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Evolution of predetermined germ cells in vertebrate embryos: implications for macroevolution

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SUMMARY The germ line is established in animal embryos with the formation of primordial germ cells (PGCs), which give rise to gametes. Therefore, the need to form PGCs can act as a developmental constraint by inhibiting the evolution of embryonic patterning mechanisms that compromise their development. Conversely, events that stabilize the PGCs may liberate these constraints. Two modes of germ cell determination exist in animal embryos: (a) either PGCs are predetermined by the inheritance of germ cell determinants (germ plasm) or (b) PGCs are formed by inducing signals secreted by embryonic tissues (i.e., regulative determination). Surprisingly, among the major extant amphibian lineages, one mechanism is found in urodeles and the other in anurans. In anuran amphibians PGCs are predetermined by germ plasm; in urodele amphibians PGCs are formed by inducing signals. To determine which mechanism is ancestral to the tetrapod lineage and to understand the pattern of inheritance in higher vertebrates, we used a phylogenetic approach to analyze basic morphological processes in both groups and correlated these with mechanisms of germ cell determination. Our results indicate that regulative germ cell determination is a property of embryos retaining ancestral embryological processes, whereas predetermined germ cells are found in embryos with derived morphological traits. These correlations suggest that regulative germ cell formation is an important developmental constraint in vertebrate embryos, acting before the highly conserved pharyngula stage. Moreover, our analysis suggests that germ plasm has evolved independently in several lineages of vertebrate embryos.

INTRODUCTION

Variations in adult morphology arising during evolution are attributable to changes in developmental processes. The embryos of many vertebrates pass through a morphologically similar stage, the pharyngula, occurring after the completion of neurulation, which is characterized by visible somites, prominent pharyngeal arches, and ventral flexure occurring at the position of the neck (i.e., cervical flexure). Embryological variation occurring after the pharyngula stage plays a major role in diversification at higher order taxonomic levels (Richardson 1999), and understanding the molecular mechanisms underlying such diversification is currently an area of intense research. However, it is likely that variation of earlier developmental processes, occurring before and during the pharyngula stage, will result in the most profound morphological variation, even though these events may also be the most highly constrained and resistant to change (Raff 1996). In this review we consider the evolution of an early developmental process, the germ cell determining mechanisms among vertebrates, and its relation to developmental processes occurring before and after the pharyngula stage. Throughout we use the two major amphibian groups, the anurans (frogs and toads) and the urodeles (salamanders and newts), as the prime examples for discussion, but we also consider several issues at the level of the vertebrates.

Anuran and urodele embryos begin to diverge before gastrulation, at least a day before the pharyngula stage. When compared within a phylogenetic context, we note that in all cases early (prepharyngula) anuran development appears to be more derived and shows significant variability among individual species, whereas urodele development retains ancestral features and is less variable within the various urodele lineages that have been examined. Because the germ line develops by very different mechanisms in anuran and urodele embryos, we next considered if this is related to the observed differences in morphogenesis in these groups.

In anuran embryos primordial germ cells (PGCs), the cells that give rise to gametes, are of endodermal origin, and they are specified by the differential distribution of maternally deposited germ cell determinants (known as germ plasm) to the presumptive germ line blastomeres. Thus, from the inception of development, anuran PGCs are considered to be predetermined by germ plasm. In urodele embryos PGCs derive from lateral plate mesoderm. Urodele embryos do not contain germ plasm, and so PGCs are specified later in development than in anurans. In urodeles PGCs form in response to extracellular inducing signals, not unlike those that produce other mesodermal cell types. This is considered regulative germ cell specification. When compared within a phylogenetic context, again the anuran mode of development appears to be derived, whereas the urodele mechanism appears to be primitive to the tetrapods. Furthermore, when considering the phylogeny of animals with and without germ plasm, the data suggest that germ plasm has evolved independently in several lineages of vertebrate embryos.

Development of the notochord is a fundamental aspect of vertebrate embryogenesis. We identified a strong correlation between the position of the notochord at the completion of gastrulation and the mode of germ cell specification carried out by embryos. Thus, regulative germ cell specification is correlated, in diverse groups, with the ancestral mode of notochord development. In contrast, a derived process of notochord development is observed in embryos that develop with a predetermined germ line. Within this context, we discuss how the emergence of germ plasm within an individual lineage may alleviate developmental constraints that are imposed on morphogenetic movements by the process of regulative germ cell specification and that regulative germ cell specification may cause the retention of ancestral embryological characters. In this way germ plasm may facilitate the evolution of novel embryological features and thereby contribute to macroevolutionary divergence of adult form.

EARLY MORPHOGENESIS DIVERGES BEFORE THE PHARYNGULA STAGE IN AXOLOTLS AND *XENOPUS*

> Nothing in biology makes sense except when seen in the light of evolution.
>
> T. Dobzhansky (1973)

The fact that vertebrate embryos from diverse taxa and very different early embryological forms (such as fish, amphibians, and mammals) converge on a similar morphology at the pharyngula stage has long been recognized (Von Baer 1828), and it has been postulated that passage through the pharyngula stage serves as a constraint on the potential for morphological variation. Indeed, after the pharyngula stage embryonic form diverges in a species-specific manner, leading to the view that passage through the pharyngula stage represents a developmental bottleneck (for discussions of the "hourglass model" describing the pharyngula stage as a developmental bottleneck, see Elinson 1987; Raff 1996; Gerhart and Kirschner 1997). Changes in developmental processes occurring before the pharyngula stage, however, may result in profound alterations to body structure, that is, macroevolution (Raff 1996). An excellent example of early developmental changes correlating with differences in adult morphology are found in the major amphibian lineages.

Pharyngula stage embryos and adults of *Xenopus laevis* (a frog) and axolotl (*Ambystoma mexicanum*, a salamander) are compared in Figure 1. Note that in *Xenopus* embryos (Fig. 1A) the cervical region is reduced and does not undergo flexure (marking the future position of the neck) as it does in axolotls (Fig. 1B, arrow) or other vertebrates. X-rays of adult *Xenopus* and axolotl females show the vastly different skeletal structure of these animals. The axolotl (Fig. 1, E and F) has a skeletal structure similar to that of most vertebrates, including two cervical vertebrae in the neck and 18 total vertebrae (arrow); *Xenopus* (Fig. 1, C and D), in contrast, has a much shortened vertebral column with only eight total vertebrae and one cervical vertebra. In addition, *Xenopus* also has the expanded pelvic girdle and lengthened hindlimbs typical of most frogs.

Anurans and urodeles diverged from a common ancestor about 250 million years ago (Cannatella and Hillis 1992; Milner 1992), and therefore it is likely that only one of the patterns of early development observed in the extant amphibians reflects the ancestral pattern. The other mode would be a derived pattern. The adult urodele body plan more closely approximates that of the common amphibian ancestor than does that of anurans (Duellman and Trueb 1986), particularly that of *Xenopus*, which is highly derived (Cannatella and De Sa 1993). Here we compare various aspects of embryonic development. For clarification, the anuran or urodele species referred to in each study are identified in text by superscripted letters that are listed in Table 1. We conclude that urodele embryos have retained more features that can be considered primitive to vertebrates, and anuran embryos are generally more derived. Although comparative aspects of amphibian morphogenesis have been reviewed before (Hanken 1986; Malacinski et al. 1997), this has not been addressed within a framework assuming a monophyletic origin of urodeles and anurans nor with insights available from more recent work.

Origin of mesoderm from deep cells, as occurs in *Xenopus*, is a derived condition, whereas urodeles retain a more conserved mode of mesoderm morphogenesis

A variety of evidence demonstrates that in *Xenopus* embryos only a very small amount of the dorsal mesoderm originates

