Zebrafish SPI-1 (PU.1) Marks a Site of Myeloid Development Independent of Primitive Erythropoiesis: Implications for Axial Patterning

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The mammalian transcription factor SPI-1 (synonyms: SPII, PU.1, or Sp11) plays a critical role in myeloid development. To examine early myeloid commitment in the zebrafish embryo, we isolated a gene from zebrafish that is a SPI-1 orthologue on the basis of homology and phylogenetic considerations. The zebrafish spil (pul) gene was first expressed at 12 h postfertilization in rostral lateral plate mesoderm (LPM), anatomically isolated from erythroid development in caudal lateral plate mesoderm. Fate-mapping traced rostral LPM cells from the region of initial spil expression to a myeloid fate. spil expression was lost in the bloodless mutant cloche, but rostral spil expression and myeloid development were preserved in the mutant spadetail, despite its complete erythropoietic failure. This dissociation of myeloid and erythroid development was further explored in studies of embryos overexpressing BMP-4, or chordin, in bmp-deficient swirl and snailhouse mutants, and chordin-deficient chordino mutants. These studies demonstrate that, in zebrafish, spil marks a rostral population of LPM cells committed to a myeloid fate anatomically separated from and developmentally independent of erythroid commitment in the caudal LPM. Such complete anatomical and developmental dissociation of two hematopoietic lineages adds an interesting complexity to the understanding of vertebrate hematopoietic development and presents significant implications for the mechanisms regulating axial patterning. © 2002 Elsevier Science (USA)

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INTRODUCTION

The zebrafish (Danio rerio) has proven to be a versatile and informative model for the study of early hematopoietic commitment (reviewed in Amatrusa and Zon, 1999). Zebrafish orthologues have been isolated for a range of molecules important in mammalian hematopoietic commitment, suggesting that, in zebrafish, embryonic and definitive hematopoiesis depends on similar molecular mechanisms and genetic controls to that of mammals.

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Zebrafish hematopoietic genes isolated by various strategies include the transcription factors scl, lmo2, gata1, gata2, cmv and runx family members and cbfb (Liao et al., 1998; Gering et al., 1998; Thompson et al., 1998; Detrich et al., 1995; Kataoka et al., 2000; Blake et al., 2000), some erythroid lineage-specific genes, including the globin gene family and β-spectrin (Chan et al., 1997; Liao et al., 2000a), the heme-synthesis genes δ-aminolevulinate synthetase, uroporphyrinogen decarboxylase, and ferrochelatase (Brownlie et al., 1998; Wang et al., 1998; Childs et al., 2000), and intracellular signaling molecules of the JAK and STAT families (Conway et al., 1997; Oates et al., 1999a,b). The optical transparency of young zebrafish embryos means that endogenous red hemoglobin pigment serves well as a visual flag for both the location and the adequacy of erythropoiesis, and based on this visual tag of hematopoi-
esis, several large-scale screens for organ-specific zebrafish mutants collected a group of anemic mutants (Ransom et al., 1996; Weinstein et al., 1996). Identification of the mutated genes underpinning these mutant phenotypes has, in some cases, implicated new genes in the erythropoietic process (e.g., ferroportin1) (Donovan et al., 2000).

Fate mapping experiments show that primitive circulating blood arises from cells in the radially symmetric zebrafish gastrula that are situated opposite the future embryonic organizer, or shield, which gives rise to the notochord (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). Recognizable erythroid blood precursors are first found in the caudal lateral plate mesoderm (LPM; Al-Ahdami and Kunz, 1977), which is ventrally located in the body plan of the segmentation-stage embryo. Since the notochord is dorsally located in the embryo, the axis running through the shield and blood-forming region in the gastrula is thought to define the future dorsoventral axis of the zebrafish body plan (Kimmel et al., 1990). Like other vertebrates, zebrafish are viewed as patterning mesoderm along this axis by a gradient of bone morphogenetic protein (BMP) signaling, with highest levels at the blood-forming side of the early embryo (ventral end of the axis), and an active repression of BMP signaling required for the organizer and somitic fates (dorsal end of axis) to develop (Jones et al., 1992; Dale et al., 1992; Piccolo et al., 1996; Neave et al., 1997; Nikaido et al., 1997). Thus, high levels of BMP signaling in the pregastrula demarcate an area of mesoderm opposite the future shield that is committed to form embryonic blood (reviewed in Amatruda and Zon, 1999). The fate of this region of erythroid hematopoietic commitment is elegantly displayed in zebrafish by the restriction of gata1 expression to the caudal LPM during somitogenesis. As somitogenesis proceeds, these bilateral bands of gata1-expressing cells move to the midline in a rostral to caudal wave to form the intermediate cell mass (ICM), an axial mesodermal element ventral to the hypochord (Detrich et al., 1995). As the vasculature condenses in the ICM around these gata1-expressing cells, they and their progeny enter the newly established circulation and constitute the primitive wave of erythrocytes.

In contrast to this quite detailed knowledge about zebrafish erythropoiesis, myelopoiesis has been less well characterized in zebrafish. Zebrafish myeloid cells include macrophages and granulocytes (Lieschke et al., 2001; Bennett et al., 2001), which function as the early immune response to bacterial infection, as well as removing cellular debris. At a molecular level, only a few myeloid lineage-specific zebrafish genes have been isolated to date. These include cfls1 and l-plasticin pertaining to macrophages (Herbold et al., 1999; Parichy et al., 2000), and mpo/mpx and cebp1 pertaining to granulocytes (Lieschke et al., 2001; Bennett et al., 2001; Lyons et al., 2001). Additionally, ikaros, rag1, and rag2 orthologues, which mark lymphopoiesis, have also been identified (Haire et al., 2000; Willett et al., 1997).

We now report a comprehensive analysis of early myeloid specification in the zebrafish based on the zebrafish spi1 gene. SPI-1 (also known as SPI1, Sf91, and PU.1) is a member of the E26 transformation-specific (ETS) family of transcription factors that also includes SPI-B and SPI-C proteins (Sharrocks et al., 1997). SPI family members are characterized by a carboxyl-terminal domain that binds to DNA sequences 5'-GAGGAA-3' (PU box). SPI-1 also contains an N-terminal transactivation domain, and a central PEST domain involved in protein-protein interactions (Pongubala et al., 1993; Kiemetz and Makl, 1996). SPI-1 plays a critical role in mammalian myelopoiesis, evidenced by the quantitative and functional deficiencies in macrophages, granulocytes, and lymphocytes manifest in Spi-1-deficient mice immediately after birth (Scott et al., 1994; McKeever et al., 1996). Recently, it has been shown that Spi-1 directly interacts with Gata-1, and mis-expression studies in Xenopus demonstrate that a potential consequence of this interaction can be to divert the fate of a Gata-1-expressing cell away from an erythroid fate (Rekhtman et al., 1999; Zhang et al., 2000). SPI family members are found in the genomes of agnathans, gnathostomes, reptiles, amphibians, and birds, as well as mammals, suggesting that their functional role is widely conserved (Shintani et al., 2000; Anderson et al., 2001).

In zebrafish, videomicroscopic examination has revealed a mobile population of phagocytes emanating from a region of LPM rostral to the heart (Herbold et al., 1999). This population of macrophages expressed a zebrafish l-plasticin, and a novel gene draculinn, isolated by pattern recognition in a screen of EST expression patterns. Despite their appearance at a rostral postgastrulation site, fate-mapping by early blastomere labeling suggested that these macrophages arise from the ventral end of the presumed dorsoventral axis of the gastrula, consistent with a common origin for erythroid and myeloid cells (Herbold et al., 1999). Recently, however, several observations across a range of species have challenged the view that a hematopoietic fate results exclusively from the cells located in the region of the gastrula opposite the organizer (Lane and Smith, 1999; Miyanaeta et al., 1998; Ohinata et al., 1990; Tracey et al., 1998; and reviewed in Shepard and Zon, 2000).

