Available online at www.sciencedirect.com

ScienceDirect

Developmental Biology 296 (2006) 561–579

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Genomes & Developmental Control

Regulation of *Drosophila* Friend of GATA gene, *u-shaped*, during hematopoiesis: A direct role for Serpent and Lozenge

Selen Muratoglu [a], Betsy Garratt [a], Kristy Hyman [b], Kathleen Gajewski [b], Robert A. Schulz [b], Nancy Fossett [a,b,*]

[a] *Center for Vascular and Inflammatory Diseases and the Department of Pathology, University of Maryland School of Medicine, Baltimore, MD 21201, USA*
[b] *Department of Biochemistry and Molecular Biology, Graduate Program in Genes and Development, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA*

Received for publication 10 February 2006; revised 12 April 2006; accepted 19 April 2006
Available online 27 April 2006

Abstract

Friend of GATA proteins interact with GATA factors to regulate development in a variety of tissues. We analyzed *cis*- and *trans*-regulation of the *Drosophila* gene, *u-shaped*, to better understand the transcriptional control of this important gene family during hematopoiesis. Using overlapping genomic fragments driving tissue-specific reporter-gene (*lacZ*) expression, we identified two minimal hematopoietic enhancers within the 7.4 kb region upstream of the transcription start site. One enhancer was active in all classes of hemocytes, whereas the other was active in hemocyte precursors and plasmatocytes only. The GATA factor, Serpent, directly regulated the activity of both enhancers. However, activity in the crystal cell lineage not only required Serpent but also the RUNX homologue, Lozenge. This is the first demonstration of GATA and RUNX direct regulation of Friend of GATA gene expression and provides additional evidence for the combinatorial control of crystal cell lineage commitment by Serpent, Lozenge, and U-shaped. In addition, we analyzed *cis*-regulation of *ush* expression in the lymph gland and identified similarities and differences between regulatory strategies used during embryonic and lymph gland hematopoiesis. The results of these studies provide information to analyze further the regulation of this conserved gene family and its role during hematopoietic lineage commitment.

Keywords: *Drosophila*; Friend of GATA; GATA; Gene regulation; Hematopoiesis; RUNX

Introduction

Hematopoiesis is a tightly controlled process by which specialized blood cells develop from a single pluripotent stem cell. Central to this process is the orchestration of lineage-specific developmental programs from diverse genetic networks (Zhu and Emerson, 2002). These networks control the temporal and spatial expression of key regulatory factors, some of which form transient protein complexes that direct lineage differentiation (Orkin, 2000). Current models depict lineage commitment as a process that involves cross-antagonism between blood cell programs, which promotes one lineage at the expense of the others (Orkin, 2000). As a result, identifying key transcriptional factors and understanding their precise regulation will serve to explain how multiple genetic networks converge to specify cell fate choice. Because of the remarkable conservation of gene function, *Drosophila* genetics provides a facile approach to this complex biological problem. Using this system, we previously showed that combinatorial interactions between conserved transcriptional regulators control lineage commitment (Fossett et al., 2003). In this study, we more fully assessed the molecular genetic mechanisms that direct these multifactor interactions.

The fly has a rudimentary blood system compared to that of vertebrates. However, there are striking similarities between vertebrate and *Drosophila* hematopoiesis. The two primary *Drosophila* blood cell types, plasmatocytes and crystal cells, have similar functions to cells of the vertebrate myeloid lineage (Rizki, 1978; Dearolf, 1998; Evans et al., 2003). Crystal cells, named for their crystalline inclusion bodies, are necessary for wound healing and encapsulation of foreign invaders (Rizki,

* Corresponding author. Center for Vascular and Inflammatory Diseases and the Department of Pathology, University of Maryland School of Medicine, 800 W. Baltimore Street, Room 215, Baltimore, MD 21201, USA. Fax: +1 410 706 8121.
E-mail address: nfossett@som.umaryland.edu (N. Fossett).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.04.455

1978). Plasmatocytes synthesize anti-microbial peptides and, similar to monocytes, differentiate into macrophage-like cells that phagocytize microbes and apoptotic cells (Rizki, 1978; Tepass et al., 1994; Dearolf, 1998). Like their vertebrate counterparts, the *Drosophila* blood cells develop from a common hematopoietic progenitor (Rizki, 1978; Dearolf, 1998; Lebestky et al., 2000; Lanot et al., 2001). Furthermore, an increasing number of conserved factors have been shown to regulate blood cell development (Fossett and Schulz, 2001; Evans et al., 2003 and references therein).

The GATA, Friend of GATA (FOG), and RUNX protein families are of particular interest because these conserved hematopoietic regulators act combinatorially to regulate lineage commitment (Tsang et al., 1997, 1998; Elagib et al., 2003; Fossett et al., 2003). The GATA family of transcription factors is named for the consensus DNA sequence (WGATAR) to which its members bind (Orkin, 1996; Parmacek and Leiden, 1999). GATA factors generally have two zinc-finger domains. The carboxyl (C)-terminal zinc-finger binds to the GATA recognition sequence, whereas the amino (N)-terminal zinc-finger both stabilizes DNA binding and interacts with FOG proteins (Orkin, 1996; Trainor et al., 1996; Tsang et al., 1997). The FOG proteins are multitype zinc-finger proteins with up to nine zinc-fingers (Cubadda et al., 1997; Tsang et al., 1997; Tevosian et al., 1999; Svensson et al., 1999; Lu et al., 1999). These factors modify the activity of GATA transcription factors, enhancing or antagonizing GATA activity depending on the gene regulatory context (Tsang et al., 1997; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999). The importance of the GATA:FOG complex has been established using animal models and from studies of heritable diseases resulting from GATA mutations that block FOG binding (Haenlin et al., 1997; Tsang et al., 1998; Deconinck et al., 2000; Fossett et al., 2000; Nichols et al., 2000; Svensson et al., 2000; Tevosian et al., 2000; Yu et al., 2002; Fossett et al., 2003). The GATA and FOG factors that have a role in *Drosophila* hematopoiesis are Serpent (Srp) and U-shaped (Ush), respectively (Rehorn et al., 1996; Sam et al., 1996; Fossett et al., 2001; Waltzer et al., 2002; Fossett et al., 2003). The *srp* gene is alternatively spliced to produce either a single C-terminal zinc-finger isoform (SrpC) or the canonical dual zinc-finger protein (SrpNC). Only SrpNC interacts with Ush (Waltzer et al., 2002; Fossett et al., 2003). RUNX class proteins are named for the pair rule gene, *runt*. These transcriptional regulators bind to DNA through the conserved Runt domain, and heterodimerization with Core Binding Factor β has been shown to increase its binding affinity (Okuda et al., 1996; Tracey and Speck, 2000; Adya et al., 2000; Rennert et al., 2003). In general, Runx activity is influenced by a variety of interacting transcriptional regulators, including GATA factors (Coffman, 2003; Elagib et al., 2003; Fossett et al., 2003; Waltzer et al., 2003). Of the three mammalian *RUNX* genes, *RUNX1* is required for hematopoiesis and is one of the most frequent targets of chromosomal translocations associated with human leukemia (Okuda et al., 1996; Speck and Gilliland, 2002; de Bruijn and Speck, 2004). *Drosophila* RUNX proteins include the founding member, Runt; the hematopoietic factor, Lozenge (Lz); and RunxA and RunxB (Rennert et al., 2003).

Positioned at the apex of *Drosophila* hematopoiesis, Srp is required for production of hemocyte precursors, which give rise to the hematopoietic lineages (Rehorn et al., 1996; Sam et al., 1996). Srp acts upstream of Lz and Glial cells missing (Gcm), which are required for the production of crystal cells and plasmatocytes, respectively (Bernardoni et al., 1997; Lebestky et al., 2000; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). In vertebrates, GATA-2, acting analogously to Srp, is required for the production and survival of pluripotent progenitors (Tsai and Orkin, 1997; Ling et al., 2004). As hematopoiesis progresses, GATA-1 can interact with either RUNX1 or FOG-1 to promote differentiation of the erythroid biopotential precursor (Tsang et al., 1997, 1998; Elagib et al., 2003). GATA-1 also interacts with FOG-1 to block eosinophil production (Querfurth et al., 2000). Our previous work in *Drosophila* shows that the functions of the GATA/RUNX and GATA/FOG protein complexes are not only conserved but that cross-talk between these complexes precisely regulates crystal cell production (Fossett et al., 2003). Specifically, SrpNC acts as a cross-regulatory switch, interacting with Lz to upregulate the crystal cell program or with Ush to block crystal cell production. Consequently, the factors that regulate *ush* gene expression also affect this contextual switch.

In addition to the functional similarity between the *Drosophila* and vertebrate FOG proteins, both *FOG* genes are expressed in a variety of hematopoietic cells during developmental stages ranging from pluripotency to terminal differentiation (Tsang et al., 1997; Fossett et al., 2001; Cantor and Orkin, 2005). *FOG* genes are also downregulated in some lineages to permit differentiation (Querfurth et al., 2000; Fossett et al., 2001). *FOG-1* is expressed at low levels in hematopoietic stem cells and at high levels in early hematopoietic progenitors, erythrocytes, and megakaryocytes. *FOG-1* is downregulated in eosinophils (Tsang et al., 1997; Cantor and Orkin, 2005). In *Drosophila*, *ush* is highly expressed in hemocyte precursors and throughout the development of the plasmatocyte lineage. *ush* is also expressed in crystal cell precursors but downregulated as these cells develop (Fossett et al., 2001). Thus, the precise regulation of *FOG* genes is evident by their intricate developmental and lineage-specific expression patterns. Moreover, as discussed above, the exacting regulation of *FOG* gene expression undoubtedly contributes to the precise formation of transient multiprotein complexes, which can redirect lineage developmental pathways. Finally, the potential to place the *FOG* genes in the context of known developmental pathways will certainly add to our understanding of FOG function. For these reasons, we initiated an analysis of the *cis*- and *trans*-regulation of *Drosophila ush* expression during hematopoiesis. We have defined the *ush* embryonic *cis*-regulatory region, showing that most of the *cis*-elements reside within the region 7.4 kb upstream of the transcription start site. Using this information, we identified two minimal embryonic hematopoietic enhancers and characterized the regulation of *ush* expression during hematopoiesis. One enhancer is active in all classes of hemocytes, whereas the other is active in hemocyte precursors

and plasmatocytes but not the crystal cell lineage. In addition, we showed that Srp directly regulates *ush* enhancer activity in all hemocytes. However, our data indicate that Srp alone is not sufficient to upregulate *ush* hematopoietic expression. Srp must also act with Lz to upregulate *ush* enhancer activity in crystal cell precursors. These findings represent the first demonstration of GATA and RUNX regulation of *FOG* gene expression. We also analyzed *cis*-regulation of *ush* expression in the lymph gland. This identified similarities and differences between regulatory strategies used by hemocytes during embryonic and lymph gland hematopoiesis. Our studies have generated information that will be used to further analyze these important transcriptional regulators.

Materials and methods

Fly strains

Fly stocks were maintained at 23°C on standard food, and w^{1118} was used as the wild-type stock. The following fly lines were used in this study and are described elsewhere: *upstream activation sequence* (*UAS*)-*ush*, *UAS-srpNC* and *twiGal4* (Fossett et al., 2001, 2003). *UAS-srpC* was a generous gift of Deborah K. Hoshizaki (University of Nevada, Los Vegas, NV). *Dmef2Gal4* was a generous gift from Elizabeth Chen (Johns Hopkins, Baltimore, MD) $ru^1\ h^1\ th^1\ st^1\ cu^1\ srp^3\ sr^1\ e^s\ ca^1$/*TM3*, $Sb^1\ Ser^1$ (srp^3) was obtained from the Bloomington Stock Center. The generation of strains carrying *ush* enhancer-*lacZ* transgenes is described below.

Generation of transgenic animals carrying ush enhancer-lacZ fusion constructs

The overlapping DNA fragments used to identify the *ush cis*-regulatory enhancers were generated by PCR amplification using BAC clone BACR48E08 (obtained from the BACPAC Resource Center at Children's Hospital, Oakland Research Institute). The sequences of oligonucleotide primers used to generate the fragments are listed in Table 1. All PCR reactions were performed with *TaKaRa LA Taq*™ Polymerase (TaKaRa). These fragments were cloned into the pCRII-TOPO cloning vector (Invitrogen) and subsequently subcloned into the P element CaSpeR-*Hsp43*-AUG-βgal (Chab) germline transformation vector (Thummel et al., 1988).

Three different methods were used to introduce site-directed mutations (SDM) into DNA fragments. First, SDM was performed using the Stratagene QuikChange II XL Site-Directed Mutagenesis Kit, according to the manufacturer's instructions (Stratagene). Second, PCR-mediated SDM was performed as described by Barettino et al. (1994), except that all of the targets were cloned into the pCRII-TOPO cloning vector (Invitrogen). Finally, standard PCR reactions were performed using oligonucleotide primers with mutated transcription factor binding sites. The oligonucleotide primers used to generate SDM in DNA fragments are as listed in Table 1.

The sequence of each recombinant vector was verified prior to injection. yw^{67c23} or w^{1118} embryos were injected with these recombinant vectors by Model Systems Genomics of Duke University or at The University of Texas, M. D. Anderson Cancer Center. Germline transformants were established according to previously described methods and were screened for tissue-specific *lacZ* expression using immunohistochemical analysis as previously described (Gajewski et al., 1997). At least five independent lines were generated and tested for each construct.