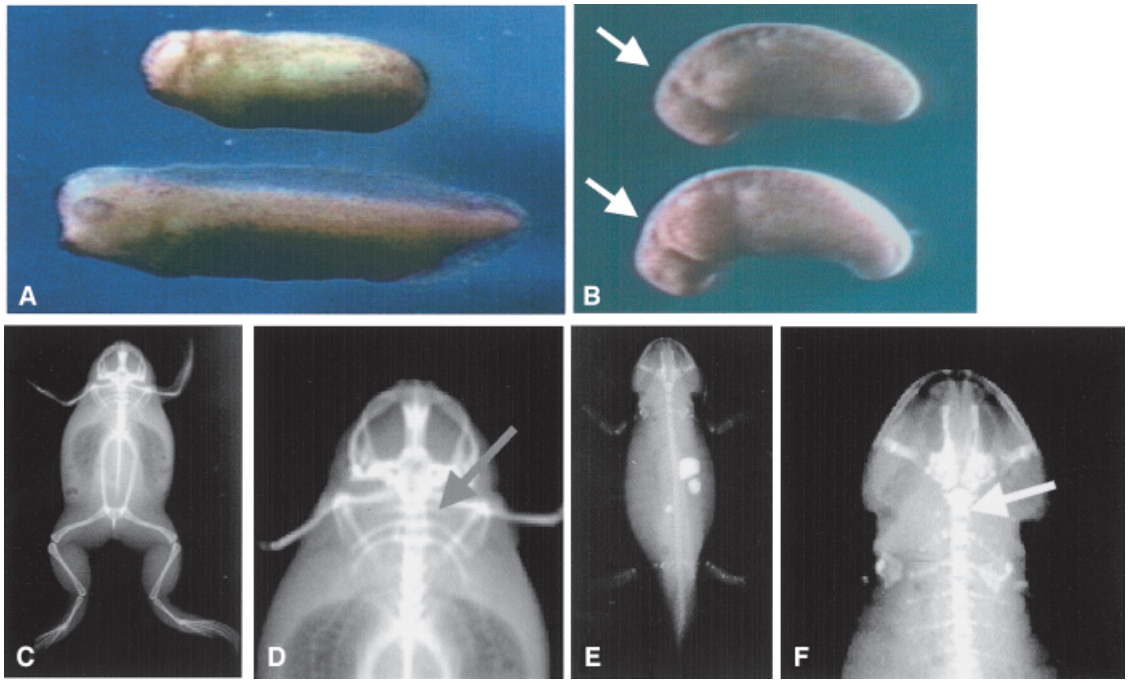

Fig. 1. Comparison of *Xenopus* and axolotl embryos and adults. (A) *Xenopus* embryos during "pharyngula" stages. (B) Axolotl embryos during pharyngula stages. Note cervical flexure at position of arrow in axolotl. *Xenopus* embryos do not undergo the prominent cervical flexure (compare A and B). (For both *Xenopus* and axolotl, top embryo at tailbud stage 26, bottom embryo at tailbud stage 28; Nieuwkoop and Faber [1994] stages for *Xenopus*. Bordzilovskaya et al. [1989] stages for axolotl. Staging is roughly equivalent for both species.) (C–F) X-rays taken through adult females to show skeletal structure. (C) Adult *Xenopus* female. Note highly irregular vertebral pattern, including extended pelvic bones. *Xenopus* retains only eight presacral vertebrae. (D) Close-up of animal shown in C, showing neck region. Note single cervical vertebra (position of arrow). (E) Adult axolotl female. Axolotls adults have 18 presacral vertebrae. (Bright regions in gut are undigested food.) (F) Close-up of neck region of animal in A showing two cervical vertebrae (arrow).

from cells that are on the surface epithelium at the gastrula stage. Almost all the mesoderm originates from deep cells underlying the surface epithelium (Keller 1975, 1976; Smith and Malacinski 1983; Minsuk and Keller 1996; for a review see Keller 2000). Those surface cells that involute around the blastopore lips give rise primarily to endoderm. In contrast, in axolotls about half of the dorsal mesoderm originates within the surface epithelium (Smith and Malacinski 1983). The presumptive paraxial mesoderm moves out of the epithelial layer as it rounds the lateral lips of the blastopore (Lundmark 1986), ingressing into the space between ectoderm and endoderm in a manner resembling the movements around the primitive streak in mammals (Shook et al. 2002).

Comparative analysis suggests that the derivation of mesoderm from surface cells is the ancestral condition for vertebrate development. For example, in addition to axolotls, mesoderm originates from surface cells in all other urodele amphibians that have been studied (Vogt 1929[b]; Pasteels 1942[c]; Delarue et al. 1992[c]; Shook et al. 2002[d,e]) and most informatively in sturgeons, a primitive chondrostean fish (Ballard and Ginsburg 1980; Bolker 1993). Indeed, the origin of mesoderm only from deep cells appears limited even among anurans, as many anuran species other than *Xenopus* develop dorsal mesoderm from surface cells (Vogt 1929[m]; Pasteels 1942[n]; Purcell and Keller 1993[o]; Delarue et al. 1994[p]; Minsuk and Keller 1996[q]), similar to urodeles. Together

Table 1. Amphibian species for which data are discussed for aspects of morphogenesis or germ cell formation are referred to in the text by superscript letters

Urodeles	Anurans
a. *Ambystoma mexicanum*	l. *Xenopus laevis*
b. Triton	m. Bombinator
c. *Pleurodeles waltlii*	n. *Discoglossus*
d. *Ambystoma maculatum*	o. *Ceratophrys ornata*
e. *Taricha granulosa*	p. *Rana pipiens*
f. *Hemidactylium scutatum*	q. *Hymenochirus*
g. *Triturus viridescens*	r. *Rana sphenocephala*
h. *Triturus torosus*	s. *Rana temporaria*
i. *Ambystoma jeffersonianum*	t. *Rana esculenta*
j. *Triturus alpestris*	u. *Bufo bufo*
k. *Triturus cristatus carnifex*	

these results suggest that the development of mesoderm from deep cells, as in *Xenopus*, is a derived condition, having evolved within the anuran lineage, as has been suggested elsewhere (Purcell and Keller 1993; Minsuk and Keller 1996).

Notochord forms part of the archenteron roof in urodeles and most other vertebrates but not in *Xenopus*

The appearance of a notochord is a hallmark of chordate development. As a consequence of the surface origin of dorsal mesoderm in axolotls, the notochord comes to occupy the roof of the presumptive archenteron, flanked on either side by the presumptive endoderm (Smith and Malacinski 1983; Brun and Garson 1984). Later, as neurulation is underway, the notochord moves into the mesodermal layer by ingression and the definitive endoderm seals to form the archenteron. This mode of notochord development has been highly conserved during vertebrate evolution. For example, in the embryos of sturgeons (Ballard and Ginsburg 1980; Bolker 1993) and of mice (Sulik et al. 1994), the notochord is a component of the gastrocoel roof. Further, this type of notochord development is a primitive feature of chordate embryogenesis, as it is observed during development of ascidian and *Amphioxus* embryos (Balinsky 1975).

In *Xenopus* embryos, the notochord is at no time a component of the gastrocoel roof. As a result of the deep origin of mesoderm, when the presumptive notochord rounds the dorsal lip it comes to overlie the endodermal roof of the presumptive archenteron. Notochord formation is variable in anuran embryos, and in some cases is intermediate between urodeles and *Xenopus* with varying amounts of the notochord associated with the archenteron roof. The process of ingression is also species variable in anurans, with several novel mechanisms having been identified (for a thorough review of this topic, see Keller 2000).

In addition to anurans, notochord development can show variability in other groups, including the amniotes. For example, in reptiles, some species (such as geckos) show the primitive mode of notochord development, whereas other species (such as snakes) do not (Hubert 1985a). Similarly, in contrast to mice, in chicks the notochord develops within the mesodermal layer, separate from the endoderm of the archenteron roof (Sausedo and Schoenwolf 1993).

Other differences that modify the body plan occur after the pharyngula stage

In addition to the early differences observed in axolotl and *Xenopus* development, some processes occurring during and after the pharyngula stage also show variation. For instance, the somites are contributors to major vertebrate structures. As above, somite patterning in various anuran species is unique and highly variable; in urodeles, somites develop the classic "rosette" structure that is common to other vertebrates (Youn and Malacinski 1981a,b[a,c,l,r]; Keynes and Stern 1988). There is no evidence for variability in somite development in urodeles. (This topic has received excellent coverage in reviews by Malacinski et al. [1997] and Keller [2000].)

During anuran development, the ventral posterior trunk is lost and/or repatterned as the gut tube coils near the head (for *Xenopus* see Lynch and Fraser 1990; Nieuwkoop and Faber 1994). This is a unique modification (Duellman and Trueb 1986); it does not occur during development of axolotls or other terrestrial vertebrates or primitive fish such as sturgeon and lungfish. The loss of posterior ventral structures has consequences for development of blood and PGCs (Masi and Johnson, unpublished data), the latter of which will be considered in detail below (see Fig. 4).

Finally, anurans undergo a radical metamorphosis that prepares adults for terrestrial life. Events similar to anuran metamorphosis do not occur in primitive fish, urodele amphibians, or amniotes. From this it can be surmised that metamorphosis, *as it occurs in anuran embryos*, is a derived condition that emerged after the divergence of the anuran and urodele amphibian lineages.

Conclusions

Several processes of embryogenesis, occurring before and after the pharyngula stage, are more primitive (less derived) in axolotls than in *Xenopus*. Axolotls exhibit the classic pharyngula stage of development, that is the hallmark for the constraint on vertebrate embryonic structure, whereas the pharyngula stage of *Xenopus* embryos deviates from the conserved state. In axolotl, the early location of presumptive mesoderm, and morphogenesis of the notochord and somites resemble these processes in other vertebrates, whereas they do not in *Xenopus*. Moreover, these processes show more variability in anurans than in urodeles. From this analysis we conclude that embryos from various anurans display significant diversity in processes that would be expected to alter the body plan, suggesting they have been liberated from at least some of the constraints imposed on embryos with more conserved features.