We were interested in better understanding the molecular basis of myeloid specification in the zebrafish embryo, and here we demonstrate the isolation of zebrafish spi1, a SPI-1 orthologue, providing us with a marker of myeloid development, as shown by fate-mapping spi1-expressing cells. Zebrafish spi1 was expressed in rostral LPM at a site where lineage-specific transcriptionally regulated hematopoietic specification had not previously been demonstrated. We show that both erythrocytes and myeloid cells are lost in cloche mutant embryos, whereas there is a selective loss of erythrocytes in the spadetail mutant line. We explored the effects of increasing and decreasing effective BMP signaling on myeloid, as distinct from erythroid development, by overexpressing BMPs and their antagonists, and by examining these lineages in loss-of-function mutants in BMP signaling genes. Our results indicate that myeloid and
erythroid development respond differently to these manipulations, a finding at odds with a common origin for these blood lineages. Collectively, these data demonstrate the dissociability of early myeloid and erythroid development in the zebrafish embryo, and lead us to hypothesize a pregastrulation origin for early myeloid cells separate from and closer to the organizer than that for erythrocytes. These studies thus contribute to the evidence mandating a revision of previous models of vertebrate hematopoietic development which postulate that all hematopoietic lineages originate from the same region of the gastrula, to one in which blood arises from multiple locations around the mesodermal margin.

MATERIALS AND METHODS

Zebrafish

Wild-type zebrafish stocks were obtained from a local pet shop and held in the Ludwig Institute for Cancer Research Aquarim Facility employing standard husbandry practices. The m39 cloche allele (thought to be a gene deletion; Liao et al., 2000b) and the null b104 spadetail allele (Kimmel et al., 1989; Griffin et al., 1998) were used. The dira<sup>−/−</sup> and swirl (swr<sup>−/−</sup>) alleles of chordin and bmp2b were used, both of which result in a morphologically unambiguous phenotype at bud stage (Mullins et al., 1996; Hammerschmidt et al., 1998a,b; Schulte-Merker et al., 1997; Kishimoto et al., 1997). The sbh<sup>−/−</sup> allele of snailhouse, a point mutation in the predomain of the bmp7 locus, was used (Dick et al., 2000).

Embryos for developmental studies were collected from tanks and held at 28°C on a plate warmer. Some embryos were raised in egg water supplemented by 0.003% 1-phenyl-2-thiourea (PTU) (Sigma, P-7629) from 12 h postfertilization (hpf) to suppress melanization (e.g., for in situ hybridization analysis). In describing zebrafish embryos, we have followed the conventions for terms of direction as recently suggested (Moorman, 2001).

Cloning a Zebrafish spil Orthologue

Degenerate oligonucleotide primers corresponding to conserved regions of human and mouse Pu.1 protein were synthesized: WVVVDKD/5'-ccgggccgccgctggtggtnaagyaaraga-3' and YGK-TGE/5'cgcccgccgcctgcgccnccrtarctct-3'; underlined lower case sequences correspond to introduced EcoRI and BamHI sites, respectively. Total RNA was isolated from either kidneys of adult zebrafish previously treated with phenylhydrazine or zebrafish embryos at 30 hpf. cDNA was synthesized with oligo(dT) primer from total RNA isolated by using standard conditions. PCR amplification was performed with degenerate primers under the following conditions: 94°C for 1 min, 40°C for 1.5 min, 72°C for 1.5 min, for a total of 40 cycles. The predicted ~170-bp fragment was gel-purified, subcloned, and sequenced to confirm its identity. The zebrafish spil cDNA fragment was subsequently used as probe to screen a randomly primed cDNA library derived from wild-type zebrafish kidney. After screening approximately 10<sup>5</sup> plaque forming units of the cDNA library made in the λ ZAP express vector, several positive clones were isolated by serial selection under high-stringency conditions (0.1× SSC and 0.1% SDS at 65°C). A clone derived from the oligo(dT)-primed kidney cDNA was isolated by using the random cDNA clone as probe. The cDNA insert in this clone (pBKCMVspil) was sequenced.

The zebrafish gene represented by this cDNA was named spil in accordance with the Zebrafish Nomenclature Guidelines (http://zf.n.org/zf_info/nomen.html) and was approved by the Zebrafish Nomenclature Committee. Acknowledging common usage of the alternate name Pu.1 for the mammalian orthologue, its alias as pu.1, is acknowledged in Genbank and zebrafish databases.

Phylogenetic Analysis

Sequence identity values for the whole molecule and subdomains were determined by using the CLUSTAL algorithm in MegAlign application of the DNASTAR suite of programs (Madison, WI; http://www.dnastar.com) with PAM250 residue weight tables and no manual adjustments. The dendrogram was constructed based on an alignment generated from CLUSTAL X (1.81) (Thompson et al., 1997) using default settings and viewed with TREEVIEW using human ELF5 as an outgroup. Bootstrap values derive from 1000 bootstrap trials.

Linkage Group Assignment and Synteny Analysis

Linkage group assignment on the LNS4 and T51 radiation hybrid panels was achieved by using primers directed to the 3' UTR of the spil cDNA: 5'-tcaatagaaacagcggtgtattgaatct-3' and 5'-cagctactcatagaggtacctcatc-3'. PCR amplification conditions are as follows: 55°C annealing for 1 min, 72°C elongation for 2 min, and 94°C denaturing for 1 min. for a total of 45 cycles. The synteny analysis was performed by using the consolidated zebrafish maps available from ZFIN (http://zfin.org/ZFIN) and data available from LocusLink (http://www.ncbi.nlm.nih.gov/LocusLink/).

Whole-Mount in Situ Hybridization Gene Expression Analysis

Whole mount in situ hybridization analyses were performed as described (Schulte-Merker et al., 1992; Liao et al., 1998; Thompson et al., 1998) by using a hybridization temperature of 70°C. Two-color in situ was performed according to Hauptmann and Gerster (1994) and Prince et al. (1998), with the exception of the Fast Red pre斯坦 washes that used 0.1 M Tris–HCl, pH 8.0. pBKCMVspil was used to generate digoxigenin- and fluorescein-labeled spil riboprobes. To generate a template plasmid for riboprobes corresponding to the first 367 nucleotides of spil, the EcoRI-StuI fragment of pBKCMVspil was subcloned into the EcoRI–Smal site of pBSKCMV. Both full-length and short spil antisense riboprobes were transcribed by using T7 polymerase and cDNA templates linearized with EcoRI. Sense riboprobes were transcribed with T3 polymerase from templates linearized with either XhoI (full-length) or KpnI (short). Controls with sense riboprobes were prepared in parallel with initial spil antisense in situ hybridization analyses and showed no staining, hence subsequently they were not routinely repeated. Probes to gata1, scl, isil, and nkx2.5 were prepared as previously described (Detrich et al., 1995; Liao et al., 1998; Thompson et al., 1998; Alexander et al., 1998). The blue NBT precipitate sometimes completely quenched the visual and fluorescent signals from the fast-red precipitate, regardless of which way they were deployed in the detection step, and careful adjustment of the staining conditions was required. The false-color images of Fig. 8 were taken in black on white on a CoolSNAP HQ Camera (Roper Scientific Photometrics, Trenton, NJ) by using RS Image 1.7.3 software (Roper Scientific) and a fluorescence equipped Leica FL-III dissecting microscope. The blue dye was imaged utilizing epilumination. The Leica FL-III green excitation filter set was used
FIG. 1. A zebrafish spi1 orthologue. (A) Comparison of functional domains of SPL1 proteins from four species, showing amino acid identity scores for each of the three functional domains. In the box linear diagrams, numbers above domain boxes indicate the last residue of each functional domain, and numbers within indicate the percent identity to zebrafish spi1. (B) Phylogenetic analysis for amino acids demonstrates that D. rerio spi1 is an orthologue of previously described SPL1 genes, and that the related gene family members SPI-B and SPI-C form separate clades. [GenBank GI (or Accession) numbers, top to bottom: 4507175, 6755473, 8745406, 2369863, 11245497, (AF321099), 8745412, 36562, 2586116, 8745407, 8745409, 11245499, 8745404, 8745414, 11245501, 6755618, 4557551]. The analysis was based over the DNA binding domain for all proteins commencing at the sequence GX(R/K)R/G/K(R/R/XR) in all but human ELF5, for which the sequence starting "TSLQSS" was used. The dendrogram was constructed by using ClustalX and Treeview building on the analysis of Spi family DNA-binding domains given in Shintani et al. (2000) and Anderson et al. (2001) using human ELF-5 as an outgroup. Bootstrap values (n = 1000) are indicated at nodes as percents. (C) Only one spi1 gene exists in the zebrafish genome. Southern analysis of zebrafish genomic DNA digested with a various restriction enzymes (Lanes: 1, EcoRI; 2, BamHI; 3, BglII; 4, PstI; 5, XhoI; 6, SmaI) and hybridized to a radiolabeled probe corresponding to either the entire 1034 nucleotides (Probe A) or the first 357 nucleotides (Probe B) of the zebrafish spi1 cDNA clone. A single band in lane 6 (probe B) indicates that this spi1 gene fragment is represented in the zebrafish genome by one copy. (D) Survey of spi1 expression in early zebrafish development by RT-PCR. Templates in control lanes (a–d) are: a, plasmid DNA; b, genomic DNA; c, water; d, RT-PCR without reverse transcriptase. Developmental ages from which RNA were prepared are 2, 8, 16, and 24 hpf and 2, 3, 5, and 7 dpf.

