1. The Inductive Signal—Evidence from Tissue Culture Experiments

The signals by which ureteric bud induces metanephrogenic mesenchyme to undergo further development have not yet been identified, although something is known of their general characteristics and a few candidate molecules now exist. The candidacy for each is supported by some experimental evidence. So far, however, no molecules meet all of the Slack criteria (Slack, 1993) for being renal inducers. In the case of the kidney, these criteria are as follows (Davies, 1996): (1) The molecule(s) must be present in the ureteric bud tips as long as induction takes place. (2) The candidate molecule(s) must be capable of inducing nephrogenesis or at least some aspect of the process in completely uninduced mesenchyme. (3) Inhibition of the molecule(s) should block induction in an intact kidney (provided there is no redundancy).

For many years, the induction of mesenchyme was believed to require direct cell–cell contact between the inducing and the induced cells. The evidence for this view came from transfilter experiments in which the mesenchyme was separated from the inducing tissue by a polycarbonate filter of sharply defined pore

size. Generally, the inducing tissue used was spinal cord rather than ureteric bud, because embryonic spinal cord is a much stronger inducer (Grobstein, 1955). When the filter pores were large enough to allow penetration by cell processes, transfilter induction took place (Fig. 8), but if the filter pores were too small to allow penetration by cell processes, then induction failed (Saxén *et al.*, 1976; Saxén, 1980). The obvious conclusion was either that the inducer was tightly bound to the cell surface, so that it could not diffuse away and act at a distance, or that a combination of its half-life in free solution and the concentration required for it to act limited the range of an effective concentration to a distance so small as to be indistinguishable from contact. Electron microscopy confirmed that contact takes place in the large-pore transfilter system and demonstrated that there were no morphological specializations at the sites of intercellular contact.

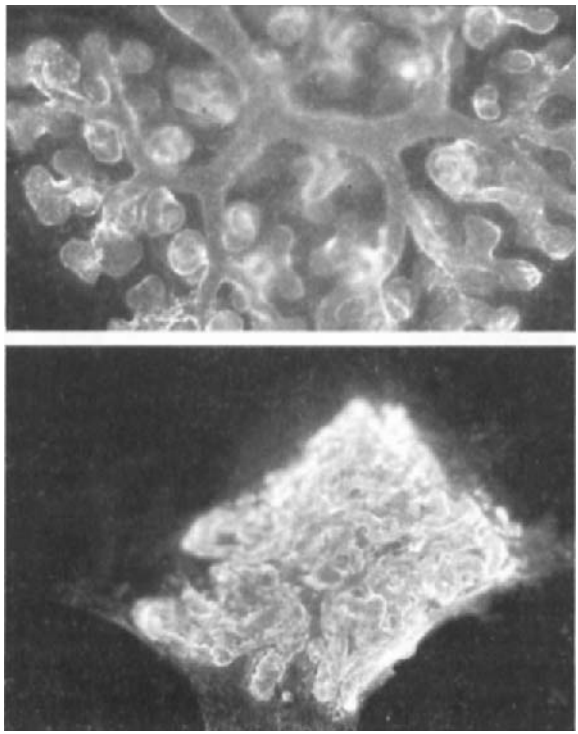

Fig. 8 Two commonly used methods of inducing nephron development in culture. In the top panel, a complete E10.5 murine kidney rudiment has been cultured on a filter at the gas–medium interface; its collecting duct system has branched and induced nephrons to form from the surrounding stroma. In the bottom panel, isolated metanephrogenic mesenchyme has been cultured on a filter underneath which is embryonic spinal cord; the spinal cord has induced the formation of many nephrons in the mesenchyme.

The classical transfilter experiments that led to the view that contact was a necessary feature of induction shared one methodological feature: A blob of agar or agarose was used to attach the inducing tissue to the underside of the filter (the mesenchyme was cultured on the filter's upper surface). This agar(ose) was regarded as a neutral "glue." However, it has become clear that many growth factors (e.g., FGF-2 and HGF) bind to sulfated glycans (particularly heparin sulfate). If the inducing molecule were to bind to the sulfated glycans present in agar, then the blob of glue may in fact have sequestered a diffusible inducing molecule before it was able to pass beyond the glue and through the filter pores. To explore this possibility, the classical transfilter experiments have been repeated but instead using a sandwich of filters instead of agar to hold the inducing tissue (again spinal cord) in some cultures. In the absence of agar, induction took place even across multiple layers of filters with pore sizes too small to admit cell processes; exclusion of these processes was confirmed by SEM (Davies and Bard, unpublished). These data lead to the view that the inducing molecules from spinal cord are capable of acting tens of cell diameters from their source. Unfortunately, transfilter induction by normal ureteric bud cannot be obtained even with large-pore filters, so that we still know nothing about the diffusibility or otherwise of signals from the natural inducer of nephrogenesis. However, induction of at least the first stages of development (see Section IV.D.2 for a discussion of multistage induction) can be performed by conditioned medium from a ureteric-bud-derived cell line, strongly suggesting that ureteric bud, too, induces at least some stages of the process by diffusible means (Barasch *et al.*, 1996).