Table 1
List of oligonucleotide primers

Primer name	Forward	Reverse	Use
Wild-type fragments			
−12475/−6925	gatacatacatttggagtgaaagagagatag	gccacaacaaggacacacaacagagcaatag	Enhancer
−7462/−25	ggcccgctccctttgtctgcactcag	tcggctaagaggtctcgtctcgtcc	Enhancer
−2190/−25	ctctcagaggactgttttttt	tcggctaagaggtctcgtctcgtcc	Enhancer
−1421/−25	gttcaggtgaaattacactgtgttctg	tcggctaagaggtctcgtctcgtcc	Enhancer
1809/7908	ttagggtattcgcttcaaactacca	cataaatgtcgcttccttcgattac	Enhancer
6640/12678	atctactccgacctacccaactcac	cgtcatcgatattgttattgtt	Enhancer
−1421/−25	gttcaggtgaaattacactgtgttctg	tcggctaagaggtctcgtctcgtcc	Enhancer
−1421/−956	gttcaggtgaaattacactgtgttctg	ttctttcccagcgataacaatctccttc	Enhancer
−1421/−1215	gttcaggtgaaattacactgtgttctg	cgtgccgcttgccgctaactctcttgc	Enhancer
−1243/−956	tgcaagagagttagcgg	ttctttcccagcgataacaatctccttc	Enhancer/EMSA
−1014/−352	tacagagagcaaaatgagagagagcgg	ctacggctaatggaattctgattattg	Enhancer
−420/−25	gatacagagatacaattaaacacaaaacg	tcggctaagaggtctcgtctcgtcc	Enhancer
−420/−172	gatacagagatacaattaaacacaaaacg	aacagaaacagaaccaaaaccaac	Enhancer
−237/−25	tcagtgcttcagtgtttgtgtatc	tcggctaagaggtctcgtctcgtcc	Enhancer
−174/−25	ttctgtttctgcgatgttatct	tcggctaagaggtctcgtctcgtcc	Enhancer
−174/−85	ttctgtttctgcgatgttatct	cggaattattcgaaacaaaaactga	Enhancer/EMSA
Mutant fragments			
−1243/−956 3xGATAm	method: PCR directed		Enhancer/EMSA
distGATA1	gtcaacaatggcaaacgag**cgc**atcgtcgcaaaggcagccagcag	ctgctggctgcctttgcgacgat**gcg**ctcgtttgccattgttgac	
distGATA2,3	gtttttcgtgg**cgc**taccccaatatatat	ttctttcccagcg**cgc**acaatctccttc	
−1243/−956 3xGATAm	tgcaagagagttagcgg	ttctttcccagcg**cgc**acaatctccttc	
−237/−25 3xGATAm	method: Stratagene SDM		Enhancer
proxGATA1	tttctgtttctgcgatgt**gcg**ctaagcgcagagctcgc	gcgagctctgcgcttag**cgc**acatcgcagaaacagaaa	
proxGATA2,3	gcgctgcgctcct**gcg**cagtgctt**gcg**cag	ctg**cgc**aagcactg**cgc**aggagcgcagcgc	
−174/−85 m competitor	method: PCR, template −237/−25 3xGATAm		EMSA
proxGATGm	ttctgtttctgc**ct**tgt**gcg**ct	tcggctaagaggtctcgtctcgtcc	
−174/−25 Runxm	method: PCR		Enhancer/EMSA
proxRunxm	ttctgtttctgcgatgttatct	ggacgagccgagacctcttagccgagactctctgc**aaa**gg**g**cgacttcc	
SrpC cDNA			cDNA cloning
XhoSrp/SrpXba	aaaactcgagtcatacgcctacccagctctacgag	tttgtctagagatagattttagatagatgagtgttctttg	

Mutated sequences are in bold and underlined.

Immunohistochemical and fluorescent antibody staining of embryos and larvae

Collection, fixation, and immunohistochemical staining of embryos were performed as previously described (Gajewski et al., 1999; Schulz and Fossett, 2005). The following primary antibodies were used: rabbit anti-Ush, 1:500 (Fossett et al., 2001); rabbit anti-Odd-skipped, 1:500 (a generous gift from James Skeath, Washington University School of Medicine, St. Louis, MO); rabbit anti-Serpent, 1:1000 (a generous gift of Mark Brennan, University of Louisville, Louisville, KY); mouse anti-β-galactosidase, 1:500 (Promega). Secondary antibodies were either biotinylated or conjugated with fluorescent dyes and are as follows: biotinylated horse anti-mouse, 1:1000 (Vector Laboratories); biotinylated goat anti-rabbit, 1:1000 (Vector Laboratories); Alexa Fluor 555 donkey anti-mouse, 1:300 (Invitrogen); Alexa Fluor 488 chicken anti-rabbit, 1:300 (Invitrogen). Biotinylated secondary antibodies were detected using the ABC-Elite-peroxidase kit (Vector Laboratories) with diaminobenzidine as a substrate. Embryos were mounted in 50% glycerol and visualized using Zeiss Axioplan optics. Embryos stained with secondary antibodies conjugated with fluorescent dyes were mounted in ProLong Gold anti-fade reagent (Invitrogen) and visualized using Zeiss confocal microscopy. The confocal images were analyzed by Volocity software (Improvision).

Larval lymph glands were dissected and fixed as previously described (Cripps et al., 1998). To assess β-galactosidase activity, the lymph glands were histochemically stained with mouse anti-β-galactosidase, 1:2000 in PBS containing 0.1% Triton X-100 (PBT) for 1 h at room temperature. After incubation, the lymph glands were washed 10 times for 1 min each in PBT. Subsequently, they were incubated in biotinylated secondary horse anti-mouse IgG, 1:2000 for 1 h at room temperature. They were again washed 10 times for 1 min each in PBT and then processed for chromogenic detection as described above for embryos. Stained preparations were mounted in 50% glycerol under cover slips and visualized using Zeiss Axioplan optics.

Gene expression analysis in mutant and Gal4/UAS embryos

Embryos were cultured and collected at 23°C. *ush* enhancer-*lacZ* activity was assayed in srp^3 homozygous embryos after a two generation cross. Males carrying *ush* enhancer-*lacZ* constructs on chromosome II were crossed to $ru^1\ h^1\ th^1\ st^1\ cu^1\ srp^3\ sr^1\ e^s\ ca^1$/*TM3*, $Sb^1\ Ser^1$ virgin females. F_1 enhancer-*lacZ*/+; srp^3/+ *trans*-heterozygous progeny were intercrossed. The F_2 progeny from this cross were collected during embryogenesis and assayed for β-galactosidase activity using immunohistochemistry. Because *ush* enhancer-*lacZ* activity is readily detectable as a single copy, 75% of the entire F_2 population will have *lacZ* expression if Srp is not required for enhancer activity. In contrast, if Srp is required for enhancer activity, 75% of the wild-type embryos will show *lacZ* expression, whereas none of the *srp* mutant embryos will exhibit *lacZ* expression in the hematopoietic or midgut tissues. srp^3 is a null allele due to a point mutation in the C-terminal zinc-finger, which blocks DNA binding (Rehorn et al., 1996). Because Srp is required for germ band retraction, homozygous mutants are easily identified by the altered morphology that results from failure of the germ band to retract.

Enhancer activity assessment in Srp gain of function backgrounds also required a two generation cross. Each enhancer line was crossed to *twiGal4* virgin females to produce *twiGal4*/+; *ush* enhancer/+ *trans*-heterozygous progeny. The F_1 virgin females were collected and then crossed to either *UAS-srpNC* or *UAS-srpC* homozygous males. The F_2 progeny from this cross were collected during embryogenesis and immunohistochemically stained with the β-galactosidase antibody as previously described (Gajewski et al., 1997). Homozygous *twiGal4* virgins were crossed to homozygous *UAS-ush* males to produce *twiGal4/+; UAS-ush/+* embryos. Lymph gland development in these embryos was assayed by analyzing the expression of Serpent and Odd-skipped using immunohistochemical staining.

Gel shift assays

Full-length *srpC* and *srpNC* cDNAs were cloned into the pCMV-TNT® vector (Promega) using the following strategy. The *srpC* coding region was PCR amplified using DNA isolated from *UAS-srpC* fly strains (Hayes et al., 2001) and the XhoSrpC and SrpCXba primers (Table 1). The resulting fragment was cloned into the pCR II-TOPO vector. *srpNC* was cloned as previously described (Fossett et al., 2003). Each *srp* clone was isolated from its respective pCR-II TOPO vector using *Kpn*I/*Xba*I restriction endonuclease digestion. The restriction fragments were isolated and subcloned into the pCMV-TNT® vector, and the sequence of each clone was verified. The *lz* cDNA was cloned into pET3C (kindly provided by Richard W. Carthew, Northwestern University, Evanston, IL). Using these cDNA clones, SrpC, SrpNC or Lz proteins were produced by TNT® coupled in vitro transcription/translation (Promega) according to the manufacturer's instructions. DNA probes corresponding to wild-type or mutant versions of the *ush* hematopoietic enhancers were prepared by PCR amplification of the respective pCRII-TOPO clones. Oligonucleotide primers used to generate the probes are listed in Table 1. The PCR products were purified by gel extraction (Qiagen). The purified fragments were then end-labeled with ^{32}P in a reaction using T4 polynucleotide kinase (Roche). The gel shift assay binding reactions were performed using 4 μl of the appropriate TNT product and respective end-labeled DNA probe. For competition binding assays, increasing amounts of excess unlabeled wild-type and mutated DNA were used. Competitor DNA was added 10 min prior to the end-labeled probe. All binding reactions were incubated on ice for 20 min. The binding reactions were separated on 0.5× TBE non-denaturing polyacrylamide gels at 4°C. Gels were dried and were visualized using the Molecular Dynamics STORM analysis system.

Results

ush embryonic cis-regulatory elements reside within a 7.4 kb region upstream of the transcription start site

The *ush* gene is expressed in a variety of tissues throughout embryogenesis. These include the anterior and posterior midgut, dorsal ectoderm, fat body, visceral and cardiogenic mesoderm, and hematopoietic tissues, specifically the hemocyte precursors, plasmatocytes and crystal cells (Fossett et al., 2000, 2001). The precise temporal and spatial regulation of this intricate expression pattern is undoubtedly controlled by a broad array of transcriptional regulators. These *trans*-acting factors bind to *cis*-elements that likely reside within a 32 kb region, which has previously been shown to contain the *ush* locus (Cubadda et al., 1997). This region contains a 15 kb upstream noncoding sequence and a 17 kb transcribed sequence that includes a 10.5 kb intron located between exons 2 and 3 (Fig. 1A).

To begin identification of the *cis*-elements and *trans*-acting factors that control *ush* expression, we conducted a screen for genomic DNA fragments that drive tissue-specific reporter-gene (*lacZ*) expression. As described in the Materials and methods section, PCR amplification was used to generate overlapping fragments from the upstream noncoding region, between positions −12475 and −25, and from the downstream transcribed region, between positions 1809 and 12678, which includes intron 2 (Fig. 1A). Using this approach, we identified a 7.4 kb enhancer, located between positions −7462 and −25, which recapitulates endogenous *ush* expression. The developmental staging of embryos showed that Ush and β-galactosidase were first detected in the amnioserosa during stage 7 (Fig. 1B panels a, b). By stage 8, both proteins were observed in the hemocyte precursors (Fig. 1B panels f, g). During stage 10, Ush and β-galactosidase were observed in the developing hemocytes, midgut, and dorsal ectoderm (Fig. 1B panels k, l, m, and data not shown). In these tissues, expression continued until the

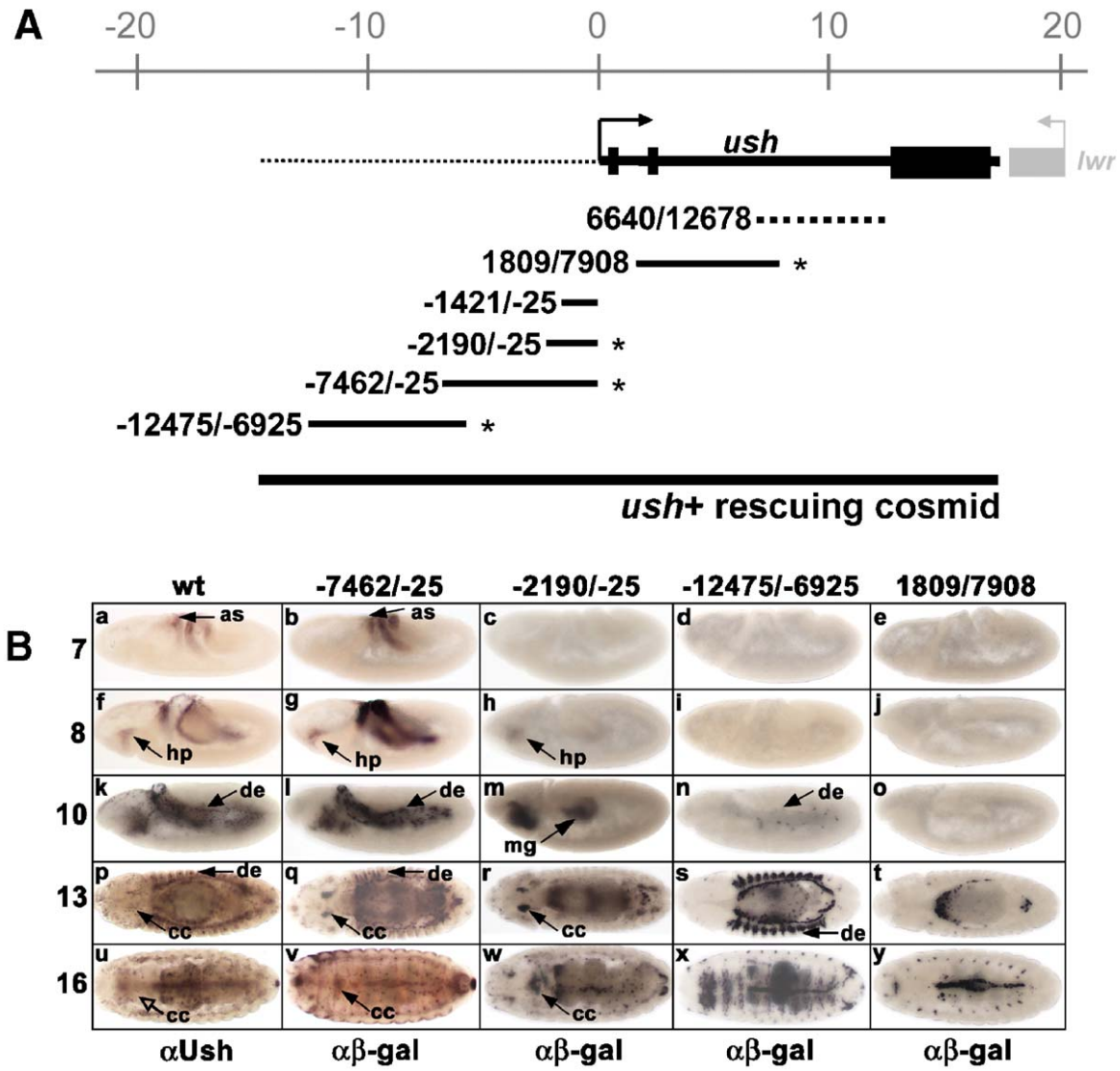


Fig. 1. Delineation of the *ush cis*-regulatory region. (A) Schematic of the *ush* locus and the screen for *cis*-regulatory elements. The locus is complemented by a 30 kb *ush*$^{+}$ cosmid, which rescues the embryonic lethal phenotype. A horizontal arrow indicates the transcription start site, designated position 0 with respect to the *ush* locus. From left to right, the three black boxes represent exons 1, 2, and 3–8. The nearby *lesswright* gene is depicted in grey. A dotted line indicates the upstream region. The DNA fragments used to screen for enhancers are indicated by black lines and are positioned and numbered relative to the transcription start site. Solid lines represent enhancers with activity; the dashed line represents a fragment lacking activity. The asterisks designate representative enhancer data presented in part B. (B) Comparison of endogenous Ush and enhancer-driver reporter-gene (*lacZ*) expression patterns during embryogenesis. The stage of embryogenesis is indicated at the left of each row. Stages 7, 8, and 10 (panels a-o) are viewed laterally, and stages 13 and 16 (panels p–y) are dorsal views. The wild-type (panels a, f, k, p, u) and *ush* enhancer (panels b–e, g–j, l–o, q–t, v–y) strains are indicated at the top of each column. The antibodies used for immunohistochemical localization are listed at the bottom of each column. Solid arrows indicate activity in representative tissues; open arrows indicate lack of activity in crystal cells. Abbreviations: *lwr*, *lesswright*; αUsh, U-shaped antibody; αβ-gal, β-galactosidase antibody; wt, wild type; as, amnioserosa; hp, hemocyte precursors; mg, midgut; de, dorsal ectoderm; cc, crystal cells.

late stages of embryogenesis (Fig. 1B panels p, q, u, v). Beginning about stage 11 and continuing through stage 13, Ush and β-galactosidase were detected in the bilateral cluster of developing crystal cells (Fig. 1B panels p, q, and data not shown). Later, during stage 16, both proteins were observed in the circulating plasmatocytes (Fig. 1B panels u, v). Despite the overall similarity, some differences were observed between the expression patterns of the endogenous protein and the enhancer-driven reporter-gene. First, *ush* expression is downregulated during crystal cell maturation (Fossett et al., 2001; Fig. 1B panel u). In contrast, enhancer activity in these cells persisted until the late stages of embryogenesis (Fig. 1B panel v). This difference may be due to the long half-life of the β-galactosidase protein. Second, *ush* is expressed in cells of the developing central nervous system (Fossett et al., 2000). However, none of the fragments tested had activity in these cells (data not shown). Thus, elements that drive this activity may be located outside the region tested. Alternatively, expression may require the combined activity of elements in two or more of the DNA fragments.

