

***GATA-1* expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene**

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SUMMARY

In this study, DNA constructs containing the putative zebrafish promoter sequences of *GATA-1*, an erythroid-specific transcription factor, and the green fluorescent protein reporter gene, were microinjected into single-cell zebrafish embryos. Erythroid-specific activity of the *GATA-1* promoter was observed in living embryos during early development. Fluorescent circulating blood cells were detected in microinjected embryos 24 hours after fertilization and were still present in 2-month-old fish. Germline transgenic fish obtained from the injected founders continued to express green fluorescent protein in erythroid cells in the F₁ and F₂ generations. The green fluorescent protein expression patterns in transgenic fish were consis-

tent with the pattern of *GATA-1* mRNA expression detected by RNA in situ hybridization. These transgenic fish have allowed us to isolate, by fluorescence-activated cell sorting, the earliest erythroid progenitor cells from developing embryos for in vitro studies. By generating transgenic fish using constructs containing other zebrafish promoters and green fluorescent protein reporter gene, it should be possible to visualize the origin and migration of any lineage-specific progenitor cells in a living embryo.

Key words: transgenesis, hematopoiesis, zebrafish, *GATA-1*, green fluorescent protein, GFP

INTRODUCTION

Transgenic technology is useful in studies of gene regulation, cell lineage analysis and transplantation experiments (Hanahan, 1989; Jaenisch, 1988). Studies on promoter function or lineage analysis require the expression of a foreign reporter gene, such as the bacterial gene *lacZ*, to identify tissues harboring a transgene. Typically, transgene expression has been identified by in situ hybridization or by histochemistry in fixed tissues. The inability to easily detect transgene expression in living animals severely limits the utility of this technology, particularly in lineage analysis. A new technique utilizing the green fluorescent protein (GFP) as a reporter gene overcomes this limitation and enables transgene expression to be observed in living embryos (Chalfie et al., 1994).

GFP, a protein from the jellyfish *Aequorea victoria*, fluoresces upon exposure to ultraviolet light without the addition of exogenous substrate (Chalfie et al., 1994). A number of modified GFPs have been created that generate as much as 50-fold greater fluorescence than wild-type GFP under standard conditions (Cormack et al., 1996; Zolotukhin et al., 1996). This level of fluorescence should allow the detection of low levels of tissue-specific expression in a living transgenic animal. Ligation of a modified GFP to a tissue-specific promoter should allow careful studies of developmental processes, such as cell migration.

The use of GFP transgenes to study gene expression during zebrafish development is particularly attractive since embryos

are easily accessible and nearly transparent. In addition, embryonic development of the zebrafish is extremely rapid; rudiments of the major organs, including a functional heart and circulating blood cells, develop within 24 hours (Kimmel, 1989). Therefore, a transgenic zebrafish embryo, carrying a tissue-specific promoter linked to a GFP reporter gene, can provide a rapid, real time in vivo system for analyzing spatial and temporal expression of developmentally regulated genes. Recently, thousands of zebrafish mutants were isolated in two large-scale chemical mutagenesis screens (Driever et al., 1996; Haffter et al., 1996). The introduction of tissue-specific promoter-GFP transgenes into these mutants will allow us to study gene expression and developmental processes, such as cell migration, in aberrant genetic backgrounds.

GATA-1, an early marker of the erythroid lineage, was initially identified through its effects upon globin gene expression (Evans and Felsenfeld, 1989; Tsai et al., 1989). Since then *GATA-1* has been shown to be a member of a multigene family that encodes transcription factors that recognize the DNA core consensus sequence, WGATAR (Orkin, 1992). *GATA* transcription factors are key regulators of many important developmental processes in vertebrates, particularly hematopoiesis, as demonstrated by mouse null mutations (Pevny et al., 1991). In chimeric mice, embryonic stem cells carrying a null mutation in *GATA-1* contributed to a white blood cell fraction and to all non-hematopoietic tissues tested, but failed to give rise to mature red blood cells.

In zebrafish, *GATA-1* expression is restricted to erythroid progenitor cells that initially occupy a ventral extraembryonic position (Detrich et al., 1995). As development proceeds, these progenitor cells enter the zebrafish embryo and form a distinct structure known as the hematopoietic intermediate cell mass (ICM). We have isolated genomic DNA fragments that contain the zebrafish *GATA-1* locus and have constructed vectors containing putative *GATA-1* promoter regions linked to a modified GFP gene. These constructs were microinjected into the cytoplasm of zebrafish single-cell embryos. Analysis of either microinjected embryos or transgenic progeny at a number of distinct developmental stages revealed that the GFP expression patterns recapitulated *GATA-1* expression in living transgenic zebrafish.

