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REVIEW

Dissecting Hematopoiesis and Disease Using the Zebrafish

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The study of blood has often defined paradigms that are relevant to the biology of other vertebrate organ systems. As examples, stem cell physiology and the structure of the membrane cytoskeleton were first described in hematopoietic cells. Much of the reason for these successes resides in the ease with which blood cells can be isolated and manipulated *in vitro*. The cell biology of hematopoiesis can also be illuminated by the study of human disease states such as anemia, immunodeficiency, and leukemia. The sequential development of the blood system in vertebrates is characterized by ventral mesoderm induction, hematopoietic stem cell specification, and subsequent cell lineage differentiation. Some of the key regulatory steps in this process have been uncovered by studies in mouse, chicken, and *Xenopus*. More recently, the genetics of the zebrafish (*Danio rerio*) have been employed to define novel points of regulation of the hematopoietic program. In this review, we describe the advantages of the zebrafish system for the study of blood cell development and the initial success of the system in this pursuit. The striking similarity of zebrafish mutant phenotypes and human diseases emphasizes the utility of this model system for elucidating pathophysiologic mechanisms. New screens for lineage-specific mutations are beginning, and the availability of transgenics promises a better understanding of lineage-specific gene expression. The infrastructure of the zebrafish system is growing with an NIH-directed genome initiative, providing a detailed map of the zebrafish genome and an increasing number of candidate genes for the mutations. The zebrafish is poised to contribute greatly to our understanding of normal and disease-related hematopoiesis. © 1999 Academic Press

Key Words: **zebrafish; hematopoiesis; genetics; disease model.**

INTRODUCTION

Each day the human bone marrow gives rise to approximately 10^{12} new blood cells (Sieff and Williams, 1995). In addition to being of paramount importance in health and disease, this astonishing production rate has long fascinated developmental biologists. Many of the key events underlying organogenesis are exemplified by hematopoiesis: tissue induction, patterning, stem cell biology, cell fate decisions, and differentiation.

A number of factors make hematopoiesis especially amenable to study. Blood cells, including pluripotent stem cells, are readily available. Assays have been developed to define progenitor and stem cell populations, and many growth factors and receptors that regulate hematopoiesis have been characterized based on these assays. Transcriptional elements and DNA-binding proteins have been extensively studied *in vitro* and *in vivo* in transgenic and knockout mice. The study of human diseases such as thalassemia, immunodeficiency, and leukemia has provided important clues to the identity of factors regulating hematopoietic development.

The developmental biology of blood has been studied in many species, and several aspects of the program are conserved throughout vertebrate evolution (Fig. 1). During gastrulation, mesoderm is induced and patterned across the dorsal-to-ventral axis. Mesodermal derivatives include notochord, muscle, kidney, mesenchyme, and blood (Woodland, 1989; Ruiz i Altaba and Melton, 1990; Zon, 1995). Members of the TGF-β-related *BMP* family have been shown to be among the factors critical for this mesodermal patterning (Dale *et al.*, 1992; Graff *et al.*, 1994; Maeno *et al.*,

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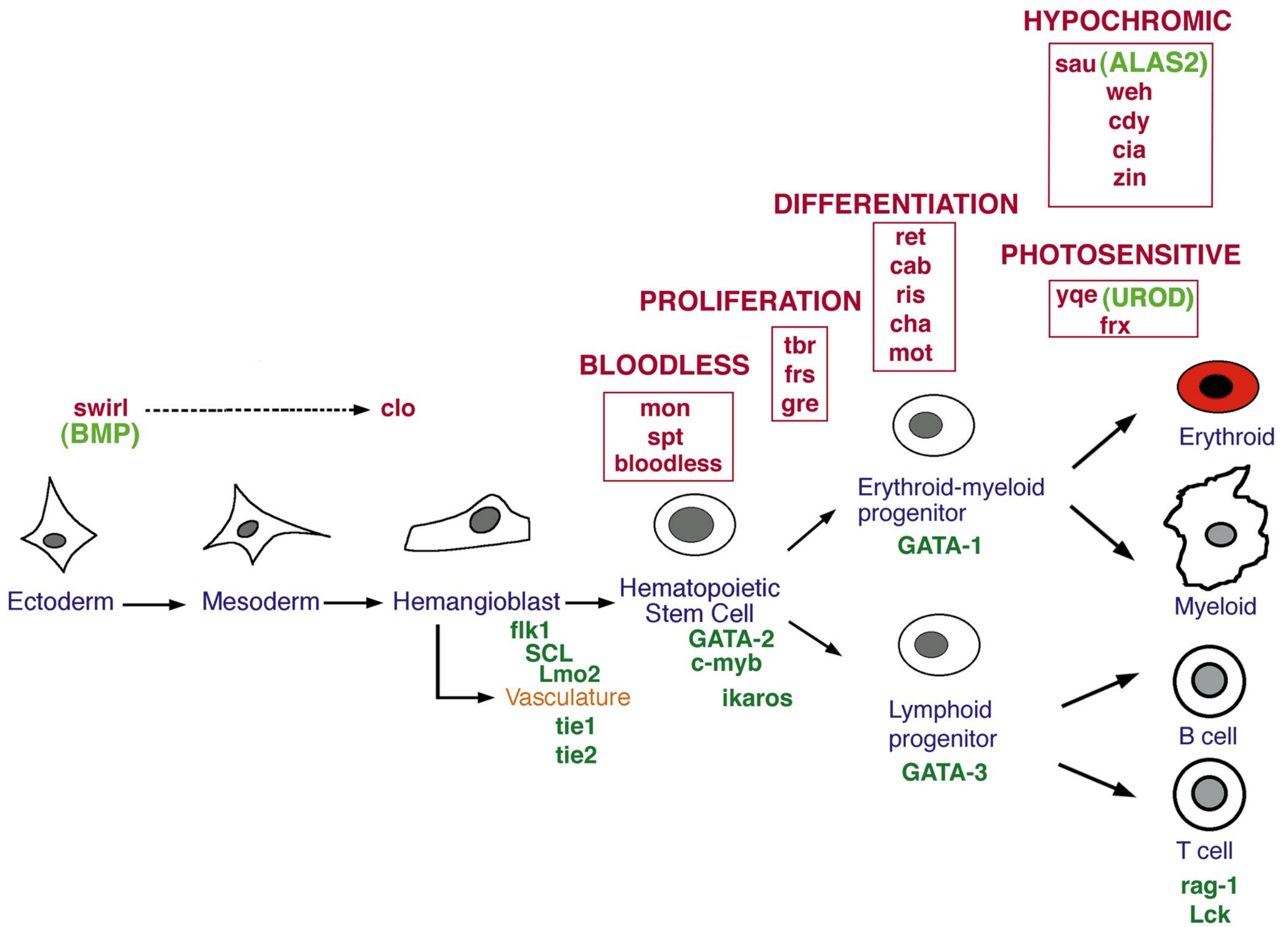

FIG. 1. The hematopoietic program in vertebrates. Blood development in vertebrates begins with the induction of ventral mesoderm, a portion of which is specified to form the putative hemangioblast. The hemangioblast gives rise to both blood and vascular tissue. The action of specific transcriptional and growth factors fosters the survival, proliferation, and terminal differentiation of hematopoietic stem cells. A number of these factors have been isolated and characterized from the zebrafish (in green). Zebrafish mutants (in red) are grouped by similar mutant phenotype. See text for details.