The region upstream of position −7462 and the region within intron 2 were less active in embryonic tissues when compared to the 7.4 kb enhancer. A fragment located between positions −12425 and −6925 lacked activity until stage 10, when weak activity was detected in the dorsal ectoderm (Fig. 1B compare panels d, i, n). By stage 13, we observed strong activity in the dorsal ectoderm and amnioserosa (Fig. 1B panel s). This activity appeared to be identical to that seen with the 7.4 kb enhancer (Fig. 1B compare panels q and s), suggesting it is regulated by elements within the 537 bp overlap between the −12475/−6925 bp fragment and the −7462/−25 bp enhancer. By stage 16, we observed strong activity in the dorsal ectoderm and posterior midgut that did not resemble either endogenous *ush* or 7.4 kb enhancer-driven reporter-gene expression (Fig. 1B compare panels u and v with x). This atypical activity may be due to the absence of repressor elements within the −12475/−6925 bp fragment. We also assayed intron 2 for enhancer activity by dividing this region into two overlapping fragments (Fig. 1A). The first fragment contained the 5′ half of intron 2, from position 1809 to 7908, and lacked activity until approximately stage 13 (Fig. 1B panels e, j, o). At this time, enhancer activity was observed in the anterior amnioserosa (Fig. 1B panel t). By stage 16, activity in the dorsal mesoderm was observed (Fig. 1B panel y). The second fragment contained the 3′ half of intron 2, from position 6640 to 12678, and had no activity (data not shown). Thus, of the four large fragments that covered most of the noncoding region, only the −7462/−25 bp fragment largely recapitulated the expression pattern of *ush* and was the only fragment with enhancer activity in the hematopoietic tissues.

Identification of ush embryonic hematopoietic enhancers

In the *Drosophila* embryo, Srp-expressing hemocyte precursors develop from the head mesoderm (Rehorn et al., 1996; Sam et al., 1996). Identified by specific markers that include the extracellular matrix component, peroxidasin, the emerging plasmatocyte population begins to migrate throughout the head mesoderm during stage 10. By mid-embryogenesis, these cells can be seen circulating throughout the endolymph (Tepass et al., 1994; Fig. 1B panels f, k, p, u). In contrast, the smaller crystal cell population, distinguished by the cell-specific expression of *lz* and members of the melanin cascade, initially develops as bilateral clusters in the head mesoderm (Lebestky et al., 2000; Fig. 1B panels p–r). After stage 13, the clusters begin to merge forming a single population that resides anterior to the developing gut (Lebestky et al., 2000).

Using this positional information together with genetic and immunocolocalization studies, we previously reported that *ush* is expressed in embryonic hemocyte precursors, plasmatocytes, and crystal cell precursors; subsequently, it is downregulated during crystal cell development. This broad expression pattern suggests that Ush, like FOG-1, has a variety of functions throughout hematopoiesis. In addition, like its vertebrate counterpart, the function of Ush depends on its association with other transcriptional regulators, notably the GATA factors and C-terminal Binding Protein (CtBP; Fossett et al., 2000, 2001, 2003). These associations produce dynamic multiprotein complexes that can alter cell fate choice (Fossett et al., 2003). The assembly of these regulatory complexes will be affected by the level of *ush* expression and, therefore, by those factors that control its expression. Thus, a greater knowledge of *ush* gene regulation will increase our understanding of its function. For this reason, we produced subfragments of the 7.4 kb enhancer to precisely define the *ush* hematopoietic *cis*-regulatory region. Two overlapping subfragments with hematopoietic activity were designated the −2190/−25 bp enhancer and the −1421/−25 bp enhancer (Fig. 1A). Using the hemocyte positional information described above, we compared the hematopoietic activity of the −2190/−25 bp enhancer (Fig. 1B panels c, h, m, r, w) and the −1421/−25 bp enhancer (Fig. 2B panels a, e, i, m, q) with the activity of the 7.4 kb enhancer (Fig. 1B panels b, g, l, q, v). All three produced indistinguishable temporal and spatial expression patterns, suggesting that the essential elements directing embryonic hematopoietic activity are located within the −1421/−25 bp enhancer.

We further subdivided the −1421/−25 bp hematopoietic enhancer into nine overlapping fragments. The analysis of these fragments identified two minimal hematopoietic enhancers. These enhancers are located within the 5′ distal (−1243/−956 bp) region and 3′ proximal (−174/−25 bp) region of the −1421/−25 bp enhancer, and are separated by a region of approximately 800 bp that lacks hematopoietic activity (Fig. 2A). The distal enhancer had activity in the hemocyte precursors and plasmatocytes but lacked crystal cell activity (Fig. 2B panels b, f, j, n, r). The proximal enhancer retained much of the activity of the parental −1421/−25 bp enhancer, with activity in the hemocyte precursors, plasmatocytes, and most notably crystal cells (Fig. 2B panels c, g, k, o, s). Thus, elements that are required for *ush* expression in the hemocyte precursors and plasmatocytes are located within the proximal and distal enhancers, whereas crystal cell-specific elements are restricted to the proximal enhancer (Fig. 2A). To more precisely locate the crystal cell-specific elements, we deleted 60 bp from the 3′ region of the proximal enhancer to produce an 89 bp truncated proximal enhancer located between positions −174 and −85 (Fig. 2A). This enhancer retained hemocyte precursor and plasmatocyte activity but lacked crystal cell activity (Fig. 2B, panels d, h, l, p, t). Thus, the crystal cell-specific elements are located between positions −85 and −25.

In addition to the embryonic hemocytes, the −1421/−25 bp enhancer was active in the midgut (Fig. 2B panels e, i, m). We analyzed the nine overlapping subfragments to determine the location of the midgut enhancer. This analysis showed that the distal −1421/−956 bp hematopoietic enhancer lacked midgut activity (data not shown). The distal −1243/−956 bp minimal hematopoietic enhancer also lacked midgut activity in the majority of embryos tested (Fig. 2B panels f, j, n). However, in three of the six lines tested, 30% of the embryo population retained midgut activity. This is in contrast to the parental −1421/−956 bp enhancer, which completely lacked midgut activity. At this time, we cannot exclude the possibility that the reduced midgut activity of the −1243/−956 bp enhancer is the

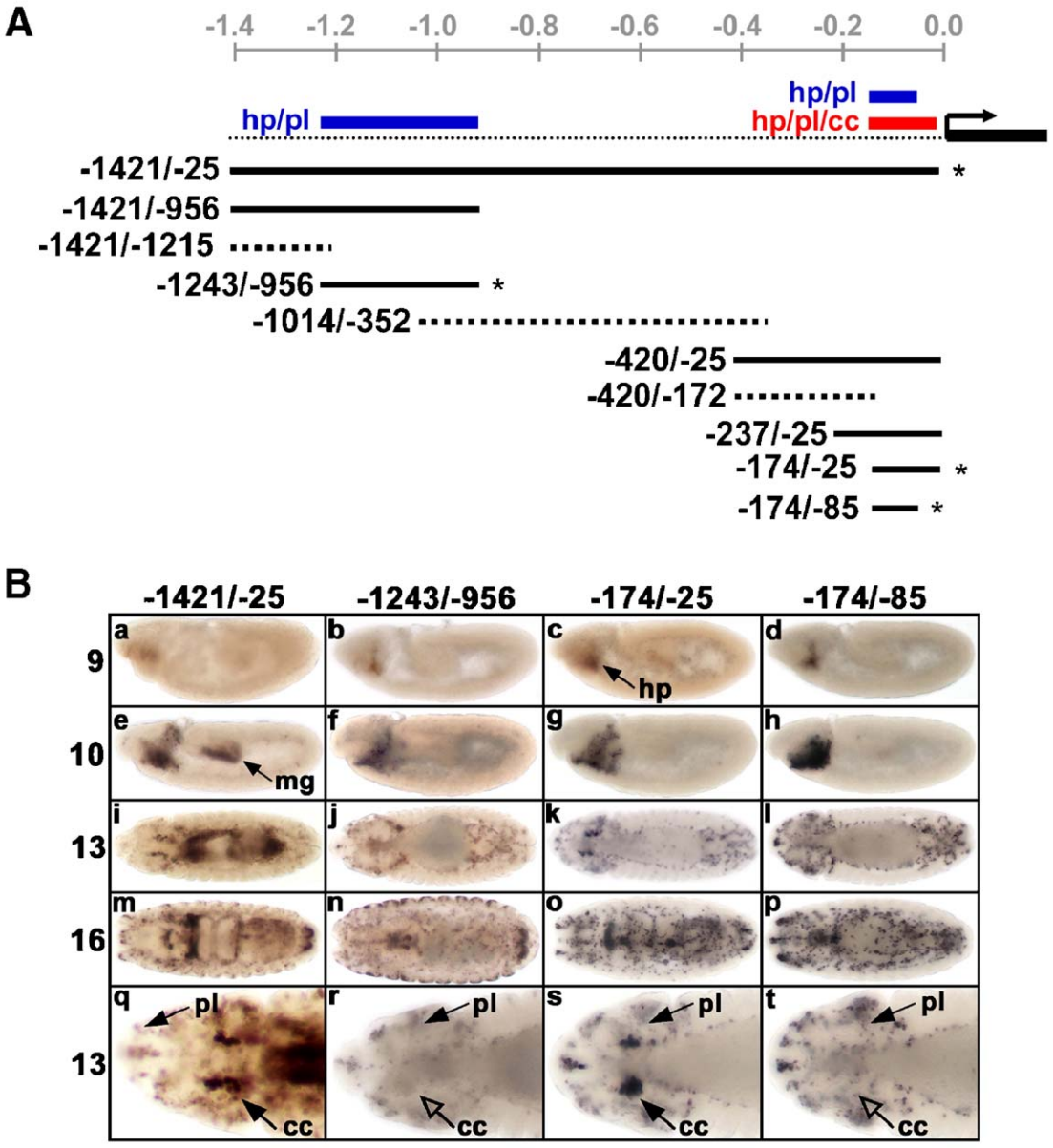


Fig. 2. Identification of minimal *ush* embryonic hematopoietic enhancers. (A) Schematic of the screen for minimal hematopoietic enhancers. The DNA fragments used to locate the minimal hematopoietic enhancers are indicated by black lines and are positioned and numbered relative to the transcription start site. Solid lines represent enhancers with activity; the dashed lines represent fragments lacking activity. The asterisks designate representative enhancer data presented in part B. Blue lines indicate the minimal region required for *ush* expression in hemocyte precursors and plasmatocytes. The red line indicates the minimal region required for *ush* expression in all classes of hemocytes. (B) Comparative embryonic enhancer activity. The stage of embryogenesis is indicated at the left of each row. Lateral views of stage 9 and 10 embryos (panels a–h), dorsal views of stage 13 and 16 embryos (panels i–p) and dorsal views of the head region of stage 13 embryos (panels q–t) are presented. The *ush* enhancer strains are indicated at the top of each column. Solid arrows indicate activity in representative tissues; open arrows indicate lack of activity in crystal cells. Abbreviations: hp, hemocyte precursors; mg, midgut; cc, crystal cells; pl, plasmatocytes.

result of repressor elements within the −1421 to −956 bp region. In contrast to the results obtained with the distal hematopoietic enhancers, the proximal −420/−25 bp enhancer was consistently active in the midgut, similar to the parental −1421/−25 bp enhancer (data not shown). However, the −237/−25 bp and the −174/−25 bp subfragments had greatly diminished midgut activity (Fig. 2B panels d, k, o, and data not shown). Finally, the −420/−172 bp fragment and the −174/−85 bp truncated proximal hematopoietic enhancer did not have activity (Fig. 2B panels h, l, p, and data not shown). Thus, full midgut enhancer activity required multiple elements located between positions −420 and −25.

Srp directly regulates ush hematopoietic expression

We analyzed the sequences within the minimal enhancers to identify *trans*-acting factors that regulate *ush* hematopoietic expression during embryogenesis. A number of observations suggested that Srp might directly regulate *ush* expression. First, Srp is required for endogenous *ush* expression (Fossett et

al., 2001). Second, twenty-two GATA binding sites are located within the −1421/−25 bp enhancer. Six of these (three each) are located in the proximal and distal minimal enhancers. Finally, two of the three sites within the distal enhancer and all three sites within the proximal enhancer are conserved between *Drosophila melanogaster* and *D. pseudoobscura* (Fig. 3A).

In order to determine if Srp directly regulates *ush* expression, we first tested enhancer activity in embryos with altered Srp function. The activities of the 7.4 kb enhancer, −1421/−25 bp enhancer, and active subfragments were assayed in embryos with Srp loss of function or gain of function genetic backgrounds. As described in the Materials and methods section, we performed a two generation cross to assay enhancer activity in a Srp loss of function background. In these studies, we used the srp^3 allele, which has a point mutation in the C-terminal zinc-finger that prevents DNA binding and results in complete loss of activity. Srp is required for a variety of developmental processes, including germ band retraction (Rehorn et al., 1996). Thus, homozygous mutants are easily identified by the altered morphology resulting from this phenotype (Fig. 3B compare panels a–d with e–h). In a homozygous srp^3 mutant background, none of the enhancers showed hematopoietic activity (Fig. 3B panels e–h, and data not shown). We obtained the same results using the hypomorphic srp^{neo45} allele (data not shown). Therefore, Srp function is required for enhancer activity.