In addition to anurans variable forms of gastrulation have been observed in fish, ranging from an amphibian-like gastrulation in primitive fish such as sturgeon and lungfish (Wourms and Kemp 1982) to a variety of different modes of gastrulation in teleosts. Thus, divergence before the pharyngula bottleneck is a recurring theme in vertebrate evolution, and it is likely to contribute to morphological diversity in adults. Also, plasticity with respect to early morphogenetic processes is a property of specific lineages of vertebrate embryos.

DEVELOPMENT OF THE GERM LINE

In bilateral animals PGCs originate outside of the gonads, to which they are transported later in development. Typically, PGCs arrive at the gonads after the rudiments of the embryonic body plan have been established, and therefore it is possible to have separate influences govern the development of the germ cells and the somatic cells. Higher animals have at least two very different means for specifying PGCs. Some species have a predetermined germ cell lineage that is specified by cytoplasmic germ cell determinants laid down in the oocyte and then differentially distributed to presumptive germ cells during embryogenesis. In other species, germ cell determination occurs later during development, and is not directly dependent on maternal molecules. In these species germ cell determination is governed by a regulative mode involving cell–cell interactions. Interestingly, the anurans use one of these mechanisms and urodeles the other.

A priori, it is not possible to predict which mechanism of germ cell determination is ancestral to the tetrapod lineage. Little descriptive data exist describing germ cell development in vertebrate embryos that could lead to robust conclusions concerning the mechanism in embryos of the tetrapod ancestor (Nieuwkoop and Sutasurya 1979), and experimental data from such species is nonexistent. Nevertheless, as a first step toward understanding this process, we discuss below available information concerning how PGCs are formed in those terrestrial vertebrate species about which germ cell development has been described in some detail.

Development of PGCs in amphibians

Germ plasm and germ cell specification in frog embryos

Among anurans most is known about germ cell development in *X. laevis*. However, because germ cell formation in *Xenopus* is typical of anurans (Blackler 1958), it is likely that most of the events characterized in *Xenopus* occur in other frogs as well. During oogenesis in *Xenopus*, electron-dense material known as germ plasm is synthesized and transported to the vegetal cortex through a specific transport mechanism involving association with the mitochondrial cloud or Balbiani body (Heasman et al. 1984; King et al. 1999). Germ plasm is inherited by the vegetal-most blastomeres of newly fertilized embryos, within the presumptive endoderm, and segregated into a few cells at the blastula stage (Houston and King 2000b; Kloc et al. 2001). Those cells that inherit germ plasm will give rise to PGCs, and sister cells that do not inherit germ plasm become typical somatic endoderm. By the time PGCs reach the gonad, material resembling the electron-dense germinal granules of germ plasm, called nuage, accumulates in a perinuclear position within the PGCs. By tracing germ plasm, or nuage, the germ line can be followed as an independent cell lineage from the inception of development through adulthood (for a review, see Nieuwkoop and Sutasurya 1979). A number of experiments in which germ plasm was destroyed (Bounoure 1939[s]; Bounoure et al. 1954[s]; Padoa 1963[t]; Smith 1966[p]; Tanabe and Kotani 1974[l]; Züst and Dixon 1975[l]; Ikenishi and Kotani 1979[l]) or transplanted into recipient embryos (Smith 1966[p]; Wakahara 1977[l]) suggest that germ plasm contains determinants that govern germ cell specification.

Recent work has identified molecules that comprise the germ plasm in *Xenopus*, and these include germ cell specific proteins and RNAs. Recent reviews detail the development of germ plasm and germ cell development in *Xenopus* extensively (Houston and King 2000b; Kloc et al. 2001).

PGC development in urodele embryos

Urodeles use mechanisms of germ cell determination that appear to have little in common with the mechanisms acting in frogs. In urodele embryos determination of germ cells is regulated by the response of cells to extracellular signals (i.e., regulative germ cell specification).

In urodele embryos vegetal pole germ plasm is absent and PGCs develop in the posterior-lateral plate mesoderm (Humphrey 1925,[f,g,h] 1929[d,i]; Nieuwkoop 1947[a]; Smith 1964[a]; Maufroid and Capuron 1972[c]; Ikenishi and Nieuwkoop 1978[a]), in contrast to the endodermal origin of PGCs in anurans. Typical of amphibian mesoderm, PGCs can be induced from cells in the animal cap region of blastula stage urodele embryos, if these are exposed to signals from the ventral vegetal blastomeres (Nieuwkoop 1969[a]; Kocher-Becker and Tiedemann 1971[g]; Boterenbrood and Nieuwkoop 1973[a]; Sutasurja and Nieuwkoop 1974[a,c,j,k]; Michael 1984[a,j]; Maufroid and Capuron 1985[c]). In addition to PGCs, these signals induce a variety of somatic mesodermal cell types, including mesenchyme, mesonephros, and blood, suggesting the signals that induce germ cells and somatic cells are not qualitatively different.

The possibility that in urodeles germ plasm is located in the equatorial region of the egg and plays a role in the production of PGCs has been discussed for many years (Nieuwkoop and Sutasurya 1979; Smith et al. 1983; Wakahara 1996). In *Xenopus*, expression of the *dazl* gene (*Xdazl*) is a marker for germ plasm (Houston et al. 1998). Using the axolotl homolog of this sequence, Johnson et al. (2001) were unable to find localized germ plasm during oogenesis or embryogenesis in axolotls. They concluded that an anuran-type germ plasm does not direct the specification of PGCs in urodele embryos. Rather, PGCs in urodeles are most likely to be induced from typical embryonic cells, such as those that also produce somatic derivatives. In support of this, axolotl PGCs

commence expression of germ cell-specific genes only after they come into close contact with the gonadal rudiments (Johnson et al. 2001), a time that closely coincides with the appearance of nuage in this species (Ikenishi and Nieuwkoop 1978). Combined with the classic experiments on several urodele species described above, we conclude that in urodeles PGCs are determined by zygotic influences, including posterior-ventral mesoderm inducing signals and subsequent cell interactions, not maternal molecules, as in anurans.

Do amniotes retain anuran or urodele germ cell determining mechanisms?

In addition to amphibians, divergent patterns of germ cell specification and development have also been identified in amniote embryos. All amniotes share a common amphibian ancestor, and therefore the embryos of the amphibian ancestor to the amniote lineage most likely had features similar to extant urodeles or extant anurans, but not both. Whether the predetermined mechanism of germ cell determination in anurans or the regulative mechanism of urodeles is retained in amniotes is considered below.

PGC development in mammalian embryos

The development of PGCs in mice occurs with striking parallels to the mechanism in urodele amphibians. In mouse embryos PGCs originate from cells located within the proximal epiblast, and the germ cell precursors are clonally related to the founder cells of the primitive blood and the allantois (Lawson and Hage 1994). PGCs require signaling from *bone morphogenetic proteins* (BMPs) (ventral mesoderm inducing agents) for their production (Lawson et al. 1999; Ying and Zhao 2001; Ying et al. 2001). Furthermore, recent work suggests that interferons may trigger a cascade of gene expression, leading to lineage restriction of the germ cells (Saitou et al. 2002). The PGC precursors migrate through the posterior primitive streak in association with extraembryonic mesoderm into the allantois, a posterior extraembryonic region (Ginsburg et al. 1990). Later they become associated with the hindgut, and from there they move through the dorsal mesentery to the genital ridges (Anderson et al. 2000). A similar site of origin of PGCs has been described for several mammalian species (Nieuwkoop and Sutasurya 1979).

Germ plasm has not been found in early mouse embryos (reviewed in Eddy 1975). On the contrary, any cell in the epiblast of a mouse embryo can give rise to PGCs if placed within the proper signaling context (Tam and Zhou 1996; Yoshimizu et al. 2001), indicating that mouse PGCs are not predetermined. Nuage appears after specification, when PGCs are in the hindgut (Eddy 1974). More recently transcription of the mouse *vasa* gene homolog, *mvh* (the *vasa* gene family is discussed in detail below), has been shown to commence after PGCs begin to colonize the genital ridge (Fujiwara et al. 1994; Toyooka et al. 2000), which is very similar to the timing of *vasa* transcriptional activation in axolotls (Bachvarova and Johnson, unpublished data). Taken together, the results summarized above indicate that urodele and mouse embryos share similar mechanisms of PGC development (for further discussion of this view, see Wakahara 1996; McLaren 1999), as they do mesoderm morphogenetic patterns.

PGC development in chicken embryos

The development of PGCs in chicks occurs very differently than in mice. In chicks the PGCs are derived from cells on the ventral surface of the area pellucida, a central region of the epiblast; these cells migrate ventrally into the plane of the hypoblast, a layer of extraembryonic endoderm (Eyal-Giladi et al. 1981; Sutasurya et al. 1983; Karagenc et al. 1996). After becoming associated with the hypoblast, the PGCs move anteriorly to colonize a region known as the germinal crescent, within the area opaca (on the periphery of the epiblast), anterior to the cranial-most region of the embryo. From the germinal crescent the PGCs enter the embryonic circulation (Nieuwkoop and Sutasurya 1979). They later exit the circulation in the vicinity of the gonad and are drawn to the developing genital ridges by chemotactic attraction (Kuwana et al. 1986).