A

zebrafish 132 166 290
TRANSACTIONAL PEST SEQUENCES DNA-BINDING

cichlid 112 160 264

chicken 110 151 256

human 108 158 264

mouse 113 167 272

B

SPI-1 human 102
SPI-1 mouse 53
SPI-1 cayman 100
SPI-1 chicken 85
SPI-1 skate 61
SPI-1 zebrafish 70
SPI-1 zebrafish 98
SPI-1 human 81
SPI-1 mouse 81
SPI-1 cayman 54
SPI-1 skate 43
SPI-1 skate 100

C

Probe A

Probe B

kb

10
5
1

1 2 3 4 5 6

D

controls hpf dpf

spi1

a b c d 2 8 16 24 3 5 7
β-actin

b d
FIG. 4. Expression of *spil* relative to other markers of lateral plate mesoderm fates. Two-color in situ hybridization gene expression analysis of wild-type zebrafish embryos (12 somite, flat mount, anterior up). Each panel in the left column shows a low-power view of the head and rostral lateral plate (A, C, E), and the corresponding three panels to the right show high-magnification views (63x water immersion lens) of particular regions of interest in which the expression of *scl* or *spil* has been detected by fluorescence of Fast Red using an RTIC filter set (B, D, F). In places, the presence of high levels of blue NBT/BCIP precipitate quenches the fluorescent signal. (A, B) *spil* (blue) and *scl* (red). Asterisk marks rostral portion of *scl*/*spil* cells, arrow shows domain of *scl*/*spil* cells, and arrowheads show *scl*/*spil* cells caudal to the domain of coexpressing cells. (C, D) *fli1* (blue) and *spil* (red). Asterisk marks rostral *fli1*/*spil* cells, and the arrow shows that, although *fli1* and *spil* are largely coexpressed, at the lateral margin, some *fli1*/*spil* cells are evident. (E, F) *nkx2.5* (blue) and *spil* (red). The heart anlage marked by *nkx2.5* (asterisk) is separate and caudal to the region of *spil* expression (arrow). Scale bar in (B), 30 μm.

FIG. 2. Expression of zebrafish *spil* in early embryonic development. Whole-mount in situ hybridization analysis of *spil* expression in early zebrafish development. Panels show direct lateral (A–E, K–M) and ventral (F–J, N–P) views of embryos at each of the developmental times (hpf) indicated, with anterior to the left. In each case, the paired lateral and ventral views are of the same embryo. The results illustrated were generated with a 1034-nt riboprobe spanning the entire *spil* cDNA; identical patterns were obtained with a 367-nt riboprobe corresponding to sequences encoding the more unique transactivation domain.

FIG. 3. Expression of *spil* relative to *gata1* in the lateral plate mesoderm. Two-color in situ hybridization gene expression analysis of wild-type zebrafish embryo (12 somite, flat mount, rostral up). (A) A low-power view spanning the entire lateral plate mesoderm (LPM). (B, C) High-magnification views (63x water immersion lens) of regions boxed in (A), in which the expression of *spil* has been detected by fluorescence of Fast Red using a RTIC filter set. (B) *spil*/*gata1* cells in the rostral LPM. Arrowheads indicate the caudal margin of the eye. (C) *spil*/*gata1* cells in the caudal LPM. Arrowhead indicates an indentation to the otherwise uniform LPM *gata1* domain, due to several cells that express neither *gata1* nor *spil*. Arrow indicates a lateral irregularity in the LPM *gata1* domain, due to several cells that express both *gata1* and *spil*. Scale bar in (B), 30 μm.
for imaging the red fluorescent dye. The paired images were then combined into a composite red on black and white image by using ImageJ 1.25s software (http://rsb.info.nih.gov/ij/).

RT-PCR Expression Analysis

Total RNA (1.5 μg) was reverse transcribed into cDNA by using 12.5 μM random hexamers, 8 mM MgCl₂, 1 mM of each dNTP, 1 U/μl RNase Inhibitor, and 10 U/μl M-MulV reverse transcriptase (New England Biolabs). Two microliters of the 20-μl reaction were used in a PCR containing 2.5 mM MgCl₂, 400 nM of each primer, and 0.2 U/μl Taq polymerase. PCR conditions were: 94°C 1 min, 52°C 1.5 min, 72°C 2 min, for 25 cycles, which preliminary experiments demonstrated to lie in the linear amplification range for these PCR products. Primer sequences were: sp1, 5′-CAGAATGGAGGGCTA CAT-3′ and 5′-CGTTCTGACTGTCAT CAA-3′; Bactin, 5′-TGGCATACACCTTCTAC-3′ and 5′-AGACCATCAGAGTCC-3′; PCR product sizes were: sp1, 200 nt; Bactin, 221 nt.

Fate Mapping by UV Activation of Caged FITC-Dextran

Photoactivation of caged fluorescein was carried out as described (Kozlowski et al., 1997). Briefly, early cleavage embryos were injected with 2% anionic DMBN-caged fluorescein-dextran (MW 10000; Molecular Probes, Eugene, OR) and stored in the dark until segmentation stages when they were transferred to a depression slide and oriented on 3% methyl cellulose with the lateral plate facing toward the objective. The slide was transferred to the stage of an Axioplan (Zeiss, Thornwood, NY) and the lateral plate was viewed with a 63× water immersion lens. The fluorescein was uncaged between 3 and 12 somites by a 5-s exposure of UV light (DAPI filter set) through a pinhole created by fully closing down the iris in the fluorescence light path. The site of photoactivation was immediately confirmed by using a low-light photomultiplier video camera (VS2000N-Rvideoscope International Ltd., Herndon, VA) and Cytos imaging software (ASI Inc., Eugene, OR) to prevent damage to the embryo from free radicals created by high-intensity fluorescence. Embryos were transferred to 24-well plates containing embryo medium and grown until the time of assay. The location of fluorescent cells was detected and scored initially by using a low-light photomultiplier video camera and Cytos imaging as above, and then embryos of interest were photographed by using Kodak Gold ASA100 print film on an Axioptool AX (Zeiss). Bright-field and fluorescent images were adjusted for contrast and merged by using Adobe Photoshop 5.5 software.