Gain of function studies were conducted using the *UAS/Gal4* binary system developed by Brand and Perrimon (1993). *twiGal4* was used to drive Srp expression throughout the mesoderm, including the hematopoietic mesoderm. In *twiGal4*-driven *UAS-srpC* or *UAS-srpNC* embryos, the activity of the 7.4 kb enhancer was dramatically increased, and expression could be seen throughout the mesoderm and mesectoderm (Fig. 3B panels i, m, and data not shown). Endogenous Ush has a similar expression pattern to the 7.4 kb enhancer in Srp gain of function embryos (Fossett et al., 2003). In contrast, the proximal −174/−25 bp enhancer showed a minimal increase in activity,

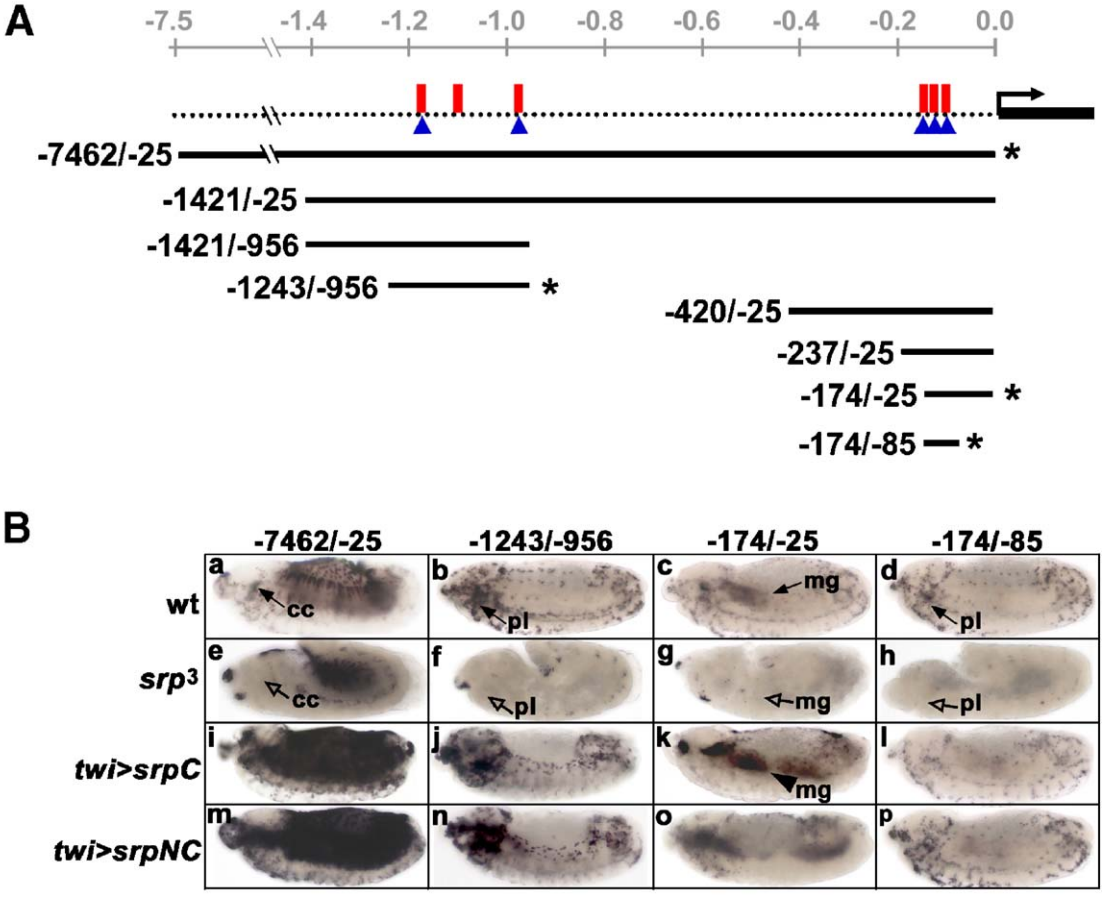


Fig. 3. Srp is required for *ush* enhancer activity in hematopoietic tissues. (A) Schematic showing the relative positions of the 7.4 kb (−7462/−25) and embryonic hematopoietic enhancers. The region between −7.4 kb and −1.4 kb is condensed. Red vertical lines indicate GATA sites within the minimal enhancers. GATA sites labeled with blue arrowheads are conserved between *D. melanogaster* and *D. pseudoobscura*. The asterisks designate representative enhancer data presented in part B. (B) *ush* enhancer activity in embryos with altered Srp function. Lateral views of stage 13 embryos are presented. The genetic backgrounds are indicated at the left of each row and are as follows: wild type (panels a–d); homozygous srp^3 (panels e–h); *twiGal4*-driven *UAS-srpC* (panels i–l); *twiGal4*-driven *UAS-srpNC* (panels m–p). The *ush* enhancer strains are indicated at the top of each column. Closed arrows indicate activity in representative tissues; arrowhead indicates expanded midgut activity and open arrows indicate lack of activity. Abbreviations: wt, wild type; cc, crystal cells; pl, plasmatocytes; mg, midgut.

whereas neither the distal −1243/−956 bp enhancer nor the −174/−85 bp truncated proximal enhancer showed increased activity in Srp gain of function embryos (Fig. 3B panels j, k, l, n, o, p). The difference between the 7.4 kb enhancer and the minimal hematopoietic enhancers may be at least partially due to *cis*-regulatory elements within the 7.4 kb enhancer that drive midgut activity. This interpretation is supported by our data showing that forced-expression of Srp slightly expanded the diminished midgut activity observed with the proximal −174/−25 bp enhancer (Fig. 3B panels k, o) and greatly expanded the activity of enhancers that produced full midgut activity, such as the −420/−25 bp enhancer (data not shown). The failure of Srp to expand the expression domain of the minimal hematopoietic enhancers suggested either that it does not directly regulate *ush* hematopoietic expression, or it acts with additional factors to drive expression in hematopoietic tissues. To distinguish between these possibilities, we determined whether Srp directly regulates the distal and proximal minimal hematopoietic enhancers. Direct activation would involve Srp binding to the GATA recognition sites within the minimal enhancers. Furthermore, these sites would be required for enhancer activity in vivo.

Using gel shift studies, we showed that both SrpC and SrpNC bind to radiolabeled DNA fragments corresponding to the distal −1243/−956 bp enhancer and −174/−85 bp truncated proximal enhancer (Figs. 4A, B lanes 1, 2, 7, and data not shown). Next, to determine if Srp would specifically bind to the GATA sequences within each fragment, we performed competition-binding assays using both wild-type and mutated fragments (Fig. 4A). Excess unlabeled wild-type fragment effectively competed for Srp binding (Fig. 4B lanes 1–5, 7–10, and data not shown). Mutation of all GATA sites within each fragment produced partial competition for binding, suggesting that both Srp isoforms bind to other sequences within the minimal enhancers (data not shown). Because vertebrate GATA factors have been shown to bind GATG, GATT, and GATC sequences with varying affinities (Pedone et al., 1997; Newton et al., 2001), we surveyed the region from position −174 to −85 for GAT core sequences. We located a GATG site between positions −161 and −158 and mutated this site along with the three GATA sites (Fig. 4A). This fragment failed to compete for Srp binding (Fig. 4B lanes 6, 11), indicating that both Srp isoforms bind to GATG sites. This result is consistent with studies that showed the N-terminal zinc-finger of vertebrate GATA factors bind to this site (Pedone et al., 1997).

To determine if the GATA sites are required for enhancer activity in vivo, we mutated these sites and then tested the resulting enhancers for hematopoietic activity. When all three GATA sites in the distal enhancer were mutated, complete loss of enhancer activity was observed (Figs. 4A, C compare panels a, e, i with b, f, j). The proximal −237/−25 bp enhancer has four conserved GATA sites and one GATG site. Mutating all three GATA sites between positions −174 and −85, but not the 5′ GATA or GATG site, also resulted in complete loss of enhancer activity (Figs. 4A, C compare panels c, g, k, with d, h, l). Thus, although both Srp isoforms bind to the GATA and GATG sites, this interaction is insufficient for enhancer activity. Together, these data indicate that while Srp directly regulates *ush* expression, it is not sufficient for *ush* hematopoietic enhancer activity. Thus, additional factors may act with Srp to upregulate *ush* expression in hematopoietic tissues.

Srp and Lz are required for ush expression in crystal cells

Consistent with its function as a repressor of crystal cell production, Ush is expressed in the crystal cell lineage and downregulated as these cells develop (Fossett et al., 2001). As we have shown in this report, *ush* enhancer activity in the crystal cell lineage appears to be driven by element(s) located between positions −85 and −25. We surveyed this region and identified a consensus RUNX sequence located between positions −66 and −60 (Fig. 5A). In addition, several other observations suggested that Lz mediates *ush* expression in the crystal cell lineage. First, Ush and Lz are coexpressed in hemocyte and crystal cell precursors (Lebestky et al., 2000; Fossett et al., 2001), providing the opportunity for Lz to regulate *ush* expression. Second, the RUNX site is present in the proximal enhancer but not the distal enhancer, consistent with the lack of crystal cell activity displayed by this enhancer. Finally, like many of the GATA sites, the RUNX site is conserved between *D. melanogaster* and *D. pseudoobscura* (Fig. 5A). Based on this information, we tested if Lz directs *ush* enhancer activity in the crystal cell lineage.

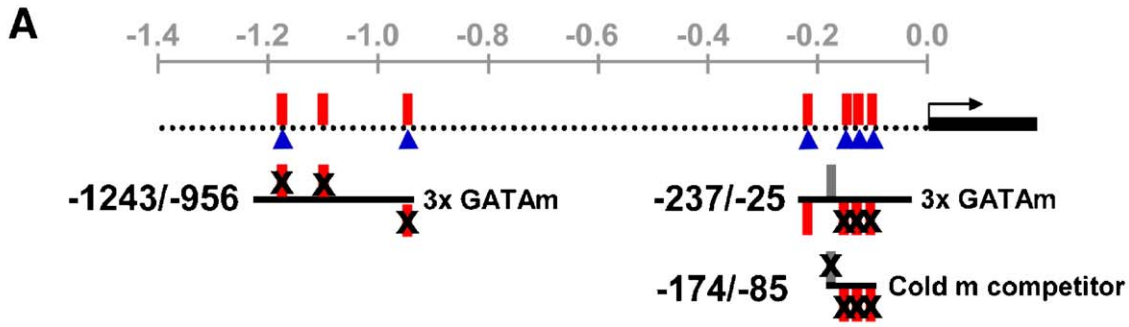
The direct activation of *ush* expression by Lz would be mediated through binding to the RUNX site. An in vitro transcribed/translated protein and a radiolabeled fragment corresponding to the proximal −174/−25 bp enhancer were used in gel shift studies to show that Lz binds to the enhancer (Fig. 5A). To establish the specificity of this interaction, we showed that excess unlabeled wild-type fragment effectively competed for Lz binding (Fig. 5B lanes 1–4). In contrast, a fragment containing a mutated RUNX site was a poor competitor (Fig. 5B lane 5). This mutated fragment was also tested for enhancer activity in vivo and was found to be active in hemocyte precursors and plasmatocytes but not crystal cells (Figs. 5A, C compare panels a, c with b, d). Together, results from these in vitro and in vivo studies indicate that Lz directly activates *ush* expression in the crystal cell lineage.

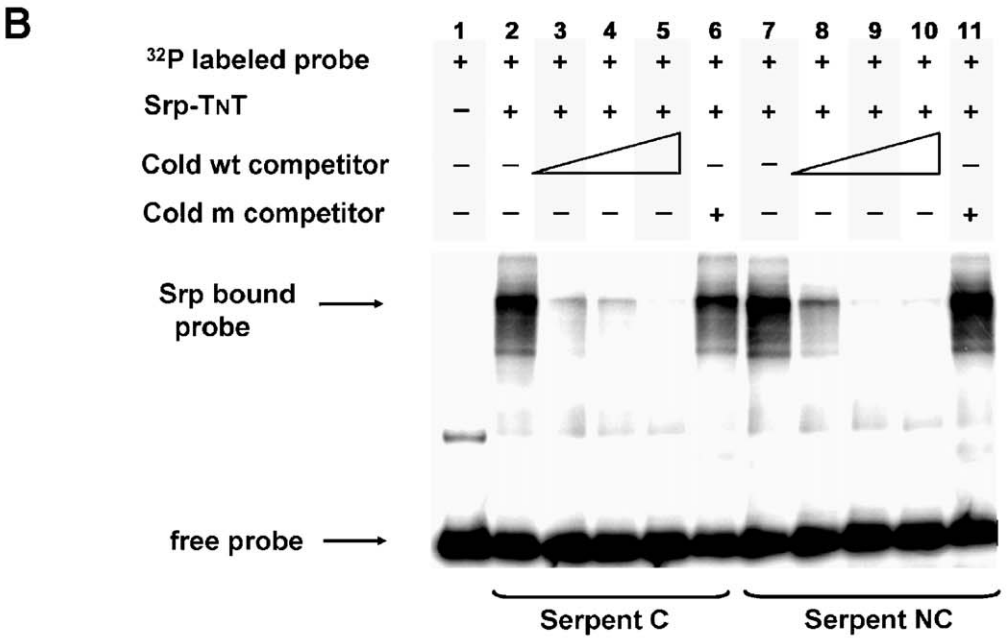
The data presented in this report indicate that Srp is required for *ush* expression in all hematopoietic tissues, including the crystal cells (Fig. 3). And, more to the point, Srp may upregulate expression in crystal cells by binding to conserved GATA sites that are located within the proximal −174/−25 bp enhancer (Fig. 4). Furthermore, *ush* expression in crystal cells also appears to require Lz binding to a conserved site, which is located 3′ and in close proximity to the cluster of conserved GATA sites (Fig. 5). Together, these findings suggest that Srp and Lz interact to positively regulate *ush* expression in the crystal cell lineage.

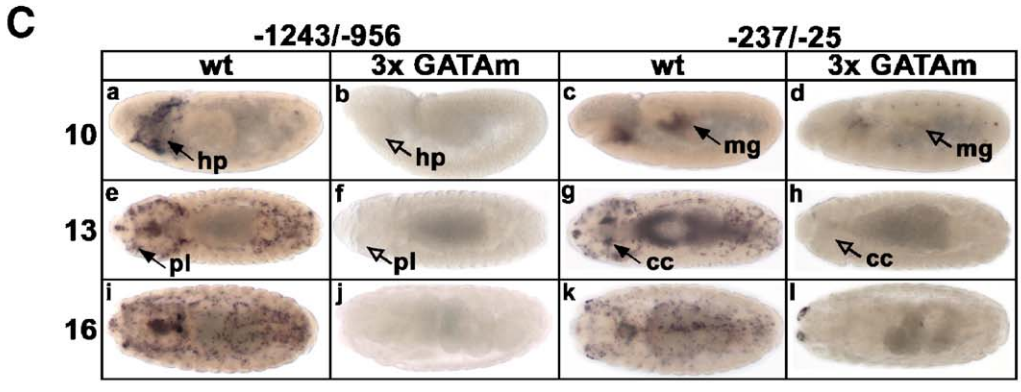
Regulation of ush expression during both hematopoietic waves

In both vertebrates and *Drosophila*, hematopoiesis consists of two spatially and temporally separated periods or waves. In

mammals, the first wave is known as primitive or embryonic hematopoiesis and takes place in the extraembryonic yolk sac. The second or definitive wave originates in the aorta/gonad/mesonephros region of the embryo proper (Cumano and Godin, 2001). To this point, we have examined *ush* expression during the first wave of *Drosophila* hematopoiesis. The first wave begins with the formation of the hematopoietic anlage from the head mesoderm. As embryogenesis progresses, hemocyte precursor cells develop in this region and later give rise to the plasmatocytes and crystal cells (Rizki, 1978; Dearolf, 1998). In keeping with the mammalian system, we will refer to the first hematopoietic wave as embryonic hematopoiesis. The second wave takes place in a specialized organ known as the lymph gland. For this reason, the second wave will be referred to as lymph gland hematopoiesis. The lymph gland develops from the cardiogenic mesoderm beginning about embryonic stage 13. By the end of embryogenesis, it has formed two bilateral lobes that flank the developing heart (Rugendorff et al., 1994; Mandal







et al., 2004). Morphological and gene expression analyses show that the lymph gland becomes highly compartmentalized by the end of the third larval instar (Jung et al., 2005) and functions until the onset of metamorphosis (Holz et al., 2003; Evans et al., 2003).