Tsunekawa et al. (2000) recently isolated a chick homolog of *vasa* and showed that *vasa* protein was present in germ cells throughout their life cycle. *Vasa* protein is found in specific structures associated with the mitochondrial cloud (a structure involved in germ plasm formation; see below) in oocytes and is segregated into a few cells during cleavage. *Vasa*-containing cells of the area pellucida then follow the path to the germinal crescent and the gonad described above for PGCs. This expression pattern suggests that germ cells are predetermined by maternally inherited germ plasm in chicks.

PGC development in reptiles

Much less is known about germ cell development in the embryos of reptiles than in widely studied experimental systems. Germ plasm has never been identified in reptilian eggs (Hubert 1985b). Further, indications are that different species of reptiles use one or the other of the two amniote patterns of PGC development described above or a combination of both patterns. For instance, in several groups (*Gekkonidae*, *Iguanidae*, *Lacertidae*, turtles) PGCs follow a pattern of development very similar to mice, in that PGCs apparently originate within the epiblast and assume a posterior location in the mesoderm adjacent to the cloaca (Hubert 1985a). PGCs in these species migrate to the gonad

via an interstitial route through the posterior lateral mesoderm. Other groups (*Scincidae*, *Chamaeleonidae*, *Agamidae*, *Cordylidae*, *Anguidae*, snakes) apparently use a mechanism similar to the chicks, in which PGCs migrate to the anterior region, which is presumably equivalent to the germinal crescent, and reach the gonads through the circulatory system. Finally, in tuataras (*Sphenodon*) both modes may be used, some PGCs showing posterior interstitial migration and other appearing anteriorly and moving through the circulation (for reviews, see Nieuwkoop and Sutasurya 1979; Hubert 1985a,b).

Conclusion

In amniotes, like amphibians, two different modes of germ cell development have been reported, one in mice in which the germ cells develop in response to extracellular signals, similar to urodeles, and another in chicks in which the germ cells enter the endoderm and are predetermined by maternal molecules, similar to frogs. Various species of reptiles apparently display one or the other of these processes. Thus, within the context that all terrestrial vertebrates share a common ancestor, two possibilities exist. First, perhaps PGCs in the embryos of the ancestral tetrapod were predetermined. In this case frog and chick embryos would represent the primitive mechanism, and urodeles and mice would have evolved novel strategies. Second, alternatively, the primitive condition was retained by urodeles and mammals, and the predetermined germ line in frogs, and in chicks, is newly evolved. To address which of these models may be more accurate requires an analysis of available information from the embryos of more primitive species.

EVOLUTION OF GERM CELL DETERMINING MECHANISMS IN EMBRYOS

> Nothing in evolution makes sense except when seen in the light of phylogeny.
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> Jay Savage (1997)

Is the anuran mechanism of germ cell specification a primitive or convergent trait: phylogenetic analysis of the presence of germ plasm in different animals

Much of our knowledge about the molecular genetic mechanisms that govern animal development has been obtained from studies using as model systems two widely divergent animals: *X. laevis*, representing vertebrates, and *Drosophila melanogaster*, representing invertebrates. These species share strikingly similar mechanisms in the way they establish the germ line.

The posterior pole of *Drosophila* eggs contains material that is structurally and functionally similar to anuran germ plasm (Mahowald and Hennen 1971) (called pole plasm in *Drosophila*). *Drosophila* pole plasm is inherited by PGCs during the early stages of development, and it has been shown to function directly as a determinant of germ cells (Illmensee and Mahowald 1974; Okada et al. 1974). More recently, at least one homologous molecule has been identified in the germ (pole) plasm of *Drosophila* and *Xenopus* (*nanos-XCAT2*; Mosquera et al. 1993; Forbes and Lehmann 1998). These observations have led to the conclusion that the role of germ plasm in PGC development is a process that has been conserved from insects to vertebrates. However, this view has not been considered within a plausible phylogenetic context.

As discussed above, germ plasm is present in birds and frogs but not in mammals or urodeles. Thus, either germ plasm or the regulative mode of germ cell determination has evolved independently in more than one lineage. The morphological evidence presented above suggests that urodeles more closely resemble both the embryonic development and adult morphology of the tetrapod ancestor than frogs, arguing against the view that germ plasm is an ancestral character of tetrapod embryos. If this is true, then similarities shared between the mechanisms of germ cell determination in anurans and chick (and higher insects as well) must be the result of independently derived traits converging on a developmental process. However, the likely nature of the ancestral trait can only be inferred by examining the trait in a sister-group. To examine this question, we constructed an abbreviated phylogenetic tree of the deuterostomes showing the distribution of animals that contain germ plasm in their eggs and those that do not (Fig. 2A).

Evidence presented below indicates that the closest sister-group to tetrapods, represented by the lungfish, does not contain germ plasm (Fig. 3). Going further down the tree, although the embryos of zebrafish and other teleosts are known to contain germ plasm, the situation in more primitive fishes, such as sturgeon, is unclear. Urochordate embryos appear to contain germ plasm (Fujimura and Takamura 2000); however, they also appear to be capable of producing germ cells by regulative mechanisms (Takamura et al. 2002). For this reason they are considered indeterminate. Echinoderms are a sister-group of the chordate phylum. Germ plasm has not been identified in early echinoderm embryos, although oocytes contain nuage (Eddy 1975; Holland 1978). Furthermore, more recent evidence from deletion studies indicates that PGCs in sea urchins are not predetermined; rather, they most likely arise in response to regulative influences during development (Ransick et al. 1996), suggesting that the regulative mode is primitive. These data were also subjected to optimization analysis with the program MacClade to reconstruct the character evolution using parsimony and to infer the ancestral mode of germ cell determination. As indicated in Figure 2A, the hypothesis inferred by this analysis is that the induced or regulative mode is the ancestral

condition and germ plasm is a derived condition that has arisen convergently multiple times in the deuterostomes. More complete data on the distribution of germ plasm among various deuterostomes, including those not represented in the figure (e.g., cephalochordates, chondrichthyes, and agnathans), are necessary to test this hypothesis. Nevertheless, the hypothesis is supported by considering the distribution of regulative and predetermined germ cell-forming mechanisms in embryos with conserved or derived traits.

For comparison, a phylogenetic tree has been constructed in Figure 2B showing the distribution of animals that exhibit a primitive or derived mode of gastrulation. For chordates we used the position of the notochord at the completion of gastrulation as an indicator of either the primitive or derived condition (see above). Comparison of Figure 2A with Figure 2B indicates a strong correlation between the presence of germ plasm and derived modes of gastrulation. This suggests that in the chordates the mode of notochord formation may be predictive for the mode of germ cell determination in those animals for which it is uncertain. This view is considered below. Moreover, it is interesting to note that ascidian embryos, which appear to contain germ plasm, undergo a primitive form of gastrulation and are also capable of producing germ cells by regulative means, as predicted by the model. This suggests that the regulative means of germ cell specification may be ancestral to the ascidian lineage.

Taken together, these results support the view that the embryos of the vertebrate ancestor did not contain germ plasm. Furthermore, the development of PGCs by regulative mechanisms is likely to represent the primitive condition in deuterostome embryos.

Expression of germ cell-specific genes in different species. Are homologous patterns of expression retained? Are similar roles retained?