MATERIALS AND METHODS

Cloning and sequencing of *GATA-1* genomic DNA

A zebrafish genomic phage library was screened with a ³²P-radio-labeled probe containing a region of zebrafish *GATA-2* cDNA that encodes a conserved zinc finger. A number of positive clones were identified. The inserts in these clones were isolated by digestion with various restriction enzymes and were subcloned into pBluescript II KS(-). DNA sequence analysis demonstrated that two phage clones contained zebrafish *GATA-1* sequences (Detrich et al., 1995).

Plasmid constructs

Construct G1-(Bgl)-GM2 was generated by ligating a 5.4 kb *EcoRI/BglIII* fragment containing the putative zebrafish *GATA-1* promoter to a modified GFP reporter gene, GM2. The promoter contains the 5' flanking sequences upstream of the *GATA-1* transcription start sites. GM2 contains 5' wild-type GFP and a 3' *NcoI/EcoRI* fragment derived from a GFP variant, m2, that emits approximately 30-fold greater fluorescence than does the wild-type GFP under standard FITC conditions (Cormack et al., 1996).

To isolate promoter sequences containing the 5' untranslated region of *GATA-1*, a 5.6 kb DNA fragment from a *GATA-1* genomic subclone was amplified by the polymerase chain reaction (PCR) using a T7 primer complementary to vector sequences, and a primer, Oligo (1), that is complementary to the cDNA sequence just 5' of the *GATA-1* translation start. The *GATA-1*-specific primer Oligo (1) contained a *BamHI* site to facilitate subsequent cloning. PCR was carried out using the Expand™ Long Template PCR System (Boehringer Mannheim) for 30 cycles (94°C, 30 seconds; 60°C, 30 seconds, 68°C, 5 minutes). After digestion with *BamHI* and *XhoI*, the amplified fragment was gel purified and ligated to the modified GFP, resulting in construct G1-GM2 (Fig. 1). The construct G1-(5/3)-GM2 was generated by ligating an additional 4 kb of *GATA-1* genomic sequences, containing *GATA-1* intron and exon sequences, to the 3' end of the reporter gene in construct G1-GM2.

Microinjection of zebrafish embryos

G1-GM2 plasmid DNA was linearized by digestion of the vector backbone with restriction enzyme *AatII*. The *GATA-1*/GFP transgene was excised from plasmid vector of G1-(5/3)-GM2 by restriction digestion with the *SacI* and isolated following electrophoresis in low melting agarose gel. DNA fragments were purified using GENECLAN II Kit (Bio101 Inc.) and resuspended in 5 mM Tris, 0.5 mM EDTA, 0.1 M KCl at a final concentration of 50 µg/ml prior to microinjection. Single-cell wild-type embryos were injected as described by Culp et al. (1991), except that tetramethyl-rhodamine dextran was included as an injection control. Injection with each construct was done independently 5-10 times and the data obtained were pooled. In addition, peptides containing the SV40 T antigen

nuclear localization signals (NLSs) were included in the early injections as described (Collas and Alestrom, 1997). However, in our hands, addition of NLSs did not improve the frequency of germline transmission of the *GATA-1*-GFP transgenes.

Fluorescent microscopic observation and imaging

Embryos and adult fish were anesthetized using tricaine (Sigma A-5040) as described previously (Westerfield, 1995) and examined under a FITC filter on a Zeiss microscope equipped with a video camera. Images of circulating blood cells were produced by printing individual frames of recorded videos. Other pictures of fluorescent embryos were generated by superimposing a bright-field image on a fluorescent image using Adobe Photoshop software. 1-month-old fish were anesthetized and then rapidly embedded in OCT. Sections of 60 µm were cut using a cryostat and were immediately observed using fluorescence microscopy. Heart puncture and blood cell collection were performed as described (Weinstein et al., 1996).

Identification of germline transgenic fish by PCR

DNA isolation, internal control primers and PCR conditions were as previously described (Lin et al., 1994). Specific primers, oligo 2 and oligo 3, used to detect GFP, generated a 267 bp product.