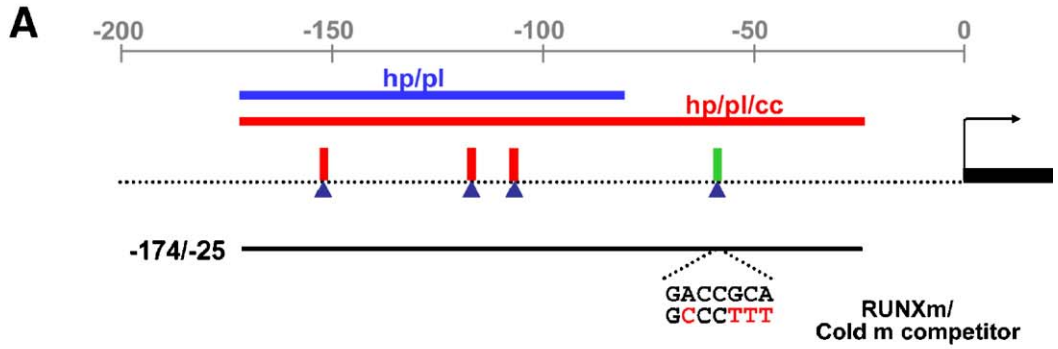
We previously showed that *ush* is expressed in the third larval instar lymph gland (Fossett et al., 2001). Using fluorescent antibody staining and confocal microscopy, we observed weak *ush* expression in the stage 16 embryonic lymph gland (Fig. 6A panel a). This is contrasted with the strong expression seen in both the cardiogenic mesoderm precursor cells (Fossett et al., 2000; L. Mandal, personal communication) and third larval instar lymph gland descendents (Fossett et al., 2001; Fig. 6A panel b). We judged the relative strength of Ush expression by visually comparing the level of staining across tissues within the same developmental time point. Using this method, we observed that the third larval instar lymph gland and the cardiogenic mesoderm had staining that was greater than or equal to the majority of Ush-expressing tissues within these time points (Fossett et al., 2000, 2001; Fig. 6A panel b). In contrast, expression in stage 16 embryonic lymph glands was considerably less than that observed in the neighboring cells of the amnioserosa (Fig. 6A panel a), as well as other Ush-expressing tissues. Together these data suggest that *ush* expression is downregulated during lymph gland specification but rebounds as the lymph gland develops. To test if downregulation of *ush* expression is necessary to permit lymph gland development, we expressed *ush* pan-mesodermally using the *twiGal4* driver and assayed for embryonic lymph gland development using the Odd-skipped (Odd) and Srp markers. Lymph gland development in either *twiGal4* or *Dmef2Gal4*-driven *UAS-ush* embryos was greatly impaired compared to wild-type controls (Fig. 6B and data not shown). Interestingly, *twiGal4*-driven *UAS-ush* embryos survive, hatch, and progress through the life cycle to adulthood (data not shown). Because *twi* expression is downregulated during the later stages of embryogenesis, ectopic Ush expression may similarly wane. This may allow for the recovery of the developing lymph gland during the early larval stages. However, impairment of lymph gland development should not affect the number of circulating hemocytes in non-immune challenged larvae because this population consists of embryonic hemocytes that persist throughout the life cycle (Holz et al., 2003). These embryonic hemocytes may compensate for the loss of lymph gland derived hemocytes, which may help to explain the survival of *twiGal4*-driven *UAS-ush* animals.

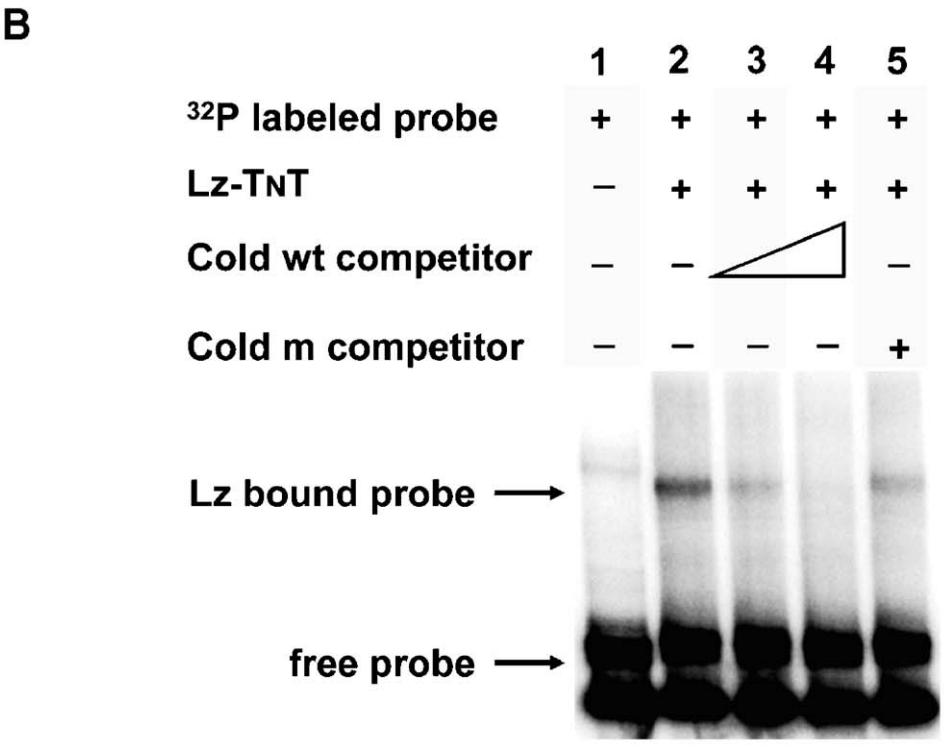
In addition to *ush*, a number of regulatory factors function during both hematopoietic waves, including Lz and Srp (Lebestky et al., 2000; Evans et al., 2003; Jung et al., 2005). However, recent studies have highlighted differences between embryonic and lymph gland hematopoiesis. The lymph gland is organized into morphologically distinct zones, characterized by the degree of hemocyte maturation and the expression of various markers (Jung et al., 2005). Moreover, the zinc-finger protein Odd is expressed during the second wave of hematopoiesis but not the first (Ward and Skeath, 2000; Jung et al., 2005). In contrast, Gcm may not be expressed during lymph gland hematopoiesis but is required for embryonic plasmatocyte development (Bernardoni et al., 1997; Bataille et al., 2005). Given these differences, we evaluated *cis*-regulation of *ush* expression in the developing embryonic lymph gland and the third instar larval lymph gland. We then compared *cis*-regulation of *ush* expression during both hematopoietic waves.

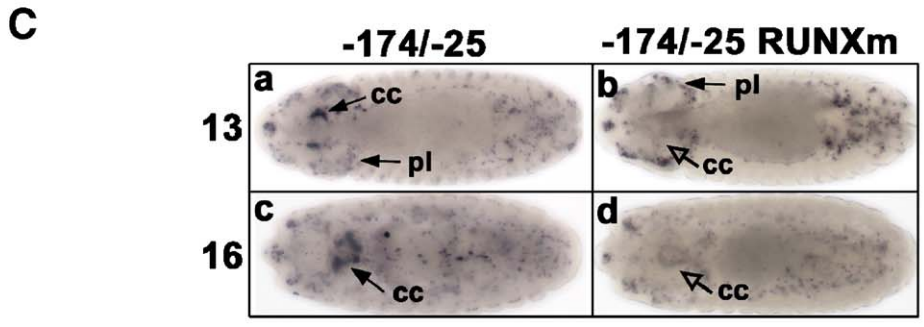
Enhancer activity in the embryonic lymph gland was determined by evaluating the colocalization of Odd and β-galactosidase. Odd is expressed throughout the lobes of the developing lymph gland, the pericardial cells, and a number of other tissues during embryogenesis (Ward and Skeath, 2000; Mandal et al., 2004). Third instar larval lymph glands were removed and then tested for enhancer-driven β-galactosidase expression using immunohistochemistry. A subset of the hematopoietic enhancers had strong activity in both the embryonic and third instar larval lymph glands (Fig. 7A). As expected, the 7.4 kb enhancer was active in both the embryonic and third instar larval lymph glands (Figs. 7A, B panels a, g). The −2190/−25 bp enhancer was also active in both the embryonic and third instar larval lymph glands (Figs. 7A, B panels b, h). In contrast, the −1421/−25 bp enhancer had reduced activity in lymph glands during both developmental stages (Figs. 7A, B panels c, i). Similarly, the distal −1243/−956 bp minimal hematopoietic enhancer and a larger version of the distal enhancer (−1421/−956 bp) also had reduced lymph gland activity (Figs. 7A, B panels d, j, and data not shown). Considering the weak activity of the −1421/−25 bp enhancer, the strong activity of the −420/−25 bp deletion product of this enhancer was quite unexpected (Figs. 7A, B panels e, k). Thus, by deleting the −2190 to −1422 region, we produced the −1421/−25 bp fragment, which had severely reduced enhancer activity.

Fig. 4. Srp directs *ush* enhancer activity through GATA recognition sites. (A) Schematic showing the mutated fragments used to evaluate Srp binding in vitro and GATA directed activity in vivo. Red vertical lines show the relative positions of 7 GATA sites within the distal −1243/−956 bp enhancer, −237/−25 bp enhancer, and −174/−85 bp truncated proximal enhancer. The grey vertical line indicates the GATG site. Blue arrowheads indicate GATA sites conserved between *D. melanogaster* and *D. pseudoobscura*. Red and grey lines that have been crossed out with the letter X indicate GATA sites that were mutated in these studies. The positions of the red and grey lines on each enhancer represent the orientation of the GAT core sites, with sites running 5′ to 3′ above the line and those running 3′ to 5′ below the line. (B) Competition gel shift assay showing Srp isoforms bind to the −174/−85 bp truncated proximal enhancer. A ^{32}P-labeled version of the −174/−85 bp truncated proximal enhancer (lane 1) was shifted by the addition of either SrpC or SrpNC (lanes 2 and 7, respectively). Srp binding to the labeled probe was abolished by the addition of 20-, 100-, and 200-fold excess of unlabeled wild-type competitor (lanes 3, 4, 5, and 8, 9, 10). The same competition reaction was repeated using 200-fold excess of the GATA/GATG mutant version of the truncated proximal enhancer (lanes 6 and 11). Arrows point to the free probe and the Srp-DNA complex. (C) Comparison of wild-type and GATA mutant enhancer activity. The stage of embryogenesis is indicated at the left of each row. Stage 10 embryos (panels a–d) are lateral views; all others (panels e–l) are dorsal views. Enhancer strains are indicated at the top of each column. Closed arrows indicate activity in representative tissues; open arrows indicate lack of activity. Abbreviations: 3xGATAm, three GATA sites mutated; m competitor, mutant competitor; wt competitor, wild-type competitor; Srp-TNT, in vitro transcribed/translated Srp protein; hp, hemocyte precursors; pl, plasmatocytes; cc, crystal cells.

Subsequently, by deleting the −1421 to −421 region, we produced the −420/−25 bp fragment, which restored enhancer activity (Fig. 7A). Together, these data indicate that the −2190/−25 bp enhancer contains multiple domains, which regulate *ush* expression in the lymph gland. Specifically, *ush* expression is upregulated by the combinatorial activity of elements located in the −2190/−1421 bp and −420/−25 bp regions, while expression is repressed by elements located in the −1421/−420 bp region. Finally, in contrast to the parental −420/−25 bp enhancer, the −237/−25 bp and −174/−25 bp deletion products had weak activity during development of the embryonic lymph gland but strong activity in the third instar larval lymph gland (Figs. 7A, B panels f, l, and data not shown). These results suggest that during early lymph gland development, full *ush*







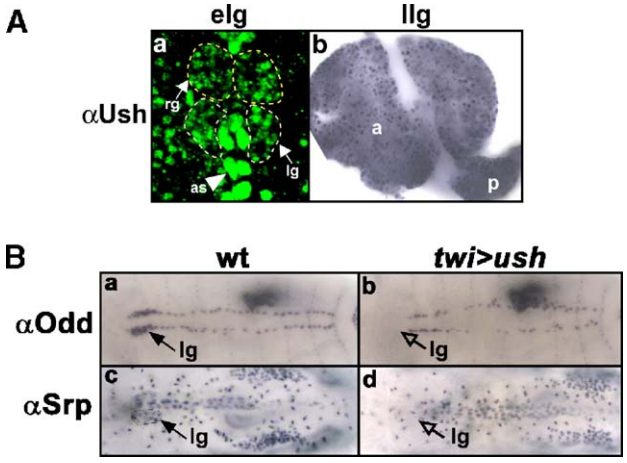


Fig. 6. Ush expression and lymph gland development. (A) The Ush expression pattern in stage 16 lymph glands. Anterio-dorsal view of fluorescent antibody stained embryonic lymph gland (panel a). Arrows indicate weak staining in the lymph gland and ring gland. Arrowhead indicates strong staining in the amnioserosa. Immunohistochemical stained third larval instar lymph gland (panel b). (B) Forced mesodermal expression of *ush* blocks lymph gland development. Dorsal views showing embryonic stage 16 lymph gland development in wild-type (panels a, c) and *twiGal4*-driven *UAS-ush* (panels b, d) genetic backgrounds. Lymph glands are visualized with anti-Odd (panels a, b) and anti-Srp (panels c, d) antibodies. Closed arrows indicate embryonic lymph gland; open arrows indicate reduced lymph gland development. Abbreviations: as, amnioserosa; elg, embryonic lymph gland; llg, larval lymph gland; αUsh, U-shaped antibody; rg, ring gland; lg, lymph gland; a, anterior lobe; p, posterior lobe; wt, wild type; *twi>ush*, *twiGal4*-driven *UAS-ush*; αOdd, anti-Odd-skipped antibody; αSrp, anti-Serpent antibody.

expression requires additional elements located between positions −420 and −237.

The ability of the −2190/−1421 bp region to relieve the lymph gland-specific repressor activity of the −1421/−420 bp fragment suggested that this region contained an additional hematopoietic enhancer. Consistent with this hypothesis, we observed clustered GATA and RUNX binding sites located between positions −1619 and −1558, which are similar to those of the proximal −174/−25 bp enhancer. To test this hypothesis, we generated nine separate transgenic lines that carried the −2190/−1421 bp fragment upstream of the *lacZ* reporter-gene and assayed these for hematopoietic-specific reporter-gene expression during embryonic and lymph gland hematopoiesis (Figs. 7A, C).