Injection of Zebrafish Embryos

Capped mRNA for microinjection was prepared by using mRNA machine kits (Ambion, Austin, Texas) using the following transcription templates, polymerase and linearizing enzyme combinations: for Xenopus chordin, pSP35T-chd (Sasa et al., 1994); SP6, Xbal; for zebrafish chordin, pCS2+chordin, SP6, NotI (Miller-Bertoglio et al., 1997); for Xenopus BMP-4, pSP64T (Maeno et al., 1998) SP6, BamHI; and for enhanced green fluorescent protein (EGFP), pSP64TK-EGFP. The final resuspension of mRNA for injection was in water, the concentration determined spectrophotometrically, and mRNA integrity checked by visualization after denaturing formaldehyde agarose gel electrophoresis. One to four cell embryos were injected with approximately 600 pl, containing 10–100 pg of mRNA by using a Leica stereomicroscope (Wetzlar, Germany) and Narishige micromanipulators (Tokyo, Japan). Figure 7 data are representative of observations made from Xenopus chordin- or BMP-4-injected embryos, and control un.injected or diluent-injected embryos collected for analysis from several independent injection days. Observations of sp1 and gata1 expression were made in embryos selected from this collection, matched for both developmental age at the time of fixation, and morphologically for an equivalent degree of dorsalization or ventralization. Figure 8 data are representative of the spectrum of effects observed in Xenopus BMP-4 and zebrafish chordin-injected embryos collected from several independent injection experiments. In these experiments, BMP-4 and chordin were coinjected with 10 pg of mRNA-encoding EGFP, and only fluorescing embryos were included in the analysis. Control embryos were injected with 10 pg of EGFP-mRNA alone and showed a <5% abnormality rate (94% EGFP-injected embryos were distributed over the various subgroups to demonstrate the wild-type expression pattern in the in situ hybridization expression analysis). Noninjected embryos were also included as unmanipulated controls in each experiment.

Electron Microscopy

Embryos for electron microscopy were fixed in 2.5% glutaraldehyde and processed as described (Stanley et al., 1994).

RESULTS

Zebrafish sp1 Transcript and Gene

A 1034-nucleotide cDNA encoding a zebrafish sp1 orthologue was isolated by degenerate PCR and library screening (Genbank Accession No. AF321099). Conceptual translation revealed an open reading frame of 290 amino acids starting at nucleotide 15, although alternate candidate initiation ATG codons were located at downstream codons 22 and 54. In vitro translation of this cDNA recovered three polypeptides in approximately equal amounts, suggesting that all translational initiation start sites could be used in vitro at least (data not shown). The 290-amino-acid sp1 protein showed 48.5–53.8% overall identity with mammalian, avian, and crocodilian PU.1, 57.2% identity with a cichlid PU.1, and only 28.2% identity with a lamprey SPI-1. Relative to mammalian and avian PU.1, homology was greatest in the DNA-binding domain, less in the central PEST sequences, and least in the N-terminal transactivation domain (amino acid identities of 83–91, 32–54, and 25–29%, respectively) (Fig. 1A). A phylogenetic analysis of SPI-family members (Fig. 1B) demonstrated that SPI-1 (PU.1) orthologues formed an independent clade to murine, human, toad, and crocodilian Spi-B orthologues (39.5–42.4% identity), and that murine and cichlid Spi-C formed a more distantly related clade again (25.2 and 21.9% identity, respectively).

Southern blot analysis of genomic zebrafish DNA digested with several enzymes using a full-length zebrafish sp1 cDNA probe resulted in multiple hybridizing bands, even at high stringencies. However, a shorter probe covering sequences confined to the transactivation domain (i.e., lacking sequences encoding the DNA-binding domain characterizing ETS family members, but still traversing 3
exons in the orthologous marine gene (Moreau-Gachelin et al., 1989) hybridized to a single band in a SmaI digest, confirming single copy representation of this gene in the zebrafish genome (Fig. 1C). The multiple hybridizing bands seen using the longer probe are likely due to separation of the multiple exons of the spi1 genomic locus encompassed by the full-length cDNA probe onto several restriction digest DNA fragments, but may also reflect cross-hybridization to other ETS family members, in particular including other SPI-family members as have been described in other fish (Shintani et al., 2000; Anderson et al., 2001). We did not pursue further the possibility of other zebrafish spi-family members suggested by this analysis.

Zebrafish spi1 was mapped to linkage group (LG) 7 on the LNS4 radiation hybrid panel between markers Z21519 and Z9521 (at 111.3 and 116.12 cR), placing it near grouchol (at 111.4 cR). Mapping on the T51 panel placed it between z11894 and z21519 (at 187.10 cR), also near grouchol (at 1865.0 cR). Human PU.1 (SPI1) lies at 11p11.2, and murine Pu.1 (Sfi1) is located on chromosome 2 at 47.5 cM. Surveying the mapped genes in the vicinity indicates that zebrafish spi lies in a LG7 region with a disjointed synteny to human chromosome 11 (Bertrand et al., 2001; Yoder and Litman, 2000), and that this region shows less conserved genomic structure in mice. The syntenic relationships are not sufficiently conserved to contribute evidence to the orthologous relationship between spi1 and SPI1.

Collectively, the structural, phylogenetic and genomic data indicate that the cDNA isolated is a zebrafish spi1 orthologue.

**Expression of spi1 during Early Zebrafish Development**

RT-PCR was used to survey spi1 gene expression during early development (Fig. 1D): spi1 expression was absent prior to gastrulation (2 and 8 hpf). Initiated during somitogenesis (16 hpf), and remained up to 7 days postfertilization (dpf). To identify the anatomic sites of early spi1 expression in zebrafish embryos, whole-mount in situ hybridization analysis of zebrafish embryos was undertaken (Fig. 2).

*spi1* expression first appeared in the rostral LPM at the 6-somite stage (12 hpf) (Figs. 2A and 2F). By 10-somites (14 hpf), rostral LPM expression had increased in intensity and extent, and single *spi1*-expressing cells began a process of dispersion, extending toward the midline underneath the developing animal axis, and laterally over the surface of the yolk (Figs. 2B and 2G). At this time, expression first appeared in the more caudal LPM (often referred to as "ventral" and/or "posterior") in a region identical with that previously described for *gata1* expression. Two-color *in situ* hybridization analysis confirmed that, in this caudal LPM domain, *spi1* and *gata1* expression overlapped on a cell-by-cell basis (Fig. 3), an exact concordance emphasized by several irregularities in the otherwise uniform stripe of LPM *gata1* expression (Fig. 3C). Rostrally, as expected, *spi1* expression was not accompanied by *gata1* (Fig. 3B).

Over subsequent hours, *spi1*-expressing cells dispersed over the surface of the spherical yolk sac from the rostral focus of LPM expression (Figs. 2C and 2H), so that by 18 hpf, the surface of the yolk was speckled with *spi1*-expressing cells, with their density usually greater over the rostral half of the yolk surface (Figs. 2D and 2I). The greatest extent of *spi1* expression occurred at 16–18 hpf, and following this, the number of *spi1*-positive cells and intensity of expression in all three regions waned (Figs. 2E and 2J). The domain of *spi1*-expression in the caudal LPM disappeared by 20 hpf (Figs. 2E and 2I). Embryos at 22–24 hpf generally retained only a few *spi1*-positive cells on the yolk surface (Figs. 2K and 2N). In a finely controlled time course experiment, caudal *spi1* expression was evident in 3/29 20-hpf embryos, but 0/46 22- to 24-hpf embryos. This reduction in *spi1* expression at 24 hpf was also evident in the semiquantitative RT-PCR analyses of gene expression (Fig. 1D). Over the period between 26 and 30 hpf, a small number of *spi1*-expressing cells were located in the posterior intermediate cell mass in the region where *gata2* expression had been previously described (also called the "posterior blood island"), and sometimes accompanied by scattered *spi1*-expressing cells in the axis of the embryo and over the yolk (Figs. 2L–2P). No further expression was seen by whole-mount *in situ* hybridization analysis of embryos up to 7 dpf, although by the more sensitive RT-PCR, transcripts could be demonstrated in embryos continuously up to this age (Fig. 1D). Identical patterns of expression were seen with riboprobes corresponding to either the first 367 nucleotides or entire 1034 nucleotides of the *spi1* cDNA, eliminating the possibility that the anatomically separate rostral and caudal sites of LPM expression might represent transcripts of different ETS-family members cross-hybridizing with the longer riboprobe.

