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Characterization of zebrafish mutants with defects in embryonic hematopoiesis

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SUMMARY

As part of a large scale chemical mutagenesis screen of the zebrafish (*Danio rerio*) genome, we have identified 33 mutants with defects in hematopoiesis. Complementation analysis placed 32 of these mutants into 17 complementation groups. The allelism of the remaining 1 blood mutant is currently unresolved. We have categorized these blood mutants into four phenotypic classes based on analyses of whole embryos and isolated blood cells, as well as by in situ hybridization using the hematopoietic transcription factors *GATA-1* and *GATA-2*. Embryos mutant for the gene *moonshine* have few if any proerythroblasts visible on the day circulation begins and normal erythroid cell differentiation is blocked as determined by staining for hemoglobin and *GATA-1* expression. Mutations in five genes, *chablis, frascati, merlot, retsina, thunderbird* and two possibly unique mutations cause a progressive decrease in the number of blood cells during the first 5 days of development. Mutations in another seven genes, *chardonnay, chianti, grenache, sauternes, weißherbst* and *zinfandel*, and two additional mutations result in hypochromic blood cells which also decrease in number as development proceeds. Several of these mutants have immature cells in the circulation, indicating a block in normal erythroid development. The mutation in *zinfandel* is dominant, and 2-day old heterozygous carriers fail to express detectable levels of hemoglobin and have decreasing numbers of circulating cells during the first 5 days of development. Mutations in two genes, *freixenet* and *yquem*, result in the animals that are photosensitive with autofluorescent blood, similar to that found in the human congenital porphyrias. The collection of mutants presented here represent several steps required for normal erythropoiesis. The analysis of these mutants provides a powerful approach towards defining the molecular mechanisms involved in vertebrate hematopoietic development.

Key words: zebrafish, hematopoiesis, blood, *GATA-1*, *GATA-2*, *Danio rerio*

INTRODUCTION

Vertebrate hematopoietic cells are derived from self-renewing multipotential stem cells that are specified early during embryonic development (reviewed by Zon, 1995). Hematopoiesis involves the processes of cell proliferation, cell differentiation, commitment to particular cell lineages and protection from apoptosis. The lineages and stages of normal hematopoietic development in humans and mice have been well-characterized by in vivo transplantation and by in vitro culture assays (Moore and Metcalf, 1970). Multipotential stem cells differentiate into progenitors committed to erythroid, lymphoid or myeloid lineages. Stem cells and progenitors are rare components of bone marrow and have been defined by functional assays rather than morphology. In the case of the erythroid lineage, progenitors differentiate into precursors that progress from proerythroblast stages to erythroblasts and finally differentiate into mature erythrocytes. The morphology of blood cell precursors and mature cells have been extensively studied by light and electron microscopy in a wide range of vertebrates, including fish (Catton, 1951, Rowley et al., 1988, Glomski, et al., 1992). The identification of genes required for hematopoietic development is of considerable interest particularly given the clinical significance of factors that regulate blood cell production.

In mammals, hematopoiesis first occurs in ventral blood islands located on the yolk sac. This embryonic wave of differ-

entiation is followed by a switch to fetal hematopoiesis in the liver and finally adult hematopoiesis in the bone marrow (Stamatoyannopoulos et al., 1987). Teleosts such as the zebrafish (*Danio rerio*) lack yolk sac blood islands and instead have a distinct site of embryonic erythropoiesis, called the intermediate cell mass (Al-Adhami and Kunz, 1977; Colle-Vandevelde, 1963; Detrich et al., 1995). The intermediate cell mass is formed during early gastrulation from two bilateral stripes of paraxial mesoderm that converge medially in the tail and posterior trunk. By 20 hours of development, these stripes have fused into a single intraembryonic hematopoietic region located between the notochord and endoderm (Detrich et al., 1995). This region contains a population of presumptive stem cells that express the hematopoietic transcription factor *GATA-2* and a more differentiated population of erythroid precursors that also express *GATA-1*. Embryonic/primitive erythroid progenitors develop to the early proerythroblast stage and are released into circulation. As development proceeds over the next 5 days, these cells undergo a nearly synchronous wave of differentiation to form embryonic erythrocytes (Al-Adhami and Kunz, 1977). During early larval development, zebrafish switch to adult/definitive hematopoiesis in the spleen and kidney rather than the bone marrow (Al-Adhami and Kunz, 1977).

Recently, several genes isolated by their biochemical or functional properties were found to be required for hematopoiesis by gene-targeting experiments in murine embryonic stem cell lines (Ness and Engel, 1994; Weiss and Orkin, 1995). Many of these genes appear to be required for fetal/adult hematopoiesis and not for the earlier embryonic wave of hematopoiesis. Mutations in the transcription factors *myb* (Mucenski et al., 1991), *EKLF* (Perkins et al., 1995), and *Pu.1* (Scott et al., 1994), the receptor tyrosine kinase *W* (c-*kit*; Bernstein et al., 1990; Nocka et al., 1990b), and the growth factor *Steel* (c-*kit* ligand; Brannan et al., 1991; Huang et al., 1990; Nocka et al., 1990a) all cause defects in definitive hematopoiesis that takes place in the fetal liver and bone marrow, but primitive embryonic hematopoietic cells of the yolk sac are normal.

Thus far, five genes have been demonstrated to be essential for both primitive and definitive hematopoiesis in mice. Two members of the GATA family of transcription factors, *GATA-1* and *GATA-2* are required for the production of normal numbers of erythroid cells. *GATA-1* deficient cells do not differentiate into embryonic erythroid cells in chimeric mice (Pevny et al., 1991). Progenitor studies of hematopoietic cells in *GATA-1*-deficient ES cell lines has demonstrated an arrest at the proerythroblast stage (Weiss et al., 1994). *GATA-2* mutant mice (Tsai et al., 1994) have a decreased number of erythroid progenitors. The transcription factors *SCL* (Shivdasani et al., 1995) and *TTG2* (Warren et al., 1994) are also required for embryonic hematopoiesis in mice. Mutants embryos with disruptions of these genes do not produce hematopoietic stem cells in the early embryo. Mice that lack the receptor tyrosine kinase FLK-1 have defects in both hematopoiesis and vasculogenesis (Shalaby et al., 1995). *FLK-1* mutant mice fail to form yolk sac blood islands and have greatly decreased numbers of vascular endothelial cells. There are also a number of human hematopoietic disorders such as congenital dyserythropoietic anemia and Diamond Blackfan anemia, for which candidate genes have not been defined (Lux, 1995).