FIG. 2. The expression pattern of hematopoietic factors during zebrafish embryogenesis. Whole-embryo *in situ* hybridization analysis showing the localization of hematopoietic factors in zebrafish embryos at 3 somites, 18 somites, 24 h, or 48 h postfertilization (hpf). In the schematic panels (left), colored regions mark the anatomic areas involved in embryonic hematopoiesis. Early markers such as *SCL* are expressed in two stripes of lateral plate mesoderm by the 3-somite stage. *GATA-1* expression, not detectable at this stage, begins at approximately 5 somites. Between 18 somites and 24 hpf the stripes converge and fuse to form the ICM (red, anterior ICM; light blue, posterior ICM; dark blue, ventral tail region). Unlike *SCL,* which is expressed throughout the ICM and in the ventral tail, *GATA-1* expression is limited to the anterior and posterior ICM (arrows, posterior ICM; arrowheads, ventral tail putative hematopoietic region). In *cloche* mutant embryos, *GATA-1* persists only in the posterior ICM. By 48 hpf, *SCL* and *GATA-1* expression in blood is decreased, though *SCL* is highly expressed in the brain. *c-myb* is expressed in the ventral wall of the dorsal aorta (double arrow). 3 somites, dorsal view; 18 somites, anterior is to the left, dorsal is up; 24 and 48 hpf, anterior is up, dorsal is to the right. (Courtesy of C. Kimmel, M. A. Thompson, D. G. Ransom, and E. C. Liao.)

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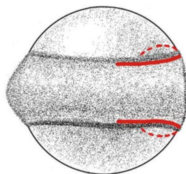
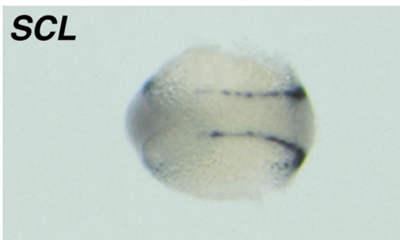
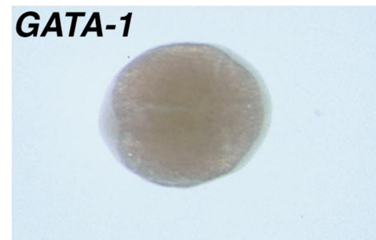
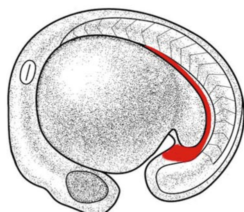
3 somites

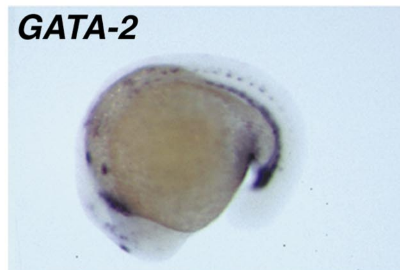


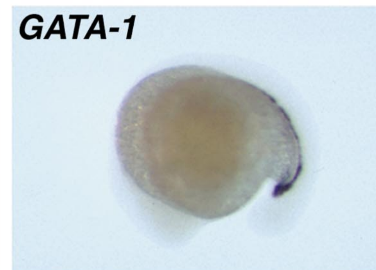




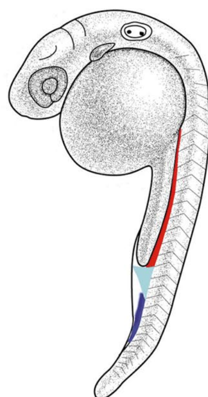
18 somites

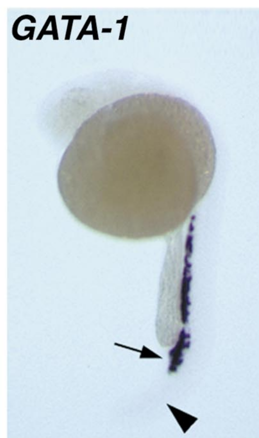


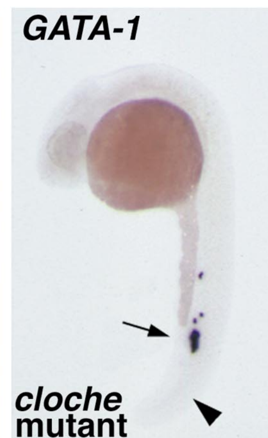




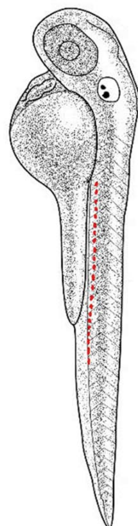
24 hpf

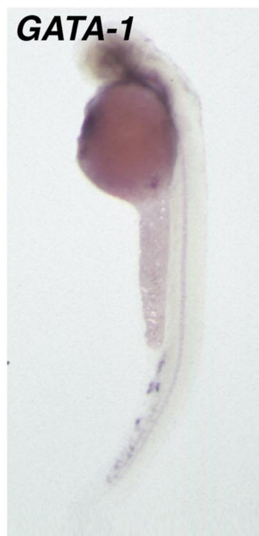


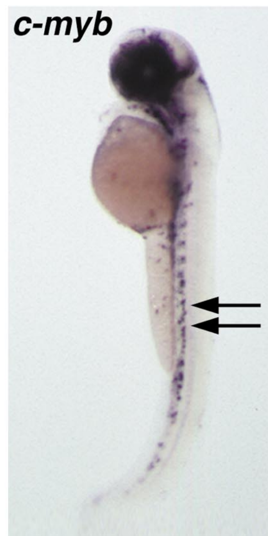




48 hpf







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1994; Schmidt *et al.*, 1995; Jones *et al.*, 1996; Zhang and Evans, 1996; Evans, 1997; reviewed in Graff, 1997; Huber and Zon, 1998; Mead and Zon, 1998). In many vertebrates, portions of the ventral mesoderm migrate onto the embryonic yolk sac and form *blood islands,* which consist of erythroid cells and vascular endothelial cells, adjacent to endoderm. The close association and similar gene expression of the blood and the vasculature have led to the hypothesis of a common precursor, called a *hemangioblast* (His, 1900; Sabin, 1920; Pardanaud *et al.*, 1987, 1996). In amphibians, the analogous region of hematopoiesis and vasculogenesis is the ventral blood island, whereas in many teleosts (such as the zebrafish) hematopoiesis arises in an intraembryonic location known as the intermediate cell mass (ICM) (Oellacher, 1872). The blood islands give rise to circulating, primitive nucleated erythrocytes expressing embryonic globins.