2. Candidate Molecules

The implications of the preceding experiments—that the inducer is diffusible and interacts with sulfated glycans—are in accord with the results of one quest for the biochemical identity of the inducing molecule (see Table V). Perantoni *et al.* (1995) have identified the critical component of a cell extract that can induce nephrogenesis in the rat as FGF-2 and have gone on to show that very high concentrations of purified FGF-2 can induce nephrogenesis in isolated rat metanephrogenic mesenchyme in culture, albeit unusually slowly. In its favor, FGF-2 is present in the ureteric bud throughout the period of induction (Dono and Zeller, 1994), and it is present in the artificial inducers of nephrogenesis such as spinal cord. However, several facts argue against FGF-2 being the natural inducer. First, FGF-2 fails to induce mouse nephrogenic mesenchyme; this implies either that rats and mice induce kidney development very differently, which would be very surprising, or that FGF-2 by chance happens to mimic the natural inducer in rats, but itself is not that inducer. Second, FGF-2 is produced by tissues that are not inducers of nephrogenesis, including nephrons themselves. Third, treatments that would be expected (from other systems) to inhibit FGF-2 action do not inhibit nephrogenesis, at least in mouse. For example, the removal of cellular sulfated

Table V Candidate Inducers of Nephrogenesis

Candidate inducer	For	Against
FGF-2	Purified FGF-2 induces nephrogenesis in isolated rat mesenchymes FGF-2 is produced by ureteric bud	FGF-2 fails to induce mouse mesenchyme FGF-2 is also produced by tissues that do not induce nephrogenesis (including nephrons themselves)
BMP-7	Produced in the right place BMP-7 will induce isolated rat nephrogenic mesenchymes Inhibition of BMP-7 inhibits nephrogenesis BMP-7−/− mice show a severe deficiency of nephrons	Produced in tissues that do not induce (mouse data)
Wnt proteins	Wnt-1-transfected cells induce mouse mesenchyme Li^+-mediated induction fits a Wnt model	No Wnt known to have the expression expected of an inducer

glycosaminoglycans by chlorate ions [which compete with sulfate ions in the synthesis of phosphoadenosine 5′-phosphosulfate, the sulfate donor utilized by sulfotransferases that mediate polysaccharide sulfation (Farley *et al.*, 1978; Rapraeger *et al.*, 1991)] or by heparitinase enzymes is known to inhibit the effects of FGF-2 in cell lines. These treatments do not, however, prevent nephrogenesis *in vitro* (Davies *et al.*, 1995). Treatment of kidney rudiments developing *in vitro* with function-blocking anti-FGF-2 also fails to block nephrogenesis (J. A. Davies, unpublished).

Another growth factor, BMP-7, is a second candidate for an inducing molecule. BMP-7 appears to meet the three criteria mentioned earlier. It is produced in the ureteric bud at the right time, it induces isolated rat mesenchyme (no mouse data are available), antibodies to BMP-7 block nephrogenesis, and transgenic BMP-7−/− mice show a severe shortage of nephrons (Fig. 9e, f). These data, therefore, make it a very strong candidate for being the inducer. However, BMP-7 is also produced by developing nephrons themselves. In mouse, developing nephrons are known not to possess inducing activity (Saxén and Saksela, 1971), a fact that is difficult to reconcile with BMP-7 being the inducing molecule. An alternative explanation for the role of BMP-7, and one equally compatible with the data, is that BMP-7 is necessary for the subsequent nephrogenic development of cells induced by another molecule or for the maintenance of the stem-cell population.

Two strands of evidence implicate Wnt proteins in the induction of nephrogenesis. First, Herzlinger *et al.* (1994) have demonstrated that cells that are not normally inducers of nephrogenesis become inducers when transfected with