Germ plasm has been identified in diverse species, deuterstomal and protostomal, and so has been considered to be conserved in the animal kingdom. In recent years a number of germ cell-specific genes have been identified in diverse species. Below we review the data to determine whether they are expressed in a pattern indicating homology. Given the importance of homology assessment, a clarification of our view of the concept follows. With developmental genes, we recognize two distinct characters: the gene itself and the expression of that gene. For example, although the *dazl* genes in *Xenopus* and chickens are homologous (related by descent),

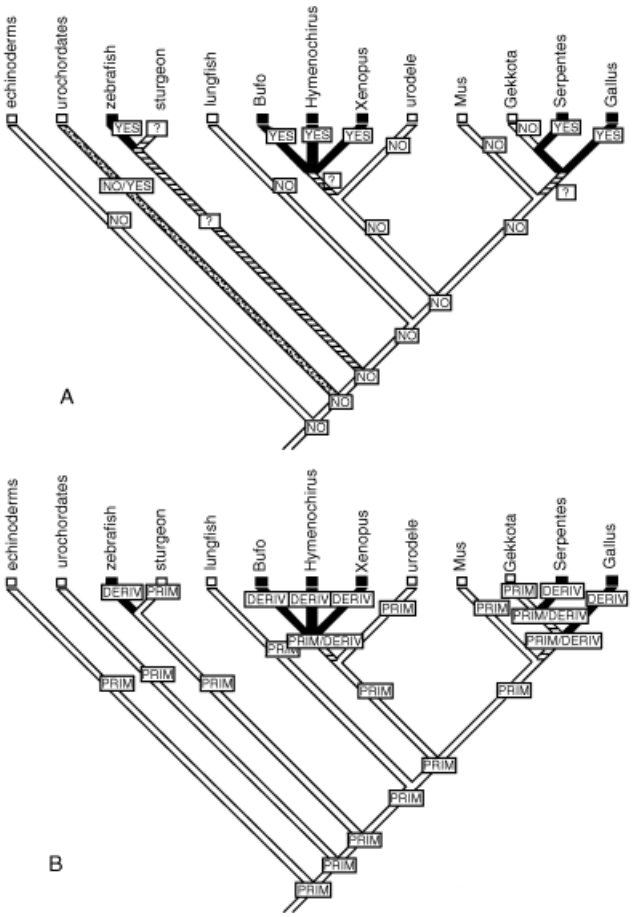

Fig. 2. Trees depicting the evolutionary relationships between type of embryology and the mode of germ cell formation by either predetermined (cell autonomous) or regulative (induced) mechanisms. Branch lengths on both trees are arbitrary and not intended to indicate evolutionary distance or relative time of divergence. (A) Evolution of germ cell determination mode as hypothesized by parsimony optimization. The black boxes represent taxa for which data suggest that the germ line is predetermined. Open boxes represent taxa for which data suggest that the germ line is formed by the induced or regulative mode. The branches are labeled for the presence (YES) or absence (NO) of predetermined germ cells. Cross hatched (?) = unknown. Stippling (NO/YES) = evidence supports both a germ plasm and a regulative mode of germ cell formation. The data for reptiles is based only on cytological evidence as discussed in the text. The hypothesis generated by MacClade analysis (Maddison and Maddison 1992) is that the ancestral mode is regulative, indicated here as NO. (B) Evolution of derived morphogenesis in vertebrate embryos as hypothesized by parsimony optimization. Species developing from embryos that have primitive gastrulation movements are represented by open boxes. Species developing from embryos with derived modes are represented by shaded boxes. DERIV, derived; PRIM, primitive. For chordates, we used position of the notochord within the roof of the archenteron at the completion of gastrulation as an indicator of primitive gastrulation movements; any other position indicates a derived mode of gastrulation. We assumed a primitive embryology for lungfish, due to overall similarity to the embryos of urodele amphibians, as well as for echinoderms, which lack a notochord. Note that the distribution of predetermined germ lines, where known, is identical to that of embryos with derived morphogenetic traits.

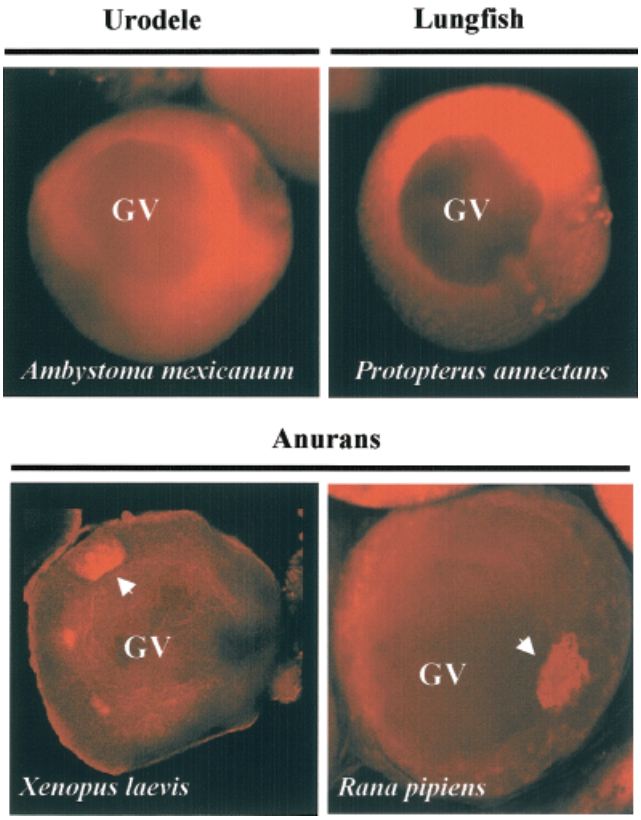

Fig. 3. Lungfish oocytes do not contain a mitochondrial cloud. Oocytes were liberated from the ovaries of *Protopterus annectans*, *Ambystoma mexicanum*, *Xenopus laevis*, and *Rana pipiens* adult females by treatment with collagenase. Oocytes were washed, and previtellogenic oocytes were incubated in MitoTracker Red (Molecular Probes, Eugene, OR) as described elsewhere to detect the presence of mitochondrial clouds (Johnson et al. 2001). Oocytes were analyzed by optical sectioning. A mitochondrial cloud is prominent in the cytoplasm from oocytes of *R. pipiens* and *X. laevis* (arrows). Like axolotl oocytes, lungfish oocytes do not contain a mitochondrial cloud. Identical results were obtained for 20 oocytes of each species, including a wide range of sizes.

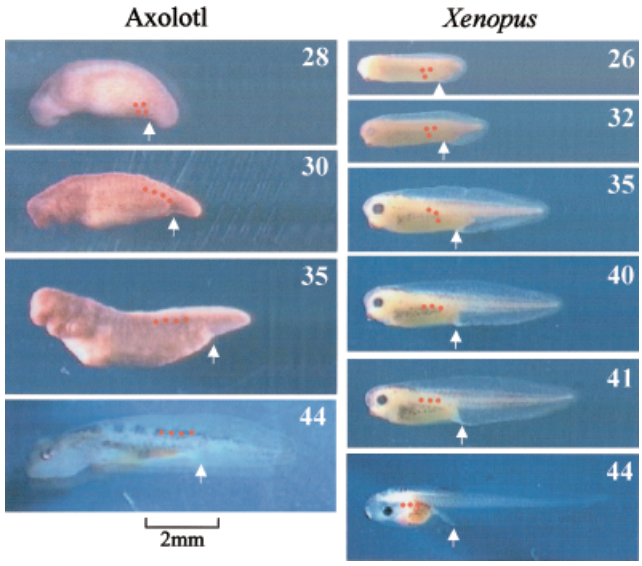

Fig. 4. Germ cells take up an anterior position during *Xenopus* development but not axolotl development. Axolotl and *Xenopus* embryos are shown at stages from tailbud to larva. The position of the cloaca is indicated by a white arrow. The approximate position of the PGCs is indicated by red dots. As far as is known, in axolotls PGC precursors move with the posterior lateral mesoderm during gastrulation into the posterior half of the trunk and end up at larval stages in the genital ridge at vertebral levels 7–18 (Humphrey 1925; Nieuwkoop 1947). In *Xenopus* the PGCs are found in the mid-posterior endoderm at the end of gastrulation and in the mid-trunk at tailbud stages (Houston and King 2000a,b.) As the trunk shortens, the germ cells enter the gonad at vertebral levels 3–8.

the expression patterns of the genes need not be (they may be convergent, independently evolved). The recognition of these characters as distinct entities is critical to the discussion of the evolution of germ cell specifying mechanisms which follows. (For a discussion of this view of homology, see Abouheif et al. 1997.)

DAZ-like genes

The *DAZ* (*Deleted in Azoospermia*) gene encodes an RNA binding protein located on the Y chromosome of human males, and mutations to *DAZ* are a major cause of male sterility (Reijo et al. 1995). The *dazl* gene family was identified on the basis of homology to *DAZ*. The *dazl* genes are autosomal (Cooke et al. 1996; Reijo et al. 1996) and show germ cell-specific expression in each of the species from which members have been identified. Recent work demonstrates that the *dazl* gene family is related to a *Drosophila* gene called *boule* (Xu et al. 2001), which regulates late stages of gonadal germ cell development in males. In fact, *Drosophila* mutants for *boule*, in which germ cells arrest during meiotic progression (Eberhart et al. 1996), can be rescued by the product of *Xenopus Dazl* (Houston et al. 1998), indicating conservation of sequence between these genes. Nevertheless, during normal *Xenopus* development the product of the *dazl* gene fulfills a quite different function.

In *Xenopus*, *Xdazl* RNA is localized to the germ plasm in eggs and embryos (Houston et al. 1998). Depletion studies show that maternal *Xdazl* RNA is required for normal PGC development (Houston and King 2000a), consistent with the known role of germ plasm. Yet, this role for *dazl* RNA is not conserved in amphibians. In axolotl eggs and embryos maternal *Dazl* RNA is not localized (Johnson et al. 2001), as would be suggested by the absence of germ plasm in this species. The earliest cell specific expression of the axolotl *dazl* gene is found in PGCs after they are in the vicinity of the genital ridges (Johnson et al. 2001), suggesting that the gene is

induced by patterning signals in the embryo. From these results it is impossible to determine the pattern of *dazl* gene expression that is ancestral to tetrapods. However, in mice homozygous for a knock-out of the *dazl* gene, germ cells are produced normally, though germ cell development arrests at or near meiosis in both sexes (Ruggiu et al. 1997). This suggests that *DAZL* may have acquired an additional role in *Xenopus* that is not present in mammals.

