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ScienceDirect
Developmental Biology 311 (2007) 636–649
DEVELOPMENTAL BIOLOGY
www.elsevier.com/developmentalbiology

Genomes & Developmental Control

The GATA factor Serpent cross-regulates *lozenge* and *u-shaped* expression during *Drosophila* blood cell development

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Received for publication 24 April 2007; revised 2 August 2007; accepted 7 August 2007
Available online 16 August 2007

Abstract

The *Drosophila* GATA factor Serpent interacts with the RUNX factor Lozenge to activate the crystal cell program, whereas SerpentNC binds the Friend of GATA protein U-shaped to limit crystal cell production. Here, we identified a *lozenge* minimal hematopoietic *cis*-regulatory module and showed that *lozenge–lacZ* reporter-gene expression was autoregulated by Serpent and Lozenge. We also showed that upregulation of *u-shaped* was delayed until after *lozenge* activation, consistent with our previous results that showed *u-shaped* expression in the crystal cell lineage is dependent on both Serpent and Lozenge. Together, these observations describe a feed forward regulatory motif, which controls the temporal expression of *u-shaped*. Finally, we showed that *lozenge* reporter-gene activity increased in a *u-shaped* mutant background and that forced expression of SerpentNC with U-shaped blocked *lozenge-* and *u-shaped-lacZ* reporter-gene activity. This is the first demonstration of GATA:FOG regulation of *Runx* and *Fog* gene expression. Moreover, these results identify components of a Serpent cross-regulatory sub-circuit that can modulate *lozenge* expression. Based on the sub-circuit design and the combinatorial control of crystal cell production, we present a model for the specification of a dynamic bi-potential regulatory state that contributes to the selection between a Lozenge-positive and Lozenge-negative state.

Keywords: Drosophila; Friend of GATA; GATA; Hematopoiesis; Linage commitment; RUNX

Introduction

Hematopoiesis is a dynamic process that produces the various blood cell lineages from a single hematopoietic stem cell and is regulated by key lineage-specific factors (Orkin, 2000; Zhu and Emerson, 2002; Warren and Rothenberg, 2003). When viewed in the context of all known genetic interactions, the complexity of the processes that control hematopoiesis can be appreciated, but not readily understood (Swiers et al., 2006). An understanding of the interactions in terms of gene activation or repression, coupled with information about *cis*-regulatory inputs, has revealed mechanistic details about the modular sub-circuits that together describe these processes and development in general (Swiers et al., 2006; Oliveri and Davidson, 2007). Moreover, simple genetic model organisms provide an opportunity to analyze these interactions in vivo, thereby providing a direct link between the genomic programs that encode them and the biological functions they control. The *Drosophila* model system has been used to identify conserved key factors and investigate their function during hematopoiesis (Dearolf, 1998; Fossett and Schulz, 2001; Evans et al., 2003; Meister and Lagueux, 2003; Sorrentino et al., 2005; Hartenstein, 2006; Crozatier and Meister, 2007). In order to more fully understand *Drosophila* hematopoiesis, we characterized the role of Serpent (Srp) cross-regulation of Lozenge (Lz) and U-shaped (Ush) in the crystal cell lineage.

The blood system of the fly lacks the complexity seen in vertebrates. Nevertheless, cross-species comparisons have shown that fundamental aspects of hematopoiesis are conserved across taxa (Fossett and Schulz, 2001; Evans et al., 2003; Fossett et al., 2003; Meister and Lagueux, 2003; Sorrentino et al., 2005; Hartenstein, 2006; Crozatier and Meister, 2007). *Drosophila* has two primary blood cell types, plasmatocytes and crystal cells, which have similar functions to the vertebrate myeloid lineages (Rizki, 1978; Dearolf, 1998; Evans et al., 2003). Crystal cells,

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0012-1606/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2007.08.015

named for their crystalline inclusion bodies, are required for wound repair and xenobiotic encapsulation (Rizki, 1978). Plasmatocytes are operational macrophages and synthesize antimicrobials (Rizki, 1978; Tepass et al., 1994; Dearolf, 1998). Like their vertebrate counterparts, these cells develop from a common hematopoietic progenitor (Rizki, 1978; Tepass et al., 1994; Dearolf, 1998; Lebestky et al., 2000; Lanot et al., 2001). Both vertebrate and *Drosophila* hematopoiesis consists of two spatially and temporally distinct periods or waves. In *Drosophila*, the first hematopoietic wave begins in the early embryonic head mesoderm. The second wave begins in embryogenesis and continues throughout larval development within a specialized hematopoietic organ, called the lymph gland (Dearolf, 1998; Lebestky et al., 2000; Lanot et al., 2001; Fossett and Schulz, 2001; Evans et al., 2003; Hartenstein, 2006; Crozatier and Meister, 2007).