We are using the zebrafish as a model system to identify and characterize mutants of hematopoiesis. The optical clarity of the zebrafish embryo allows observation of the relative number and color of blood cells from the onset of circulation (Mullins et al., 1994). These observations serve as qualitative measurements of progenitor proliferation and erythroid differentiation. Blood cells obtained from zebrafish embryos display stage-specific cell morphological and histochemical characteristics that can be used to assess the developmental steps affected by the mutations.

Three zebrafish mutants have recently been investigated for their defects in hematopoietic development. Mutations in the gene *cloche* (*clo*^{m39}), affect early hematopoietic stem cell or progenitor populations. Homozygous *clo*^{m39} mutant embryos do not express detectable levels of *GATA-1* and *GATA-2* in the intermediate cell mass (Stanier et al., 1995) and lack the endocardial lining of the heart. This represents the first identification of a gene that is essential for hematopoiesis in the zebrafish embryo. Other mutants such as *spade tail* (Ho and Kane, 1990) and *sort-of-bloodless* (W. Detrich and L. Zon, personal communication) express *GATA-2* in early erythroid progenitors, but lack differentiated erythroid cells that express *GATA-1*. These mutants demonstrate that the zebrafish can be used to characterize the functions of genes that regulate early stem cell or progenitor differentiation.

In a large scale screen for mutations affecting development of the zebrafish (described by Haffter et al., 1996), we have isolated 33 mutants with defects in the formation or differentiation of hematopoietic cells. In this report we present a phenotypic analysis of these mutants. The zebrafish mutations presented here provide powerful tools for defining the molecular components involved in normal vertebrate hematopoietic development.

MATERIALS AND METHODS

Fish strains, mutational screen and complementation analysis

Zebrafish were bred and maintained as described by Mullins et al. (1994). The isolation of mutants and complementation analysis was performed as described by Haffter et al. (1996).

Whole-mount staining of embryos

Whole embryo staining for heme expression was performed using o-dianisidine histochemistry according to previously described methods (Detrich et al., 1995; Iuchi and Yamamoto, 1983). After staining, embryos were postfixed in 4% paraformaldehyde for 1 hour.

Whole-mount in situ hybridization with digoxigenin-labeled RNA probes was performed as previously described (Schulte-Merker et al., 1992). Synthesis of *GATA-1* and *GATA-2* antisense RNA probes was performed as described by Detrich et al. (1995). Embryos were dehydrated in methanol and cleared in 2:1 benzyl benzoate: benzyl alcohol solution before mounting.

Photographs were taken on a Microphot-FXA photomicroscope or a SMZ-U dissecting microscope (Nikon) and processed using Photoshop 3.0 (Adobe).

Isolation and histological staining of embryonic blood

Blood cells were collected by cardiac puncture on anesthetized embryos in a solution of 0.02% tricaine (Sigma), 1% bovine serum albumin in calcium- and magnesium-free phosphate-buffered saline, pH 7.4. Collected cells were concentrated by centrifugation at 700

rpm for 3 minutes onto slides using a Cytospin 3 (Shandon). Slides were then immediately fixed in methanol for 30 seconds and stained by the Wright-Giemsa method (Nelson and Morris, 1984).

Detection of autofluorescent heme bi-products in erythrocytes

Mutant embryos were examined under 330 to 380 nm ultra violet (UV) light using a Microphot photomicroscope (Nikon).

RESULTS

Isolation of mutants with defects in hematopoietic development

We have identified 33 mutants with defects in the development of embryonic and larval hematopoietic cells. Complementation analysis placed 32 of these mutants into 17 complementation groups (Table 1). One mutant remains to be fully tested. Most of the 17 genes represented by the complementation groups were given names of wines, which reflects the classification of both by red and white colors. The exception is *moonshine* that was named for the rays of light reflected by the increased number of iridophores (Kelsh et al., 1996).

The blood displays a smaller number of morphological landmarks than most other developing tissues that are visible under the dissecting microscope. However, all of the circulating blood cells are visible during embryonic development. We have used the number of blood cells and the relative concentration of hemoglobin, as judged by the intensity of red cell color, as the primary criteria for the classification of blood mutants.

In addition, individual embryos were bled and peripheral blood cells were deposited on slides by centrifugation and stained by the Wright-Geimsa method (Nelson and Morris, 1984) to evaluate the morphology of circulating cells in greater detail. The classification of normal teleost blood cells into developmental stages based on morphology is well established (Catton, 1951, Rowley et al., 1988, Glomski et al, 1992). The morphology of erythroid cells in zebrafish has also been previously described and follows the common vertebrate pattern (Al-Adhami and Kunz, 1977). Proerythroblasts are round cells with large open nuclei and basophilic cytoplasm that gradually differentiate into mature erythrocytes which retain a compact nucleus and have cytoplasm filled with hemoglobin.

We also examined the mutants using o-dianisodine staining (Detrich et al., 1995; Iuchi and Yamamoto, 1983), which detects heme present in hemoglobin, and whole embryo in situ analysis to detect the expression of the hematopoietic transcription factors *GATA-1* and *GATA-2* (Detrich et al., 1995). Based on these analyses, we categorized the zebrafish mutants with hematopoietic defects into 4 phenotypic classes described below.