Recently, the *DAZ*-like gene (*Zdazl*) has also been identified in zebrafish (*Brachydanio rerio*) (Maegawa et al. 1999). Maternal *Zdazl* RNA shows a localized pattern of expression during oogenesis that closely resembles *Xdazl* in *Xenopus*. Zebrafish embryos contain germ plasm (Knaut et al. 2000); however, unlike *Xdazl* in *Xenopus*, *Zdazl* RNA is not found in the germ plasm or the germ cell precursors early in development. Zebrafish germ cells originate at the periphery of the developing blastoderm in the animal hemisphere (Olsen et al. 1997; Yoon et al. 1997; Knaut et al. 2000), whereas *Zdazl* RNA is found within the vegetal yolk cell during early stages (Maegawa et al. 1999). Thus, despite the similar location of the maternal *Dazl* transcript, its role in germ cell development is not conserved between *Xenopus* and zebrafish.

Finally, in *Caenorhabditis elegans* the *dazl* gene is not expressed in early embryos, and it has been shown to affect meiotic progression in oocytes, not spermatocytes (Karashima et al. 2000). This is opposite to its function in *Drosophila*. Furthermore, even though the germ line is predetermined in *C. elegans* (Seydoux and Strome 1999), maternal *dazl* RNA does not appear to mediate early germ cell development, as it does in *Xenopus*.

Vasa genes

The *vasa* gene was first identified in a screen for the grandchildless phenotype to identify components of the germ plasm in *Drosophila* (Hay et al. 1988; Lasko and Ashburner 1988). *Vasa* encodes an RNA unwindase that is a member of the DEAD box family of RNA binding proteins. *Vasa* RNA is broadly distributed in *Drosophila* eggs; however, the protein is a component of germ plasm and is required for normal development of the PGCs (Hay et al. 1988; Lasko and Ashburner 1988).

Genes homologous to *vasa* have been identified in *Xenopus* (Komiya et al. 1994) and axolotls (Drum and Johnson, unpublished data). The *vasa* gene shows diverse patterns of expression in these amphibians. For example, in *Xenopus* embryos maternal *vasa* RNA is found early in germ cell precursors, as well as in somatic cells, and the zygotic gene also does not show germ cell-specific expression (Ikenishi and Tanaka 2000). The protein encoded by the *vasa* gene, on the other hand, segregates with the germ plasm through the early stages of development (Ikenishi 1998), and antibody injection studies show that the maternal protein is required for PGC development (Ikenishi and Tanaka 1997). Maternal *vasa* RNA in axolotls is not germ cell specific; rather, it is randomly distributed in early embryos (Drum and Johnson, unpublished data). The zygotic gene is not activated until late in development, much later than in *Xenopus*, and in a cell-specific manner, in PGCs in the gonads (Bachvarova and Johnson, unpublished data). The distribution of the Vasa protein in axolotls has not yet been investigated. Nevertheless, the expression of maternal *vasa* RNA, as well as early zygotic expression, shows different patterns in the amphibians.

Vasa gene homologs have been isolated from several species of teleost fish, including zebrafish (Olsen et al. 1997; Yoon et al. 1997), medaka (Shinomiya et al. 2000), rainbow trout (Yoshizaki et al. 2000), tilapia (Kobayashi et al. 2000), and several others (Knaut et al. 2002). In zebrafish maternal *vasa* RNA is a component of germ plasm in the egg and is distributed to early germ cells; however, the protein it encodes is not (Knaut et al. 2000). This pattern is the inverse of *vasa* expression in *Drosophila* and is different from *Xenopus*. Recently, Knaut et al. (2002) examined the expression profile of *vasa* genes in embryos from diverse teleosts by in situ hybridization. They show that *vasa* RNA is localized in butterfly fish, the most basal species they examined, as well as in ostariophysans (a clade including tetra, Fegrades danio, zebrafish, and carp). In contrast, *vasa* RNA is not localized in euteleosts (including trout, rainbow fish, and medaka), even though these embryos, like other teleosts, contain germ plasm. They conclude that *vasa* RNA localization is basal to the teleosts, and this feature has been lost in the euteleost lineage.

In a series of expression experiments in *Xenopus* oocytes, Knaut et al. (2002) demonstrated that the 3′-untranslated region from butterfly fish and ostariophysans *vasa* RNAs are sufficient to direct translocation of injected RNAs to the germ plasm. However, *Xenopus vasa*-like RNA (*XVLG1*) is not a component of the germ plasm, indicating that *vasa* localization has not been conserved between teleosts and anuran amphibians. Interestingly, an RNA encoding a related RNA helicase, *DEADsouth*, is localized to the germ plasm in *Xenopus* oocytes, and sequences within the 3′-untranslated region of this RNA share a weak homology with that of zebrafish vasa. Because *DEADsouth* is from a different family of helicases than the *vasa* family (MacArthur et al. 2000), this suggests that the *cis*-acting signals that govern localization of RNAs to the germ plasm have evolved more than once in the vertebrates. This supports the likelihood that germ plasm has evolved multiple times in vertebrate lineages and suggests that germ plasm can evolve quite readily.

Recently, a *vasa* homolog has been isolated from *Ciona*, a urochordate (Fujimura and Takamura 2000). Ascidian embryos develop from mosaic eggs, and maternal *vasa* RNA expression in *Ciona* appears to marks PGCs in the

endodermal strand posterior cells (Takamura et al. 2002). However, extirpation studies demonstrate that *Ciona* embryos can compensate for the loss of early PGCs by producing them anew within the embryo. Thus, ascidian embryos may have both predetermined and regulative germ cells. Because PGCs cannot be replaced upon removal in vertebrate embryos (Nieuwkoop and Sutasurya 1979), it is uncertain how this process relates to vertebrate germ cell development.

Finally, in amniotes, chick *vasa* protein is maternally inherited and segregates with the germ cells from the inception of their development, as described above. In mouse embryos *vasa* RNA and protein are not maternally inherited; they are first expressed in PGCs after they colonize the genital ridge (Fujiwara et al. 1994; Toyooka et al. 2000). Also, homozygotes for a null allele of the mouse *vasa* gene, *mvh*, demonstrate that *vasa* gene products are not required to produce PGCs. Rather, *mvh* is required only in male gonadal germ cells to complete meiosis (Tanaka et al. 2000). Therefore, the role of the *vasa* genes from mouse and *Xenopus* has not been conserved.

Nanos genes

The *nanos* gene family encodes zinc finger RNA binding proteins. *Nanos* was originally described in *Drosophila*, where it is required for posterior embryonic development (Irish et al. 1989). Maternal Nanos protein is incorporated into germ cells early in embryogenesis, and germ cells of embryos deficient for maternal Nanos protein display a variety of defects, including failure to complete migration to the gonads (Kobayashi et al. 1996; Forbes and Lehmann 1998). However, *nanos* is not required for the specification of germ cells. Similarly, RNAs from *nanos* homologs of *C. elegans* segregate with the germ cell progenitors and are also required for germ line maintenance after specification (Subramaniam and Seydoux 1999). Furthermore, *nanos*-deficient embryos display a phenotype similar to *Drosophila* in that germ cells fail to incorporate into the gonad. Interestingly, a *nanos* homolog has been identified in the leech *Helobdella robusta* (Pilon and Weisbat 1997), and its maternal transcript is broadly distributed in the egg cytoplasm (Kang et al. 2002). Its zygotic transcript only appears in presumptive germ cells when they can first be identified in segmental mesoderm, relatively late in development (Kang et al. 2002). Thus, although *nanos* is likely to be involved in leech PGC development, a role for maternal *nanos* transcripts has not been conserved in protostomes.

Among vertebrates, *nanos* homologs have been identified in zebrafish (Koprunner et al. 2001) and in *Xenopus* (Mosquera et al. 1993). In both species maternal RNA encoded by the homolog of the *nanos* gene becomes associated with germ plasm, though this is regulated by different mechanisms. In *Xenopus*, RNA encoding the *nanos* homolog, *XCAT2*, is localized to the germ plasm during early oogenesis, through the germ plasm specific localization pathway mediated by the mitochondrial cloud (Zhou and King 1996). Thus, *XCAT2* RNA is a component of germ plasm and is exclusive to the presumptive germ line blastomeres from the inception of development in *Xenopus*. In zebrafish, RNA from the *nanos* homolog, *nos1*, is distributed throughout the oocyte cytoplasm. During early embryogenesis a fraction of *nos1* RNA becomes associated with germ plasm (Koprunner et al. 2001). Nos1 transcripts associated with the germ plasm are protected from degradation, whereas RNA in other regions of cytoplasm is destroyed. Eventually, by 50% epiboly, *nos1* RNA is found exclusively within the germ plasm, and it is then partitioned to PGCs. Interestingly, *nos1* is not required for PGC specification but plays an essential role in migration of PGCs to the gonad, similar to the phenotypes of *nanos* mutants in *Drosophila* and *C. elegans*.