During further vertebrate development, overlapping waves of hematopoiesis occur in distinct anatomic sites. Primitive erythropoiesis is replaced by definitive hematopoiesis, which consists of all blood lineages including myeloid and lymphoid and gives rise to the fetal and adult peripheral blood cells. Recent work has provided a common theme for vertebrates in which definitive hematopoiesis is largely derived from an intraembryonic location known as the aorta–gonad–mesonephros (AGM) region (Dieterlen-Lievre, 1975; Turpen and Smith, 1989; Muller *et al.*, 1994; Chen and Turpen, 1995; Cumano *et al.*, 1996; Medvinsky and Dzierzak, 1996; Dieterlen-Lievre *et al.*, 1997), although evidence suggests that yolk sac cells have stem cell capability and may be utilized *in vivo* for definitive hematopoiesis (Auerbach *et al.*, 1996; Turpen *et al.*, 1997; Yoder *et al.*, 1997a,b). Definitive hematopoietic cells do not differentiate *in situ* in the AGM, but travel to a fetal site of hematopoiesis. The sites of hematopoiesis are not well conserved throughout vertebrate development. In mammals, fetal blood formation occurs in the liver and spleen while the adult site is the bone marrow. In teleosts, the pronephros generates larval blood, and the kidney (pronephros and mesonephros) is maintained as the site of adult hematopoiesis.

All blood cells ultimately arise from *hematopoietic stem cells,* which are pluripotent cells capable of self-renewal, maintenance, and differentiation into all hematopoietic lineages (Weissman *et al.*, 1978; Spangrude *et al.*, 1988, 1991; Uchida *et al.*, 1994; Li and Johnson, 1995; Morrison *et al.*, 1995). These cells can be purified from hematopoietic sources using a variety of techniques based on physical characteristics (Spangrude *et al.*, 1988; Fritsch *et al.*, 1995; Goodell *et al.*, 1996) or positive and negative selection using monoclonal antibodies (Muller-Sieburg *et al.*, 1986; reviewed in Weissman *et al.*, 1989; Civin and Small, 1995; Novelli *et al.*, 1998). Hematopoietic stem cells give rise to *progenitors,* cells that lack the capacity for self-renewal. These progenitors in turn differentiate into *precursors,* which are committed to a specific hematopoietic lineage and are morphologically recognizable in the hematopoietic sites. Cultured *in vitro,* progenitors form colonies specific for the erythroid, granulocyte/macrophage, granulocyte, macrophage, eosinophil, basophil, or megakaryocytic lines. The development of each of these lineages requires the action of multiple growth factors and cytokines such as the *c-kit* ligand and erythropoietin (reviewed in Shivdasani, 1997; Orkin, 1998), and the intrinsic program is dependent on blood-specific differentiation factors (Shivdasani and Orkin, 1996).

Despite substantial advances, many fundamental questions about hematopoiesis remain only partially answered. What factors govern mesodermal patterning and the commitment to form blood? Is there a common precursor for blood and vascular tissue? What factors control the maintenance, proliferation, and eventual specification of hematopoietic stem cells? What is the precise relationship between embryonic and adult hematopoiesis? Finally, what are the critical steps leading to each unique blood cell lineage? In the remainder of this review, we will discuss how each of these questions is being approached through genetic, embryologic, and molecular studies in the zebrafish.

USE OF THE ZEBRAFISH SYSTEM TO STUDY BLOOD FORMATION

The zebrafish is an ideal genetic system for developmental studies of organogenesis (Ingham, 1997; Bahary and Zon, 1998; Chitnis and Dawid, 1999; Detrich *et al.*, 1999; Johnson and Bennett, 1999; Malicki, 1999; Moens and Fritz, 1999). A large number of fish can be raised in a relatively small amount of space, and the females lay hundreds of eggs at weekly intervals. The embryos are transparent, and development can be monitored under the dissecting microscope. By 24 h postfertilization, the heart is beating and embryonic circulation has begun. Although zebrafish are stably diploid, haploid progeny which are viable for several days can be generated. Gynogenetic diploids can be made via application of early pressure (Streisinger *et al.*, 1981; Beattie *et al.*, 1999; Corley-Smith *et al.*, 1999; Pelegri and Schulte-Merker, 1999). The haploid and gynogenetic diploid embryos can be used for genetic screens or for mutation mapping (Johnson *et al.*, 1995, 1996).

Zebrafish Blood

Factors critical for normal hematopoiesis and vasculogenesis have been identified through expression cloning in *Xenopus* and gene targeting approaches in mice (reviewed in Orkin, 1995; Shivdasani and Orkin, 1996; Huber and Zon, 1998; Turpen, 1998). The isolation of zebrafish homologs of these genes, including the transcription factors *tal-1/SCL* (hereafter, *SCL*), *GATA-2*, *GATA-1* and *c-myb,* has permitted *in situ* hybridization studies defining the anatomy of hematopoiesis and vasculogenesis (Fig. 2) (Fou-

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quet *et al.*, 1997; Liao *et al.*, 1997, 1998; Sumoy *et al.*, 1997; Thompson *et al.*, 1998). At the 3-somite stage (approximately 11 h postfertilization (hpf)), *GATA-2* and *SCL* are expressed in two stripes of lateral plate mesoderm, marking the earliest appearance of blood and vascular tissue in the zebrafish. *GATA-1* expression first appears slightly later at the 5-somite stage, and by 12 somites globin expression begins. At 18 somites (approx 18 hpf) the stripes have fused anteriorly and have begun to converge medially, and by 23 hpf, shortly before the onset of circulation, the two stripes have merged to form the tubular structure known as the ICM. Though intraembryonic, this tissue is analogous to the extraembryonic blood islands on the yolk sac of higher vertebrates.