(I) Bloodless mutants

The 7 stronger alleles of *moonshine* (*mon*), including mon^{tb222b} and mon^{tc246b}, are the only mutations among the 33 that can be classified as bloodless at the onset of circulation. All of the mutant *mon* alleles are recessive, cause severe anemia and are lethal after 5 to 7 days except for mon^{tc239b}. Mutant mon^{tc239b} embryos have several dozen blood cells at the start of circulation but never produce more than approximately 100 cells. These less severely affected larvae also form swim bladders but do not survive to adulthood. Mutants of the other seven alleles of *mon* have few if any cells in circulation initially but have 50 to 100 blood cells by 5 days of development. In addition to the hematopoietic defect, the *mon* mutants exhibit defects in fin morphology and in pigment cell proliferation and migration. The tail fins of mutants have jagged edges, visible in 2-day and older embryos but are not obviously necrotic. The *mon* mutants also have an abnormal pattern of iridophore proliferation and migration while melanophores are normal (described in Kelsh et al., 1996). The intensity of the blood

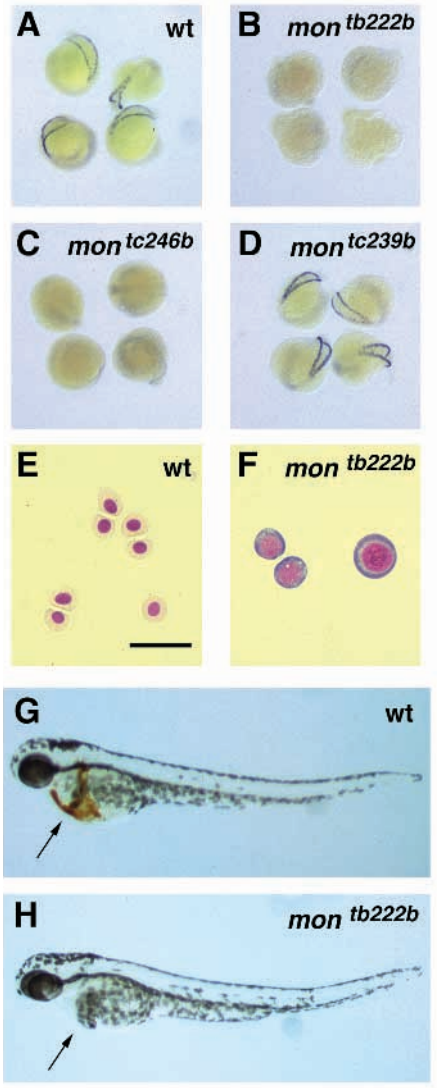

Fig. 1. Mutations in the gene *moonshine* (*mon*) cause early defects in hematopoietic cell development leading to severe anemia. The relative strength of different alleles correlates with expression of *GATA-1* mRNA detected by whole-mount in situ hybridization. (A-D) Whole-mount in situ hybridization for *GATA-1* expression in wild-type and mutant embryos at the 18 somite stage. (A) In wild-type embryos, *GATA-1* is expressed in two stripes of hematopoietic cells that converge toward the dorsal midline to form the intermediate cell mass. (B) Embryos with the strongest allele, mon^{tb222b}, express barely detectable levels of *GATA-1*. (C) The intermediate allele mon^{tc246b}, results in a slightly increased *GATA-1* expression. (D) The weakest allele, mon^{tc239b}, has no obvious effect on *GATA-1* expression at that stage. All embryos derived from a cross between mon^{tc239b} heterozygous carriers looked the same as the embryos shown in D. (E-F) Isolated blood cells from wild-type and mutant larvae stained by the Wright-Giemsa method. (E) Blood cells from 5-day old wild-type larvae show the characteristic morphology of differentiated red blood cells. (F) The few blood cells circulating in 5-day old mon^{tb222b} mutant larvae are large and basophilic. (G-H) O-dianisidine staining for hemoglobin (arrow) in 2-day old wild-type and mutant embryos. (G) Hemoglobin in red blood cells is strongly stained in wild-type embryos. (H) In contrast, *mon* mutant embryos do not express detectable levels of hemoglobin. Scale bar (E,F) 20 μm.

Table 1. Phenotypes of mutants with defects primarily restricted to hematopoietic cells

Gene	Alleles	Recessive dominant viability	Hematopoietic phenotypes
(I) Bloodless mutants			
moonshine (*mon*)	*tb222b, tc239b, tc246b, te345, tg234, tu244b, ty57, tz276*	Recessive embryonic lethal	Bloodless at the onset of circulation. After 4 days of development there are 50 to 100 blood cells in circulation. Fin edges are jagged but not necrotic. Iridophores are increased in number and migrate abnormally into the tail fin. Also described in Kelsh et al. (1995). Relative allele strengths: *tb222b, te345, tg234, tu244b, ty57, tz276, > tc246b > tc239b*
(II) Mutants with decreasing blood cell counts:			
chablis (*cha*)	*tu242e, tu245*	Recessive embryonic lethal	Decreasing number of blood cells beginning after 2 to 3 days of development. No cells in circulation after 4 to 5 days.
frascati (*frs*)	*tg221, tg280a, tm130d, tq223*	Recessive embryonic lethal	Decreasing number of blood cells beginning after 2 to 4 days of development. Relative allele strengths: *tg221, tq223 > tg280a > tm130d.*
merlot (*mot*)	*tm303c, tu275*	Recessive embryonic lethal	Decreasing number of blood cells after 2 to 3 days of development decreasing to 50 to 100 cells after day 4. *tm303c > tu275.*
retsina (*ret*)	*tr217, tr265*	Recessive embryonic lethal	Decreasing number of blood cells beginning after 3 to 4 days of development. Few, if any, cells in circulation after 5 days.
thunderbird (*tbr*)	*ty118b*	Recessive adult viable	Decreasing number of blood cells after 3 days of development to 25% of normal.
riesling (*ris*)	*tb237*	Recessive embryonic lethal	Decreasing number of blood cells beginning after 3 days of development. No cells in circulation after 4 days.
cabernet (*cab*)	*tl236*	Recessive embryonic lethal	Decreasing number of blood cells blood after 4 days of development to 75% of normal levels. Pericardial edema after 5 days of development.
grenache (*gre*)	*tr219*	Recessive embryonic lethal	Hypochromic blood after 4 days of development. Number of blood cells reduced to 50% of normal levels.
(III) Mutants with hypochromic blood and decreasing bloood counts			
chardonnay (*cdy*)	*te216*	Recessive adult viable	Hypochromic blood after 2 days of development. Variably decreasing number of blood cells to between 50% to 75% of normal levels.
chianti (*cia*)	*tu25f*	Recessive adult viable	Hypochromic blood after 3 days of development. Number of blood cells reduced to between 50% to 75% of normal levels.
pinotage (*pnt*)	*tq209*	Recessive adult viable	Hypochromic blood after 2 days of development. Number of blood cells reduced to between 25% to 50% of normal levels.
sauternes (*sau*)	*tb223, ty121*	Recessive embryonic lethal (*tb223*), adult viable (*ty121*)	Hypochromic blood and variably decreasing number of blood cells to between 25% to 50% of normal levels. Cardiomegaly in approximately 50% of *ty121* homozygous adults. Relative allele strengths: *tb223 > ty121.*
weißherbst (*weh*)	*th238, tp85c*	Recessive embryonic lethal	Hypochromic blood, but nearly normal number of blood cells after 2 days of development. Blood cell number decreases to 50% of normal levels after 4 days of development.
zinfandel (*zin*)	*te207*	Dominant adult viable	Hypochromic blood after 2 days of development.Variably decreasing number of blood cells to between 50% to 75% of normal levels. Cell number reduced by 90% in early larval period.
(unresolved)	*te264*	Recessive embryonic viable	Hypochromic blood after 2 days of development. Variably decreasing number of blood cells to between 50% to 75% of normal levels.
Photosensitive mutants			
freixenet (*frx*)	*tu271*	Recessive embryonic lethal	Porphyria syndrome. Photosensitive, autofluorescent erythrocytes. In constant light, there are no cells in circulation after 2 days of development. Lysed red blood cells form dark inclusions in the liver.
yquem (*yqe*)	*tp61*	Recessive embryonic lethal	Porphyria syndrome. Photosensitive, autofluorescent erythrocytes. In constant light, there are no cells in circulation. Pericardial edema and fin necrosis syndromes when exposed to light.