Preparation of embryonic cells and flow cytometry

Embryos were disrupted in Holfereter's solution using a 1.5 ml pellet pestle (Kontes Glass, OEM749521-1590). Cells were collected by centrifugation at 400 g for 5 minutes. After digestion with 1× Trypsin/EDTA for 15 minutes at 32°C, the cells were washed twice with PBS, and passed through a 40 µm nylon mesh filter. Fluorescence-activated cell sorting (FACS) was performed under standard FITC conditions.

cDNA synthesis and PCR

Total RNA was extracted from FACS-purified cells using the RNA isolation kit, TRIZoL (Bio101). Reverse transcription and PCR (RT-PCR) were performed using the Access RT-PCR System from Promega (Catalog no. A1250). Specific primers, oligo 4 and oligo 5, used to detect the zebrafish *GATA-1* cDNA, generated a 410 bp product (30 cycles: 94°C, 30 seconds; 60°C, 30 seconds, 72°C, 1 minute).

Oligonucleotide sequences

- (1) 5'CCGGATCCTGCAAGTGTAGTATTGAA3' (*GATA-1*, promoter antisense);
- (2) 5'AATGTATCAATCATGGCAGAC3' (GM2 sense);
- (3) 5'TGTATAGTTCATCCATGCCATGTG3' (GM2 antisense);
- (4) 5'ATGAACCTTTCTACTCAAGCT3' (*GATA-1*, cDNA sense)
- (5) 5'GCTGCTTCCACTTCCACTCAT3' (*GATA-1*, cDNA antisense)

Whole-mount RNA in situ hybridization

Sense and antisense digoxigenin-labeled RNA probes were generated from a *GATA-1* cDNA clone (a gift from Dr L. Zon) or a *GATA-1* genomic subclone containing the second and third exon coding sequence using a DIG/Genius™ 4 RNA Labeling Kit (SP6/T7) (Boehringer Mannheim). RNA in situ hybridizations were performed as described (Westerfield, 1995).

RESULTS

Genomic structure of the zebrafish *GATA-1* gene

A lambda phage zebrafish genomic library was screened for *GATA* family genes using a ³²P-labeled probe containing the zinc finger consensus sequence of the zebrafish *GATA-2* cDNA. Of the positive clones isolated, two were shown to contain zebrafish *GATA-1* by DNA sequence analysis. Restriction

tion enzyme mapping indicated that the two overlapping clones contained approximately 35 kb of the *GATA-1* locus.

To define the promoter of the zebrafish *GATA-1* gene, transcription initiation sites for the zebrafish *GATA-1* were mapped by primer extension. As in chicken, mouse, human and other species, multiple transcription initiation sites were identified. In zebrafish, these sites are located between 130 and 187 bases upstream of the translation start (data not shown).

Comparison of the *GATA-1* genomic structure between human, mouse and chicken suggested that the intron-exon junction sequences of this gene are likely to be conserved throughout vertebrates. Oligonucleotide primers flanking potential *GATA-1* introns were designed and used to sequence the zebrafish genomic clones. Sequence analysis revealed that the zebrafish *GATA-1* gene consists of five exons and four introns, which lie within a 6.5 kb genomic region (Fig. 1A). Although the number of exons and introns and junction sequences are well conserved between zebrafish and other vertebrates, the zebrafish *GATA-1* introns are smaller than in other species.

Transient expression of GFP driven by the *GATA-1* promoter in zebrafish embryos

Based on the structure of the zebrafish *GATA-1* gene, three GFP reporter gene constructs were generated (Fig. 1B). Construct G1-(Bgl)-GM2 was generated by ligation of a modified GFP reporter gene (GM2) to a 5.4 kb *EcoRI/BglII* fragment that contains the 5' flanking sequences upstream of the *GATA-1* transcription start sites. Construct G1-GM2 contained 5.6 kb of DNA upstream of the translation start of *GATA-1*. The third construct, G1-(5/3)-GM2, was generated by ligating an additional 4 kb of *GATA-1* DNA fragment containing intron and exon sequences, to the 3' end of the reporter gene in construct G1-GM2. Each construct was microinjected into the cytoplasm of single-cell zebrafish embryos. GFP reporter gene expression in the embryos was examined at a number of distinct developmental stages using fluorescence microscopy.