Although the biochemical functions of *nanos* RNA may be highly related, or equivalent, in diverse species, the expression patterns of these genes have not been conserved in vertebrate embryos. Apparently, the association of *nanos* RNA with germ plasm has evolved along separate pathways in *Xenopus* and zebrafish. *Nanos* homologs have not been identified in vertebrate species that develop without germ plasm, precluding comparison of *nanos* structure and function in the two types of organisms.

Summary

Based on the data discussed above, we draw the following two conclusions. First, for germ cell-specific genes there are distinct differences among organisms in whether the protein, the RNA, or neither is localized in the germ plasm, indicating that germ plasm has a different molecular makeup in different organisms. Second, there are differences in the way that germ plasm is formed in the oocytes of different species, as exemplified by *Xenopus* and zebrafish. This suggests that germ plasm has not been conserved across vertebrate phyla.

Did oocytes of the tetrapod ancestor contain germ plasm?

To shed light on whether the presence of maternal germ plasm is ancestral to terrestrial vertebrates, we analyzed oocytes of the lungfish, the closest living relative of the tetrapod ancestor (Zardoya et al. 1998), to determine whether they contain germ plasm.

Much is known about how germ plasm is formed in *Xenopus* oocytes. Heasman et al. (1984) showed that electron-dense germinal granules become associated with an aggregate of mitochondria, the mitochondrial cloud, in the cytoplasm of previtellogenic oocytes. During later stages of oogenesis, the germ plasm is transported along with the mitochondrial cloud to the vegetal cortex (King et al. 1999). Structures similar to

the mitochondrial cloud of *Xenopus* are found in the oocytes of chickens (Tsunekawa et al. 2000), which suggests a central role for the mitochondrial cloud in the formation of germ plasm. Consistent with this, axolotl oocytes, which do not contain germ plasm, do not contain a mitochondrial cloud (Johnson et al. 2001).

We were interested in determining whether axolotls (representing urodeles) or *Xenopus* (representing anurans) were more likely to reflect the mode of germ cell determination found in lungfish. To do this we asked if previtellogenic lungfish oocytes contain a mitochondrial cloud. Lungfish ovaries contain oocytes at all stages of oogenesis, similar to amphibians, and it was therefore easy to identify those at stages before and shortly after the onset of yolk uptake. Small oocytes were incubated with a mitochondria specific dye and analyzed by optical sectioning, as shown in Figure 3. Similar to axolotls, lungfish oocytes do not contain a mitochondrial cloud. Previtellogenic oocytes from *X. laevis* and *Rana pipiens* were used in this experiment as positive controls to demonstrate the mitochondrial cloud in anuran oocytes (arrows). These results support the likelihood that lungfish embryos produce PGCs in a mode similar to axolotls. In support of this, lungfish embryos resemble, morphologically, the embryos of urodeles not anurans (Kemp 1981). Further, we isolated the *vasa* gene from lungfish, and it demonstrates conservation of gene sequence between lungfish and axolotl, with the *Xenopus* sequences having diverged (Drum and Johnson, unpublished data). Together these results imply that specification of PGCs through extracellular signals in the absence of germ plasm, as occurs in urodeles and mammals, represents the primitive condition for terrestrial vertebrates and that the germ plasm in frog oocytes is independently evolved.

Conclusion

The role of germ plasm in animal development has presented an enigma to developmental biologists for many years because the eggs and early embryos of some species contain germ plasm and those of other species do not. On the one hand, the ultrastructure of germ plasm is very similar in different species, consisting of large aggregates of mitochondria and electron-dense germinal granules, suggesting that it has been conserved. On the other hand, more recent results demonstrate that the molecular composition of germ plasm and action of germ cell-specific genes has not been conserved. In addition, the evidence we present here suggests that germ cells in the embryos of lungfish, the closest sister-group to the tetrapods, do not contain germ plasm. Together, these results support the likelihood that germ plasm has evolved at least several times in animal lineages, including the terrestrial vertebrates.

GERM CELL DEVELOPMENT AND DEVELOPMENTAL CONSTRAINTS

Based on the material reviewed above, a reasonable hypothesis is that regulative germ cell specification, such as has been identified in sea urchins, urodele amphibians, and mouse embryos, represents the ancestral condition for germ cell development during vertebrate embryogenesis. This raises the question of why germ plasm may have arisen several times, in different lineages of vertebrate eggs and embryos. We address this issue below.

Absence of germ plasm correlates with primitive vertebrate embryology

Above we compared early morphogenesis in a variety of species to identify characteristics primitive to the tetrapod lineage. One of the processes that is highly conserved in early vertebrate embryos is the development of the notochord, which, in the primitive case, originates within the plane of presumptive endoderm lining the gastrocoel roof and later moves into the mesodermal layer. Then we presented evidence that, in general, embryos typical of the primitive mode of morphogenesis develop PGCs through a regulative mechanism, in the absence of germ plasm; in the case of mice and axolotls the PGCs develop in response to ventral-posterior signaling. On the other hand, those embryos in which the presumptive notochord does not reside within the gastrocoel roof form germ cells using germ plasm localized in the egg. This correlation suggests a cause and effect relationship between the evolution of predetermined germ cells and derived modes of gastrulation. Interestingly, many different patterns of gastrulation have been reported in anuran embryos, yet the PGCs in all anuran embryos are of endodermal origin and contain germ plasm (Blackler 1958[l,s,u]; Nieuwkoop and Sutasurya 1979). This suggests that the evolution of germ plasm in anurans *preceded* the evolution of novel modes of morphogenesis, and *germ plasm may then be a cause, not a consequence, of embryological variation*.

Mode of germ cell development as a constraint acting during gastrulation

Germ cell development acting as a constraint on embryonic morphogenesis is at first difficult to accept. However, the retention of a pool of PGCs, which will later produce gametes, is a fundamental constraint on any sexually reproducing organism, because the inability to pass inherited traits to subsequent generations will terminate an individual's lineage. Therefore, changes in developmental processes that endanger the maintenance of PGCs will not be retained. Events that stabilize the germ line, on the other hand, might relieve such restraints and establish conditions for the evolution of novel embryologies.

The notochord provides structural integrity to the early embryo and serves as the scaffold around which the vertebrae later form; therefore, it is not surprising that altering its position from the primitive location would have ramifications for embryonic and adult structure. Less obvious is why its primitive positioning would correlate with an ancestral mode of germ cell development. We consider a possible reason below.

The notochord secretes embryonic patterning signals, including molecules (e.g., follistatin, noggin, and chordin) that are known to antagonize BMP signals (Smith and Harland 1992; Hemmati-Brivanlou et al. 1994; Sasai et al. 1994). These molecules establish a BMP signaling gradient with lowest levels of BMP activity near the notochord and highest levels distal to the notochord. In mice BMP signals are required to produce PGCs, and in axolotl germ cells arise in the posterior lateral mesoderm where BMP levels are high. It is reasonable to assume that the PGCs can only be produced, or maintained, within a specific range of BMP levels, hence at a fixed distance from the notochord. We speculate that the position of the notochord within the epithelial layer of the presumptive gut provides conditions favorable for germ cell development by providing specific levels of BMP inhibitors (or other growth factors) in the posterior lateral mesoderm. Conversely, if the notochord were repositioned, in some location other than the early gut epithelium, BMP levels would be altered and may not be permissive for PGC specification. This would restrain the ability to evolve morphogenetic movements that alter notochord positioning, because this might compromise germ cell development. In this way regulative germ cell specification would act as a constraint on the evolution of notochord development and associated aspects of morphogenesis. On the other hand, PGCs specified by maternal determinants arise in a different location and are presumably less dependent on specific levels of growth factors to maintain their germ cell identity. Thus, the evolution of predetermined germ cells (i.e., the acquisition of germ plasm) would establish conditions that allow for the evolution of novel modes of morphogenesis and novel adult morphologies.

Repositioning of PGCs permits novel morphologies

The position of the germ cells in axolotl and *Xenopus* embryos is compared in Figure 4 (orange dots indicate germ cells). In a tailbud axolotl embryo (stage 28), the germ cells are located in the posterior mesoderm, lateral to the cloaca (Nieuwkoop 1947; Maufroid and Capuron 1972). During subsequent trunk elongation, the PGCs become distributed in the dorsal lateral mesoderm along the posterior half of the trunk. By stage 40 (early larva), they are found just ventral and medial to the mesonephric duct from somite levels 8 to 18 (Humphrey 1925; Nieuwkoop 1947; Johnson et al. 2001). Finally, they begin to colonize the gonads at about stage 42, when they commence differentiation into definitive PGCs, as defined by the commencement of germ cell-specific transcription and the appearance of nuage (Ikenishi and Nieuwkoop 1978; Johnson et al. 2001).