Cells from the ICM migrate anteriorly prior to the onset of circulation and have been hypothesized to populate the dorsal mesentery and the ventral wall of the dorsal aorta (Detrich *et al.*, 1995). Subsequently, a subset of these cells exits onto the yolk sac and undergoes a purely erythroid differentiation to establish the embryonic circulation. This seeming restriction in developmental potential may be the result of local environmental factors, as hematopoietic stem cells derived from yolk sac are capable of giving rise to all lineages (Yoder *et al.,* 1997b). At the posterior end of the yolk tube the ICM curves posteriorly and ventrally to form a wedge-shaped area that we have designated the "posterior ICM" (Thompson *et al.*, 1998) (Fig. 2). The cells in this area enter circulation slightly later than the cells in the anterior ICM and may represent a distinct cell population (Al-Adhami and Kunz, 1977; Detrich *et al.*, 1995). Expression of *SCL* and *GATA-2* but not *GATA-1* also extends into the ventral tail at 24 hpf (Fig. 2, arrowheads) (Liao *et al.*, 1998; Thompson *et al.*, 1998).

Between 24 hpf and 4 days postfertilization (dpf), the distribution of hematopoietic markers undergoes changes reflecting the evolving hematopoietic program. By 24 hpf globin protein is detectable in the primitive erythroid cells and circulation has begun. By 48 hpf, *GATA-2* is no longer expressed in blood but persists in neurons; the expression of *GATA-1* is likewise severely reduced. *SCL* expression is reduced to a few circulating cells; some *SCL* expression may be maintained in the tail. *c-myb* (Fig. 2, double arrow) is expressed in cells in the ventral wall of the dorsal aorta at 48 hpf. By analogy to other vertebrates, these cells are likely to represent definitive hematopoietic stem cells in the aorta–gonads–mesonephros region. At 4 dpf, *rag-1* expression is seen in the developing thymus, while globin, *c-myb,* and *SCL* expression becomes more prominent in the ventral tail (Fig. 3) (Willett *et al.*, 1997; Liao *et al.*, 1998; Trede and Zon, 1998; A. Brownlie and L. Zon, unpublished results). Transfusion experiments suggest that the ICM is the source of blood from 1 to 4 dpf (Weinstein *et al.*, 1996); thus this 4-dpf ventral tail globin+/*c-myb*+/*SCL*+ population may represent a second wave of hematopoiesis. In summary, hematopoiesis in zebrafish is similar to that of other vertebrates, being characterized by successive waves of tissue specification, cell migration, and expression of specific transcription factors.

Hematopoietic Mutants

A diverse array of blood mutants was obtained as part of a large-scale chemical mutagenesis screen of the zebrafish genome (Driever *et al.*, 1996; Haffter *et al.*, 1996). To date, there are over 50 described mutants with defects in hematopoiesis. The mutants studied in our laboratory comprise 26 complementation groups (Ransom *et al.*, 1996; Weinstein *et al.*, 1996; L. Zon *et al.*, unpublished data). Most mutant phenotypes were detected by visual inspection of embryos, and the defects were further characterized by analysis of cell morphology and *in situ* hybridization with a variety of well-characterized markers. The phenotypes of these hematopoietic mutants have been found to span the proposed stages of hematopoietic differentiation and fall into five classes: bloodless, blocked progenitor proliferation, blocked progenitor differentiation, hypochromia, and photosensitive blood (Fig. 1).

DEFINITION OF THE HEMATOPOIETIC PROGRAM USING THE ZEBRAFISH

Developmental Pathways from Ventral Mesoderm to Blood

Making Ventral Mesoderm

As mesoderm is being formed by inductive effects of prospective endoderm, dorsal–ventral patterning establishes segregation of ventral mesodermal tissue into future blood. Work in a variety of model systems has indicated a highly conserved system specifying dorsal–ventral cell fates in the vertebrate embryo (Holley and Ferguson, 1997). Members of the TGF-β superfamily (*dpp* in *Drosophila* and its vertebrate homologs, *BMP2/4*) have been found to play a role in the specification of ventral cell fates in the developing embryo (Dale *et al.*, 1992; Maeno *et al.*, 1996; Newfeld *et al.*, 1996). Antagonizing the ventralizing signal are products of the dorsal organizer, including *chordin* (*sog* in *Drosophila*), *noggin, follistatin,* and members of the *DAN/gremlin* family (Graff *et al.*, 1994; Holley *et al.*, 1995, 1996; Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996; Hsu *et al.*, 1998; Stanley *et al.*, 1998). Recent data firmly establish the importance of this pathway in the zebrafish (Driever, 1995; Mullins *et al.*, 1996; Neave *et al.*, 1997; Nikaido *et al.*, 1997; Bauer *et al.*, 1998; reviewed in Holley and Ferguson, 1997; Solnica-Krezel, 1999). Injection of *noggin* or dominant-negative *bmp4* receptor causes an expansion of dorsal cell fates at the expense of intermediate and ventral mesoderm (Hammerschmidt *et al.*, 1996b; Neave *et al.*, 1997). Conversely, injection of the zebrafish *chordin* antagonist, *tolloid,* causes expansion of ventral structures, including blood (Blader *et al.*, 1997). Finally, mutations in the ze-