GFP expression was observed in embryos injected with either construct G1-GM2 or construct G1-(5/3)-GM2 as early as 80% epiboly, approximately 8 hours postfertilization (pf). At that time, GFP-positive cells were restricted to the ventral region of the injected embryos (Fig. 2A). At 16 hours pf, GFP expression was clearly visible in the developing intermediate cell mass (ICM),

the earliest hematopoietic tissue in zebrafish (Fig. 2B,C). After 24 hours pf, GFP-positive cells were observed in circulating blood and were continuously observed in circulating blood for several months. During the first 5 days pf, examination of circulating blood revealed two distinct cell populations with different levels of GFP expression (Fig. 2D). One cell type was larger and brighter; the other was smaller and less bright. No significant difference in GFP expression levels was detected between embryos injected with either construct G1-GM2 or G1-

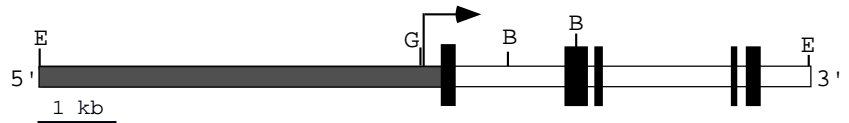
A.

IVS-1
AGACACAGTCCAG (GTGAGTCCAA...1.6 kb...ATTAAACAG) TTCGCCAAGTC
R H S P V R Q V

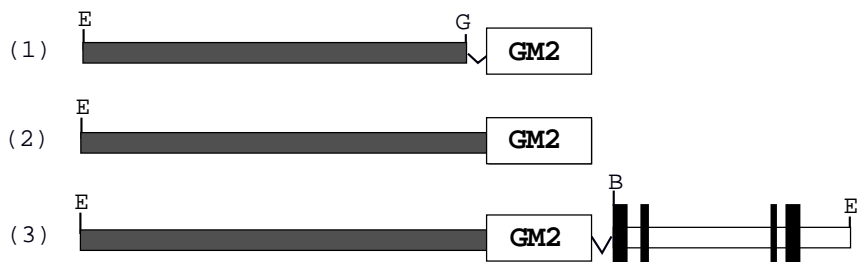
IVS-2
CTTTCGCCACCTG (GTATGTTGTG...0.07 kb...AATTTTACAG) AGGCTCGTGAA
L S P P E A R E

IVS-3
AAAAAGAGGCTG (GTATGTAAAA...1.7 kb...CCTGCATCAG) ATTGTCAGCAA
K K R L I V S K

IVS-4
AAACTGCACAAT (GTGAGTATAC...0.08 kb...CTTTTTCAG) GTCAACAGGCCCT
K L H N V N R P



B.



C.

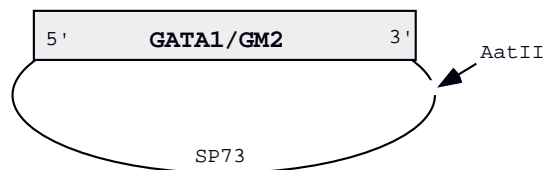


Fig. 1. Genomic structure of the zebrafish *GATA-1* gene and constructs utilized for generating transgenic zebrafish. (A) The exon-intron structure of the zebrafish *GATA-1* gene. Codons and corresponding amino acids surrounding each intron are shown. Conserved nucleotides at the ends of each intron are underlined. (B) Maps of the *GATA-1*/GM2 reporter constructs. (1) G1-(Bgl)-GM2; (2) G1-GM2; (3) G1-(5/3)-GM2. Solid blocks represent exons and gray blocks represent the *GATA-1* promoter. (C) The GFP insert and vector backbone with the restriction site of AatII, which was used to linearize the DNA for injection. B, *Bam*HI; E, *Eco*RI; G, *Bgl*II. The arrow indicates a transcription start site.

