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The NK homeodomain transcription factor Tinman is a direct activator of *seven-up* in the *Drosophila* dorsal vessel

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Abstract

A complex regulatory cascade is required for normal cardiac development, and many aspects of this network are conserved from *Drosophila* to mammals. In *Drosophila*, the *seven-up* (*svp*) gene, an ortholog of the vertebrate *chick ovalbumin upstream promoter transcription factors* (*COUP-TFI* and *II*), is initially activated in the cardiac mesoderm and is subsequently restricted to cells forming the cardiac inflow tracts. Here, we investigate *svp* regulation in the developing cardiac tube. Using bioinformatics, we identify a 1007-bp enhancer of *svp* which recapitulates its entire expression in the embryonic heart and other mesodermal derivatives, and we show that this enhancer is initially activated by the NK homeodomain factor Tinman (Tin) via two conserved Tin binding sites. Mutation of the Tin binding sites significantly reduces enhancer activity both during normal development and in response to ectopic Tin. This is the first identification of an enhancer for the complex *svp* gene, demonstrating the effectiveness of bioinformatics tools in assisting in unraveling transcriptional regulatory networks. Our studies define a critical component of the *svp* regulatory cascade and place gene regulatory events in direct apposition to the formation of critical cardiac structures.

Keywords: Heart; Inflow tract; Seven-up; COUP-TF; Tinman; NKX2.5; Transcriptional regulation

Introduction

A key focal issue in developmental biology is that distantly related species, such as mice and fruit flies, have gene products which are conserved in function. The genes which encode these proteins in *Drosophila* frequently have regulatory pathways in common with higher animals having have much larger and more complex genomes (reviewed in Davidson, 2001; Carroll et al., 2005). This commonality makes it possible for research in simpler species to facilitate or even direct studies in higher vertebrates, such as has been apparent in the study of the transcriptional regulation of cardiac development (Cripps and Olson, 2002; Brand, 2003). Identifying parallel pathways in diverse model systems has become easier with increasingly available and comprehensive genomic databases, as well as computational tools for genome sequence comparison and analysis.

Fruit flies have a partially open circulatory system which is a linear cardiac tube, termed the dorsal vessel, through which hemolymph is transported anteriorly and deposited near the brain (Rizki, 1978; Wigglesworth, 1984). All cardial cells initially express the NK homeodomain transcription factor gene *tinman* (*tin*), which is essential for cardiac specification (Bodmer, 1993; Azpiazu and Frasch, 1993). Tin binds the consensus sequence 5′TYAAGTG-3′ (Chen and Schwartz, 1995), and numerous Tin target enhancers have been identified, most containing two copies of the consensus Tin binding sequence (Gajewski et al., 1997; Xu et al., 1998; Cripps et al., 1999; Kremser et al., 1999; Gajewski et al., 2001; Han and Olson, 2005).

tin expression in the mature embryonic dorsal vessel is down-regulated in seven bilateral pairs of cells. These cells instead express the orphan nuclear receptor gene *seven-up* (*svp*; Bodmer and Frasch, 1999). *svp* expression is first detected in the dorsal vessel at late stage 11, and *svp* function in those cells is required to subsequently suppress the expression of *tin* (Lo and Frasch, 2001).

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The posterior-most three sets of Svp cells form the embryonic and larval inflow tracts called ostia (Rizki, 1978; Molina and Cripps, 2001), and studies have demonstrated a role for *svp* function in the normal formation of the ostia (Ponzielli et al., 2002). As inflow tracts, ostia are functionally similar to atria in vertebrate hearts. *Chick ovalbumin upstream promoter transcription factor II* (*COUP-TF II*), a vertebrate ortholog of *svp*, is expressed in the developing atria, and mouse knockout analyses have shown that atrium formation is severely retarded in the absence of *COUP-TFII* function (Pereira et al., 1999). The nature of this conservation in function between Svp and COUP-TFII provides opportunity to use *Drosophila* to illuminate their upstream and downstream targets, and to define the roles of Svp and COUP-TFII in cardiac development.

Our current understanding of how *svp* expression in the dorsal vessel is initiated at the transcriptional level has come from genetic analyses of *svp* expression in particular mutant backgrounds. The segmentally repeated onset of *svp* transcription results from ectodermal Hedgehog (Hh) signaling (Ponzielli et al., 2002), and *svp* transcription is further limited to the seven posterior-most segments by the actions of Hox genes of the Bithorax and Antennapedia Complexes (Ryan et al., 2005; Perrin et al., 2004). How these factors directly impact a *svp* cardiac enhancer is not known since no such enhancer has been identified, probably due the extremely large size of the *svp* transcription unit (Mlodzik et al., 1990; Adams et al., 2000). More importantly, how Hox and Hh actions are confined to the cardiac mesoderm is yet to be understood.