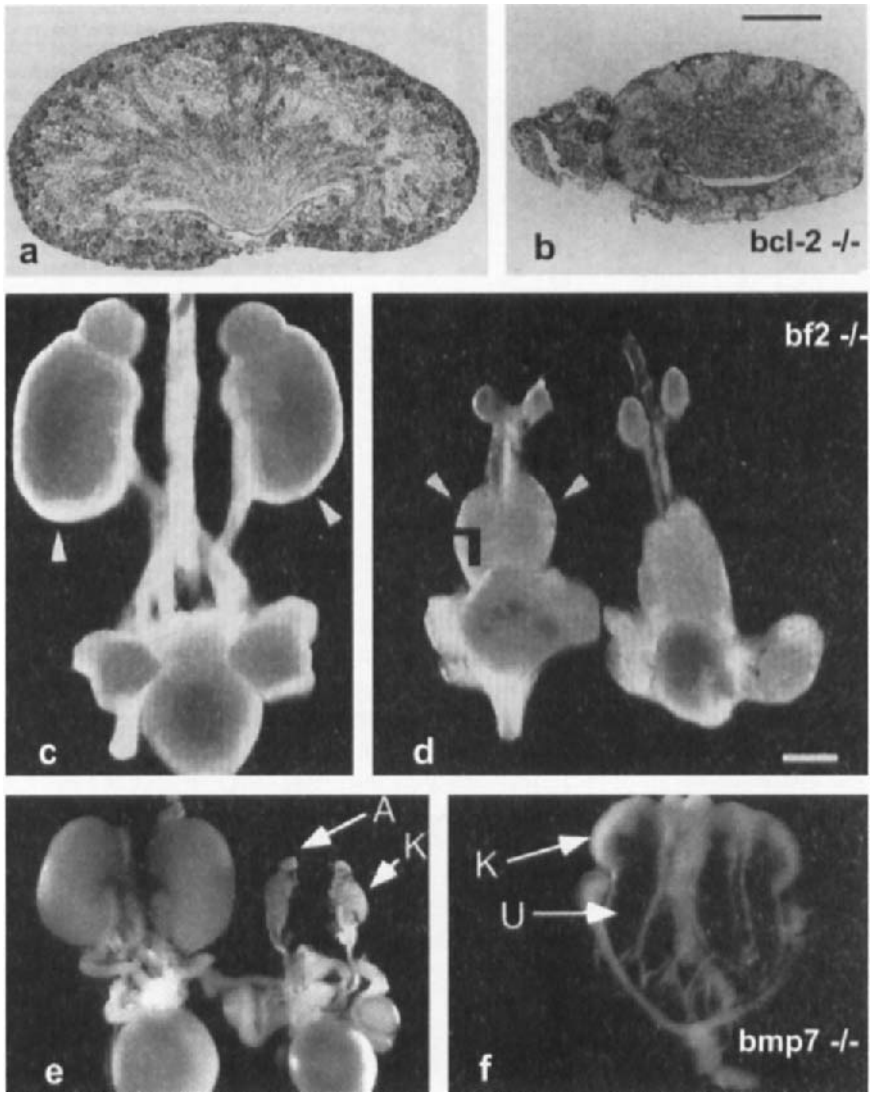

Fig. 9 Transgenic kidneys. *bcl-2* (a,b) BF-2 (c,d) and BMP-7 are three genes whose absence leads to kidneys that are much smaller than wild-type ones in adult mice. (a, b) Sections of a normal mouse kidney and the very much smaller kidney from a *bcl-2* −/− mouse. Bar = 0.2 mm. (Courtesy of Nagata *et al.* (1996). *Am. J. Pat.*, **148,** 1601–1611.) (c, d) Kidneys (arrow heads) from a normal (c) and from a BF-2 −/− (d) mouse. Bar = 1.5 mm. (Courtesy of Hatini *et al.* (1996). *Genes & Development*, **10,** 1467–1478.) (e) Kidneys (arrow heads) from an E19 BMP-7 −/− mouse (A: adrenal, K: kidney) compared with those from a normal mouse. (f) The acute hydroureter phenotype (distended renal pelvis and ureter) shown by the majority of newborn BMP-7 −/− mice. (Courtesy of Dudley *et al.* (1996), *Genes & Development*, **9,** 2795–2807.)

Wnt-1 cDNA. Wnt-1 itself is not present in developing kidneys (Wilkinson *et al.*, 1987) and so cannot be the inducer, but the promiscuous interactions within the Wnt signaling pathway suggest that another Wnt protein might be the inducer. So far, however, no Wnt proteins have been found to have the expected distribution of an inducer of nephrogenesis (present in ureteric buds but not in other renal tissues), although they are clearly involved in events downstream of induction (see Section IV.E.2).

The second strand of evidence for Wnt signaling comes from the observation that lithium ions induce the early stages of nephrogenesis in isolated mouse mesenchymes (Davies and Garrod, 1995). The primary biological effect of lithium ions is known to be inhibition of the enzyme, glycogen synthase kinase 3β (GSK-3β, the mammalian homologue of the *Drosophila* gene *shaggy/zeste-white 3*; Klein and Melton, 1996). GSK-3β lies downstream of a Wnt signaling pathway, and binding of Wnt proteins to their receptors indirectly results in the inhibition of GSK-3β. Li^+-mediated inhibition of GSK-3β therefore can be viewed as a means of mimicking Wnt-mediated signaling (though it might of course have other effects too). It therefore provides more circumstantial evidence in favor of Wnt proteins. Against the Wnt story, however, is the difficulty in reconciling it with the transfilter data described earlier that suggest that the inducer is a diffusible agent that binds to charged carbohydrate.

There are, then, at least three candidate inducers, each with some evidence in its favor and some evidence against. The confusion of the data and their interpretation probably reflects the complexity of the system and the fact that a succession of inductive and permissive factors appears to be necessary for complete nephrogenesis. The increasing evidence that there are, in fact, two stages of induction (see Section IV.D.3) and therefore perhaps two distinct inducers might help to clarify apparent contradictions in the data described earlier.

3. How Many Stages Are There in Induction?

The fate of isolated, uninduced metanephrogenic mesenchyme is to exist for a few days with very little cell division and then to die by apoptosis; this is true both in culture (Koseki *et al.*, 1992) and in mutants in which the ureteric bud fails to develop (e.g., WT1−/− and c-ret−/−; Kreidberg *et al.*, 1993; Pachnis *et al.*, 1994; Schuchardt *et al.*, 1994). The fate of mesenchyme that has been invaded by a ureteric bud is to proliferate sufficiently such that it can eventually produce 1000–2000 nephrons (discussed earlier). Once the metanephrogenic mesenchyme has been invaded by the bud, it has to set aside its death wish and multiply quickly.

A decade ago, it seemed likely that induction was a single-stage process, with the MM just growing while those cells in contact with the bud were induced to form nephrons. This view is no longer tenable as we know that all of the MM cells are rapidly induced to switch out of an apoptotic and into a growth phase, whether or not they are in contact with the bud (e.g., Koseki *et al.*, 1992). Only

later do small groups of cells then enter the nephrogenic pathway. Furthermore, this early change in growth pattern is reflected in changes taking place in the expression profile of the MM (e.g., the low-affinity NGF receptor is replaced by TrkB and -C; see below for more details).

The change in behavior of the metanephrogenic mesenchyme cells upon being invaded by the ureteric bud is now regarded as a change in phenotype from uninduced metanephrogenic mesenchyme into nephrogenic stem cells and, hence, induction is a two-stage process. The first stage consists of an interaction that induces the metanephrogenic mesenchyme into becoming stem cells, and the second induces groups of stem cells to become nephrons. Alternative models in which a single inductive event causes a cell to divide into one daughter committed to nephrogenesis and another remaining mesenchymal to maintain the mesenchymal population are simply incompatible with the massive amount of growth that takes place.