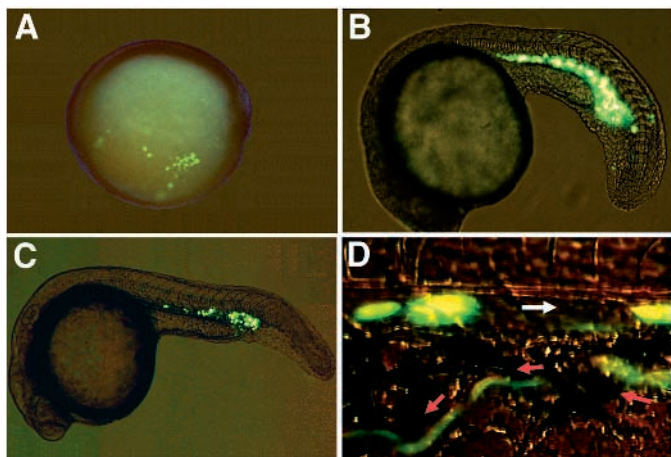


Fig. 2. Zebrafish *GATA-1* promoter activity in living zebrafish embryos. (A) Transient GFP expression (fluorescent green) driven by a zebrafish *GATA-1* promoter construct (G1-GM2) in an 8-hour-old embryo. (B,C) Transient GFP expression in the intermediate cell mass of zebrafish embryos (construct G1-GM2). (D) Transient GFP expression in circulating blood cells of a 2-day-old zebrafish embryo. A white arrow indicates circulation in the dorsal aorta and red arrows indicate circulation in the the ventral axial vein.

(5/3)-GM2. However, injection of construct G1-(Bgl)-GM2 yielded very weak GFP expression in developing embryos. This result indicated that either the *GATA-1* transcription initiation site was removed by digestion with *Bgl*III, or that the 5' untranslated region of the zebrafish *GATA-1* gene is required for high level tissue-specific expression of GFP. It is not surprising that a construct lacking the 5' untranslated region did not generate much GFP expression in microinjected embryos since these regions are often needed for transcript stability and may also contain binding sites for regulators of gene expression.

At least 75% of the embryos injected with the G1-GM2 or G1-(5/3)-GM2 constructs showed some degree of ICM-specific GFP expression (Table 1). The number of GFP-positive cells in the ICM or in circulating blood ranged from a single cell to a few hundred cells. Less than 7% of these embryos showed GFP expression in non-hematopoietic tissues and this was usually limited to fewer than 10 cells per embryo. These observations indicated that a genomic DNA fragment extending approximately 5.6 kb upstream from the *GATA-1*

Table 1. Transient expression of GFP in embryos microinjected with different *GATA-1*-GM2 constructs

| Constructs | Nos observed embryos | Nos embryos with GFP expression in ICM (%) | Nos embryos with strong GFP expression in ICM (%)* | Nos embryos with non-specific expression of GFP (%)† |
|----------------|----------------------|--|--|--|
| G1-GM2 | 336 | 274 (81.5%) | 177 (52.7%) | 15 (4.5%) |
| G1-GM2(5/3) | 248 | 187 (75.4%) | 150 (60.5%) | 16 (6.5%) |
| G1(BglIII)-GM2 | 370 | 0 (0%) | 0 (0%) | 19 (5.1%) |

*Strong GFP expression means that each embryo has more than 10 green fluorescent cells in the ICM.

†Non-specific expression of GFP was usually observed in notochord, muscle and enveloping layer cells, and was limited to no more than 10 cells per embryo.

translation start site sufficed to recapitulate the embryonic pattern of *GATA-1* expression in zebrafish.

GFP expression in germline *GATA-1*/GFP transgenic zebrafish

Microinjected zebrafish embryos were raised to sexual maturity and mated. Progeny were tested by PCR to determine the frequency of germline transmission of the *GATA-1*/GFP transgene. To date, 9 of 672 founder fish have transmitted GFP to the F₁ generation. Examination of these fish by fluorescence microscopy revealed that seven of eight lines expressed GFP in the ICM (Fig. 3A,B) and in circulating blood cells (Fig. 3E,F). GFP expression patterns in the ICM were consistent with the RNA in situ hybridization patterns previously observed for *GATA-1* mRNA expression in zebrafish (Detrich et al., 1995; also see Fig. 3C,D). In the two lines where F₂ transgenic fish have been obtained, GFP expression in blood cells was observed in 50% of the progeny when a transgenic F₂ was mated to a non-transgenic fish. This indicated that GFP was transmitted to progeny in a Mendelian fashion. Southern blot analysis showed that GFP transgene insertions occurred at different sites in these two lines. The apparent copy number of the transgene in the two lines are 4 and 7 (data not shown).