In this paper, we demonstrate use of bioinformatics to locate a 1007-bp region containing the *svp* cardiac enhancer (SCE). We show that Tin is a direct regulator of cardiac *svp* expression via a pair of Tin sites which are critical for correct temporal and spatial activity of the enhancer. These studies define important regulatory processes in cardiac development, which promise to provide critical insight into vertebrate cardiac development.

Materials and methods

Fly stocks and crosses

Flies were maintained on Carpenter's medium (Carpenter, 1950) at 25°C unless indicated. Transgenic lines were generated according to Rubin and Spradling (1982) using the Delta2–3 helper plasmid (Robertson et al., 1988), and at least three independent lines of each construct were tested for enhancer activity.

The *24Bgal4* (Brand and Perrimon, 1993) line was obtained from the Bloomington *Drosophila* Stock Center. A stock carrying the tin^{EC40} allele (Bodmer, 1993) maintained over a *TM3*, *lacZ* balancer was obtained from Zhe Han (University of Texas Southwestern Medical Center). Homozygous *tin* mutant embryos from this stock were identified based on the absence of β-galactosidase accumulation. The *svp-lacZ* enhancer trap allele svp^{3} (Mlodzik et al., 1990) was obtained from the Bloomington *Drosophila* Stock Center, and was used as a marker of *svp* expression in Fig. 1 only.

For studying the effects of ectopic Tin upon *svp* enhancer activity, virgin females homozygous for the mesodermal driver *24Bgal4* were crossed with males homozygous for either the wild-type or mutant *SCE-lacZ*. Double-heterozygous virgin females from these crosses were then mated at 25°C to males homozygous for the *UAS-tin* insert, and progeny embryos were collected and processed for immunofluorescence. To ensure that all three genetic elements had been brought together in a particular embryo, only those embryos showing ectopic expression of *tin* and some pattern of β-galactosidase accumulation were studied. A large number of such embryos were generated and representative images are shown in Fig. 5.

Bioinformatics

Putative Tin binding sites were initially located using Flyenhancer (www.flyenhancer.org; Markstein et al., 2002) to identify genes containing two copies of the sequence 5′-TYAAGTGG-3′ within a 300-bp window. Having established that such sites lay in the vicinity of the *svp* gene, the entire *svp* transcribed region was extracted from Flybase (Grumbling et al., 2006) and all consensus Tin binding sites were mapped. Potential Tin binding regions were studied for conservation using the genome sites for sequences of other *Drosophila* species, located using GenomeVista (Couronne et al., 2003; Bray et al., 2003). To create a detailed representation of conserved sequences, ClustalW 1.8 analysis at the http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html site was used to generate an MSF file, which was then put into the Boxshade 3.21 at www.ch.embnet.org to create the comparison shown in Fig. 2B and Fig. S1.

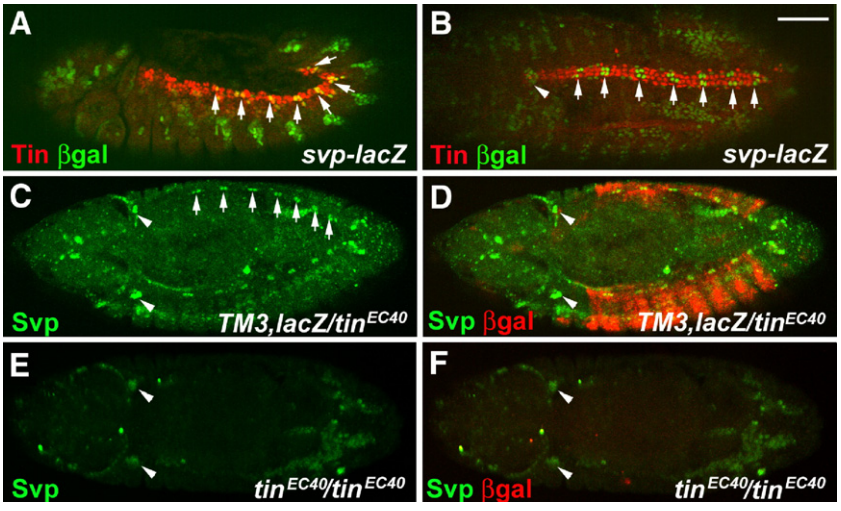

Fig. 1. Tinman is required for *seven-up* expression. (A, B) While *tin* (red) and *svp–lacZ* (green) are initially co-expressed at stage 12 (A), their expression is subsequently mutually exclusive (B, stage 16). (C, D) In the presence of *tin* function, seven bilateral pairs of Svp cells form (arrows); counterstain for β-galactosidase (red in panel D) identifies this embryo as a *TM3*, $lacZ/tin^{EC40}$ heterozygote. (E) In tin^{EC40} homozygotes, Svp accumulation in the cardiac tissue is absent; counterstain for β-galactosidase (F) identifies this sample as mutant homozygote. Expression of *svp* in the ring gland primordia (arrowheads) is not dependent upon Tin. Panel A is a sagittal view, all other panels are dorsal views. Bar: 100 μm.