Srp, similar to vertebrate GATA-2, is positioned at the apex of hematopoiesis and, as such, is required for the production of hemocyte precursors (Rehorn et al., 1996; Sam et al., 1996). In this role, Srp acts upstream of Glial cells missing (Gcm) and the RUNX factor Lz, which are required later for plasmatocyte and crystal cell production, respectively (Bernardoni et al., 1997; Lebestky et al., 2000; Kammerer and Giangrande, 2001; Alfonso and Jones, 2002). Of the conserved hematopoietic regulators, the GATA, Friend of GATA (FOG), and RUNX protein families are of particular interest because pair-wise interactions between GATA and FOG or between GATA and RUNX regulate both vertebrate and *Drosophila* hematopoiesis (Tsang et al., 1997, 1998; Querfurth et al., 2000; Elagib et al., 2003; Fossett et al., 2003; Waltzer et al., 2003; Cantor and Orkin, 2005, Ferjoux et al., 2007). Moreover, Srp acts as a contextual switch between RUNX activation and FOG repression of the crystal cell lineage (Fossett et al., 2003). GATA transcriptional regulators generally have two zinc-finger domains. The C-terminal zinc-finger binds the DNA recognition sequence, WGATAR (Cantor and Orkin, 2005). The N-terminal zinc-finger interacts with FOG proteins; and the GATA:FOG complex modifies transcription by either activating or antagonizing activity, depending upon the gene regulatory context (Crispino et al., 1999; Lu et al., 1999; Svensson et al., 1999; Tevosian et al., 1999; Chang et al., 2002; Letting et al., 2004; Hong et al., 2005; Cantor and Orkin, 2005; Lowry and Mackay, 2006). 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). The FOG protein Ush interacts with SrpNC, but not SrpC, which lacks the N-terminal zinc-finger (Waltzer et al., 2002; Fossett et al., 2003). RUNX proteins bind DNA through the conserved Runt domain (Tracey and Speck, 2000; Speck and Gilliland, 2002; Rennert et al., 2003; Anglin and Passaniti, 2004). In general, RUNX activity is influenced by a variety of interacting factors, including GATA factors (Coffman, 2003; Elagib et al., 2003; Fossett et al., 2003; Waltzer et al., 2003, Ferjoux et al., 2007). Of the three mammalian *Runx* genes, *Runx1* is required for hematopoiesis (Otto et al., 2003) 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). Currently, there is a lack of information about the role of *Runx1* in hematopoietic gene regulatory networks (Swiers et al., 2006; Otto et al., 2003).

Srp, Lz and Ush act combinatorially to regulate crystal cell production. Both SrpC and SrpNC can interact with Lz to activate the crystal cell program (Fossett et al., 2003; Waltzer et al., 2003), whereas only SrpNC interacts with Ush to repress crystal cell production (Fossett et al., 2003). This suggests that Srp acts as a contextual switch, mediating cross-talk between crystal cell activation and repression pathways. In order to increase our understanding of the mechanistic basis for this contextual switch, and how it regulates crystal cell production, we investigated the *cis*- and *trans*-regulation of *lz* and *ush*. Collectively, our data provide evidence for a Srp cross-regulatory sub-circuit that regulates *lz* and *ush* expression. Based on these results, we present a model for the specification of a dynamic bi-potential regulatory state that contributes to the selection between a Lz-positive and Lz-negative state.

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: ush^{1}/*SM6,Roi,eve–lacZ*; *upstream activation sequence* (*UAS*)*–ush*; *UAS–srpNC*; *UAS–srpNC*^{V421G}; and *twi–Gal4* (Fossett et al., 2001, 2003). The following strains were generous gifts from colleagues: *lz–Gal4;UAS–GFP* (J. Pollock, Duquesne University); *UAS–lz* (J. Canon and U. Banerjee, University of California, Los Angeles, CA); *UAS–srpC* (D. K. Hoshizaki, University of Nevada, Las Vegas, NV); and *UAS–srpNC;UAS–lz* (K. M. Gajewski, University of Texas, M. D. Anderson Cancer Center, Houston, TX). *UAS–srpNC,UAS–ush* stock was constructed using genetic recombination between *UAS–transgenes* located on chromosome II. The generation of strains carrying *lz–lacZ* transgenes is described below.

Generation of transgenic animals carrying lz–lacZ fusion constructs

Overlapping DNA fragments of the *lz* 1.5 kb 5′ UTR genomic DNA region were analyzed for their ability to direct *lacZ* reporter-gene expression in crystal cells. This was accomplished by generating PCR fragments that were either cloned directly into the P-element CaSpeR-Hsp43-AUG-βgal (Chab) germline transformation vector (Thummel et al., 1988), or first cloned into pCR-II TOPO cloning vector (Invitrogen) and subsequently into Chab vector. Site-directed mutations (SDM) were introduced into DNA fragments as described previously (Muratoglu et al., 2006). The oligonucleotide primers used to generate point mutations in DNA fragments are available upon request.

The DNA sequence of each recombinant vector was verified prior to injection. w^{1118} embryos were injected with the recombinant vectors by Model Systems Genomics, Duke University, or Rainbow Transgenic Flies, Inc., Newbury Park, CA. Germline transformants were established according to previously described methods and were screened for tissue-specific *lacZ* expression using immunohistochemical staining analysis as previously described (Gajewski et al., 1997). At least six independent lines were generated and tested for each construct.

Determination of the lz–Gal4 chromosomal insertion site

Plasmid rescue was used to identify the *lz* genomic sequences that flank the pGawB insertion (Pirrotta, 1986). Briefly, genomic DNA was isolated from *lz–Gal4* fly lines. The DNA was cut with *Msp*I or *Dra*I, each having a single restriction site within the pGawB plasmid insert. Ligation of this restriction digest produced a circular template for PCR. Specific primers for the pGawB plasmid, but facing the flanking sequences, were used to produce a pGawB/