Blood cells were collected from 48 hour transgenic fish by heart puncture and a blood smear was observed by fluorescence microscopy. Two distinct populations of fluorescent cells were identified (Fig. 4A). As in the circulation of embryos that tran-

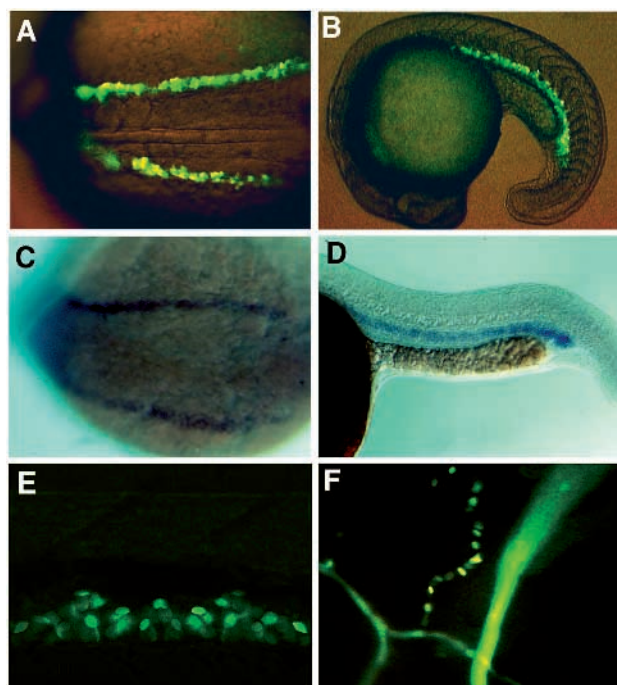


Fig. 3. Comparison of *GATA-1* mRNA and GFP expression driven by the *GATA-1* promoter in germline transgenic zebrafish. (A,B) GFP expression driven by the G1-GM2 construct in a 12 hour and a 22 hour transgenic zebrafish embryo, respectively. (C,D) *GATA-1* mRNA detected by RNA in situ hybridization in a 12 hour and a 24 hour zebrafish embryo, respectively. (E) GFP expression in circulating blood cells in the ventral axial vein of a 3-day-old transgenic embryo. (F) GFP expression in circulating blood cells of an adult transgenic zebrafish.

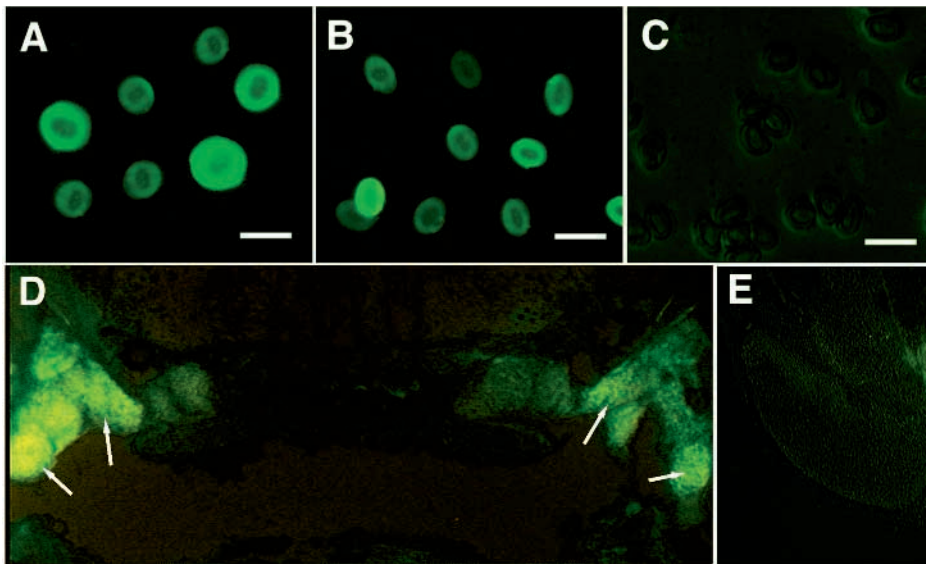


Fig. 4. GFP expression in blood cells of transgenic zebrafish harboring the G1-GM2 construct. (A,B) GFP expression in blood cells isolated from 48 hour embryos and adult transgenic fish, respectively. (C) GFP-negative blood cells from a non-transgenic fish (D) GFP expression in a cross section of head kidney (arrows) from a 1-month-old transgenic zebrafish. (E) Cross section of liver from the same transgenic fish.

siently expressed GFP, we observed one cell population that was larger and brighter and another that was smaller and less bright. Although the blood cells collected from adult transgenic zebrafish showed some variability in fluorescence intensity, they appeared to have uniform size (Fig. 4B). Blood cells collected from non-transgenic fish showed no fluorescence (Fig. 4C).