Our analysis of the parental −2190/−25 bp enhancer showed that in all of the transgenic lines 100% of the stage 8 to 10 embryo population had enhancer-driven β-galactosidase activity in hemocyte precursors (Fig. 1B panels g, l). Similarly, 100% of the stage 11 to 15 embryo population had activity in both the plasmatocyte and crystal cell lineages (Fig. 1B panel g and data not shown). The same results were obtained with the −1421/−25 bp enhancer and with all three proximal enhancers (−420/−25 bp, −237/−25 bp and −174/−25 bp enhancers; Fig. 2B panels a, c, e, g, l, k, q, s and data not shown). In contrast, the activity of the −2190/−1421 bp fragment differed significantly from this pattern. As a result, we classified β-galactosidase activity as aberrant when greater than 40% of the embryo population within a given developmental stage deviated from the pattern observed with the parental −2190/−25 bp enhancer. Using this criterion, we determined that eight of the nine −2190/−1421 bp enhancer transgenic lines had activity in stage 8 to 10 hemocyte precursors (Fig. 7C panels a, b; Table 2). In contrast, only two lines were active in both the crystal cell and plasmatocyte lineages (Fig. 7C panel c; Table 2). We observed that four lines had activity in only one of the blood cell lineages; two lines had activity in crystal cells only and two lines had activity in plasmatocytes only (Fig. 7C panels d, e; Table 2). Two lines lacked activity in both lineages (Fig. 7C panel f; Table 2). Finally, five of the eight lines had ectopic activity in the dorsal ectoderm that was not evident in the parental −2190/−25 bp enhancer (Table 2 and data not shown). Together these data suggest that an additional embryonic hemocyte precursor enhancer lies upstream of position −1421. However, sustained expression in developing hemocytes requires the −1243/−956 bp and −174/−25 bp enhancers. Furthermore, elements within the −1421/−25 bp region are required to block non-specific expression.

During lymph gland hematopoiesis, −2190/−1421 bp fragment-driven β-galactosidase expression was not detected in the embryonic lymph gland or in the anterior lobe of the third larval instar lymph gland (Fig. 7C panels g–j and data not shown). However, expression was detected in a subpopulation of cells in the posterior lobe of the third larval instar lymph gland (Fig. 7C panels g–j). Taken together, our analyses of the −2190/−25 bp enhancer indicate that the −2190/−1421 bp region is necessary but not sufficient for *ush* expression during early lymph gland development and hemocyte maturation in the anterior lobe of the third larval instar lymph gland. As such, we have designated the −2190/−1421 bp fragment an activator region rather than an enhancer (Fig. 7A). However, an additional hemocyte precursor enhancer within the −2190/−1421 bp region, possibly the same

Fig. 5. The proximal −174/−25 bp enhancer contains a conserved RUNX site that binds Lz and is required for activity in crystal cells. (A) Schematic of the proximal −174/−25 bp enhancer. The red vertical lines show the relative position of GATA sites, and the green vertical line shows the relative position of the RUNX site. Blue arrowheads indicate the sites that are conserved between *D. melanogaster* and *D. pseudoobscura*. The blue horizontal line indicates the region of the enhancer required for hemocyte precursor and plasmatocyte activity. The red horizontal line indicates the region required for activity in all three hemocyte classes. The sequence is that of the wild-type (in black) and mutant (in red) RUNX site. (B) Competition gel shift assay showing that Lz binds to the conserved RUNX site within the proximal enhancer. A ^{32}P-labeled version of the proximal −174/−25 bp enhancer (lane 1) was shifted by the addition of Lz (lane 2). Lz binding to the probe was abolished by the addition of 50- and 100-fold excess unlabeled wild-type competitor (lanes 3 and 4). The competition reaction was repeated using 100-fold excess of the RUNX-mutant version of the proximal enhancer (lane 5). Arrows point to the free probe and the Lz-DNA complex. (C) Comparison of wild-type and RUNX-mutant enhancer activity. The stage of embryogenesis is indicated at the left of each row. All embryos are dorsal views. Enhancer strains are indicated at the top of each column. Embryos carrying wild-type (panels a, c) and RUNX-mutant (panels b, d) proximal enhancers are presented. Closed arrows indicate activity in representative tissues; open arrows indicate lack of activity in crystal cells. Abbreviations: RUNXm, RUNX-mutant; m competitor, mutant competitor; wt competitor, wild-type competitor; Lz-TNT, in vitro transcribed/translated Lz protein; pl, plasmatocytes; cc, crystal cells.

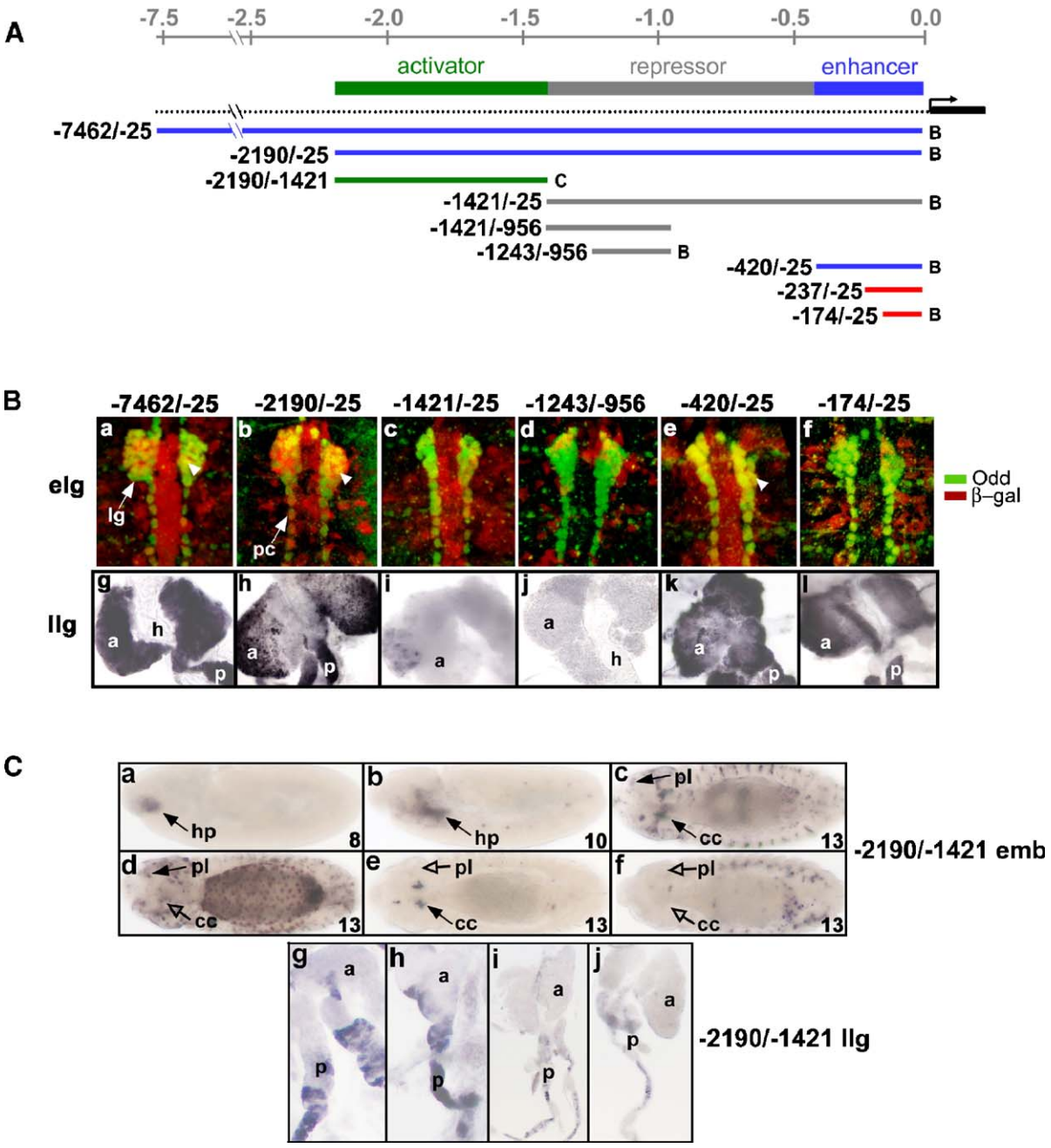


Fig. 7. Identification of enhancers that regulate *ush* expression in the embryonic and larval lymph glands. (A) Schematic summarizing the lymph gland activity of the hematopoietic enhancers. The region between −7.5 kb and −2.5 kb is condensed. The hematopoietic enhancers are positioned and numbered relative to the transcription start site. Enhancers depicted with blue lines have strong activity in the developing embryonic and third instar larval lymph glands. Those depicted in red have strong activity in the larval lymph gland but weak activity in embryonic lymph gland. Those in grey have weak lymph gland activity during both developmental stages. The green line depicts the activator region. The letter "B" or "C" to the right of each line indicates data that are presented in part B or C, respectively. The multicolored green, grey, and blue line depicts the regulatory domains within the −2190/−25 bp enhancer. (B) Embryonic and larval lymph gland activity of representative hematopoietic enhancers. The hematopoietic enhancer strains are designated at the top of each column. The top panels (a–f) show the expression of Odd (green) and enhancer-driven dβ-galactosidase (red) proteins in stage 16 embryonic lymph glands. Colocalization of these proteins is shown in yellow. All embryos are anterior-dorsal views. Arrows indicate the lymph gland and pericardial cells. Arrowheads indicate areas with the greatest degree of colocalization between the β-galactosidase and Odd proteins. Bottom panels (g–l) show enhancer-driven β-galactosidase expression in larval lymph glands. (C) Activity of the −2190/−1421 bp region during both hematopoietic waves. The top panels (a–f) show −2190/−1421 bp fragment-driven β-galactosidase expression during embryogenesis. The stage of embryogenesis is indicated in the lower right corner. Lateral views of stage 8 and 10 embryos (panels a, b) and dorsal views of stage 13 embryos (panels c–f) are shown. Solid arrows indicate activity in hemocytes; open arrows indicate lack of activity in hemocytes. The bottom panels (g–j) show −2190/−1421 bp fragment activity during third larval instar lymph gland hematopoiesis in four different transgenic lines. Abbreviations: lg, lymph gland; elg, embryonic lymph gland; llg, larval lymph gland; Odd, Odd-skipped; β-gal, β-galactosidase; pc, pericardial cells; a, anterior lobe; p, posterior lobe; h, heart; emb, embryogenesis; hp, hemocyte precursors; pl, plasmatocytes; cc, crystal cells.

Table 2
−2190/−1421 bp fragment driven β-galactosidase activity

Transgenic line	Percentage of embryo population with β-galactosidase activity					
	Stages 8–10 hp	Stages 11–15 pl, cc lineages	Stages 11–15 neither lineage	Stages 11–15 pl only	Stages 11–15 cc only	Stages 8–15 ectopic de
#25	0[a]	0[a]	100[a]	0	0	100[a]
#19	100	0[a]	0	0	100[a]	0
#37	100	0[a]	0	0	100[a]	0
#40	100	33[a]	42[a]	3	21	42[a]
#62	100	43[a]	13	43[a]	0	50[a]
#85	100	43[a]	0	45[a]	12	0
#57	100	56[a]	6	32	6	6
#84	100	65	0	29	6	87[a]
#46	100	96	0	4	0	100[a]

This table shows the percentage of the embryo population with β-galactosidase activity in hematopoietic tissues for all nine transgenic lines. This table also shows the percentage of the embryo population with ectopic dorsal ectoderm activity for each line. Abbreviations: hp, hemocyte precursors; pl, plasmatocytes; cc, crystal cells; de, dorsal ectoderm.

[a] Aberrant expression: greater than 40% of the population differs from the parental −2190/−25 bp enhancer activity.

enhancer that appears to direct expression during embryonic hematopoiesis, may control β-galactosidase expression in the posterior lobes of the mature lymph gland.

We found both similarities and differences in the regulation of *ush* expression during the two hematopoietic waves. *ush* expression appears to be controlled by a shared proximal enhancer located between positions −174 and −25, which upregulates expression during both embryonic and third instar larval lymph gland hematopoiesis (Figs. 2A, 7A). We have also identified three differences in the regulatory process. First, the proximal −174/−25 bp minimal hematopoietic enhancer is active throughout the embryonic wave (Fig. 2B panels c, g, k, o). In contrast, during the second wave this minimal enhancer is only fully operational in the mature lymph gland, as additional upstream element(s) are required for full expression in the developing lymph gland (Figs. 7A, B panels e, f, l, and data not shown). Second, a distinct distal enhancer located between positions −1243 and −956 upregulates *ush* expression during embryonic but not lymph gland hematopoiesis (Figs. 2A, B panels b, f, j, n, r; 7A, B panels d, j). Third, proximal enhancer activity is repressed by the −1421/−420 bp region during lymph gland but not embryonic hematopoiesis. Thus, both shared and divergent regulatory programs are used during each hematopoietic wave.

Discussion

The *Drosophila FOG* gene, *ush*, is expressed in a variety of embryonic tissues and during hematopoiesis (Fossett et al., 2001). We surveyed the *ush* locus for DNA fragments that drive tissue-specific reporter-gene expression. Results obtained from this screen identified a 7.4 kb *ush* embryonic *cis*-regulatory region that included a proximal and distal minimal hematopoietic enhancer. These enhancers were then used to identify *trans*-acting regulators of *ush* hematopoietic expression. We showed that Srp upregulates enhancer activity in all hemocytes, whereas Lz together with Srp is required to upregulate enhancer activity during crystal cell lineage commitment (Fig. 8A). Our findings represent the first demonstration of direct regulation of *FOG* gene expression by GATA and RUNX factors. We also identified similarities and differences in the *cis*-regulation of *ush* during embryonic and lymph gland hematopoiesis (Fig. 8B). The information obtained from these studies will be used to identify additional regulators of *ush* hematopoietic expression and assign novel functions to those that are currently known.

Srp regulation of ush expression

Our results indicate that Srp directly activates *ush* hematopoietic expression through binding to the GATA recognition sequences within the *ush* proximal and distal enhancers. The direct activation of *FOG* gene expression by GATA factors may be evolutionarily conserved. This hypothesis is also supported by vertebrate tissue culture studies that showed endogenous FOG-1 gene expression increased in response to exogenous GATA-1 protein and from the observed overlap of vertebrate *GATA* and *FOG* gene expression in a variety of developing tissues (Tsang et al., 1997; Tevosian et al., 1999; Svensson et al., 1999; Lu et al., 1999; Gaines et al., 2000; Cantor and Orkin, 2005). However, our studies show that Srp function alone may not be sufficient to upregulate *ush* hematopoietic expression. *twiGal4*-driven *UAS-srp* is expressed throughout the mesoderm (Fossett et al., 2003). If Srp was the sole activator of the *ush* hematopoietic enhancers, we would expect to see pan-mesodermal enhancer activity. Instead, we observed a minimal increase in the activity of the proximal −174/−25 bp enhancer and no increase in the activity of the distal −1243/−956 bp enhancer and the −174/−85 bp truncated proximal enhancer. Moreover, these results complement our previous findings that *ush* is expressed considerably later than *srp* during hemocyte precursor development (Fossett et al., 2001). Taken together, these results indicate that additional factors work with Srp to upregulate *ush* expression during hematopoiesis (Fig. 8A). This is consistent with studies showing that multiple classes of transcription factors activate prototypical *cis*-regulators (Howard and Davidson, 2004; Levine and Davidson, 2005). Furthermore, the requirement for multiple activators of *ush* prevents the unrestricted coexpression of Srp and Ush in hemocytes. This additional regulatory feature, together with alternative splicing, may limit the formation of the SrpNC:Ush complex. Strict control of complex formation is critical considering the impact this complex has on hemocyte development and function. In this regard, the SrpNC:Ush complex blocks expression of an important component of programmed cell death, *croquemort* (Franc et al., 1999; Waltzer et al., 2002), and, as discussed above, limits crystal cell production (Fossett et al., 2003).