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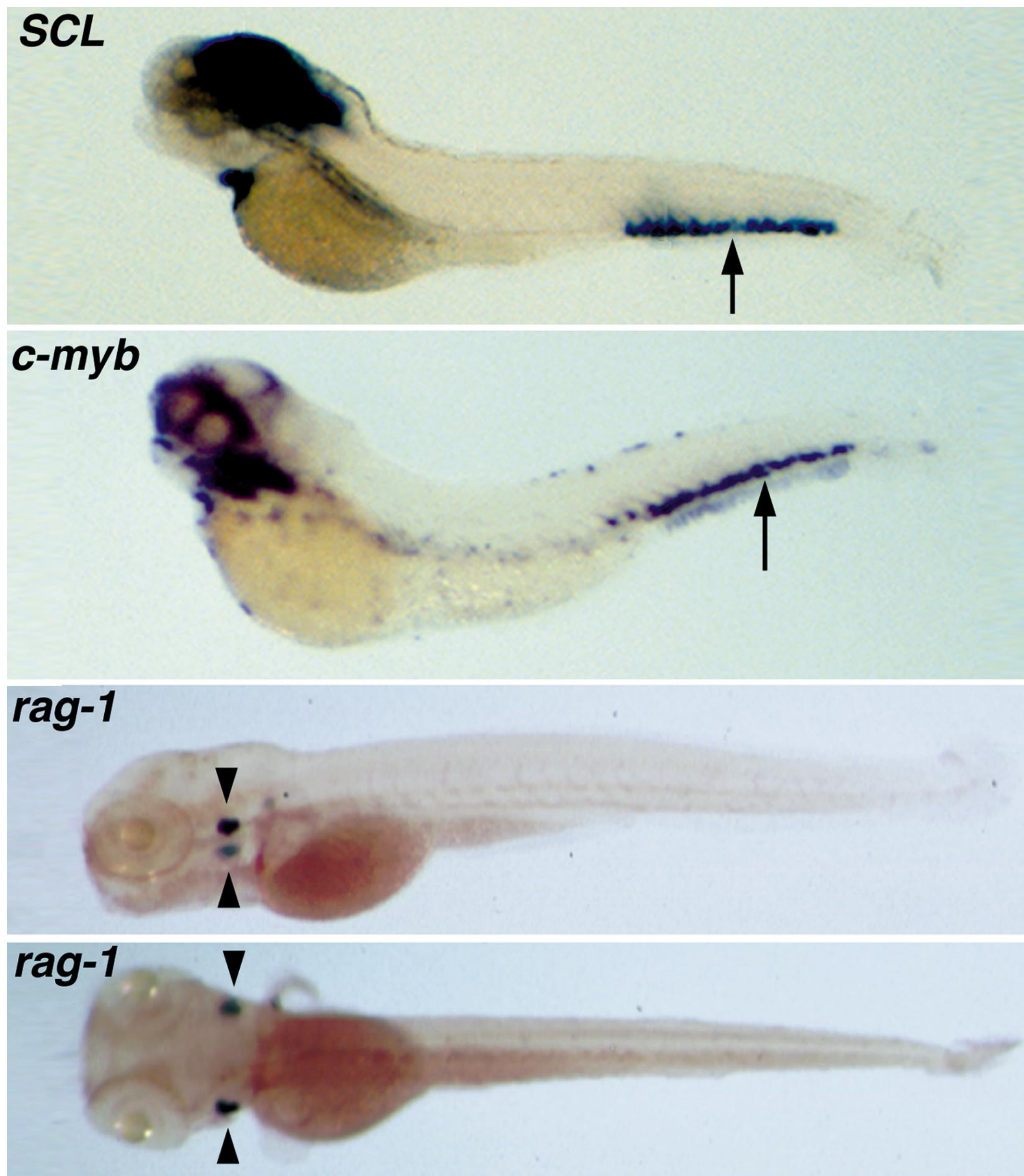

FIG. 3. Hematopoiesis in zebrafish embryos at 4 days postfertilization. Expression of *SCL, c-myb,* and *rag-1* at 4 dpf. *SCL* and *c-myb* are expressed in the ventral tail region (arrows) as well as the nervous system. *rag-1* expression is confined to the thymus (arrowheads). (Courtesy of E. C. Liao and N. Trede.)

brafish homologs of *chordin* and *BMP2* (the *chordino* and *swirl* mutants, respectively) were uncovered by screening for mutations that disrupt dorsoventral patterning (Mullins *et al.*, 1996), confirming the importance of the TGF-β-related pathway for this process (Kishimoto *et al.*, 1997; Schulte-Merker *et al.*, 1997; Nguyen *et al.*, 1998). More recently, mutations in zebrafish *BMP7* were also shown to cause a dorsalized phenotype (M. Mullins, personal communication). Given that these molecules are conserved between zebrafish and other vertebrates, it is likely that other components of the axial patterning system, such as the *SMAD* family (Sekelsky *et al.*, 1995; Graff *et al.*, 1996; Newfeld *et al.*, 1996; Savage *et al.*, 1996; Suzuki *et al.*, 1997; Wilson *et al.*, 1997; Attisano and Wrana, 1998; Nakayama *et al.*, 1998; Weinstein *et al.*, 1998) and the ventralizing homeobox proteins (Gawantka *et al.*, 1995; Ault *et al.*,

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1996; Ladher *et al.*, 1996; Mead *et al.*, 1996; Schmidt *et al.*, 1996; Lemaire *et al.*, 1998; Onichtchouk *et al.*, 1998) will also be critical for mesodermal patterning in zebrafish.

The expression of *GATA-1* and *GATA-2* is markedly increased in the tail of *chordino* mutants, whereas *swirl* homozygous mutants fail to express *GATA-1* (Hammerschmidt *et al.*, 1996a; Mullins *et al.*, 1996). Another zebrafish mutant called *spadetail* (*spt*) also has defects in both embryonic patterning and early hematopoiesis (Kimmel *et al.*, 1989; Ho and Kane, 1990; Thompson *et al.*, 1998; E. Liao and L. Zon, unpublished results). The gene responsible for *spt* belongs to the T-box family of transcription factors (Griffin *et al.*, 1998). *spt* mutants are defective in the dorsal convergence of paraxial mesoderm and the formation of somites. Ventral tail expression of *SCL* and *GATA-2* persists, and expression of the vascular marker *flk1* (VEGF receptor-2) is extensive (though disorganized), but the expression of *GATA-1* in the ICM and *c-myb* in the aorta–gonads–mesonephros region is almost completely abolished. Taken together, these data suggest the profound effect of patterning on the hematopoietic program.

The Hemangioblast

Speculation about a common origin for blood and vascular tissue dates back nearly a century and has been based on the close proximity in the yolk sac of cells that will give rise to blood and blood vessels, respectively (His, 1900; Sabin, 1920). Several lines of evidence support this hypothesis. Recently, Choi and co-workers have described the isolation of blast-colony-forming cells (BL-CFC) from embryoid bodies which arise during culture of embryonic stem cells (Kennedy *et al.*, 1997). When cultured in the presence of appropriate cytokines, BL-CFC can give rise to both hematopoietic and endothelial cells (Choi *et al.*, 1998; Robb and Elefanty, 1998). Furthermore, hematopoietic stem cells and endothelial cells have similar gene and surface marker expression, including CD34, *SCL,* and *flk1. SCL* has been shown to be essential for embryonic hematopoiesis (Robb *et al.*, 1995; Shivdasani *et al.*, 1995). Through analysis of chimeric *SCL* −/− mice expressing a transgene targeting lacZ to vessels, *SCL* was found to play a role in angiogenesis (Visvader *et al.*, 1998). Finally, mice bearing null mutations of *flk1* have profound defects in both hematopoiesis and vasculogenesis (Shalaby *et al.*, 1997).

What signals lead to the differentiation of this putative hemangioblast? The answer may lie in analysis of the zebrafish mutant *cloche* (*clo*). *clo* mutant embryos are defective in both blood and endothelial cell development (Stainier *et al.*, 1995). The heart is enlarged due to the lack of an endothelial layer, and the atrium balloons to the shape of a bell (*cloche,* in French). The expression of blood-related markers *GATA-1* (Fig. 2, 24 hpf)*, SCL,* and *Lmo2* is severely reduced or absent in *clo* embryos, as are the vascular markers *flk1, fli1,* and *tie1* (Liao *et al.*, 1997, 1998; Thompson *et al.*, 1998). Thus, *clo* differs significantly from *spt* (in which expression of *SCL* and vascular markers are maintained) and likely represents a gene essential for the genesis, survival, or differentiation of the hemangioblast. Interestingly, injection of *SCL* rescues much of the *clo* phenotype, including expression of *GATA-1, flk1,* and *tie1* (Liao *et al.*, 1998). Thus, *SCL* appears to act downstream of *cloche* to specify hematopoietic and vascular differentiation. The identification of the *clo* gene should provide significant information regarding the signal events upstream of the hemangioblast.