phenotype correlates with the strength of the fin and the iridophore phenotypes. The weakest allele *mon*^{tc239b} produces the weakest blood, fin and iridophore phenotype.

We examined the expression patterns of the hematopoietic transcription factor *GATA-1* in all of the 33 mutants and also tested the *mon* mutants for *GATA-2* expression. The more severely affected *mon* mutants were the only ones with detectable differences in *GATA-1* expression (Fig. 1A-D). Levels of *GATA-2* expression in *mon* mutant embryos at the 18-somite stage are equivalent to those in wild type. 29% of the embryos from a mating between carriers of a strong allele (*mon*^{tb222b}) did not express detectable amounts of *GATA-1* (Fig. 1B). 23% of the embryos derived from a mating between carriers of an intermediate allele (*mon*^{tc246b}) expressed reduced levels of *GATA-1* (Fig. 1C), and all embryos from a cross between carriers of a weak allele (*mon*^{tc239b}) exhibited wild-type levels of *GATA-1* (Fig. 1D). The expression of *GATA-1* therefore correlates with the strength of the phenotype.

All of the *mon* mutants have 50 to 100 cells in circulation by 5 days of development in comparison to the 1000 to 3000 differentiated red blood cells found in wild-type larvae. These

wild-type cells have a typical nucleated erythrocyte morphology in Wright-Giemsa stained preparations of isolated blood (Fig. 1E). The rare mutant cells such as those found in *mon*^{tb222b} mutants have a proerythroblast morphology with basophilic cytoplasm and non-condensed nuclei (Fig. 1F). The *mon* mutants also have no detectable expression of hemoglobin based on o-dianisidine staining. 2-day old wild-type embryos have robust circulation and express large amounts of hemoglobin (Fig. 1G) while *mon*^{tb222b} mutants have no detectable staining for hemoglobin (Fig. 1H). In summary, these data suggest that mutations in the gene *moonshine* affect the ability of hematopoietic stem cells to differentiate into erythroid progenitors that express *GATA-1* and also affect a subset of pigmented neural crest cells.

(II) Mutants with decreasing blood counts

Fourteen mutations cause severe anemia with initially normal levels of erythroid cells in circulation which then rapidly decrease in number. In these mutants, the level of hemoglobin expression decreases consistent with the peripheral blood cell number. Such a correlation is well documented in humans with iron deficiency anemia due to bleeding (Lux, 1995). The largest number of mutations are in this category, including *chablis* (*cha*, 2 alleles), *frascati* (*frs*, 4 alleles), *merlot* (*mot*, 2 alleles), *retsina* (*ret*, 2 alleles), *thunderbird* (*tbr*, 1 allele), *riesling* (*ris*, l allele), *cabernet* (*cab*, l allele) and *grenache* (*gre*, l allele). All of these mutants are difficult to distinguish from wild-type sibs during the first 2 days of development. They all have normally pigmented blood visible in living embryos and o-dianisidine stained whole-mounts indicating that their erythroid cells, although reduced in number, produce hemoglobin. All of these mutations are recessive and lethal in the first 1 to 2 weeks of larval life except a mutation in the gene *thunderbird, tbr*^{ty118b}, which is homozygous viable.

Mutations in the genes *frs* and *ret* cause severe anemia, visible after hatching, but display different types of morphological defects in the remaining erythroid cells (Fig. 2). Both groups of mutants have wild-type levels of *GATA-2* and *GATA-1* expression in 1-day old embryos (data not shown). The 4 mutations in *frs* vary with respect to the onset of the decrease in blood count. The phenotypes of the strongest mutations *frs*^{tg221} and *frs*^{tq223} are visible around hatching. The phenotypes of the slightly milder mutant alleles, *frs*^{tg280a} and *frs*^{tm130d}, are visible after 3 to 4 days of development. All of these mutations are lethal and result in few if any cells in circulation in 4-day old larvae. 2-day old wild-type embryos have cells in circulation with the color and morphology characteristic of erythroblasts with smaller more condensed nuclei than proerythroblasts (Fig. 2A). These cells also express large amounts of hemoglobin as measured by o-dianisidine staining (Fig. 2B). Blood collected from 2-day old *frs*^{tq223} mutant embryos revealed cells at various stages of erythroblast maturation and none that have the morphology of differentiated erythrocytes. The cells are large with basophilic cytoplasm and many of the nuclei are abnormally fragmented into patches of chromatin (Fig. 2C). Whole-mount staining with o-dianisidine indicates that these cells, though reduced in number still express some hemoglobin (Fig. 2D). Rare *frs* mutant fish live 3 to 4 weeks and are severely anemic.