In *Xenopus* the PGCs are much internalized compared with axolotls because they are present deep within the endodermal layer, not within the more superficial mesodermal layer. At stage 26 the posterior development of *Xenopus* embryos closely resembles that of axolotl at stage 28 (Fig. 4). Note the position of the cloaca (Fig. 4, arrow) originating at about somite level 18 in both species (Nieuwkoop 1947; Nieuwkoop and Faber 1994). Between stages 35 and 42, all structures ventral to the most posterior trunk somites degenerate or are transformed into tailbud tissues, whereas the dorsal mesoderm becomes somitic mesoderm of the tail. The definitive trunk retains only eight vertebrae, derived from the most anterior somites, typical for anurans but unusual among vertebrates (Duellman and Trueb 1986). The postanal gut degenerates, and the cloaca is repositioned to a more anterior location. (Note the inversion of the angle to the cloacal opening that at first points in a rostral direction and then inverts to point caudally by stage 40 [also see Lynch and Fraser 1990; Nieuwkoop and Faber 1994].) During the loss of posterior structures the PGCs are located in endodermal tissue in front of the cloaca, and therefore they are not eliminated.

In *Xenopus* the gonads form in the remaining trunk mesoderm (corresponding to vertebral levels 3 to 8), located just behind the head (Wylie et al. 1976; Wylie and Heasman 1976), and PGCs reach the gonads at stage 42 (Kloc et al. 2001) (Fig. 4). Thus, in *Xenopus* the gonads are found at a much more anterior level (vertebral levels 3 to 8) than in the axolotl.

The loss of posterior-ventral tissues and the tight anterior coiling of the gut tube during larval stages, as well as the ensuing further loss of posterior structures that accompany metamorphosis (such as derivatives of the tail somites), are processes that occur in many anuran species and are unique to anuran development (Duellman and Trueb 1986). We propose that the evolution of endodermal PGCs capable of anterior displacement, such as in *Xenopus*, relieved developmental constraints imposed on embryos with primitive features. In this scenario, primitive embryology is restrained by the process that generates PGCs in the posterior-lateral and ventral structures, making it unlikely that the posterior region could be reduced or lost in animals with regulative germ cells, such as axolotls and mice. Further, it might be expected that more anteriorized morphologies are found among animals with embryos that contain localized germ plasm than among those animals that develop with regulative, posterior, germ cells. This relationship holds true in anurans (Fig. 1), and it can be argued for teleosts as well.

In fish a hallmark of primitive adult morphology is the presence of pelvic appendages, which gave rise to hindlimbs during evolution of the tetrapods. Three examples of fish with pelvic appendages are shown in Figure 5, the Black Tip Reef Shark (Fig. 5A), Gulf Sturgeon (Fig. 5B), and lungfish (Fig. 5C). In contrast, many teleost species with derived adult morphology have lost the pelvic appendages, as demonstrated in zebrafish (Fig. 5D), Damsel fish (Fig. 5E), Cream Angelfish (Fig. 5F), and Jack (Fig. 5G). Sturgeon and lungfish develop from embryologies that have much in common with urodele amphibians; further, our data suggest that lungfish embryos do not contain germ plasm. Based on the arguments presented above, we suggest that primitive fish are under constraints that prevent the loss of posterior structures, including the pelvic appendages, because they regulate germ cell development in the posterior-lateral mesoderm. On the other hand, zebrafish embryos contain germ plasm (Knaut et al. 2000), and they develop from a highly derived embryogenesis (Collazo et al. 1994) that accommodates the loss of the posterior structures. A more exaggerated outcome of an anteriorized type of development is found in the other teleost species shown above.

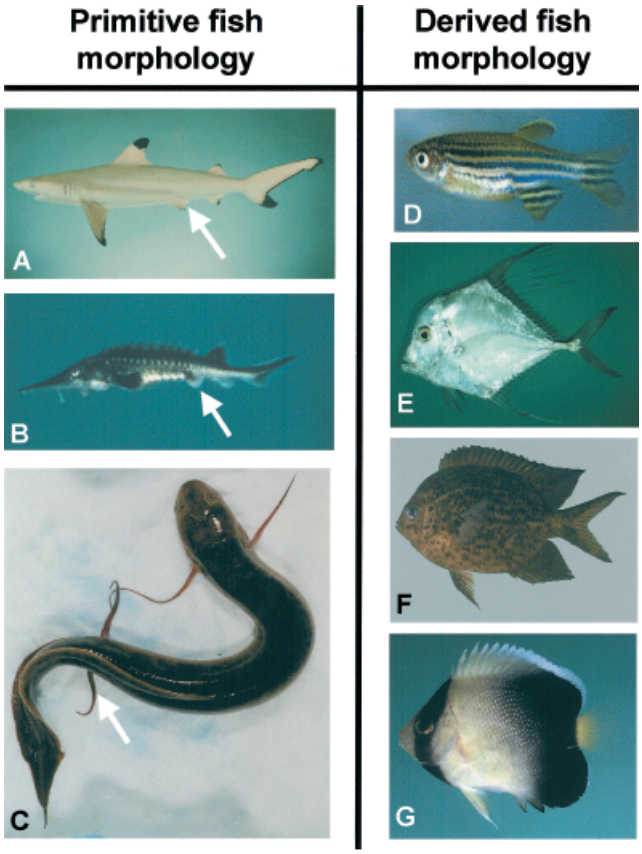

Fig. 5. Primitive fish morphology retains posterior structures that are lost in derived fish. In the left column are fish showing primitive morphologies, (A) Black Tip Reef Shark (*Carcharhinus melanopterus*), (B) Gulf Sturgeon (*Acipenser oxyrhynchus*), and (C) African lungfish (*Protopterus annectans*), as defined by the retention of pelvic appendages (arrows). In the right column are fish demonstrating derived anteriorized morphologies: (D) zebrafish (*Brachydanio rerio*), (E) Jack (*Alectis indicu*), (F) Damsel fish (*Acanthochromis* sp.), and (G) Cream Anglefish (*Apolemichthys xanthurus*). In D through G note the absence of pelvic appendages and diminished posterior trunk axis.

Role of germ plasm as a force in evolution

Frogs, birds, and teleost fish contain the greatest number of individual species among vertebrate groups (or clades), and the embryos of representative species from each group contain germ plasm. This trend of speciose clades correlated with the presence of germ plasm is especially evident when the above-mentioned clades are compared with their sister clades, whose embryos undergo regulative germ cell determination. In addition to increased species numbers, we also see another correlation with the presence of germ plasm, the anteriorization of the body plan. We suggest that a major effect of germ plasm in vertebrate embryos is to permit the evolution of embryologies that anteriorize the adult body plan, as a consequence of the release of the constraint maintaining the generalized primitive morphology. This would occur because embryos that do not require posterior-ventral signaling for germ cell production may more readily absorb mutations to axial patterning genes (such as *Hox* genes) that produce novel anteriorized phenotypes. Similar mutations could not be absorbed in more primitive embryos because they would terminate the germ line. The relative species diversity in each clade may reflect a tendency for natural selection to favor the evolution of anteriorized morphologies or may simply reflect morphological release in the absence of negative selection.

The functional components of germ plasm are germ cell-specific molecules, such as RNA binding proteins or transcription factors and the RNAs that encode them. These molecules are expressed in the oocytes of all species but are aggregated in specific locations only in oocytes with germ plasm. The localization of these germ cell-specific molecules to specific regions of the oocyte may cause them to act as determinants by programming germ cell-specific transcription in the nuclei of the lineage of cells to which they are distributed during development. In this way germ cell specification occurs earlier in embryos with germ plasm than in those embryos that lack it. Thus, the emergence of a predetermined germ line within a specific lineage may be the result of aggregation of germ cell-specific molecules with a specific structure (or organelle), such as the mitochondrial cloud, that localizes during oogenesis.

We propose a recurring two-step process that contributes to the evolution of divergent embryonic forms. The first step

involves the evolution of a localized functional germ plasm, via the association of germ cell specific molecules with the mitochondrial cloud, that can be transmitted to a specific set of blastomeres in the embryo. Second, as a consequence of a fixed (protected) germ line, the embryo can absorb mutations to embryonic patterning genes that affect the morphogenetic movements associated with gastrulation. The mutation process will tend to give rise to diverse and derived adult forms. Diversity can then be accomplished within individual lineages by the accumulation of specific mutations that are accommodated by prior steps in evolution. We suggest that this embryologically based mechanism for variation in adult morphology is a major contributor to species radiation and morphological diversity in vertebrates and thus to the process of macroevolution.

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