We examined the rostral *spi1* expression domain relative to other markers of rostral LPM fates by two-color *in situ* hybridization at the 12-somite stage (Fig. 4). The transcript-
Rostral Zebrafish spil Expression Marks a Myeloid Cell Fate

spil expression directs a hematopoietic cell fate in other vertebrates, and in particular, specifies a myeloid rather than an erythroid fate (Rekhstman et al., 1999), so we hypothesized that it would serve an analogous function in zebrafish. Since spil is expressed first in the rostral LPM, we determined the developmental fate of rostral LPM cells by activation of caged fluorescein-dextran (Table 1 and Fig. 5), following the fate of caudal LPM cells as a control. Cells located in the caudal LPM early in somitogenesis frequently ended up as circulating erythrocytes, consistent with previous observations from gata1 and globin gene expression (Detrich et al., 1995; Chan et al., 1997). In contrast, uncaged fluorescein-marked rostral LPM cells (Figs. 5A and 5B) contributed to a range of tissues (Fig. 5C), and in 33% (5/15) cases, could be identified as large round nonerythroid cells in the nascent circulation (Fig. 5D). These cells exhibited a range of behaviors consistent with a macrophage identity, including extension of pseudopodia, rapid changes in cell shape, and phagocytosis of debris and erythrocytes (not shown), indicating that they are similar or identical to the macrophages identified by videomicroscopy by Herbmel et al. (1999). Thus, cells in the spil expression domain of the rostral LPM give rise to myeloid cells during embryogenesis.

spil Expression in Hematopoietic-Failure Mutants

The mutant cloche (clo) fails to initiate hematopoiesis and vasculogenesis due to a genetic lesion lying upstream of the early hematopoietic fate specification transcription factor, scl (Liao et al., 1998). Embryos homozygous for the severe clo<sup>599</sup> allele show loss of early scl expression in both the rostral and caudal LPM, and erythroidic failure at 24 hpf is reflected in the loss of gata1 expression in caudal LPM during somitogenesis (Thompson et al., 1998). We evaluated spil expression in clo<sup>599</sup> mutant embryos. There was total loss of rostral and caudal spil expression (Figs. 6A and 6B), supporting the hypothesis that spil marked cells with a hematopoietic fate.

The mutant spadetail (spdt)<sup>666</sup> has defective trunk mesodermal development consequent to a lesion in the tbx16 gene (Ruvinsky et al., 1998; Griffin et al., 1998), which, despite expression of earlier hematopoietic lineage commitment markers such as lmno2 and gata2, results in erythropoietic failure evidenced by reduced expression of gata1 and cmyb in the ICM (Figs. 6C and 6D) (Thompson et al., 1998). We used spil expression to evaluate myeloid development in spdt mutants. Although spdt mutants showed loss of caudal LPM spil expression, the rostral domain of spil expression was preserved (Figs. 6E and 6F), consistent with normal rostral development in these mutants. Furthermore, electron microscopic examination of spdt mutants at 48 hpf confirmed the presence of myeloid granulocytic cells (3/3 surveyed embryos), evidenced by their distinctive intracytoplasmic granules (Figs. 6G and 6H).
FIG. 6. spil expression in the hematopoietic failure mutants cloche (clo) and spadetail (spt). (A, B) Whole-mount in situ hybridization demonstration of location of spil expression in wild-type (A) and clo<sup>−/−</sup>/clo<sup>−/−</sup> (B) zebrafish (20 hpi), showing loss of expression in the clo mutant. At this age, clo homozygotes could not be recognized on the basis of coincident morphological features, but the expected
Rostral spil Expression and Myeloid Cell Fate Occur Independently of BMP Signals Required for Erythropoiesis

The initiation of myelopoiesis despite the failure of erythropoiesis observed in spadetail embryos led us to hypothesize that it may be possible for myelopoiesis to initiate, despite the absence of signals necessary for erythropoiesis. We tested this hypothesis by evaluating spil and gata1 expression in embryos with decreased or increased levels of BMP signaling by overexpression of both the BMP-antagonist chordin and BMP-4.

Embryos affected by chordin overexpression were readily identified after 16 hpf by their diminutive tail development, and such embryos retained a widely dispersed population of spil-expressing cells over the rostral yolk, despite a marked reduction in the extent of gata1 expression (Figs. 7A–7D). Embryos strongly affected by BMP-4 overexpression retained a small number of dispersed spil-expressing cells when assessed at 12–14 hpf (Figs. 7E–7H), although the rostrocaudal origin of these cells is unknown. At 24 hpf, rostral spil expression was completely absent, despite obvious reduction in head development and enlarged tails of the embryos, with expansion of gata1 expression in the posterior ICM (Figs. 7I–7L). Thus, the spil-positive myeloid cells of the rostral domain behaved in these assays much as the notochord does, being reduced by an increase, and unaffected by a decrease in BMP signaling (Nikaïdo et al., 1997).

We extended these observations by assessing the effect of chordin and BMP-4 overexpression on other LPM fates along the rostrocaudal axis, using the rostral domain of spil as a marker of rostral LPM fate; nkx2.5 as a marker of the heart anlage, an intermediate LPM fate; gata1 as a marker of caudal LPM fate, with krox20 as an reference point for rostral–dorsal development and myod as an anchor for caudal–dorsal development. Embryos were graded morphologically into groups of those either severely, moderately, or mildly affected, collected at approximately 14–16 hpf or 24–26 hpf, and subjected to two-color in situ hybridization analysis for various combinations of these markers.

Moderately BMP-4-affected embryos scored at 14–16 hpf, with normal caudal morphology but diminutive head development, displayed loss or marked reduction in rostral domain of spil, but showed a preserved (or possibly increased) posterior domain of spil expression (Figs. 8A–8C), indicating that spil expression in these two domains was independent of each other. In such embryos, the extent and pattern of gata1 and myod expression was largely unaffected (Fig. 8L), and nkx2.5 expression was reduced to a single spot of rostral expression (Fig. 8I), instead of the normal pattern of two lateral stripes (Figs. 8I and 8K, embryo "a"). More severely affected embryos, with grossly disturbed morphology, showed preservation or even expansion of a distored posterior domain of spil expression (Fig. 8D), which was analogous to the expanded distored domains of gata1 expression observed in such embryos (Fig. 8E), although it proved technically very difficult to demonstrate this together in the same embryos, due to quenching of the red gata1 signal by the exstensive blue spil signal. In such severely affected embryos, nkx2.5 expression was sometimes absent, despite expanded gata1 expression in the same embryo (Fig. 8K). Hence, the most rostral fate (marked by rostral spil) was more sensitive to abrogation by excess BMP-4 signaling than the intermediate fate (marked by nkx2.5), and neither was expanded in circumstances when caudal fates were (caudal spil and gata1, and myod; data not shown).

Moderately chordin-affected embryos scored at 14–16 hpf, with normal rostral morphology but diminutive tail development, displayed preservation of the full extent of rostral spil expression (Fig. 8L), despite marked reduction in the extent of caudal spil expression (Fig. 8I) and gata1 expression (Fig. 8N). The normal rostral–dorsal development of such embryos was confirmed by preservation of the typical pattern of krox20 expression (Fig. 8M). Even in embryos with diminutive gata1 expression, nkx2.5 expression remained bilateral and approximately normal in degree, although in some embryos, its rostrocaudal dimension was shortened (Fig. 8N). Of 24 embryos affected to varying degrees, 23/24 were gata1+/nkx- and 0/24 lacked expression of one or other marker (1 was unscorable), suggesting that chordin antagonism of BMP signaling could not completely abrogate gata1 expression, even in the most severely affected embryos. Similar observations were made in embryos scored at 24–26 hpf: extensive rostral spil expression was retained in embryos with a mere nubbin of a tail (Figs. 7D and 8O), with retention of nkx2.5 expression to a far greater degree than for gata1, which was reduced to a small region underlying the small stubby tail (Fig. 8P). Hence, the most rostral LPM fates can proceed independently of caudal BMP signaling, and the more rostral the fate, the less it is affected by chordin overexpression.