The idea of multistage induction is gaining increasing experimental support. It has long been known that a rise in DNA synthesis precedes nephrogenesis by many hours (Saxén *et al.*, 1983). Following spinal cord induction, for example, there is a bout of DNA synthesis after some 8 hr of contact with an inducer, whereas the first signs of nephrogenesis can only be seen at 18 hr (Davies and Garrod, 1995). This pattern of timing is at least compatible with the idea of a first induction to stem cells followed by a second to nephrogenesis itself. Much more significant support for the model, however, has come from the observation that conditioned medium produced by immortalized ureteric bud cells can induce metanephrogenic mesenchyme into the stem-cell state (as assessed by the cessation of apoptosis and commencement of vigorous mitosis; Barasch *et al.*, 1996) without inducing progression into nephrogenesis itself. The second induction requires contact with the basolateral surfaces of the ureteric bud; cell-conditioned medium is not enough (Barasch *et al.*, 1996), although in the case of the rat, ureteric-bud-conditioned medium plus FGF-2 plus TGFα does induce nephrogenesis (Karavanova *et al.*, 1996). These two inductions could be mediated by different molecules. Alternatively, they could be mediated by a single molecule with threshold-dependent effects; a low concentration would effect the switch to stem cells, while a higher concentration would be required for nephrogenesis. This model would account for the fact that cells undergoing nephrogenesis are located close to the developing collecting-duct system whereas cells farther away multiply without differentiating.

An unexplained feature of nephron induction, so far observed with both living (e.g., spinal cord) and pharmacological (e.g., Li^+) inducers, is the requirement that the inducer be present for at least 12–15 hr. Shorter exposures are insufficient (Davies and Garrod, 1995). This is a long time compared with that required for the transcription and translation of new genes (1–2 hr), for example, and raises the question of what the delay is for. The length of the period, 12–15 hr, is compatible with a connection to the cell cycle and perhaps suggests that cells must pass through a cycle before they become committed to epithelial differen-

tiation. There is, so far, no evidence that every induced cell passes through a cell cycle during the inductive phase, but it is known that inhibition of cell cycling (DNA synthesis) using mitomycin C blocks the induction of epitheliogenesis (Nordling *et al.*, 1978), although we do not know the precise stage at which the process halts.

Transition to the stem-cell state appears to be accompanied by the decline in apoptosis and the appearance of molecules absent from uninduced mesenchyme (I use the slightly vague phrase "appears to be" because the correlation of the molecules with the stem-cell state so far is based on distribution and timing rather than formal correlation with cell cycling). These include the transcription factors Pax-2 and Hox-C9 and the signaling molecules HGF, c-MET, and p75-NGFR. HGF and its receptor c-MET have the potential to form an autocrine loop because both are expressed in the same cells (Woolf *et al.*, 1995). The existence of such a loop is supported by the effect of adding antibodies that interfere with the HGF–c-MET interaction, which results in markedly increased apoptosis in what should be the stem-cell population. The antibodies also block subsequent differentiation, although it is not clear whether this results from a failure of earlier proliferation. There must be some redundancy *in vivo*, however, as HGF knockout mice have normal kidneys. Antisense inhibition of p75-NGFR synthesis in culture also blocks renal development (Sariola *et al.*, 1991), although once again the knockout mouse shows no renal defect (Lee *et al.*, 1992).

Induction, or at least the progression of cells from the induction to condensation stages, can be blocked reversibly by the addition of the chemokine LIF to culture medium [Bard and Ross (1991)]. This blockage only works for ureteric-bud-mediated induction, however; induction by surrogate tissues such as spinal cord takes place normally. The effect of LIF therefore might be to abolish the inducing activity of the ureteric bud. Normally, data on the distribution of the LIF receptor could be used to indicate the likelihood of this, but the only known receptor for LIF does not appear to be expressed anywhere in the developing kidney, making the effect of the chemokine even more mysterious. LIF receptor knockouts have no reported renal phenotype (Ware *et al.*, 1995), nor do transgenic mice that lack IL6 or CNTF, both of which also bind to components of the LIF receptor (Dedera *et al.*, 1996; Masu *et al.*, 1993). The one deduction that can be made on the basis of the signal data at hand is that few receptors can be as promiscuous as those in the kidney!

4. The Relationship between Stromal and Nephrogenic Cells

In the most common model of lineage in the developing kidney (see Section IV.B), cells have to "decide" some time after their induction (one of their inductions) whether to follow a nephrogenic or a stromogenic course (or differentiate into the juxtaglomerular apparatus, etc.). So far, nothing is known about the mechanisms underlying this decision, although it is possible that almost any of the mutants that block nephrogenesis (Table III) may do so by shunting all of the

cells into a stromal fate; analysis of mutants tends not to include the use of molecular markers that will indicate stromal differentiation, partly because there are few of these. The same argument applies to the effects of factors that block nephrogenesis *in vitro*, such as LIF (see Section IV.D.3).

A mechanism that used to be thought feasible for explaining the splitting of mesenchyme into different fates was based on contact with the inducing tissue: If a mesenchyme cell made such a contact with it, it underwent mesenchyme-to-epithelial transition (MET), otherwise it remained mesenchymal and became stroma. Several experiments have, however, cast doubt on this view, particularly the discovery of pharmacological means to carry out induction (see Section IV.D). Isolated mesenchymes induced with either Li^+ ions or bFGF produce a mosaic pattern of developing nephrons and surrounding stroma, despite the fact that these small molecules will have reached all of the cells in the culture. If the nephrogenic and stromogenic cells arise from lineages that were separate even at the beginning of kidney development (model 2 in section IV.B), all that needs to be explained is the sorting out of a mixed population into a mosaic pattern. This may be explained rather easily by known changes in cell adhesion molecule expression (e.g., of NCAM; Klein *et al.*, 1984). If, as is usually assumed, the MM consists of one cell type that gives rise to both stroma and nephrons, we must explain how the choice of fate is made.

One possibility is a system of lateral inhibition mediated by signaling molecules in interacting feedback loops like that based on *Notch* and *Delta*, which divides ectodermal cells of *Drosophila* into neurogenic and epithelial fates (Simpson *et al.*, 1992). The vertebrate homologues of *Notch*, mouse *notch1* and *notch 2*, are indeed expressed in the developing kidney at about the right time and place (to the resolution of published data). Transgenic knockout of *notch1* results in lethality too early in development for any metanephric effects to be assessed; a system based on these molecules would involve only nearest neighbor interactions anyway and so would not be ideal for the establishment of islands of many tightly aggregated cells of similar fates. Partitioning between fates remains one of the most baffling problems of renal development.