Two mutations in the gene *retsina, ret*^{tr217} and *ret*^{tr265} cause a similar decrease in blood count beginning after 3 to 4 days of development. These mutations are lethal during the first week of development. 3-day old wild-type larvae have blood cells with differentiated erythroblast morphology including condensed nuclei and less basophilic cytoplasm (Fig. 2E). These larvae have 1000 to 3000 thousand cells in circulation and stain strongly for hemoglobin (Fig. 2F). The remaining blood cells of *ret*^{tr217} mutant larvae exhibit earlier proerythroblast morphology with large cells and large open nuclei (Fig. 2G). This suggests that they are blocked in erythroid cell differentiation. Whole-mount o-dianisidine staining indicates that the remaining blood cells of *ret* larvae express hemoglobin similarly to those of *frs* mutants. Based on the decreasing number of blood cells in this class of mutants and the levels of hemoglobin in the remaining circulating cells (Fig. 2D,H), these mutations may not directly affect the regulation of globin gene expression, but rather affect erythroid cell proliferation and differentiation.

(III) Mutants with hypochromic blood and decreasing blood count

Embryos of 9 mutants initially have normal numbers of blood cells which then decrease, accompanied by reduced levels of hemoglobin expression. These hypochromic blood phenotypes are caused by mutations in any of six genes including *chardonnay* (*cdy* 1 allele), *chianti* (*cia*, 1 allele), *pinotage* (*pnt*, 1 allele), *sauternes* (*sau*, 2 alleles), *weißherbst* (*weh*, 2 alleles), *zinfandel* (*zin*, 1 allele) and 1 unresolved mutation, *te264*. Three of these mutations, *cdy*^{e216}, *cia*^{tu25f} and *pnt*^{tq209}, are homozygous viable in the adult. The other mutations vary in viability as described below.

Embryos with either of two mutations in the gene *weißherbst*, *weh*^{th238} and *weh*^{tp85c} are difficult to score before they are 2 days old. Around the hatching stage, however, these mutants clearly have hypochromic blood with nearly normal numbers of blood cells. Their blood cell count then decreases in number to approximately 50% of wild-type levels. Both of these mutations are recessive and lethal by 5 to 7 days of development. 2-day old wild-type embryos have circulating erythroblasts with pale blue cytoplasm and condensing nuclei (Fig. 3A). These normal cells express large amounts of hemoglobin as detected by o-dianisidine staining (Fig. 3B). In contrast the *weh*^{tp85c} mutant blood cells are not as differentiated and have the morphology of younger proerythroblasts (Fig. 3C). They are much larger than wild type and have large open nuclei typical of more immature erythroid cells. Whole mount o-dianisidine staining demonstrates that even though there are nearly wild-type numbers of cells in circulation the mutant cells express much less hemoglobin (Fig. 3D).

The single mutant allele of *zinfandel* (*zin*) also causes a hypochromic blood phenotype in the embryo. It is dominant and can be passaged through either male or female carriers. *zin* heterozygotes and homozygotes are indistinguishable in phenotype. 2-day old *zin* mutant embryos have distinctly pale colored blood. By 3 days of development, *zin* mutant carriers have hypochromic cells in circulation and slightly reduced blood counts. Blood cells isolated from 2-day old *zin* mutants are oval, larger, paler and have less condensed nuclei than normal 2-day old erythrocytes in Wright-Giemsa stained preparations (Fig. 3E). There is also an increased fraction of large cells with basophilic cytoplasm characteristic of earlier erythroblasts in the *zin* mutant embryos. The o-dianisidine

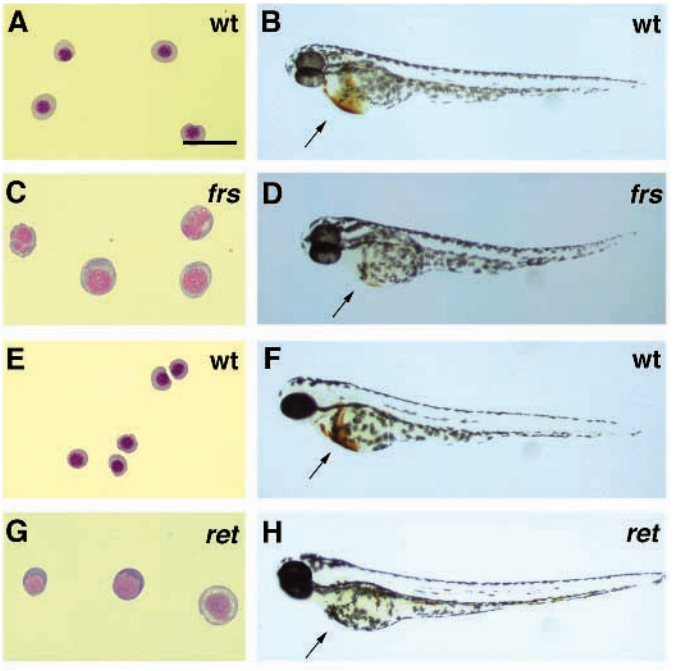

Fig. 2. Mutations in the genes *frascati* (*frs*) and *retsina* (*ret*) cause a reduction in the number of circulating blood cells. The remaining blood cells also have an abnormal cell morphology. (A,C,E,G) Isolated blood cells from wild-type and mutant larvae stained by the Wright-Giemsa method. (B,D,F,H) O-dianisidine staining for hemoglobin (arrows) in wild-type and mutant embryos. (A) Blood cells from 2-day old wild-type embryos have the normal morphology of erythroblasts. (B) Hemoglobin in red blood cells is strongly stained in wild-type embryos. (C) The circulating cells of 2-day old frs^{tq223} mutant embryos are larger than normal erythroid cells and many have abnormally fragmented nuclei. (D) The number of blood cells in 2-day old frs^{tq223} mutant embryos is greatly reduced which is reflected in the lower amount of hemoglobin. (E) Blood cells of 3-day old wild-type larva are small erythroblasts. (F) The 3-day old wild-type larva also has a large number of red blood cells producing hemoglobin. (G) The circulating cells of a 3-day old ret^{tr217} mutant larva are larger and more basophilic than normal. (H) The 3-day old ret^{tr217} mutant larva expresses very little hemoglobin, based on o-dianisidine staining. Scale bar (A,C,E,G) is equal to 20 μm.