Biology of the Stem Cell and the Relationship of Primitive and Definitive Hematopoiesis

Blood formation depends on the prior events of mesodermal patterning and the generation of hemangioblasts. Mutations affecting these prior events will thus have effects on hematopoiesis, as is the case with the mutations *spt* and *clo.* In contrast, a mutation specifically interfering with the generation or maintenance of primitive or definitive hematopoietic stem cells would be expected to exhibit defects in blood but not vasculature or other mesoderm-derived structures. Zebrafish candidates for such a stem cell mutation include *bloodless* (which has also been called *sort-of-bloodless*) and *moonshine* (*mon*) (Zon, 1995; Ransom *et al.*, 1996). *bloodless* embryos are bloodless at the onset of circulation. The phenotype of *bloodless* is dominant with incomplete penetrance, and some heterozygous mutant animals recover blood production and can be raised to adulthood. *GATA-1* expression in the anterior ICM of *bloodless* animals is severely diminished, while expression of *GATA-2, SCL,* and *Lmo2* in the ventral tail is preserved (S. J. Pratt, A. Oates, E. Liao, N. Trede, and L. Zon, unpublished results). *moonshine* (also isolated as the bloodless mutant *vampire* (Weinstein *et al.*, 1996)) is a recessive mutation with eight identified alleles, seven of which are embryonic lethal as homozygotes. The strongest alleles are bloodless at the onset of circulation; others have a maximum of 50–100 circulating cells by day 4. *GATA-1* expression is also affected and correlates with the severity of the phenotype, with the most severe alleles lacking detectable *GATA-1.* Rare homozygous mutant animals that survive to adulthood exhibit defects in erythroid differentiation (D. G. Ransom and L. I. Zon, unpublished data). Thus, *mon* likely represents a defect in both embryonic and adult erythropoiesis. *mon* mutants also exhibit increased proliferation of iridophores (a neural crest derivative) in the posterior tail. This combination of neural crest and hematopoietic defects is reminiscent of the phenotype of mice bearing mutations in the *c-kit* (stem cell factor) gene; however, linkage analysis excludes zebrafish SCF/*c-kit* as the *mon* mutation.

Do the two posterior regions, the posterior ICM and the ventral tail, represent novel sites of hematopoiesis, perhaps stem-cell compartments? The posterior ICM expresses all of the blood-related markers tested to date; however, the cells in this region enter circulation slightly later than those in the anterior ICM (Detrich *et al.*, 1995). In *clo* mutants, a small amount of *SCL, GATA-1, c-myb,* and *flk1*

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expression persists in the posterior ICM, again suggesting these cells may differ from those in the anterior ICM. The ventral tail, which does not express *GATA-1,* expresses other blood and vascular markers both early (18–24 hpf) and late (4 dpf) in embryogenesis. *c-myb,* which in mice is required for definitive but not primitive hematopoiesis (Mucenski *et al.*, 1991), is coexpressed at high levels along with *SCL* in the ventral tail at 4 dpf (Liao *et al.*, 1998) (Fig. 3). Thompson and Ransom *et al.* examined *b316* embryos, which bear a deletion that includes the zebrafish *c-myb* gene (Thompson *et al.*, 1998), and found normal levels of *GATA-1* expression. Thus, as in mice, *c-myb* is dispensable for primitive erythropoiesis. (The *b316* deletion is lethal after 24 hpf, so no effect on definitive hematopoiesis could be determined.) It is not yet established that the ventral tail region is, in fact, truly hematopoietic. Interestingly, *GATA-1,* normally absent from the tail, is expressed in the ventral tail of anemic *sauternes* embryos at 3 dpf (Brownlie *et al.*, 1998) (see below). This suggests that these cells have at least the potential to undergo hematopoietic differentiation in response to an embryonic anemia. The nature of these posterior cells, and their relation to the ICM and aorta–gonads–mesonephros regions, will be clarified by cell-lineage and transplantation experiments.

From Stem Cell to Mature Blood Cell

Once produced, hematopoietic stem cells successively undergo differentiation to progenitors. Definitive hematopoiesis is characterized by the production from these progenitors of the erythroid, lymphoid, myeloid, and megakaryocytic lines. In contrast, primitive (yolk-sac) hematopoiesis consists mostly of erythroid cells with distinct morphology and specific embryonic globin chains. However, as described above, yolk-sac hematopoietic stem cells are capable of giving rise to diverse hematopoietic lineages. Thus, analysis of embryonic hematopoiesis may reveal intrinsic factors necessary for all of the steps leading from generation of the hematopoietic stem cell to the phenotypic expression of mature blood cells.

The large-scale zebrafish mutagenesis screen revealed a large number of mutants with embryonic anemias and progressively decreasing blood counts (Ransom *et al.*, 1996; Weinstein *et al.*, 1996). Based on the morphology of the surviving cells and the expression of terminal differentiation markers such as globin, the mutants can be divided into the following classes (see also Fig. 1):

Mutations affecting progenitor proliferation or differentiation. These mutants have normal expression of *GATA-1* and *GATA-2* and normal numbers of circulating cells on day 1 of embryonic development. Between days 2 and 5, however, the number of circulating cells markedly decreases. In the mutants *frascati* (*frs*), *grenache* (*gre*), and *thunderbird* (*tbr*), most erythroid cells die by 2 dpf, suggesting a block in the survival or proliferation of erythroid progenitors. While homozygous *frs* mutations are generally embryonic lethal, homozygous *tbr* animals eventually recover blood formation and survive to adulthood (Ransom *et al.*, 1996). Other mutants in this class display decreasing blood counts beginning at varying stages, ranging from 2–3 dpf (*chablis* (*cha*) and *merlot* (*mot*)) to 3–5 dpf (*retsina* (*ret*), *reisling* (*ris*), and *cabernet* (*cab*)). Interestingly, peripheral blood smears from several of these mutants demonstrate arrest at the polychromatophilic normoblast stage, suggesting a defect in differentiation of erythroid progenitors.