Collectively, these gain-of-function experiments indicate that rostral myeloid specification can occur despite marked
diminution or even abrogation of the BMP signals required for erythroid specification, and vice versa, that caudal erythroid specification is not dependent on the signal(s) required for spi1-marked myeloid specification. To confirm and extend these observations, we next examined the development of myeloid and erythroid cells in zebrafish carrying loss-of-function mutations in chordin and bmp genes.

Loss of chordin function during early embryogenesis gives rise to the chordino (dine) mutant phenotype, which shows a reduction in rostral notochord and head structures and a dramatic increase in the production of erythroid cells (Hammerschmidt et al., 1996a,b; Mullins et al., 1996). This phenotype is thus overtly similar to, but slightly weaker than, that seen after BMP-4 overexpression. Examination of spi1 expression in dine mutants showed a rostral domain that is compressed along the rostrocaudal axis and broadened mediolaterally, and a normal caudal domain (Figs. 9A and 9B). The expression of early hematopoietic and vascular markers scl and r11 was similarly compressed rostrally, and was greatly expanded in the caudal embryo (Figs. 9C–9F), consistent with the previously demonstrated increase in erythroid cells (Hammerschmidt et al., 1996a,b; Mullins et al., 1996). These data indicate that spi1-positive myeloid cells do not require chordin for their development, and are specified under conditions that cause the absence or reduction of the anterior notochord.

Loss of zebrafish bmp2b function results in the swirl (swr) mutant phenotype, which shows a diminutive tail and lacks erythroid cells and other caudal–ventral structures, but retains a notochord (Mullins et al., 1996; Kishimoto et al., 1997). The swra3 allele results from a 6-amino-acid C-terminal extension of the mature protein that gives a dominant negative effect, resulting in a mild heterozygous dominant phenotype. Loss of bmp7 function results in the smallhouse (snh) mutant, the snha48 allele of which is due to a point mutation in the bmp7 prodomain. This phenotype resembles a partially bmp2b-rescued swr mutant (Dick et al., 2000), and has reduced or absent gata1 expression (Mullins et al., 1996), depending on the phenotypic strength which varies between mating pairs. Thus, these phenotypes show similarity to that seen after chordin overexpression, and comprise a phenotypic series of embryonic defects due to increasingly reduced BMP signaling. In both of these mutants, spi1 expression is present but its onset is temporally delayed (Figs. 10A–10C). At later stages, increasingly severe snh and swr mutant phenotypes show first a loss of the caudal domain of spi1 expression and then progressive diminution in the extent of rostral spi1 expression (Figs. 10D–10I). In stage- and phenotype-matched swra3 embryos from the same clutches, gata1 expression paralleled that of the spi1 caudal domain and was severely reduced or absent (data not shown). Thus, a reduction of BMP signaling to levels that abolish erythroid development does not significantly affect rostral spi1 expression, consistent with the overexpression experiments presented above.

**DISCUSSION**

We have cloned a homologue of mammalian SPI-1 (PU.1) that meets several criteria for identification as the zebrafish orthologue: there was high sequence conservation in the DNA binding domain; zebrafish spi1 segregated with other SPI-1 orthologues and not with other SPI family members in a cladistic analysis of this domain; and spi1 and SPI1 are positioned on zebrafish LG7 and human chromosome 11, respectively, in the context of a conserved but disjointed syntenic relationship between these chromosomes. Fate-mapping of rostral LPM cells from the region of spi1 expression to large nonerythroid cells in the nascent circulation confirms spi1 as an early marker of myeloid differentiation, suggesting that its biological role in mammals and zebrafish has been conserved.

**spi1 Marks a Rostral Site of Early Myeloid Development in Zebrafish Embryos**

The expression pattern of spi1 indicates that myeloid differentiation starts at a rostral site, anatomically separate from early gata1-marked erythroid development. Previous videomicroscopic studies in zebrafish identified mobile phagocytes emanating from a site just anterior to the developing heart, and described two markers of this population of phagocytic cells: draculin and I-plastin (Herbomel et al., 1999). Although draculin is expressed in the myeloid cells dispersing from the rostral LPM over the yolk sac during segmentation, it is also expressed throughout the lateral plate primordium from midgastrulation and persists in both differentiated macrophages and erythrocytes, suggesting a nonlineage-restricted general role in hematopoietic commitment, rather than one focused on myeloid fate per se. I-plastin, a marker of maturing macrophages, is expressed only as these cells migrate onto the yolk sac (Herbomel et al., 1999). In contrast, we have isolated a gene (spi1) which preferentially marks myeloid fate, and this has enabled us to distinguish between cells committing to myelopoiesis and those committing to another hematopoietic fate. At the onset of spi1 expression at 12 hpf (8 somite), expression is confined to a tight group of cells in a specific region of the rostral LPM that we have demonstrated is fated to a myeloid outcome. This makes it the earliest myeloid-specific marker identified in zebrafish to date. Later, spi1 expression is observed in the caudal LPM. We have not determined the functional role of spi1 expression in the caudal LPM. Given the interaction between Spi-1 and Gata-1 in mammalian systems (Rechtman et al., 1999; Zhang et al., 2000), spi1 may serve to regulate or even limit the degree of erythroid commitment in this region of the zebrafish embryo by an interaction with gata1. Another possibility, given that neonatal phenotypes of Spi-1-deficient mice implicate mammalian Spi-1 in the development of granulocytic, macrophage, and lymphoid lineages (Scott et al., 1994; McKercher et al., 1996), is that the different domains of its expression may reflect roles specific to the various myeloid lineages, and that the ICM may give
FIG. 7. Caudal erythroid commitment (marked by gata1 expression) and rostral myeloid commitment (marked by spi1 expression) can proceed independently of each other. Whole-mount in situ hybridization preparations displaying regions of gata1 and spi1 expression in embryos either untreated (control) or injected with mRNA encoding the Xenopus BMP-4 or chordin proteins as indicated to the left. (A–D) Nontreated (A, C) and chordin mRNA-injected (B, D) embryos at 18–20 hpf showing that chordin-expressing embryos have reduced gata1 expression (arrow A, B) in the caudal intermediate cell mass (ICM) but retain rostral spi1 expression in the lateral plate mesoderm (LPM) (arrow, C, D). (E–H) Nontreated (E, G) and BMP-4 mRNA-injected (F, H) embryos at 12–14 hpf showing that BMP-4-expressing embryos have reduced extent of spi1 expression (arrow, G, H) but a markedly expanded domain of gata1 expression (arrow, E, F). (I–L) Nontreated (I, K) and BMP-4 mRNA-injected (J, L) embryos at 24 hpf showing BMP-4-expressing embryos have reduced extent of spi1 expression (arrow, K) but a markedly expanded domain of gata1 expression in the posterior ICM (arrow, I, J). Images are illustrative of 169 Xenopus chordin-injected (53% severe, 47% moderate-mild) and 204 BMP-4-injected (44% severe, 56% moderate-mild) and control uninjected or diluent-injected embryos, collected for analysis from several independent injection days (BMP-4, n = 11; chordin, n = 6).

rise to a small number of previously undetected myeloid cells in addition to a multitude of erythrocytes.

**Rostral Myeloid Development Is Not Dependent on Erythroid Fate-Determining Signals**

**Evidence for the spatial separation of cells committed to different rostrocaudal LPM fates before gastrulation.** Specification of distinct hematopoietic fates could be established as early as pregastrula stages, or it might be the result of interactions at a later stage between a developmentally naive lateral plate and inducing substances with restricted rostrocaudal distribution in a neighboring tissue, such as the somites. The fate-mapping studies of Herbomel et al. (1999) based on cleavage (16-cell stage) cell labeling suggest that myeloid and erythroid lineages both appear to be derived from the part of the pregastrula embryo opposite the shield, at the ventral end of the proposed early dorsoventral axis.