E. The Downstream Effects of Nephron Induction

After they have been induced to begin their nephrogenic program, cells must aggregate, undergo an epithelial–mesenchymal transition, and, once epithelial, differentiate into the various specialized regions of a mature nephron. We now consider the limited data on these events.

1. Condensation

The first morphological consequence of nephron induction is the formation of tight aggregates of nephrogenic cells some 5–6 cells in diameter within the

already dense metanephric mesenchyme, and these soon undergo a mesenchyme-to-epithelial transition. In cultured kidney rudiments, condensation lasts approximately 6 hr, beginning some 18 hr after first contact with an inducer (Davies and Garrod, 1995). The mechanisms responsible for aggregation are not known, but work on other examples of mesenchyme condensation (see Bard, 1992) suggests the following possibilities (which are not mutually incompatible):

1. Increased intercellular adhesion cause by the expression of new adhesion molecules
2. Local disappearance of interstitial matrix
3. Directed migration
4. Localized cell division
5. Generation of tractional forces

The first model is supported by the fact that adhesion molecule expression does indeed change just before aggregation begins. For example, expression of the homophilic neural cell adhesion molecule, N-CAM, is strongly up-regulated, as is expression of the proteoglycan, syndecan-1 (Vainio *et al.*, 1992). Inhibition of N-CAM function, by antibody or transgenic knockout techniques (Klein *et al.*, 1988; Cremer *et al.*, 1994) does not, however, prevent normal nephrogenesis. The removal of syndecan-1's sulfated glycosaminoglycan side chains also fails to block nephrogenesis (Davies *et al.*, 1995), although there is as yet no information about the effects of inhibiting the synthesis of the syndecan-1 protein core. These few experiments cannot, of course, rule out the adhesion molecule hypothesis for aggregation, as the cells might also express additional, undiscovered adhesive systems. An explanation based on the specific and local expression of such adhesion molecules does imply the need for a prior mechanism specifying the population of cells that will undergo this change.

The evidence to support the second and third of these mechanisms is weak. Before condensation, mesenchymal cells are separated by a prominent interstitial matrix consisting of fibronectins, collagens I and III, and glycosaminoglycans, whereas in the condensates themselves cells are in very close apposition and all signs of interstitial collagens and fibronectins disappear (Ekblom, 1981; Ekblom *et al.*, 1981; Laitinen *et al.*, 1991). The rapid removal of these components suggest localized activity of degradative enzymes, but it is not clear that this activity is enough to account for condensation itself. In other systems, such as the condensation of somatopleure mesenchyme to form cartilage, localized loss of extracellular matrix components seems to play an important role in bringing cells closer together to form the initial condensation (Toole, 1972), although N-CAM production can also be important here [for a review, see Hall and Miyake (1995)]. It will be interesting to determine whether there is any relationship between the formation of nephrogenic and cartilaginous condensations.

Evidence in favor of mitosis as the cause of condensation is also lacking. Localized cell multiplication without migration can produce an illusion of condensation as more and more cells fill a given space, and the necessity for cell

cycling in nephrogenesis referred to earlier is compatible with this mechanism. However, there is as yet no evidence that the condensation phase is associated with particularly high levels of mitosis, nor would mitosis provide a rapid enough increase in cell concentration to fit with the speed of condensation morphogenesis.

If the evidence supporting cell-adhesion molecules, migration, growth, and loss of extracellular matrix seems too weak to support them as mechanisms of condensation, what other possibilities are there? One that has yet to be excluded is cell traction, a mechanism based on the balance of two forces: the adhesions that cells make to one another and to their environment and the contractile abilities of the cells. Harris *et al.* (1984) have shown that these forces can cause uniform cell culture to break up into aggregates. One reason for suggesting that tractional forces may play a role in the formation of nephrogenic condensations comes from the following simple experiment (J.B.L. Bard, unpublished): If kidney rudiments are cultured on inert substrata, they adhere, spread, and form about 20–30 nephrons. If, however, such rudiments are cultured in hanging-drop culture where there is no substratum, no more than two or three nephrons form, even though other tissues such as salivary glands develop normally under these conditions. Such a result demonstrates the need for substratum adhesions if condensations are to form (Bard, 1990), but does not, of course, prove that this need is manifested through traction.

An article on the effects of knocking out the transcription factor BF-2 (Hatini et al., 1996) has added a new perspective to the condensation story. This gene, one of the smallish family of "winged-helix" or "fork-head" transcription factors that has an evolutionarily conserved DNA-binding domain found in *Drosophila* homologues (Lai *et al.*, 1993), is expressed in two populations of mesenchymal cells: those that will form the medullary stroma and a second population in the cortex that does not participate in nephrogenesis and may become the juxtaglomerular cells that will make renin. Until this research was published there was no reason to suppose that the stromal cells had any effect on nephrogenesis, but, quite unexpectedly, the small kidneys of the BF-2−/− mouse were found to contain relatively few but very large mesenchymal aggregates that failed to form nephrons (Fig. 9c, d). There was also an abnormally small number of branches in the collecting-duct system. The large mesenchymal condensates were up to 20 cells in diameter and expressed *wnt-4*, a marker of the late condensation stage (Gavin *et al.*, 1990), but their further differentiation seemed to be blocked.

The abnormalities in the BF-2 knockout show that signals from stromal cells play a role in the condensation process. It is not, however, clear whether these factors are the same as those whose loss restricts the general growth of the BF-2−/− kidney. In short, we have no complete explanation as to how a balance of chemical signaling and physical forces leads to the formation of nephrogenic condensations within the relatively dense and adhesive metanephric mesenchyme, and this puzzle is one of the more intriguing facets of kidney develop-

ment. It is, however, clear that a great deal more needs to be done with the kidneys of the BF-2 knockout mouse to determine whether its expression patterns of known kidney growth factors are altered.