Hypochromic mutants. All of the mutants described above display normal hemoglobin expression in the circulating cells. In contrast, the hypochromic mutants—*chardonnay* (*cdy*), *chianti* (*cia*), *sauternes* (*sau*), *weissherbst* (*weh*), *clear blood* (*clb*), and *zinfandel* (*zin*)—exhibit decreased hemoglobin and other features of human anemias, including in some cases reduced cell volume (microcytosis). In support of this, *zin* maps to the globin locus (Chan *et al.*, 1997, and A. Brownlie, manuscript in preparation) and thus represents a thalassemia-like defect. Taken together, these hypochromic mutants may represent defects in genes underlying all of the steps necessary for hemoglobin production—iron metabolism, heme synthesis, and globin expression.

Porphyria-like mutants. A final group of mutants to emerge from the screen comprises the mutants *dracula* (*drc*), *desmodius* (*dsm*), *freixenet* (*frx*), and *yquem* (*yqe*). These mutants were identified on the basis of photosensitive or autofluorescent blood and thus display a phenotype similar to the erythropoietic porphyrias in humans.

Thus, a large-scale mutagenesis has resulted in the isolation of a diverse group of mutants with defects in many key steps in hematopoietic differentiation. It is worth pointing out that these screens, which focused only on the first 5 days of embryogenesis, might not have detected mutations that affected definitive hematopoiesis but spared primitive hematopoiesis.

Zebrafish as Models of Human Disease

Ultimately, the promise of the zebrafish is as a genetic system that will bridge the gap between the *Caenorhabditis elegans/Drosophila* and mouse/human models. As such, it is worthwhile to ask whether zebrafish may serve as models of human disease. Several important results provide encouragement in this regard. Brownlie and co-workers recently described the positional cloning of the hypochromic mutant *sauternes* (Brownlie *et al.*, 1998). *sau* mutant embryos have severely reduced hemoglobin levels and microcytic anemia and display immature circulating erythrocytes and abnormally persistent expression of *GATA-1* and the embryonic globin chain β_{e2}. *sau* was found to encode the erythroid-specific isoform of δ-aminolevulinate synthase (ALAS2), a critical enzyme in the heme biosynthetic pathway. In humans, mutations in ALAS2 cause X-linked congenital sideroblastic anemia (CSA), a disease with many features of the *sau* phenotype. Thus, *sau* represents the first animal model of CSA.

Another example of zebrafish as a model of human

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disease is the demonstration that the photosensitive, porphyria-like mutant *yquem* is caused by a defect in the gene encoding uroporphyrinogen decarboxylase (UROD) (Wang *et al.*, 1998). The discovery, through biochemical analysis, that *yqe* mutants were deficient in UROD activity led to the identification of mutation in the *urod* gene in *yqe* animals. The phenotype of *yqe* mutants is similar to that of humans with hepatoerythropoietic porphyria , caused by an autosomal recessive *urod* mutation. This report illustrates the use of a candidate gene approach in cloning the genes defective in zebrafish mutants with phenotypes resembling known human diseases.

Biology and Genomics

The full benefits of the zebrafish large-scale mutagenesis screen will be realized through isolation of the mutant genes and characterization of their function. In order to do this, an infrastructure of cellular, molecular, and genetic techniques has been developed specifically for the zebrafish system. This work, which is the product of many laboratories, has been reviewed in detail elsewhere (Amemiya *et al.*, 1999; Beattie *et al.*, 1999; Foernzler and Beier, 1999; Gong, 1999; Kwok *et al.*, 1999; Liao and Zon, 1999; Meng *et al.*, 1999a; Parker *et al.*, 1999; Postlethwait *et al.*, 1999a,b; Talbot and Schier, 1999; Walker, 1999; Brownlie and Zon, 1999). Here, we highlight how these tools are being used to dissect hematopoiesis in the fish.

Analysis of Zebrafish Blood

Embryonic and adult peripheral blood cells can be obtained by bleeding animals at various stages of development. Globin chains have been purified and characterized from adult fish, and the α- and β-globin genes characterized (Chan *et al.*, 1997). Monoclonal antibodies that recognize myeloid or erythroid and myeloid cells are available (A. Donovan, M. Kieran, and L. Zon, unpublished data). In addition, preparations from the adult kidney can be used for morphologic studies. Adult kidney can be used as a source for progenitor assays, in which cells are cultured *in vitro* in the presence of cytokines, giving rise to differentiated, mature blood cells. Stem-cell transplants from adult zebrafish kidney to embryonic yolk sac can be used to rescue embryonic anemias, analogous to bone-marrow transplantation in humans (S. Lin, personal communication; Guo and Zon, unpublished observations). Finally, studies of hemostasis in zebrafish have demonstrated a blood-clotting system similar to that in mammals (Jagadeeswaran *et al.*, 1999).

Functional Genomics and Positional Cloning

Positional cloning efforts are now under way for many of the zebrafish mutant genes. Mutant genes already cloned by this method include *one-eyed pinhead,* required during gastrulation (Zhang *et al.*, 1998), as well as *sauternes* (Brownlie *et al.*, 1998). Including *sau,* our laboratory has completed the positional cloning of three genes responsible for hematopoietic mutants. Two other mutant genes have been cloned by a combination of positional cloning and candidate gene approaches (see below; L. Zon, unpublished results). This task has been made possible by rapidly improving genomic techniques in zebrafish (Postlethwait *et al.*, 1999a). Large-insert genomic YAC, PAC, and BAC libraries have been prepared for use in screening and positional cloning projects (Zhong *et al.*, 1998; Amemiya *et al.*, 1999; G. Silverman *et al.,* unpublished results). Rapid assignment of a gene of interest to a particular linkage group can be performed using somatic-cell and radiation-hybrid panels (Ekker *et al.*, 1999; Kwok *et al.*, 1999). For fine-scale mapping the genetic map is available, comprising simple-sequence repeat and RAPD markers (Knapik *et al.*, 1998; Postlethwait *et al.*, 1998). Under an NIH-sponsored initiative, the resolution of the map is expected to soon exceed 1 cM. Once a candidate region has been identified, SSCP and AFLP approaches can be used to rapidly generate more markers (Foernzler and Beier, 1999; Ransom and Zon, 1999). Meiotic mapping and positional cloning are much easier to perform in zebrafish than in any other vertebrate system, owing to the large brood size and the ease of scoring phenotypes in embryos (Talbot and Schier, 1999). As the technology of zebrafish genomics continues to improve, we can expect a host of further examples.