Our observations support the notion of distinct genetic control for the specification of the myeloid and erythroid lineages in the pregastrulation zebrafish embryo. Overexpression of BMPs has previously been interpreted to result in the respecification of the cells of the early zebrafish gastrula to a more ventral fate, whereby the most dorsal fates, such as notochord, are lost, and the number of cells expressing ventral fates, such as erythrocytes, is dramatically increased in the resulting embryo (Neave et al., 1997; Nikaido et al., 1997). In embryos strongly affected by BMP-4 overexpression, we observe that, despite a dramatic increase in erythrocytic fate, there is a near total loss of myeloid cells, suggesting that specification of myeloid cells cannot occur under the same high levels of BMP signaling that favor erythroid production. Conversely, in embryos overexpressing the BMP antagonist chordin, and in embryos with reduced BMP signaling due to loss-of-function mutations in bmp2b and bmp7, the production of myeloid cells is not affected, despite the near total loss of the
erythroid lineage, suggesting that that myeloid cells can be specified and develop normally without the high levels of BMP signaling typical of the region of the early gastrula opposite the shield. Thus, in these assays, myeloid cell specification does not exhibit the characteristics expected of a tissue derived from the same region of the gastrula as erythroid cells, seemingly at odds with the allocation of the pregastrula origins of this lineage as obtained by cleavage stage cell labeling to a position at the ventral end of the proposed early doroasontal axis (Herbomel et al., 1999). We note that a clone of cells descended from a blastomere labeled at the 16-cell stage will undergo considerable dispersion through radial intercalation before the onset of gastrulation (Wilson et al., 1995), potentially spreading to regions of the pregastrula where cells experience lower levels of BMP signaling than at the ventral end of the proposed doroasontal axis.

Indeed, careful examination of the results of the fate-mapping experiments (Herbomel et al., 1999) indicate that it is possible to mark one lineage of embryonic blood cells without the other, suggesting that even at this early stage, their precursors may be spatially distinct. In cases where only one lineage was labeled, cells of the heart were sometimes colabeled, and this occurred more frequently for myeloid (2/3) than erythroid (1/3) cells, suggesting that myeloid precursors in the pregastrula are closer to the precursors of the heart than are erythroid precursors. This hypothesis is consistent with our results in segmentation stage embryos showing myeloid cells positioned in the anterior LPM rostrally and immediately adjacent to the heart field, whereas the most rostral erythroid cells are found ventral to somite 6 in the trunk.

In further support of this hypothesis, fate mapping by following single cells at gastrula stages has demonstrated the origin of the head vasculature in a location adjacent to the notochord anlage (Warga and Nusslein-Volhard, 1999). This observation combined with our data showing that split-expressing myeloid precursors overlap with the flt1-positive cells of the nascent head vasculature in the rostral LPM also implies a common pregastrulation origin for both head vasculature and myeloid cells near the dorsal end of the proposed early doroasontal axis. Combined, these data argue for a genetically distinct specification of spatially separated hematopoietic lineages before the onset of gastrulation. Thus, our observations do not support a model in which the default differentiation pathway of hematopoietically fated ventral tissues is erythroid, and other hematopoietic fates are produced by lineage specification away from an erythroid developmental pathway; for example, by split competing out a gata1 effect at the stem cell level.

Our findings do not rule out the activity of additional permissive factors in the environment of the developing LPM that may be required for the progression or survival of the myeloid lineage in the postgastrulation embryo. It will be interesting to determine whether there are instructive signals that vary along the rostrocaudal axis of the LPM of postgastrulation embryos that are responsible for the precise localization of its different fates; for example, there may be some role for the CNS or lateral epidermis in positioning the boundary between myeloid and heart fate. Such tissue

FIG. 8. Actions of BMP and chordin on the LPM to perturb the balance of rostrocaudal LPM fates. Whole-mount in situ hybridization preparations of zebrafish embryos displaying expression domains of various markers (indicated in lower lefthand corner of each panel), in ECFP mRNA-injected (A, F, G, I), Xenopus BMP-4 mRNA-injected (B-E, H, J, K) and zebrafish chordin mRNA-injected (L-P) embryos (injected mRNA indicated in top righthand corner of each panel). Embryos are variously orientated to optimally display the range of expression patterns of interest. (A–N) Embryos are at approximately 14 hpf; (O, P) Approximately 21 hpf stages of development. (A–D) split expression in control ECFP-injected (A), moderately (B and C) and severely (D) affected BMP-4-injected embryos. (A–C, approximately lateral views with head to the left; the five dysmorphic embryos in D are in various orientations.) (E, F) gata1 expression in five severely affected BMP-4-injected embryos (E, various orientations), showing ectopic expression and expansion of gata1 expression compared with the wild-type expression pattern observed in ECFP-injected embryos (F). (G, H) Group of four moderately BMP-4-affect ed embryos (H) displaying normal expression of gata1 and myod (red arrowheads) (various orientations), compared with the wild-type expression pattern observed in ECFP-injected embryos (G). (I, J) Group of five moderately BMP-4-affect ed embryos (J) showing normal gata1 expression (red arrowheads) but markedly diminished nknx2.5 expression (black arrowheads) (various orientations), compared with the wild-type expression pattern observed in ECFP-injected embryos (I). (K) Three severely affected BMP-4-injected embryos showing expanded gata1 expression and loss of nknx2.5 expression, including a relatively unaffected embryo (upper left, marked "a") for comparison with rostral nknx2.5 strips (black arrowheads) and caudal gata1 stripes (red arrowheads). (L) Dorsal view (head to top) of a chordin-injected embryo showing extensive rostral split and diminished caudal split expression domain. (M) Lateral view (head to left) showing relationship of nknx2.5 and krox20 in moderately chordin-affect ed embryo. (N) Dorsal views (head to top) of three moderately chordin-affect ed embryos showing markedly reduced extent of caudal gata1 expression (black arrowheads) and less affected more rostral nknx2.5 expression (black arrows), with a minimally affected embryo (lower left, marked "a") for comparison. The wild-type expression pattern of these genes in ECFP-injected embryos is shown in two colors (G, I), (O, P) Dorsal views (head to top) of moderately affected chordin-injected embryos showing preserved dispersed split expression (O), bilateral nknx2.5 expression (P, arrow), and small spot of gata1 expression in tail stub (P, arrowhead). Images are representative of the spectrum of effects observed in 137 BMP-4 and 78 zebrafish chordin-injected embryos scored at ~14 hpf (19/137 and 41/78 severely affected, respectively), and 154 BMP-4 and 66 chordin-injected embryos scored at ~24 hpf (21/154 and 13/66 severely affected, respectively), collected from several independent injection days (BMP-4, n = 3; chordin, n = 2) and divided between the various groups of in situ hybridization analyses. In these experiments, control embryos were injected with 10 pg of mRNA encoding ECFP with a <5% abnormality rate (94 embryos, which were divided into groups to demonstrate the wild-type pattern for the various in situ hybridization expression analyses). Scale bars, 0.3 mm.
interactions are important in the separation of heart and erythroid fates (Marvin et al., 2001; Tzahor and Lassar, 2001; Schneider and Merciola, 2001).

**BMP signaling influences on rostrocaudal LPM fates.**

One interpretation of our observations is to think of the effects of BMP signaling, at least in so far as they affect a ventral, mesodermal structure like the LPM, as having differential effects along a rostrocaudal i.e., anterior-posterior axis. Our observations of the effects of BMP-4 and chordin overexpression on the rostral spil, nkk2.5, and caudal gata1. spil expression domains are consistent with this. BMP-4 overexpression first suppresses the most rostral fate (rostral spil), diminishes, and then in stronger phenotypes abolishes an intermediate LPM fate (the heart, marked by nkk2.5). In weak phenotypes, BMP-4 has little effect on caudal gata1/spil expression domains, but at high doses, expands both. In contrast, the most caudal LPM domains (marked by gata1/spil) are the most sensitive to overexpression of the BMP antagonist chordin, or to loss of endogenous bmp gene function. The intermediate LPM domain of the heart anlage marked by nkk2.5 is less sensitive to the abrogation of BMP signals by chordin overexpression, or swr or snh genetic background, and the most rostral LPM domain (rostral spil) is highly resistant to any reduction of BMP signaling, even in the most severely affected embryos.