In 2-day-old transgenic zebrafish, weak GFP expression was observed in the heart. GFP expression was also observed in the eyes and, in three of seven transgenic lines, in some neurons of the spinal cord (data not shown). Expression in the eyes peaked between 30 and 48 hours pf and became extremely weak by day 4. Using *GATA-1* and GFP as probes, we performed RNA whole-mount in situ hybridization on transgenic zebrafish embryos that have GFP expression in eyes or spinal neurons. We detected GFP transcripts but not *GATA-1*. These results suggest that *GATA-1* has no detectable expression in these localities under the conditions used.

Examination of GFP expression in tissues of 1-month-old fish showed that the head kidney contained a large number of fluorescent cells (Fig. 4D). This result suggests that the kidney is the site of adult erythropoiesis in zebrafish. It has been reported that *GATA-1* is expressed in the testes of mice (Ito et al., 1993), however, we did not find expression of GFP in testes dissected from adult fish. It is possible that our transgene constructs still lack an enhancer required for testis expression of *GATA-1*. No detectable level of GFP expression was observed in other tissues, including brain, muscle and liver (Fig. 4E).

It was unknown whether GFP expression is detrimental to development and growth of zebrafish. To determine this, we exposed GFP-positive embryos to microscopic fluorescent light for 2-3 hours per day for the first 2 weeks of development. No apparent light sensitivity or toxicity have been noticed from analyses of these transgenic fish.

FACS analysis of *GATA-1*/GFP transgenic fish

GFP expression in *GATA-1*/GFP transgenic fish provided us with an opportunity to isolate a pure population of the earliest erythroid progenitor cells for in vitro studies by fluorescence-activated cell sorting. F₁ transgenic embryos were collected at

the onset of GFP expression and cell suspensions were prepared. Approximately 3.6% of the cells prepared from whole transgenic fish showed fluorescence as compared to 0.12% in the non-transgenic controls (Fig. 5A,B). Based on the

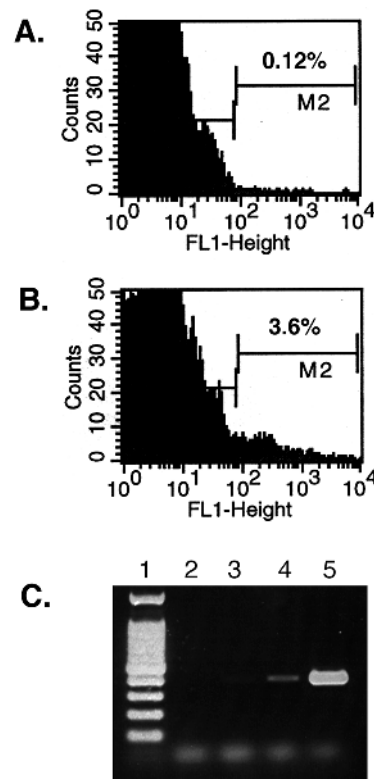


Fig. 5. Isolation and characterization of *GATA-1*/GFP-positive cells from transgenic embryos. FACS profiles of cells isolated from non-transgenic (A) and transgenic (B) 12 hour embryos. Fluorescent cells were collected from window M2. (C) RT-PCR analysis of RNA isolated from the *GATA-1*/GFP-positive cells using *GATA-1*-specific primers. RNA sources: lane 1, molecular weight marker; lane 2, same as lane 5 except no RT reaction; lane 3, FACS-isolated *GATA-1*/GFP-negative cells; lane 4, whole embryos; lane 5, FACS-isolated *GATA-1*/GFP-positive cells.

number of embryos used, FACS analysis suggested that there are approximately 300 erythroid progenitor cells per embryo at 14 hours pf.

To determine whether the FACS-purified cells are enriched for *GATA-1*, we isolated RNA from these cells and determined *GATA-1* mRNA levels by RT-PCR. Our results indicated that these cells were highly enriched for *GATA-1* mRNA (Fig. 5C).

DISCUSSION

Vertebrate hematopoiesis is a complex process that proceeds in distinct phases and at various anatomic sites during development (Zon, 1995). Although studies on in vitro model systems have generated some insight into hematopoietic development (Cumano et al., 1996; Kennedy et al., 1997; Medvinsky and Dzierzak, 1996; Nakano et al., 1996), the origin of hematopoietic progenitor cells during vertebrate embryogenesis remains controversial. Therefore, an in vivo model should be useful to determine precisely the cellular and molecular mechanisms involved in hematopoietic development. It is difficult to carefully observe hematopoietic processes in mammals since embryogenesis occurs internally.