Srp and Lz regulation of ush expression in crystal cells

Our previous studies showed that SrpNC acts as a cross-regulatory switch during crystal cell lineage commitment.

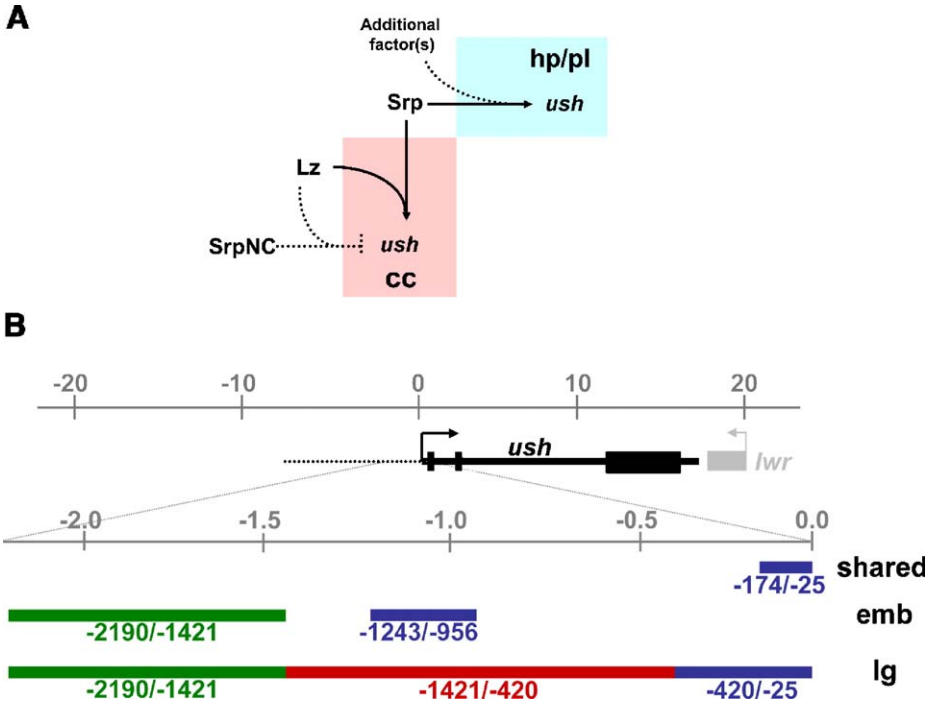


Fig. 8. *cis*- and *trans*-regulation of *ush* hematopoietic expression. (A) Model showing Srp regulation of *ush* hematopoietic expression. Srp and additional factors drive *ush* expression in hemocyte precursors and plasmatocytes, whereas Srp and Lz activate *ush* expression in crystal cells. The downregulation of *ush* expression by SrpNC and Lz promotes crystal cell maturation and proliferation. Solid lines indicate that direct regulation of enhancers by transcriptional regulators has been demonstrated; dotted lines indicate that direct regulation has not been demonstrated. The model is a simplification of the regulation of *ush* expression and does not necessarily include all of the factors required for proper temporal and spatial expression. (B) Schematic showing the relative positions of hematopoietic *cis*-regulatory regions. A horizontal arrow indicates the transcription start site, designated position 0 with respect to the *ush* locus. From left to right, the three black boxes represent exons 1, 2, and 3–8. The nearby *lesswright* gene is depicted in grey. A dotted line indicates the 7.4 kb upstream region. The 2.2 kb hematopoietic regulatory region is shown below the *ush* locus in expanded form. The relative positions of enhancers (blue), repressors (red), and activators (green) are shown. These *cis*-regulatory regions are also positioned vertically from top to bottom according to whether they are shared or are specific for embryonic or lymph gland hematopoiesis. Abbreviations: hp, hemocyte precursors; pl, plasmatocytes; cc, crystal cells; *lwr*, *lesswright*; emb, embryonic hematopoiesis; lg, lymph gland hematopoiesis.

SrpNC acts with Lz to activate the crystal cell program and with Ush to block crystal cell production (Fossett et al., 2003). Thus, the regulation of Ush expression will have a direct affect on the cross-regulatory activity of SrpNC. Interestingly, our new findings indicate that *ush* expression is maintained during crystal cell lineage commitment by the combined actions of Srp and Lz. It seems counterproductive for the crystal cell activator, Lz, to be a positive regulator of the crystal cell repressor, Ush. However, this negative regulatory strategy may be an important control to limit crystal cell production. In this model, Srp and Lz activate *ush* expression in Lz-positive hemocyte precursors (Fig. 8A). Ush then binds to the alternatively spliced Srp isoform, SrpNC, converting it from a crystal cell lineage coactivator with Lz to a repressor (Fossett et al., 2003). As a result, the Srp:Lz-directed crystal cell program is subject to negative regulation. In support of this model, only 60% of Lz-positive hemocyte precursors become terminally differentiated crystal cells (Bataille et al., 2005). Furthermore, these data suggest that Lz is not only required for crystal cell specification and differentiation but also to limit crystal cell production by positively regulating *ush* expression. Interestingly, Lz also acts as both a transcriptional repressor and activator within a single cell of the eye primordium (Canon and Banerjee, 2003). Because the RUNX proteins have been shown to be both positive and negative transcriptional regulators, this may be a conserved characteristic of the RUNX family. In general, RUNX activity may be dependent on the specific coregulator and the developmental context (Coffman, 2003).

Previously, we showed that Ush is downregulated by the combined actions of Lz and SrpNC (Fossett et al., 2003). This would limit the formation of the SrpNC:Ush repressor complex making more SrpNC available to interact with Lz, thereby positively regulating crystal cell lineage commitment. Together with our previous findings, these new results indicate that the *Drosophila* GATA, FOG, and RUNX factors are part of a highly interactive cross-regulatory network that rigorously controls crystal cell number. Finally, given that GATA-1, FOG-1, and RUNX1 function during vertebrate megakaryopoiesis (Tsang et al., 1998; Song et al., 1999; Gaines et al., 2000; Nichols et al., 2000; Michaud et al., 2002; Walker et al., 2002; Elagib et al., 2003), the cross-regulatory control of *FOG* gene expression by GATA and RUNX factors may be conserved across taxa.

Srp and Lz bind to GATA and RUNX sites that are contained within a 97 bp region of the proximal −174/−25 bp enhancer. The juxtaposition of these sites suggests that Srp and Lz physically interact to upregulate *ush* expression. This is consistent with a previous report that showed these proteins form a complex in vitro (Waltzer et al., 2003). Thus, it is possible that a Srp:Lz complex binds to the adjacent GATA and

RUNX sites. Alternatively, each protein may bind separately to the adjacent sites, facilitating their subsequent interaction. In either case, the proximity of the sites likely promotes the cooperative, cell-specific upregulation of *ush*. Moreover, this arrangement of elements may be present in other GATA:Runx responsive genes that regulate *Drosophila* crystal cell and vertebrate megakaryocyte development.

SrpNC and Lz could also mediate repression of *ush* by binding to the GATA and RUNX sites. This interpretation is supported by our previous studies that showed SrpNC and Lz blocked *ush* expression but SrpC and Lz did not. These results suggest that repression by SrpNC and Lz likely requires additional corepressors (Fossett et al., 2003). However, repression mediated through the proximal enhancer may also require input from additional *cis*-elements. This would be analogous to the repression of the proximal enhancers by the −1421/−420 bp region that occurs during lymph gland hematopoiesis. Alternatively, SrpNC and Lz may not directly block expression but rather act upstream of direct regulators of the *ush* gene (Fig. 8A).

cis-regulation of ush during both hematopoietic waves

We observed three basic differences in the *cis*-regulation of *ush* during both hematopoietic waves, indicating that divergent gene regulatory networks control aspects of embryonic and lymph gland hematopoiesis. First, our results demonstrated that although the proximal −174/−25 bp enhancer is completely operational during both embryonic hematopoiesis and third larval instar hematopoiesis, the region located between positions −420 bp and −25 bp is required for full *ush* expression in the embryonic lymph gland (Fig. 8B). The −420/−25 bp enhancer has eleven conserved GATA sequences as compared to three within the proximal −174/−25 bp enhancer (data not shown). In view of this difference, an increase in Srp binding may be required for full enhancer activity in the embryonic lymph gland. Srp has been shown to be expressed in the early embryonic lymph gland (Mandal et al., 2004). Second, the 1 kb region located between positions −1421 bp and −420 bp represses proximal enhancer activity during lymph gland but not embryonic hematopoiesis. While activity can be restored by factors that bind to elements located between −2190 bp and −1421 bp, this region is largely unable to drive expression in the absence of the proximal enhancers (Fig. 8B). Thus, *trans*-activators recruited by the −2190/−1421 bp region are able to circumvent lymph gland-specific repression by acting synergistically with the proximal enhancer machinery. Finally, other than Srp, the distal −1243/−956 bp enhancer is activated by factors largely specific for embryonic hematopoiesis and different from those that regulate the proximal −174/−25 bp enhancer. Alternatively, the −1243/−956 bp region may bind lymph gland-specific repressors that downregulate its activity during this hematopoietic wave.

These differences in *ush* regulation are not surprising considering the divergent developmental strategies of the embryonic and larval hematopoietic tissues and their respective *ush* expression patterns. During embryogenesis, hemocyte precursors develop from the head mesoderm (Rizki, 1978; Dearolf, 1998). *srp* expression is first detected in these cells during embryonic stage 5 (Rehorn et al., 1996; Sam et al., 1996). *ush* expression is first observed in the hemocyte precursors during stage 8, increases during embryogenesis, and remains constant in plasmatocytes throughout embryogenesis (Fossett et al., 2000, 2001). In contrast, the lymph gland hemocyte precursors develop from the cardiogenic mesoderm beginning around embryonic stage 13 (Rugendorff et al., 1994; Mandal et al., 2004). Furthermore, unlike embryonic hematopoiesis, *ush* displays a bimodal expression pattern during this second hematopoietic wave. *ush* is strongly expressed in the cardiogenic mesoderm (Fossett et al., 2000) but downregulated during lymph gland specification. However, expression rebounds by the third larval instar (Fossett et al., 2001). This complex expression pattern undoubtedly requires regulatory controls not necessary during embryonic hematopoiesis. In particular, downregulation of *ush* may limit its interaction with the GATA factors, Pannier (Pnr) and/or Srp, which are required for lymph gland development (Mandal et al., 2004). In our studies, forced pan-mesodermal expression of *ush* and the resulting inhibition of lymph gland development may have resulted from increased formation of Pnr:Ush and/or SrpNC:Ush repressor complexes. It should be noted that Pnr acts early to specify the cardiogenic mesoderm, which gives rise to the lymph gland. However, continued expression of Pnr within the developing lymph gland dramatically reduces the number of cells that comprise this tissue. In contrast, ectopic expression of Srp (presumably both SrpC and SrpNC) increases the number of lymph gland cells (Mandal et al., 2004). Thus, Ush may act early with Pnr or later with SrpNC or during both embryonic time points to block lymph gland production. Unfortunately, *twiGal4*-driven *UAS-srp* or *UAS-pnr* embryos die before hatching, likely due to the disruption of a number of mesodermal derivatives. This precludes an assessment of their effect on lymph gland development in the larvae. The production of lymph gland-specific drivers will unable us to ascertain how dysregulation of Srp, Pnr, and Ush affect lymph gland development across the embryonic and larval stages.

Further support for wave-specific hematopoietic gene networks comes from observations suggesting that both Gcm and Odd are expressed differentially during embryonic and lymph gland hematopoiesis. Odd is expressed in the lymph gland but not embryonic hemocytes (Ward and Skeath, 2000; Mandal et al., 2004). In contrast, Gcm is expressed in the hemocyte precursors and is required for plasmatocyte development (Bernardoni et al., 1997). However, Gcm does not appear to be expressed in the lymph gland (Bataille et al., 2005). Moreover, these wave-specific hematopoietic factors may influence *ush* expression. This is especially likely in the case of Gcm, which is required for the production of the *ush*-expressing plasmatocytes. Finally, the anterior lobe of the mature lymph gland is highly compartmentalized, containing at least three different zones. Ush is strongly expressed in the cortical and medullary zones but is repressed in the posterior signaling center (Jung et al., 2005). In contrast, the embryonic

head mesoderm apparently lacks an organized signaling center and the accompanying negative regulation of *ush* expression (Lebestky et al., 2003; Jung et al., 2005). This initial investigation of *ush cis*-regulation provides the necessary resources to identify the diverse factors that control expression across both hematopoietic waves.

Cell fate choice is controlled by genetic networks that converge to regulate lineage-specific developmental programs. The key components of these networks are transcriptional regulators that form transient multiprotein complexes. These complexes activate or repress gene expression by recognizing specific *cis*-regulatory elements. This versatile interplay between transcription factor complexes and *cis*-elements constitutes a gene-regulatory code for pluripotent progenitor diversification. The studies presented here provide a framework for the continued investigation of this process using a system ideally suited for this purpose.

Acknowledgments

We thank D. Hoshizaki for the *UAS-srpC* fly strain and E. Chen for the *Dmef2Gal4* fly strain. We are grateful to M. Brennan and J. Skeath for anti-Serpent and anti-Odd-skipped antibodies, respectively. We also thank R. Carthew for the *lz* cDNA clone. The technical assistance of T. Vachris, C. D'Ardenne, and S.T. Mon is gratefully appreciated. We are also grateful to I. Mikhailenko and S. Ishigami for assistance with confocal microscopy. This work was supported by grants to N.F. from the American Heart Association and the National Institutes of Health (DK072229) and to R.A.S from the National Institutes of Health (HL059151) and (HL071540).

References

Adya, N., Castilla, L.H., Liu, P.P., 2000. Function of CBFbeta/Bro proteins. Semin. Cell Dev. Biol. 11, 361–368.

Alfonso, T.B., Jones, B.W., 2002. *gcm2* promotes glial cell differentiation and is required with *glial cells missing* for macrophage development in *Drosophila*. Dev. Biol. 248, 369–383.

Barettino, D., Feigenbutz, M., Valcarcel, R., Stunnenberg, H.G., 1994. Improved method for PCR-mediated site-directed mutagenesis. Nucleic Acids Res. 22, 541–542.

Bataille, L., Auge, B., Ferjoux, G., Haenlin, M., Waltzer, L., 2005. Resolving embryonic blood cell fate choice in *Drosophila*: interplay of GCM and RUNX factors. Development 132, 4635–4644.