Thus, it is tempting to suggest that BMP signaling in the early zebrafish embryo establishes the anterior–posterior axis of the mesoderm, not the dorsal–ventral axis. Consistent with this, in supposedly "ventralized" BMP-4-overexpressing embryos, the caudal-dorsal marker myod was retained, suggesting that the embryo was posteriorized. Conversely, in supposedly "dorsalized" chordin-overexpressing embryos, somite formation and myod expression were reduced (data not shown), suggesting that these embryos are anteriorized. The connection between anterior and dorsal in the zebrafish fate map has been previously appreciated (Kimmel et al., 1990), and an explicit separation of dorsoventral and anterior–posterior axes has recently been achieved for the Xenopus gastrula that is in agreement with our findings (Lane and Smith, 1999; Lane and Sheets, 2000), suggesting that the assignment of the early axes may need to be reexamined. Our characterization of zebrafish spil identifies for the first time in zebrafish a reagent that can distinguish rostral–ventral fates, and will be useful in the fine fate-mapping studies necessary to test this hypothesis.

**The necessity of BMP and chordin signals for LPM fates, assessed by analysis of mutants.** Interestingly, loss of the BMP-antagonizing function of chordin in chordino mutant embryos did not greatly affect spil expression or other LPM fates, indicating that chordin is not required directly for myeloid specification. This observation indicates that any role for chordin in myelopoiesis may be restricted to that of a general BMP antagonist in the pregastrula. Moreover, the chordino mutant is not as severely affected as a strong BMP-4 overexpression phenotype, perhaps because additional BMP antagonists, noggin and ogon, are likely active in this mutant embryo (Miller-Bertoglio et al., 1999) and may function in the absence of chordino to reduce BMP signaling to levels compatible with myeloid development. The loss of caudal gata1/spil expression in the weaker phenotypes of the bmp mutants swr and snh before any effect on rostral spil is observed indicates the greater dependence of these caudal fates on the presence of BMP signals, but the delay and near abrogation of rostral spil expression in more severe phenotypes underscores the dependence of these LPM fates on the presence of at least some BMP signals.

**Conservation of a Rostral Origin for Embryonic Myeloid Cells in Vertebrate Evolution**

The question arises as to whether the anatomical separation of the first sites of erythroid and myeloid commitment is peculiar to zebrafish, or if it represents a general aspect of vertebrate development. In Xenopus, studies using a leukocyte-marking monoclonal antibody and head–body chimeras have demonstrated that a macrophage population exists that does not arise from the ventral blood island nor the dorsolateral plate, but from a region of the rostral embryo anterior to the cardiac territory (Ohnata et al., 1990; Miyanaga et al., 1998). Also of interest is the observation that the early expression pattern of Xamll (a Xenopus CBFA2/AML1 homologue) includes lateral plate mesoderm cells anterior to those of the ventral blood island that will later form erythroid cells (Tracey et al., 1998).

In mice, several markers have been used to examine the first site of macrophage appearance and suggest that an early precirculation rostral site of macrophage production may occur in mice as well. The earliest cells stained with the antibody F4/80 (which identifies a macrophage antigen prevalent in all known murine macrophage populations) appeared in the visceral yolk sac blood islands, but it is also mentioned that in one 9-day conceptus, scattered F4/80-positive cells were also seen in the regions of the head, heart, and dorsal aorta (Morris et al., 1991). Mitf gene expression, also an early marker of macrophage development in the mouse, is described as appearing in the yolk sac, head, and a band above the developing heart simultaneously (Lichanska et al., 1999). Expression of c-fms, the receptor for colony-stimulating factor-1 (the macrophage-specific hematopoietic growth factor) appears first in the yolk sac at 9.5 days. Lichanska et al. (1999) observe that the appearance of c-fms-expressing cells in the head of the embryo is never delayed relative to their appearance in the yolk sac, and comment that occasional embryos showed clustering in a band overlying the developing heart. A zebrafish orthologue of c-fms has been cloned, and the expression pattern is reported to follow that of macrophage precursors (Parichy et al., 2000).

Spil expression has also been examined in the developing mouse, but was not detectable by in situ hybridization before the onset of liver hematopoiesis (Lichanska et al., 1999), and in particular, followed the appearance of c-fms...
FIG. 9. Expression of spi1, fli1, and scl in wild-type and chordino (din) zebrafish embryos. (A, B) Flat-mount 12-somite wild-type (A) and chordino (B) embryos stained with spi1 riboprobe. Embryos were opened in the middle of their backs, deyolized, and flat mounted to display ventral views of the discontinuous rostral (top) and caudal (bottom) ends of the embryo. In chordino, the number of rostral spi1 cells is not reduced, and they are more dispersed. (C–F) Wild-type (C, D) and chordino (D, F) embryos stained with scl (blue in C, D) or fli1 (blue in E, F) and spi1 (red in C–F) riboprobes. Although the rostral LPM is somewhat reduced in size in chordino, it still makes cell types expressing these three markers, while the caudal LPM is greatly enlarged. The extent of caudal of spi1-expression was not reduced in chordino mutants, although it was altered in shape; this domain of expression is more completely shown in (D) and (F), rather than in (B). Scale bars, 100 μm.
expression. This contrasts with our observations in zebrafish, where by in situ hybridization, spil expression precedes cfms expression (G.J.L., unpublished data). However, it is possible that the failure to detect early Spi-1 expression in the mouse whole-mount in situ hybridization preparations reflected the sensitivity of the technique, rather than the absolute absence of gene expression.

Taken collectively, although the evidence derives from different molecular markers in the different species mentioned above, these observations strongly suggest that a
focus of early myeloid commitment located immediately rostrally of the developing heart is not a unique feature of zebrafish development, but characterizes early myeloid development in other vertebrates as diverse as frogs and mammals.

A Revised Gastrula Fate Map for the Ventral Mesoderm in Zebrafish

In the current zebrafish fate map, hematopoietically fated precursor cells derive from a location in the gastrula opposite that of the future shield (Kimmel et al., 1990; Warga and Nusslein-Volhard, 1999). We propose a refinement to the zebrafish fate map that separates these fates in the pregastrulation embryo (Fig. 11), and also suggest that this arrangement holds true for the early embryos of other vertebrates. We propose that in the zebrafish gastrula, cells destined for more rostral LPM positions (fated to become myeloid cells) are located near the margin and closer to the shield than the cells destined for caudal LPM locations (fated to become erythroid cells), and furthermore, that these two populations have cells of the heart field interposed between them (Stainier et al., 1993). Through gastrulation, the myeloid and erythroid precursor cells locate to their respective rostral and caudal positions along the LPM, still separated by the cardiac anlage. Then after gastrulation, according to their rostrocaudal location, and possibly now under local rather than programmed influences, these hematopoietically fated cells progress toward erythroid or myeloid outcomes. BMP signaling is required for both erythroid and myeloid outcomes, although at high levels it represses the latter. In contrast, chordin antagonizes the effect of BMP signaling, favoring a myeloid outcome in hematopoietically fated domains, although it itself is not directly required for a myeloid outcome.

Combining our observations with those made in other vertebrates mentioned above leads us to also propose a general model of early myeloid development in vertebrate embryos, in which the myeloid lineage arise from cells in the early embryo that are anatomically isolated from the erythroid lineage and adjacent to the heart anlage, and show a differential response to BMP signals from erythroid cells. These predictions now warrant evaluation in other vertebrate embryos.

Although the processes of erythropoiesis and myelopoiesis ultimately colocalize in the adult, there seems to be no necessity to presume that they will be anatomically colocalized in the precirculation embryo. Although both cell types benefit from the mobility afforded by the circulation once it is established, in the precirculation embryo, there is little necessity that the system generating erythrocytes for oxygen transportation would necessarily be sited with that generating leukocytes for debris removal and front-of-the-line host defense. Indeed, in the early zebrafish embryo, the anatomical separation of these two developmental pathways has now been demonstrated.

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REFERENCES


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