Zebrafish have a number of features that facilitate the study of vertebrate hematopoiesis. Because development is external and embryos are nearly transparent, the migration of labeled hematopoietic cells can be easily monitored. In addition, many mutants that are defective in hematopoietic development have been generated (Ransom et al., 1996; Weinstein et al., 1996). Zebrafish embryos that significantly lack circulating blood can survive for several days, so downstream effects of mutations upon gene expression deleterious to embryonic hematopoietic development can be characterized. Since the cellular processes and molecular regulation of hematopoiesis are generally conserved throughout vertebrate evolution, results from zebrafish embryonic studies may also provide insight into the mechanisms involved in mammalian hematopoiesis.

In this study, we have used the zebrafish animal model and the GFP reporter gene to study the dynamics of *GATA-1* promoter activity during embryogenesis and in the adult. Our results suggest that monitoring GFP expression may be a more sensitive method than RNA in situ hybridization by which to determine gene expression patterns. For instance, in our *GATA-1*/GFP transgenic fish, GFP expression in circulating blood allowed us to distinguish two types of cells; one cell type was larger, brighter and less abundant than the others. We believe that these cells may be erythroid precursors while the more abundant, smaller cells are likely to be fully differentiated erythrocytes. Preliminary cell transplantation experiments with embryonic blood cells have shown that they contain a cell population that has long-term proliferative capacity (unpublished data).

In 2-day-old transgenic zebrafish, GFP expression was observed in the heart. In adult transgenic zebrafish, GFP expression was observed in the kidney. By histological methods, it was shown that the heart endocardium is a transitional site for hematopoiesis in embryonic zebrafish and that the kidney is the site of adult hematopoiesis (Al-Adhami and Kunz, 1977; Willett et al., 1997). Our results support these observations.

The GFP expression that we have seen in the eyes and neurons of embryonic transgenic fish may be due to a lack of a transcriptional silencer in our transgene constructs or a gain of enhancer activity from the vector sequences contained in the injected constructs. It seems unlikely that the GFP expression in the eyes is due to positional effects caused by the sites of insertion since all seven transgenic lines have GFP expression in embryonic fish eyes.

Using fluorescence-activated cell sorting, we have been able to isolate pure populations of hematopoietic progenitor cells from the ICM of transgenic zebrafish. Since we can sort $\sim 10^7$ cells per hour, 10^5 to 10^6 purified ICM cells can be obtained in a few hours. These cells, which are derived from the earliest site of hematopoiesis in the zebrafish, can be used in a variety of in vitro studies. For instance, these pure cell populations can provide mRNA for differential screens for identifying novel hematopoietic genes. Erythroid precursors obtained from the ICM might also be established in tissue culture, allowing identification of the growth factor required by these cells.

Using the *GATA-2* promoter, we recently generated transgenic fish that harbor neuron-specific and early ventral cell expression of GFP (Meng et al., 1997 and unpublished data). These results suggest that transgenic fish that express GFP in other types of tissues can be generated in a straightforward manner. The FACS approach that we present here can therefore be used as a general method for isolating pure cell populations from developing embryos based solely on gene expression patterns. Given the large number of zebrafish mutants available, cells isolated in this manner should be useful in transplantation experiments.

To the best of our knowledge, this study is the first report that describes the generation of a germline transgenic vertebrate that harbors tissue-specific expression of GFP. While we applied this method to the study of hematopoietic development, this approach should be generally applicable to the study of any developmentally regulated process. This approach can also be applied to the identification of *cis*-acting promoter elements that are required for tissue-specific gene expression (Meng et al., 1997). The analysis of promoter activity in a whole animal is desirable since dynamic temporal and spatial changes in a cellular microenvironment can be only poorly mimicked in vitro. The ease of generating and maintaining a large number of transgenic zebrafish lines makes obtaining statistically significant results practical. Finally, transgenic zebrafish that express GFP in specific tissues provide useful markers for identifying novel mutations in future genetic screens. Given the genetic resources and embryological methods available for zebrafish, we believe that transgenic zebrafish harboring tissue-specific GFP expression will be invaluable for dissecting developmental processes.

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