staining indicates that the blood cells of *zin* mutant embryos do not express detectable levels of hemoglobin suggesting that they have a defect in globin production (Fig. 3F).

Two recessive mutations in the gene *sauternes*, sau^{tb223} and sau^{ty121}, were identified that also cause hypochromic blood phenotypes. The sau^{tb223} mutation is embryonic lethal while the sau^{ty121} mutation is adult viable. Both of these mutations are visible when circulation begins. The blood is hypochromic and initially normal in amount. By 3 days of development, the blood count has decreased to 25 to 50% of wild-type levels. After 3 days of development wild-type embryos have mature erythrocytes in circulation (Fig. 3G) and stain strongly for hemoglobin (Fig. 3H). In contrast, blood cells from sau^{ty121} mutant embryos have condensed nuclei like normal erythrocytes but are pale blue rather than red and there are numerous erythroblasts (Fig. 3I). O-dianisidine staining confirms that the sau^{ty121} mutants (Fig. 3J) express less hemoglobin than wild-type embryos though relatively more than *zin* mutant embryos.

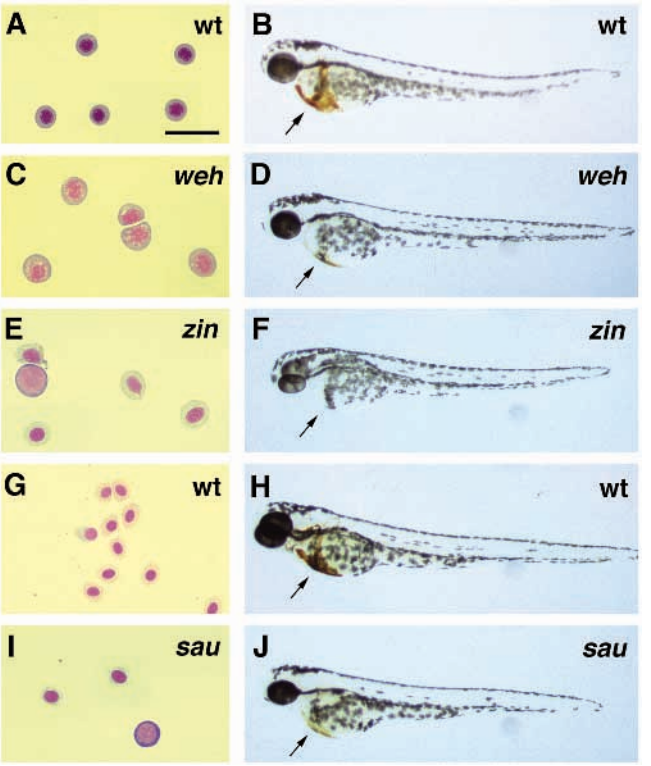

Fig. 3. Mutations in the genes *weißherbst* (*weh*), *zinfandel* (*zin*) and *sauternes* (*sau*) result in embryos with hypochromic blood and decreasing blood cell counts. (A,C,E,G,I) Isolated blood cells from wild-type and mutant larvae stained by the Wright-Giemsa method. (B,D,F,H,J) O-dianisidine staining for hemoglobin (arrows) in wild-type and mutant embryos. (A) Blood cells from 2-day old wild-type embryos have the normal morphology of late erythroblasts. (B) Wild type embryos express large amounts of hemoglobin in their differentiating red blood cells. (C) Blood cells from 2-day old weh^{tp85c} mutant embryos are larger with open nuclei more typical of early proerythroblasts. (D) 2-day old weh^{tp85c} mutant embryos have approximately 50% of the normal number of blood cells which express much lower levels of hemoglobin compared to wild-type blood cells. (E) Blood cells of 2-day old zin^{te207} mutant embryos are large but are more differentiated than the blood cells of *weh* mutant embryos. (F) 2-day old zin^{te207} heterozygotes (shown here) and homozygotes both lack detectable hemoglobin. (G) 3-day old wild-type embryos have mature erythrocytes with condensed nuclei and red staining hemoglobin. (H) A wild-type, 3-day old, embryo has large amounts of blood expressing hemoglobin. (I) Blood cells of 3-day old sau^{y121a} mutant embryos are relatively normal in morphology, but they are reduced in number to between 25% and 50% of normal levels with a few large basophilic cells. (J) 3-day old sau^{y121a} mutant embryos have fewer blood cells and thus a reduced total amount of hemoglobin expression. The scale bar in panel (A) is equal to 20 microns for panels (A,C,E,G,I).

The mutations causing hypochromic blood may represent embryonic thalassemias – defects in one of the *globin* genes, producing a globin chain imbalance that destroys erythrocytes (Nagel, 1995). Alternatively, these may be mutations in genes that regulate hematopoietic cell differentiation.