Candidate Genes

The phenotype of a particular mutant may give clues as to the identity of the mutated gene, as was the case with *yquem* (Wang *et al.*, 1998). In other cases, mapping of a mutant can suggest candidates, based on the conserved synteny between human, mouse, and zebrafish chromosomes. In these instances candidates can be tested directly, obviating the need for positional cloning; a recent example is the cloning of the hedgehog pathway gene *you-too* (Karlstrom *et al.*, 1999). The success of the candidate-gene approach depends on the availability of the zebrafish homolog of a particular gene of interest. This approach will be greatly facilitated by the more than 10,000 zebrafish expressed-sequence tags (ESTs) that have already been deposited with GenBank (S. Johnson *et al.,* Washington University). Ultimately, the zebrafish genome project will result in the characterization of 100,000 ESTs, allowing rapid cDNA cloning of a gene of interest. Conversely, researchers who have mapped a mutation in a human gene have the possibility of rapidly determining whether a mutation in the analogous zebrafish gene already exists, either as a known mutant or as a deletion in the syntenic area of the zebrafish (Zon, 1999). Strains bearing defined genomic deletions already exist for many parts of the zebrafish genome, and complete coverage is anticipated (Fritz *et al.*, 1996; Walker, 1999). This will facilitate the use of zebrafish as novel models for human diseases.

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Transgenesis and Gene Regulation

Pseudotyped retroviral vectors have been successfully used for insertional mutagenesis in zebrafish (Lin *et al.*, 1994; Gaiano *et al.*, 1996a,b). Transient or stable transgenic expression can be achieved through microinjection of embryos. As an example of approaches to defining the mechanisms of hematopoietic specification, Lin and co-workers used the zebrafish *GATA-1* promoter to drive expression of green fluorescent protein. By altering key promoter sequences and observing the expression pattern in living embryos, these investigators defined the sequences governing both positive and negative regulation of *GATA-1* expression (Meng *et al.*, 1999b) (Fig. 4). This technique has also been used to probe genetic relationships, such as the rescue of *clo* embryos by expression of *SCL* (Liao *et al.*, 1998).

Tissue-Specific Screens

Large-scale mutagenesis screens require a substantial investment of time and personnel and the ability to maintain large numbers of fish. A viable alternative to this approach is the use of small-scale screens looking for a particular phenotype or loss of a specific molecular marker. Recently, our laboratory has completed a screen for mutations in the development of the zebrafish lymphoid system (N. Trede, manuscript in preparation). By using the zebrafish *rag-1* gene to screen gynogenetic diploid fish, prepared from the eggs of F_1 females derived from an ENU-mutagenized stock, several mutants defective in lymphoid development were recovered. Characterization of these mutants will give insight into the mechanisms of lymphopoiesis and thymic development during embryogenesis. Other marker-based screens, such as for abnormal expression of *GATA-1* or the myeloid marker Pu.1, are being carried out. Another method of uncovering novel genes underlying hematopoiesis is the use of *in situ* hybridization to discover cDNAs with specific expression patterns (Thisse *et al.,* 1999). Using this approach, Thisse and co-workers isolated more than 30 cDNAs, corresponding to both known and novel genes, that are expressed specifically in blood or vasculature. These genes are excellent candidates for the hematopoietic mutants, and analysis of the genes will extend our knowledge of the factors underlying hematopoietic specification during development.

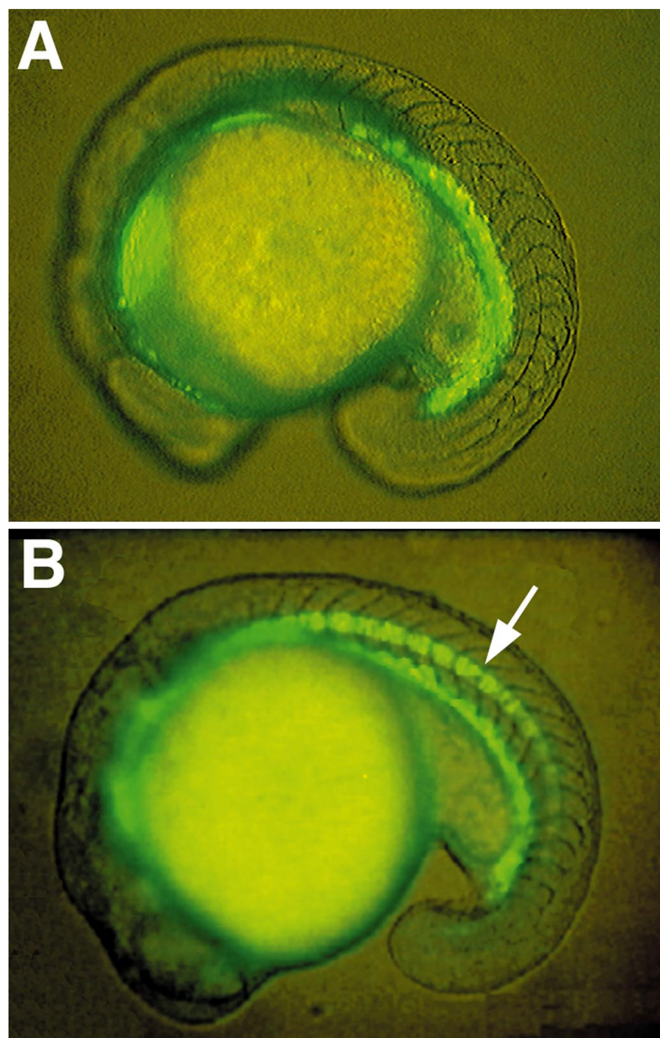

FIG. 4. Positive and negative *cis*-acting elements regulating hematopoietic expression of *GATA-1.* Transgenic zebrafish expressing green fluorescent protein from the GATA-1 promoter were generated by microinjection of *GATA-1*–GFP constructs and selection of transgenic founder lines. 18-somite-stage progeny are shown. In (A), the transgene contains the full-length GATA-1 promoter . GFP expression is confined to hematopoietic progenitors. In (B), deletion of a negative *cis*-acting element leads to ectopic GFP expression in notochord (arrow); hematopoietic expression is not affected. (Courtesy of Dr. Shuo Lin.)

LOOKING AHEAD

If the large-scale mutagenesis screens represent the "first generation" of investigations into the mechanism of organogenesis in zebrafish, what can we expect as the field grows and matures? Many exciting developments hold the promise of rapid progress yet to come. Fine mapping has been completed for most of the hematopoietic mutants discussed above, and cloning of the underlying genes can be expected to contribute greatly to our understanding of the developmental biology of hematopoiesis. Further screens directed at specific lineages will uncover mutants in the definitive hematopoietic program. Included among such mutants will likely be important new models for human diseases, including immunodeficiencies, aplastic anemia, and leukemias. The success of transgenic approaches, not only in furthering understanding of gene regulation but also in rescuing mutant phenotypes, suggests the utility of the zebrafish system for testing gene therapy techniques. As

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these critical, initial steps are completed, dominant suppressor screens will be instituted to take full advantage of the genetics of the zebrafish system. These studies will undoubtedly uncover novel pathways and molecules underlying the formation of blood and other organs and bring zebrafish to its full potential as a model system.

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