Bernardoni, R., Vivancos, V., Giangrande, A., 1997. *glide/gcm* is expressed and required in the scavenger cell lineage. Dev. Biol. 191, 118–130.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Canon, J., Banerjee, U., 2003. In vivo analysis of a developmental circuit for direct transcriptional activation and repression in the same cell by a Runx protein. Genes Dev. 17, 838–843.

Cantor, A.B., Orkin, S.H., 2005. Coregulation of GATA factors by the Friend of GATA (FOG) family of multitype zinc finger proteins. Semin. Cell Dev. Biol. 16, 117–128.

Coffman, J.A., 2003. Runx transcription factors and the developmental balance between cell proliferation and differentiation. Cell Biol. Int. 27, 315–324.

Cripps, R.M., Black, B.L., Zhao, B., Lien, C.L., Schulz, R.A., Olson, E.N., 1998. The myogenic regulatory gene Mef2 is a direct target for transcriptional activation by Twist during *Drosophila* myogenesis. Genes Dev. 2, 422–434.

Cubadda, Y., Heitzler, P., Ray, R.P., Bourouis, M., Ramain, P., Gelbart, W., Simpson, P., Haenlin, M., 1997. *u-shaped* encodes a zinc finger protein that regulates the proneural genes *achaete* and *scute* during the formation of bristles in *Drosophila*. Genes Dev. 11, 3083–3095.

Cumano, A., Godin, I., 2001. Pluripotent hematopoietic stem cell development during embryogenesis. Curr. Opin. Immunol. 13, 166–171.

de Bruijn, M.F., Speck, N.A., 2004. Core-binding factors in hematopoiesis and immune function. Oncogene 23, 4238–4248.

Dearolf, C.R., 1998. Fruit fly "leukemia". BBA 1377, M13–M23.

Deconinck, A.E., Mead, P.E., Tevosian, S.G., Crispino, J.D., Katz, S.G., Zon, L.I., Orkin, S.H., 2000. FOG acts as a repressor of red blood cell development in *Xenopus*. Development 127, 2031–2040.

Elagib, K.E., Racke, F.K., Mogass, M., Khetawat, R., Delehanty, L.L., Goldfarb, A.N., 2003. RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. Blood 101, 4333–4341.

Evans, C.J., Hartenstein, V., Banerjee, U., 2003. Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis. Dev. Cell 5, 673–690.

Fossett, N., Schulz, R.A., 2001. Functional conservation of hematopoietic factors in *Drosophila* and vertebrates. Differentiation 69, 83–90.

Fossett, N., Zhang, Q., Gajewski, K., Choi, C.Y., Kim, Y., Schulz, R.A., 2000. The multitype zinc-finger protein U-shaped functions in heart cell specification in the *Drosophila* embryo. Proc. Natl. Acad. Sci. U. S. A. 97, 7348–7353.

Fossett, N., Tevosian, S.G., Gajewski, K., Zhang, Q., Orkin, S.H., Schulz, R.A., 2001. The Friend of GATA proteins U-shaped, FOG-1, and FOG-2 function as negative regulators of blood, heart, and eye development in Drosophila. Proc. Natl. Acad. Sci. U. S. A. 98, 7342–7347.

Fossett, N., Hyman, K., Gajewski, K., Orkin, S.H., Schulz, R.A., 2003. Combinatorial interactions of Serpent, Lozenge, and U-shaped regulate crystal cell lineage commitment during *Drosophila* hematopoiesis. Proc. Natl. Acad. Sci. U. S. A. 100, 11451–11456.

Franc, N.C., Heitzler, P., Ezekowitz, R.A., White, K., 1999. Requirement for *croquemort* in phagocytosis of apoptotic cells in *Drosophila*. Science 284, 1991–1994.

Gaines, P., Geiger, J.N., Knudsen, G., Seshasayee, D., Wojchowski, D.M., 2000. GATA-1- and FOG-dependent activation of megakaryocytic alpha IIB gene expression. J. Biol. Chem. 275, 34114–34121.

Gajewski, K., Kim, Y., Lee, Y.M., Olson, E.N., Schulz, R.A., 1997. D-mef2 is a target for Tinman activation during *Drosophila* heart development. EMBO J. 16, 515–522.

Gajewski, K., Fossett, N., Molkentin, J.D., Schulz, R.A., 1999. The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*. Development 126, 5679–5688.

Haenlin, M., Cubadda, Y., Blondeau, F., Heitzler, P., Lutz, Y., Simpson, P., Ramain, P., 1997. Transcriptional activity of *pannier* is regulated negatively by heterodimerization of the GATA DNA-binding domain with a cofactor encoded by the *u-shaped* gene of *Drosophila*. Genes Dev. 11, 3096–3108.

Hayes, S.A., Miller, J.M., Hoshizaki, D.K., 2001. serpent, a GATA-like transcription factor gene, induces fat-cell development in *Drosophila melanogaster*. Development 128, 1193–1200.

Holz, A., Bossinger, B., Strasser, T., Janning, W., Klapper, R., 2003. The two origins of hemocytes in *Drosophila*. Development 130, 4955–4962.

Howard, M.L., Davidson, E.H., 2004. *cis*-Regulatory control circuits in development. Dev. Biol. 271, 108–109.

Jung, S.H., Evans, C.J., Uemura, C., Banerjee, U., 2005. The *Drosophila* lymph gland as a developmental model of hematopoiesis. Development 132, 2521–2533.

Kammerer, M., Giangrande, A., 2001. Glide2, a second glial promoting factor in *Drosophila melanogaster*. EMBO J. 20, 4664–4673.

Lanot, R., Zachary, D., Holder, F., Meister, M., 2001. Postembryonic hematopoiesis in *Drosophila*. Dev. Biol. 230, 243–257.

Lebestky, T., Chang, T., Hartenstein, V., Banerjee, U., 2000. Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. Science 88, 146–149.

Lebestky, T., Jung, S.H., Banerjee, U., 2003. A Serrate-expressing signaling center controls *Drosophila* hematopoiesis. Genes Dev. 17, 348–353.

Levine, M., Davidson, E.H., 2005. Gene regulatory networks for development. Proc. Natl. Acad. Sci. U. S. A. 102, 4936–4942.

Ling, K.W., Ottersbach, K., van Hamburg, J.P., Oziemlak, A., Tsai, F.Y., Orkin, S.H., Ploemacher, R., Hendriks, R.W., Dzierzak, E., 2004. GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. J. Exp. Med. 200, 871–882.

Lu, J.R., McKinsey, T.A., Xu, H., Wang, D.Z., Richardson, J.A., Olson, E.N., 1999. FOG-2, a heart- and brain-enriched cofactor for GATA transcription factors. Mol. Cell. Biol. 19, 4495–4502.

Mandal, L., Banerjee, U., Hartenstein, V., 2004. Evidence for a fruit fly hemangioblast and similarities between lymph-gland hematopoiesis in fruit fly and mammal aorta-gonadal-mesonephros mesoderm. Nat. Genet. 36, 1019–1023.

Michaud, J., Wu, F., Osato, M., Cottles, G.M., Yanagida, M., Asou, N., Shigesada, K., Ito, Y., Benson, K.F., Raskind, W.H., Rossier, C., Antonarakis, S.E., Israels, S., McNicol, A., Weiss, H., Horwitz, M., Scott, H.S., 2002. In vitro analyses of known and novel RUNX1/AML1 mutations in dominant familial platelet disorder with predisposition to acute myelogenous leukemia: implications for mechanisms of pathogenesis. Blood 99, 1364–1372.

Newton, A., Mackay, J., Crossley, M., 2001. The N-terminal zinc finger of the erythroid transcription factor GATA-1 binds GATC motifs in DNA. J. Biol. Chem. 276, 35794–35801.

Nichols, K.E., Crispino, J.D., Poncz, M., White, J.G., Orkin, S.H., Maris, J.M., Weiss, M.J., 2000. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. Nat. Genet. 24, 266–270.

Okuda, T., van Deursen, J., Hiebert, S.W., Grosveld, G., Downing, J.R., 1996. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. Cell 84, 321–330.

Orkin, S.H., 1996. Development of the hematopoietic system. Curr. Opin. Genet. Dev. 6, 597–602.

Orkin, S.H., 2000. Diversification of haematopoietic stem cells to specific lineages. Nat. Rev., Genet. 1, 57–64.

Parmacek, M.S., Leiden, J.M., 1999. GATA transcription factors and cardiac development. In: Harvey, R.P., Rosenthal, N. (Eds.), Heart Development. Academic Press, San Diego, pp. 291–306.

Pedone, P.V., Omichinski, J.G., Nony, P., Trainor, C., Gronenborn, A.M., Clore, G.M., Felsenfeld, G., 1997. The N-terminal fingers of chicken GATA-2 and GATA-3 are independent sequence-specific DNA binding domains. EMBO J. 16, 2874–2882.

Querfurth, E., Schuster, M., Kulessa, H., Crispino, J.D., Doderlein, G., Orkin, S.H., Graf, T., Nerlov, C., 2000. Antagonism between C/EBPbeta and FOG in eosinophil lineage commitment of multipotent hematopoietic progenitors. Genes Dev. 14, 2515–2525.

Rehorn, K.P., Thelen, H., Michelson, A.M., Reuter, R., 1996. A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila*. Development 122, 4023–4031.

Rennert, J., Coffman, J.A., Mushegian, A.R., Robertson, A.J., 2003. The evolution of *Runx* genes I. A comparative study of sequences from phylogenetically diverse model organisms. BMC Evol. Biol. 3, 4.

Rizki, T.M., 1978. The circulatory system and associated cells and tissues. In: Ashburner, M., Wright, T.R.F. (Eds.), The Genetics and Biology of *Drosophila*, vol. 2b. Academic Press, New York, pp. 397–452.

Rugendorff, A.E., Younossi-Hartenstein, A., Hartenstein, V., 1994. Embryonic origin and differentiation of the *Drosophila* heart. Roux's Arch. Dev. Biol. 203, 266–280.

Sam, S., Leise, W., Hoshizaki, D.K., 1996. The *serpent* gene is necessary for progression through the early stages of fat-body development. Mech. Dev. 60, 197–205.

Schulz, R.A., Fossett, N., 2005. Hemocyte development during *Drosophila* embryogenesis. In: Baron, M.H. (Ed.), Developmental Regulation of Hematopoiesis: Methods and Practical Approaches, Methods in Molecular Medicine series. Humana Press, New York, pp. 109–122.

Song, W.J., Sullivan, M.G., Legare, R.D., Hutchings, S., Tan, X., Kufrin, D., Ratajczak, J., Resende, I.C., Haworth, C., Hock, R., Loh, M., Felix, C., Roy, D.C., Busque, L., Kurnit, D., Willman, C., Gewirtz, A.M., Speck, N.A., Bushweller, J.H., Li, F.P., Gardiner, K., Poncz, M., Maris, J.M., Gilliland, D.G., 1999. Haploinsufficiency of *CBFA2* causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia. Nat. Genet. 23, 166–175.

Speck, N.A., Gilliland, D.G., 2002. Core-binding factors in haematopoiesis and leukaemia. Nat. Rev., Cancer 2, 502–513.

Svensson, E.C., Tufts, R.L., Polk, C.E., Leiden, J.M., 1999. Molecular cloning of FOG-2: a modulator of transcription factor GATA-4 in cardiomyocytes. Proc. Natl. Acad. Sci. U. S. A. 96, 956–961.

Svensson, E.C., Huggins, G.S., Lin, H., Clendenin, C., Jiang, F., Tufts, R., Dardik, F.B., Leiden, J.M., 2000. A syndrome of tricuspid atresia in mice with a targeted mutation of the gene encoding *Fog-2*. Nat. Genet. 25, 353–356.

Tepass, U., Fessler, L.I., Aziz, A., Hartenstein, V., 1994. Embryonic origin of hemocytes and their relationship to cell death in *Drosophila*. Development 120, 1829–1837.

Tevosian, S.G., Deconinck, A.E., Cantor, A.B., Rieff, H.I., Fujiwara, Y., Corfas, G., Orkin, S.H., 1999. FOG-2: A novel GATA-family cofactor related to multitype zinc-finger proteins Friend of GATA-1 and U-shaped. Proc. Natl. Acad. Sci. U. S. A. 96, 950–955.

Tevosian, S.G., Deconinck, A.E., Tanaka, M., Schinke, M., Litovsky, S.H., Izumo, S., Fujiwara, Y., Orkin, S.H., 2000. FOG-2, a cofactor for GATA transcription factors, is essential for heart morphogenesis and development of coronary vessels from epicardium. Cell 101, 729–739.

Thummel, C.S., Boulet, A.M., Lipshitz, H.D., 1988. Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. Gene 74, 445–456.

Tracey, W.D., Speck, N.A., 2000. Potential roles for RUNX1 and its orthologs in determining hematopoietic cell fate. Semin. Cell Dev. Biol. 11, 337–342.

Trainor, C.D., Omichinski, J.G., Vandergon, T.L., Gronenborn, A.M., Clore, G.M., Felsenfeld, G., 1996. A palindromic regulatory site within vertebrate *GATA-1* promoters requires both zinc fingers of the GATA-1 DNA-binding domain for high-affinity interaction. Mol. Cell. Biol. 16, 2238–2247.

Tsai, F.Y., Orkin, S.H., 1997. Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood 89, 3636–3643.

Tsang, A.P., Visvader, J.E., Turner, C.A., Fujiwara, Y., Yu, C., Weiss, M.J., Crossley, M., Orkin, S.H., 1997. FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. Cell 90, 109–119.

Tsang, A.P., Fujiwara, Y., Hom, D.B., Orkin, S.H., 1998. Failure of megakaryopoiesis and arrested erythropoiesis in mice lacking the GATA-1 transcriptional cofactor FOG. Genes Dev. 12, 1176–1188.

Walker, L.C., Stevens, J., Campbell, H., Corbett, R., Spearing, R., Heaton, D., Macdonald, D.H., Morris, C.M., Ganly, P., 2002. A novel inherited mutation of the transcription factor RUNX1 causes thrombocytopenia and may predispose to acute myeloid leukaemia. Br. J. Haematol. 117, 878–881.

Waltzer, L., Bataille, L., Peyrefitte, S., Haenlin, M., 2002. Two isoforms of Serpent containing either one or two GATA zinc fingers have different roles in *Drosophila* haematopoiesis. EMBO J. 21, 5477–5486.

Waltzer, L., Ferjoux, G., Bataille, L., Haenlin, M., 2003. Cooperation between the GATA and RUNX factors Serpent and Lozenge during *Drosophila* hematopoiesis. EMBO J. 22, 6516–6525.

Ward, E.J., Skeath, J.B., 2000. Characterization of a novel subset of cardiac cells and their progenitors in the *Drosophila* embryo. Development 127, 4959–4969.

Yu, C., Niakan, K.K., Matsushita, M., Stamatoyannopoulos, G., Orkin, S.H., Raskind, W.H., 2002. X-linked thrombocytopenia with thalassemia from a mutation in the amino finger of GATA-1 affecting DNA binding rather than FOG-1 interaction. Blood 100, 2040–2045.

Zhu, J., Emerson, S.G., 2002. Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene 21, 3295–3313.