(IV) Photosensitive mutants

All of the blood mutants were tested for photosensitivity and

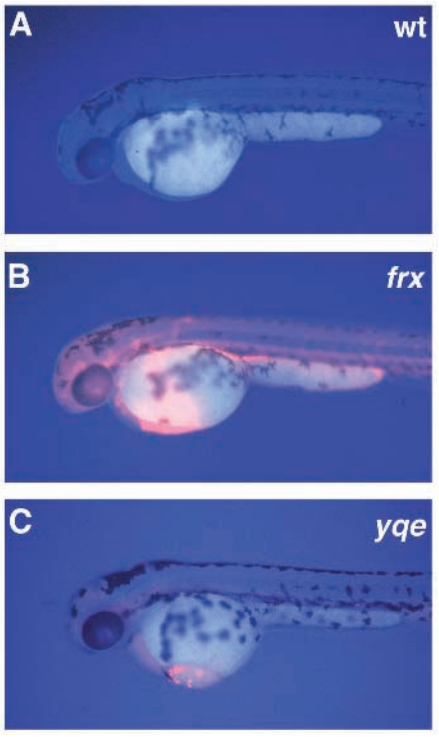

Fig. 4. Mutations in the genes *freixenet* (*frx*) and *yquem* (*yqe*) cause erythropoietic porphyria phenotypes. Mutant and wild-type embryos were examined under 330 to 380 nm UV light for autofluorescence. (A) A wild-type 2-day old embryo does not have autofluorescent blood. (B) The blood of 2-day old frx^{tu271} homozygotes is brightly autofluorescent under the same conditions. (C) The blood of 2-day old yqe^{tp61} homozygotes is also autofluorescent. In addition, the yqe^{tp61} mutants rapidly develop pericardial edema when exposed to bright light.

autofluorescence in a screen for porphyria syndromes. The offspring of identified heterozygotes were grown either in constant white light or in darkness and compared after 3 to 5 days of development. In a second experiment, all mutants were examined under UV light. This screen identified members of a class of mutants that have phenotypes analogous to the human congenital erythropoietic porphyrias. The blood of wild-type embryos is unaffected by UV light (Fig. 4A) whereas the red blood cells of two porphyria mutants, *freixenet* (frx^{tu271}; Fig. 4B) and *yquem* (yqe^{tp61}; Fig. 4C) are autofluorescent and bright orange to red.

frx mutant embryos that are exposed to light do not survive beyond 5 to 7 days of development. As little as 2 hours of exposure to ambient laboratory light is sufficient to eliminate all red blood cells from *frx* embryos that are older than 48 hours. When *frx* homozygous embryos are exposed to UV light the erythrocytes are orange-red in color (Fig. 4B). After 60 seconds of exposure, the blood cells of *frx* embryos stop circulating and the embryos die while wild-type siblings are unaffected. If *frx* larvae are exposed to white light they accumulate dark inclusions in their livers after 5 days of development caused by the accumulation of lysed red blood cells. Under UV illumination, the guts of 5-day old *frx* larvae are also fluorescent, presumably due to clearance of protoporphyrins from the blood stream.

Mutations in the gene *yquem* (yqe^{tp61}) also cause a porphyria syndrome. Under UV light their blood cells are autofluorescent like in *frx* mutants (Fig. 4C). When grown in constant light, *yqe* mutant embryos have no circulating cells while 1- and 2-day old *frx* mutant embryos have 300 to 500 blood cells. In addition, *yqe* mutant embryos show marked cardiac edema and die after 3 to 5 days as a consequence of exposure to light. Both of these mutants display phenotypes that are reminiscent of the erythropoietic porphyria syndromes, which in humans result from defects in heme biosynthesis (Sassas and Kappas, 1995).

DISCUSSION

We are using the zebrafish as a model system to study hematopoietic cell development in a vertebrate embryo. We have used a mutational approach to identify genes that are indispensable and specifically required for hematopoietic development. The 33 mutants described here display a broad range of phenotypes during the first 5 days of development (Table 1). We have classified these mutations based on complementation analysis and specific phenotypic traits.

Hematopoietic defects in the zebrafish mutants

The hematopoietic program involves the induction of ventral mesoderm followed by events that regulate cell proliferation, cell differentiation and cell death. The mutations described here characterize several of these landmarks in the development of the embryonic hematopoietic system. The types of cells remaining in these anemic mutants suggest that all of these genes influence the differentiation of erythroblasts to normal erythrocytes. This does not exclude the possibility that these genes may primarily function at much earlier steps in differentiation. Studies with marker genes expressed at different developmental stages are needed to define the steps affected by these mutations.

The blood mutants can be classified as early or late disruptions of the normal hematopoietic program based on their expression of *GATA-1* and *GATA-2* (Detrich et al., 1995). Most mutants have wild-type levels of *GATA-1* expression, suggesting that these mutants represent later defects in embryonic hematopoiesis. Of the mutations described here, only strong alleles of *moonshine* (*mon*) cause a lack of *GATA-1* expression. Since *mon* embryos have wild-type levels of *GATA-2* expression, but not *GATA-1* expression, we conclude that this mutant has early hematopoietic cells that are blocked in differentiation. It is tempting to speculate, that *mon* may directly control the level of *GATA-1* expression, although further studies would be required to confirm this hypothesis.

With regard to erythroid cell proliferation, there are a number of mutations including those in the genes *chablis*, *frascati*, *grenache*, *merlot*, *pinotage*, *retsina*, *thunderbird*, *weißherbst* and *zinfandel* that result in initially normal blood cell counts followed by severe anemia. This sudden drop in cell number indicates a destruction of the mutant blood cells. In mutants of the genes *weißherbst* and *zinfandel*, the number of circulating cells in 2-day old embryos is almost equivalent to wild type, but the level of globin expression is substantially decreased and the blood appears hypochromic. These mutations may be defects in *globin* genes or in *globin* gene regulation similar to the thalassemias found in humans (Nagel, 1995; Orkin, 1995b). Thalassemias are syndromes that result from abnormal globin synthesis. Unpaired or mutant globin chains can precipitate in erythrocytes and cause cell lysis (Nagel, 1995). Studies of peripheral blood cells of embryos mutant for *weißherbst* or *frascati* revealed cells that are blocked at the proerythroblast stage of erythroid differen-

tiation. Regarding cell death, mutants such as *frascati* have blood cells with abnormal characteristics of the nucleus and vacuolated cytoplasm. It will be interesting to investigate the mechanism of cell death involved in the elimination of cells that fail to undergo the normal maturation process.