2. Epitheliogenesis

Once condensation has been completed, the cells in the aggregate undergo a mesenchymal-to-epithelial transition, a process that in culture lasts for about 24–36 hr (Davies and Garrod, 1995). During this process, markers characteristic of mesenchyme cells (e.g. fibronectin, vimentin) are lost, and those characteristic of epithelia (e.g., E-cadherin, desmoglein, cytokeratins) are gained according to a precisely timed sequence [see Davies (1996) for a summary of the timing].

There appears to be a checkpoint of development between condensation and epitheliogenesis, presumably to ensure that further development does not take place until condensation is complete. The signaling molecule Wnt-4 might be involved in this process. Wnt-4 is produced by condensing cells and is subject to positive feedback, so that it stimulates its own synthesis (Stark *et al.*, 1994). In a Wnt-4 knockout mouse, cells do not progress beyond the condensation stage, although the condensates themselves become rather large, suggesting that in the absence of Wnt-4 either a signal indicating that condensation has gone on long enough is missing or the cell multiplication that would normally take place to elongate nephrons takes place, even though nephrogenesis itself is blocked.

The molecular processes that regulate the large-scale change in gene expression accompanying mesenchyme-to-epithelial transition remain completely unknown. Several potentially significant transcription factors are first detectable around this time, including Hox-B3, Hox-B7, LFB-3, and Pax-8, but, on the basis of the timing of their expression or the behavior of $-/-$ mice, none seems to be a master regulator of epitheliogenesis. One of the functions of Pax-8 that is compatible with the binding-site data (discussed earlier) may be the down-regulation of WT1 in most of the condensate while allowing its upregulation in the podocyte layer (Pritchard-Jones *et al.*, 1990).

The process of acquiring an epithelial phenotype also involves the acquisition of a new set of cell–cell and cell–matrix adhesion molecules, including the E- and K-cadherins, α_6-integrin matrix receptors, and laminin A in the basement membrane (Klein *et al.*, 1990; Xiang *et al.*, 1994; Ekblom *et al.*, 1990; Korhonen *et al.*, 1990, 1992; Sorokin *et al.*, 1990). Antibodies to E-cadherin fail to block nephrogenesis (Vestweber *et al.*, 1985), although this may simply reflect redundancy with K-cadherin. Antibodies that inhibit the interactions between α_6-integrin and laminin A block nephrogenesis in culture (Klein *et al.*, 1988), suggesting a critical role for integrin-mediated attachment of cells to their basement membrane. Data have, however, shown that nephrogenesis apparently takes place normally in α_6-integrin-deficient transgenic mice (Georges-Labouesse *et al.*, 1996).

3. Nephron Maturation

In the S-shaped body, the proximodistal polarity that is set up by unknown mechanisms at or before the comma stage becomes manifest in the differentiation states of the cells. Cells at the most proximal end flatten (Dorup and Maunsbach, 1982) and alter their gene expression, losing *c-myc*, *Hox-c9*, *LFB-1*, and *LFB-3*, but retain expression of *WT1*. Loss of these transcription factors is accompanied by what can be considered partial reversion to a mesenchymal phenotype: Glomerular podocyte cells express the "mesenchymal" markers α_3-integrin and vimentin and cease to express c-MET and cytokeratins, but they continue to express the "epithelial" markers α_6-integrin and desmosomal components (Holthofer *et al.*, 1984; Korhonen *et al.*, 1990, 1992; Garrod and Fleming, 1990; Sonnenberg *et al.*, 1993; Woolf *et al.*, 1995). The result of this is an arrangement of cells more organized into a sheet than a typical mesenchyme but more leaky than most epithelia, making an excellent primary filter for urine production.

The remaining cells of the S-shaped tubule remain classically epithelial but differentiate into regional segments, with segment identity being expressed (in terms of known molecular markers) in a proximodistal temporal sequence. All of the cells reduce their expression of *WT1* and also lose *N-myc*, *MFH-1*, and *Pax-2* (Lazzaro *et al.*, 1990; Poleev *et al.*, 1992; Mugrauer *et al.*, 1991; Miura *et al.*, 1993; Armstrong *et al.*, 1992; Dressler *et al.*, 1992; Rothenpieler and Dressler, 1993). If *Pax-2* loss is prevented in transgenic mice, differentiation of the tubule is inhibited and a pathological condition similar to nephrotic syndrome is produced (Dressler *et al.*, 1992).

At about this time, the developing nephrons interact with the vascular system of the developing kidney. Before they are of any physiological use, nephrons have to be connected to a blood supply at their glomerular end (the source of urinary fluid) and the collecting duct at their distal end. The first indications of a blood supply to the glomeruli are present very early in nephron development, when capillaries appear in the glomerular cleft (Loughna *et al.*, 1996). Most evidence indicates an angiogenic origin for these vessels (i.e., by the sprouting of preexisting vessels), the most significant observation being a lack of capillary development in kidney rudiments removed from E11 mice and cultured away from any external sources of endothelium (Bernstein *et al.*, 1981). Kidney rudiments elicit a strong outgrowth of capillaries if cultured on avian chorioallantoic membrane (Sariola *et al.*, 1983) and express vascular endothelial growth factor (VEGF; Landels *et al.*, 1994), which is known to be a major enhancer of angiogenesis in other systems (Breier *et al.*, 1992; Millauer *et al.*, 1992). VEGF is expressed by the glomerular epithelium, whereas its *flt-1* receptor is expressed by the endothelia themselves (Breier *et al.*, 1992; Simon *et al.*, 1995), suggesting a paracrine loop that might also be responsible for attracting growing capillaries to the correct site (although how they generate the complex glomerular architecture is still a mystery).

Connection to the collecting duct is achieved by fusion of the nephron and collecting duct epithelia, a process that again is not understood but that presumably includes the localized destruction of two basement membranes and the rearrangement of cell–cell contacts.

F. Development, Renal Function, and Kidney Disease

At this stage, when the basic structure of the kidney is in place, the reader might expect to find a section on the acquisition of the functional abilities of the organ that would in turn lead to an additional section on how these properties go awry in congenital kidney disorders. As almost nothing is known about the regulation of either of these two facets of the kidney story, these sections are conflated to this brief note.

We assume that the functional abilities of the various parts of the nephron derive from the pattern-formation processes that segment it into its proximal, loop, and other segments, but as yet have no knowledge of these events nor of their downstream effects. As for the congenital renal diseases, we should not confuse our success in identifying mutant genes with an understanding of why such mutations lead to abnormal kidneys. While we have a fair understanding of how mutated collagen IV genes help explain the abnormal glomerular filtration that characterizes the Alport syndrome, we have little idea as to how the other genes documented in Section III wreak their effects.

Here, it is interesting to look at Wilms' tumor, the first of the congenital renal disorders to be graced with a named gene, *WT1*. While it is true that mutations in this zinc-finger transcription can account for some 15% of the tumors, it is not obvious why a mutation here should lead to a single cell forming a 2-kg tumor composed of a disorganized mass containing what seem to be the appropriate cell populations. Moreover, a decade or so after the cloning of WT1 we still await WT2, WT3, WT4, etc. (to account for the remaining 85% of the tumors). The only appropriate position to take is that a complex chain of events is involved in initiating kidney development and that the breakage of a single link can flip the system onto an abnormal trajectory that is recognized as a cancer.

The multigenic basis for renal disease is shown most sharply in PKD where at least six genes have been mapped or cloned, in which mutations can lead to cysts. Here, the use of gene targeting to make mouse models of the disease (e.g., Moyer *et al.*, 1994) is likely to provide the most profitable line of investigation in elucidating the molecular basis of the disorder. A further advantage of this approach is that by studying a congenital abnormality we learn a great deal about normal development.

Nevertheless, in spite of an approach that will, in due course, provide dividends to both the renal embryologist and the "real" nephrologist, an interesting tension remains between them. The former looks at the early stages of kidney

formation, particularly the emergence of the structures that define the organ, while the latter wants to know more about the ways in which mutations in kidney differentiation lead to congenital diseases. The practical nephrologist thus is likely to be disappointed with the general focus of contemporary work, which is mainly on early organogenesis rather than on later differentiation. A possible consolation, however, is that the study of the differentiation of the functional abilities of the kidney is empty, if not easily accessible, territory begging to be invaded by those seriously interested in medical research.

V. The Future

A. Where We Are Now

The increase in our knowledge about kidney development in the decade since Saxén's monograph (1987) has been dramatic and has demonstrated the remarkable power of molecular genetic technology in opening up a field in which progress was becoming frustrated. In 1987, we knew little more about the genetic control of kidney development than that the transcription factor WT1 was important in regulating kidney development and that perhaps 15 or 20 regulatory genes of one sort or another were expressed during nephrogenesis. By now, researchers in the field have identified almost 300 genes that are expressed during the various stages of kidney development. We also know some of the signals and their downstream effects, we understand a great deal more about the various stages of kidney development and the interactions that underpin them, and we have mastered many new technical approaches to kidney development. The optimists among us can feel that a good job has been done over the past decade.

Nevertheless, there is a sense in which those dyed-in-the-wool pessimists who claimed that the advent of molecular technology would bring a surfeit of facts but little understanding can feel vindicated. They can point to the fact that we still understand almost nothing about what all of those genes do and that, even in a case like *WT1* where we know how much hangs on the activation of a single, key transcription factor, we still have little idea of what its downstream effects are and still await the cloning of those genes that account for the 85% of Wilms' tumors where *WT1* is not involved. It is even possible for them to have the gloomy satisfaction of saying that all of the millions of dollars spent on kidney research have done little more than demonstrate how much more complicated kidney development is than anyone expected.

We hold no truck with these views and think that those who hold them wanted to believe that elucidating the genetic basis of kidney organogenesis should be easy. In our view, the immense progress over the past decade has provided the study of kidney development with a far stronger factual and intellectual basis than it had then and has given it a substantial base from which we can properly and confi-

dently explore the processes that govern kidney development. Indeed, we confidently expect that the next decade will provide the answers to a whole set of questions and therefore use this last section to plot out the territory that we expect to be mapped during this period.

B. The Assay Problem

Progress in understanding the molecular and cellular underpinnings of any aspect of the developing kidney phenotype is, within limits, dependent on the assay systems available. Before going any further, it is probably sensible to remind readers of the practical context in which such work is going to be done.

The standard pathway for initiating change usually centers around a signal, a receptor, a signal transduction pathway if the receptor is not nuclear, and one or more transcription factors. The activation of these factors leads to a translation phase that, in turn, alters the cell phenotype, initiating a morphogenetic change that results in a new structure. There are many links in the chain of progress.

Given the wealth of molecules that have been identified as being involved in kidney development on the basis of their expression pattern, selection of a likely candidate gene for a particular role in many aspects of the process is not difficult. Proving that the gene has its hypothesized role is, however, turning out to be difficult, and here the Slack criteria [(i) expression at the right time and place; (ii) absence of the molecule blocks the phenotypes; (iii) addition of the molecule under these conditions restore the phenotype (Slack, 1993)] provide a first step in the analysis. Nevertheless, it is clear that the rules cannot always be adequate: They do not cover redundancy (where more than one molecule provides a single link in the chain), can only highlight a single link and not the whole chain, and may not always be practical to apply.