A number of mutants described in this report are severely anemic as embryos and larvae but develop into viable adults. These include mutants in *chardonnay, chianti, pinotage, sauternes, thunderbird* and *zinfandel*. This suggests that we may have identified genes that are specifically required for hematopoiesis in the embryo and not in the adult. Alternatively, these genes may be required for normal blood cell development in both embryos and adults. Further studies of adult fish that are homozygous for these mutations are underway to determine if they display defects in the adult hematopoietic system.

Comparison of zebrafish mutants to mouse and human mutants

The hematopoietic mutants generated in this large scale screen may represent defects in novel genes or defects in homologous genes known to be required for normal hematopoiesis in mice or humans. Mutation in several known mouse genes cause phenotypes with similarities to the zebrafish mutants. Based on the synchronous wave of erythroid differentiation of circulating blood in the zebrafish, blocked erythroid maturation that is first visible after 2 days of development might be the phenotype of a *GATA-1* defect (Pevny et al., 1991; Weiss et al., 1994). It is more difficult to predict a phenotype for zebrafish mutants deficient in *GATA-2* expression. A defect in the *GATA-2* gene, which in mice affects erythroid cell proliferation (Tsai et al., 1994), could cause relatively late phenotypes such as decreasing erythroid cell number in the zebrafish embryo. Alternatively, based on the expression in the putative hematopoietic stem cells, a mutation in *GATA-2* could result in an earlier defect in the hematopoietic system of the zebrafish. Zebrafish mutants with defects visible on the first day or two of development could be analogous to the murine *SCL* (Shivdasani et al., 1995) and *TTG2* mutants (Warren et al., 1994). Although the zebrafish mutants described here do not have vascular defects, it is possible that particular mutant alleles of *FLK-1* could produce a hematopoietic phenotype.

The combination of hematopoietic and neural crest mutant phenotypes in *mon* mutants is reminiscent of the *W* and *Steel* mutations in mice, which are defects in *c-kit* and the *c-kit* ligand, respectively (Bernstein et al., 1990; Brannan et al., 1991; Huang et al., 1990; Nocka et al., 1990a,b). Mice with mutations in either of these two genes have dramatic reductions in the proliferation and migration of pigmented neural crest cells (melanocytes) as well as defects in the differentiation of definitive adult hematopoietic stem cells. However, the analogy between these genes and *mon* is not complete. *mon* mutants have no obvious defects in melanophore pigmentation (Fig. 1G) compared to wild type (Fig. 1H). Also, *W* and *Steel* mutants do not exhibit severe defects in embryonic erythropoiesis like those caused by mutations in *mon.*

Other murine mutations that predominantly affect definitive and not embryonic hematopoiesis are less likely to have counterparts among the zebrafish mutants described here. These included mutations in the transcription factors *myb* (Mucenski et al., 1991), *EKLF* (Perkins et al., 1995; Huang et al., 1990; Nocka et al., 1990a), and *Pu.1* (Scott et al., 1994).

These zebrafish mutants have defects that also resemble diseases of the hematopoietic system in human congenital disorders. As described above, some of the hypochromic mutants may represent embryonic thalassemias (Higgs et al., 1990). We have isolated and characterized the embryonic *globin* genes from zebrafish and we are testing polymorphisms for linkage to these mutants (A. Brownlie and L. Zon, unpublished data). Other hypochromic blood mutants may be the counterpart to heme biosynthetic or iron utilization disorders, such as the congenital sideroblastic anemias or iron deficiency (Lux, 1995). For instance *frx* and *yqe* mutant embryos have porphyria phenotypes with intense fluorescence in the red blood cells and severe hemolysis upon exposure to light. These mutations may therefore be the equivalent of a congenital erythropoietic protoporphyria or erythropoietic porphyria, although formal biochemical studies of the enzymes and porphyrin bi-products would be required to secure such a diagnosis (Sassa and Kappas, 1995). Some zebrafish mutants had abnormally large erythroid cells reminiscent of the dyserythropoietic or megaloblastic erythroid cells characteristic of such human disorders as congenital dyserythropoietic anemia, Diamond Blackfan anemia, and B12 or folate deficiency (Lux, 1995). It should however be noted that only fetal and adult hematopoiesis have been extensively characterized in human hematopoietic diseases. The zebrafish mutants described here may represent defects of embryonic erythropoiesis, and it is possible that embryonic signaling cascades and enzymes (and not the adult homologs) are affected in these mutants. Nevertheless, the genes identified by the mutations described here are likely to play the same or similar roles in the hematopoietic system of other vertebrates.

Analysis of the large scale screen for blood mutants

We have placed 32 of the 33 mutations affecting the hematopoietic system into complementation groups representing 17 genes. The allelism of *te264* remains unresolved to date. Thus the allele frequency in this group of mutants is relatively low. The large scale mutational screen was not specifically designed to identify mutations affecting the hematopoietic system. Instead, a large variety of mutations affecting different tissues and organs was isolated (Haffter et al., 1996). The mutations described here either affect the number of circulating cells and/or the intensity of the red color of the blood cells. Mutations affecting myeloid or lymphoid lineages specifically or mutations affecting the red blood cell lineage without affecting the cell number or the intensity of the red color were not identified in the screen. This would suggest that additional mutants may be found by performing a screen aimed at systematically identifying the genes involved in the different aspects of zebrafish hematopoiesis.

In this report, we have shown that the zebrafish offers a powerful new method to screen for mutations in genes required for the differentiation of erythroid cells. It will be interesting to determine if any of these mutations function in a cell autonomous manner or whether factors secreted by other tissues are involved in erythroid cell differentiation, given the fact that terminal differentiation of erythroblasts takes place in circulation. Further studies are also necessary to determine what myeloid or lymphoid lineages may be affected by these

mutations. We have begun to search for zebrafish molecular markers to identify the various lymphoid and myeloid cell types in order to further characterize these mutants. Characterization of the genes affected by the mutations described in this report will further our understanding of the molecular mechanisms underlying hematopoiesis in the vertebrate embryo.

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