The expression test is the easiest and is always the baseline for selecting a candidate gene. Inhibition of expression is generally possible, in principle, through the use of transgenic technology, but the technique has severe limitations, even when it gives an abnormal phenotype. Although the abnormal phenotype confirms an important role for that gene, it gives little clue as to what that role is or even, without a great deal more work, the exact stage at which it executes that role. A finer level of resolution here, for the kidney at least, is provided by using antisense oligonucleotides to block translation in cultured rudiments or by adding antibodies to block the function of genes whose activity is external to the cell. However, in the absence of good downstream markers, it is still difficult to discern exactly what role a candidate gene has in a pathway. Moreover, as rudiments are small, it may not be easy to discern either quantitative changes in downstream markers or their presence if they are expressed only at a low level. The use of the remarkable ability of the kidney to grow in culture has limitations when the problem to be solved is that of identifying the function of a gene.

There are two other tools that are available, at least in principle. The first is the use of material from human congenital disorders, and much has already been made of Wilms' tumor. The second is the use of cell lines. If one could make a cell line that, for a short period at least, was able to display an appropriate phenotype for the problem under investigation, then it would be possible to block expression with antisense technology or antibodies, add excess protein, transfect the cells with appropriate genes, and up-regulate the expression of that gene, as well as recognize the presence of downstream genes expressed at low levels or quantitative change in their expression levels. These strategies would be particularly useful in the case of cell lines derived from the kidneys of knockout mice. In a sense, therefore, a set of problems in elucidating the genetic basis of kidney formation reduces to that of making highly state-specific cell lines. As has been discussed earlier, this approach has not been particularly successful so far for relatively early kidney development. Uninduced MM undergoes apoptosis, whereas induced MM progresses so far in culture that the cells are not useful for investigating the mechanisms that underlie that progress, and this is no matter what the genetic constitution of the MM cells.

Nevertheless, some of the tricks gleaned from studying kidney development *in vitro* may be helpful here. The use of various activators of induction (lithium, FGF, etc.) may enable early processes to be investigated, whereas the ability of LIF to block the differentiation of metanephrogenic mesenchyme at the stem-cell stage may provide stable cell lines for investigating slightly later signals and their effects. Even induced metanephrogenic mesenchyme is likely to be helpful as its downstream abilities are likely to be concentration- and substrate-dependent. In our view, the efforts required to make cell lines will be repaid many times over.

C. Likely Successes

One fortunate aspect of development in general is that its signals and receptors are used in many systems, and the tools for their investigation are readily accessible. It is, therefore, highly likely that we should soon know those molecules that signal inductive interactions in the kidney for both collecting-duct formation and MM stem-cell formation and differentiation. We also will probably know the signal from the stromal cells that helps regulate the formation of the nephrogenic condensation and that is probably upstream of Wnt-4. Knowledge of signals implies the appropriate receptor, and, given the availability of blocking antibodies that will work *in vitro*, we can look forward to substantial progress in our knowledge of the genetic pathways regulating the various interactions involved in kidney development before too many years have passed.

A second area in which progress is likely to be made in the short term is the elucidation of the various lineages within the developing kidney. As already

discussed, the work of Herzlinger and her colleagues has cast doubt on the traditional story that the duct gives rise to the collecting ducts while the MM forms nephrons, stroma, and (probably) the juxtaglomerular cells. It is also likely, but unproved, that neural crest and endothelial cells within the stroma form neurons and capillaries, respectively. The use of cell markers and confocal microscopy should provide the tools for confirming these lineage relationships and it should not be too difficult to do the experiments.

Progress in these areas will be relatively easy, and their exploration is also likely to clarify some aspects of the developmental phenotype that are still opaque. These include the early partitioning of the MM into stromal and stem-cell components, the origin of the juxtaglomerular complex, the morphogenesis of the nephron and neuronal organization within the kidney, mechanisms of growth, and reasons why stem cells are lost and nephron formation thus ceases around birth.

All of these problems fall under the rubric "reductionist," in that their solutions are simple facts. There are more such problems, but they are going to be much harder to solve. The most obvious of these are concerned with elucidating the downstream effects of cell signaling: These include the identification of the appropriate cascade of transcription factors and the genes that they cause to be expressed. This in turn will involve determining how these new genes change the cell phenotype. There is no shortage of such pathways to be elucidated, and this enterprise will certainly involve the whole repertoire of assays and approaches discussed in the preceding section, particularly the use of cell lines appropriate for each pathway. This will not be easy for any aspect of kidney development, but likely firsts here are the role of WT1 in effecting MM competence and the downstream effect on MM of the inducer from the ureteric bud.

Integration of these genes into regulatory pathways is the first step in shifting from the reductionist to the synthetic. Once we begin to see how signal pathways lead to new cell phenotypes, we will be able to work on the next set of such synthetic problems, those dealing with how these changes lead to new structures. Here, very little is known about any aspect of kidney morphogenesis, whether it be the localization of stem cells, formation of the bifurcations that generate the collecting-duct system, coalescence of nephrogenic cells into aggregates that undergo the mesenchyme-to-epithelium transition, or the first step in nephron formation or, indeed, any other step in their formation. These problems are not for the short term, partly because they are difficult and partly because they are unfashionable and therefore unlikely to attract much interest or financial support.

There is, however, one reason for supposing that the final set of such synthetic problems, those concerned with the generation of kidney function, may start to attract some attention soon: They are medically important, and the relatively pure research problems just discussed may provide some of the tools for investigating congenital kidney disease caused by either abnormal morphogenesis or differentiation.

D. Conclusions

Researchers whose field is kidney development find themselves living in interesting times. The past decade has provided us with a wealth of genetic and phenotypic information, questions are now well-defined, tools and assays are in place, and candidate genes abound. There is a clear program of work for the next decade that we are certain will lead to new insights into how the kidney develops and how the molecular basis of nephrogenesis goes awry in congenital kidney disease.

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