

The zinc finger proteins Pannier and GATA4 function as cardiogenic factors in *Drosophila*

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SUMMARY

The regulation of cardiac gene expression by GATA zinc finger transcription factors is well documented in vertebrates. However, genetic studies in mice have failed to demonstrate a function for these proteins in cardiomyocyte specification. In *Drosophila*, the existence of a cardiogenic GATA factor has been implicated through the analysis of a cardinal cell enhancer of the muscle differentiation gene *D-mef2*. We show that the GATA gene *pannier* is expressed in the dorsal mesoderm and required for cardinal cell formation while repressing a pericardial cell fate. Ectopic expression of Pannier results in cardinal cell overproduction,

while co-expression of Pannier and the homeodomain protein Tinman synergistically activate cardiac gene expression and induce cardinal cells. The related GATA4 protein of mice likewise functions as a cardiogenic factor in *Drosophila*, demonstrating an evolutionarily conserved function between Pannier and GATA4 in heart development.

Key words: Cardinal cells, Combinatorial regulation, GATA factor, Heart development, *pannier*, *tinman*

INTRODUCTION

The *Drosophila* heart is a linear organ that pumps haemolymph throughout the animal in an open circulatory system. During embryogenesis, bilateral groups of heart progenitor cells are produced within the dorsal mesoderm and these cells migrate to the midline where they fuse to form the heart tube (Lawrence et al., 1995; Azpiazu et al., 1996; Jagla et al., 1997; Gajewski et al., 1998). The dorsal vessel is composed of inner contractile cardiac cells that are flanked by non-contracting pericardial cells. The central muscle cells express the differentiation factor D-MEF2 that activates the transcription of various structural protein genes, facilitating the contractile nature of the heart (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995).

The specification of cardiac progenitors within the dorsal mesoderm requires the coordinated activities of several regulatory genes. The homeobox gene *tinman* (*tin*) plays an essential role in the patterning of the early mesoderm and multiple muscle defects are observed in *tin* mutant embryos (Azpiazu and Frasch, 1993; Bodmer, 1993; Gajewski et al., 1997, 1998; Yin and Frasch, 1998). These include an absence of heart precursor cells and a resulting lack of dorsal vessel formation. *tin* expression in the dorsal mesoderm is under the inductive control of Decapentaplegic (Dpp), a TGF β -related signaling molecule produced by dorsal ectodermal cells

(Frasch, 1995; Xu et al., 1998). Due to the deficiency of *tin* expression in *dpp* mutant embryos, cardiac mesoderm fails to develop. A second secreted molecule, Wingless (Wg), also plays a vital role in heart formation. By an unknown mechanism, Wg functions as a required co-regulator with Tin in the specification of heart precursor cells (Lawrence et al., 1995; Wu et al., 1995; Park et al., 1996; Jagla et al., 1997; Gajewski et al., 1998). Accordingly, the heart fails to form in *wg* mutant embryos. Another gene needed indirectly for the production of cardiac progenitors is *heartless* (*htl*), which encodes a fibroblast growth factor receptor homologue (Shishido et al., 1993). *Htl* functions in the directional migration of the invaginated mesoderm along the overlying ectoderm (Beiman et al., 1996; Gisselbrecht et al., 1996; Shishido et al., 1997). In mutant embryos, mesodermal cells do not migrate dorsally into the Dpp signaling domain, *tin* expression is not maintained and there is a failure of cardiac mesoderm development.

The earliest events of vertebrate heart development are remarkably similar to those observed in *Drosophila* as bilaterally symmetric heart progenitors are specified within the anterior lateral plate mesoderm and these cells migrate to the ventral midline where they form a linear beating heart tube (Bodmer, 1995; Olson and Srivastava, 1996). Inductive processes are also utilized in the specification of vertebrate cardiac precursors as signaling factors of the bone morphogenetic protein class,

Fig. 1. *pnr* is expressed in the cardiogenic mesoderm. (A) Lateral view of a late stage 10 embryo showing the broad domain of *tin* expression in the dorsal mesoderm. (B) Lateral view of a stage 11 embryo stained for *lacZ* expression under the control of the *D-mef2* heart enhancer. (C) Lateral view of a late stage 10 embryo showing *pnr* expression in the dorsal ectoderm. (D) Cross-section of a late stage 10 embryo showing *pnr* RNA in both the dorsal ectoderm and cardiogenic mesoderm. (E) Cross-section of a late stage 10 embryo showing *pnr* enhancer activity in the cardiogenic mesoderm and amnioserosa. (F) Lateral and (G) ventral views of a late stage 10 embryo demonstrating *pnr* enhancer function in the amnioserosa and dorsal mesoderm. The arrowhead points to the absence of *lacZ* expression in the dorsal ectoderm. Abbreviations: as, amnioserosa; cm, cardiogenic mesoderm; de, dorsal ectoderm; dm, dorsal mesoderm; hp, heart precursors.

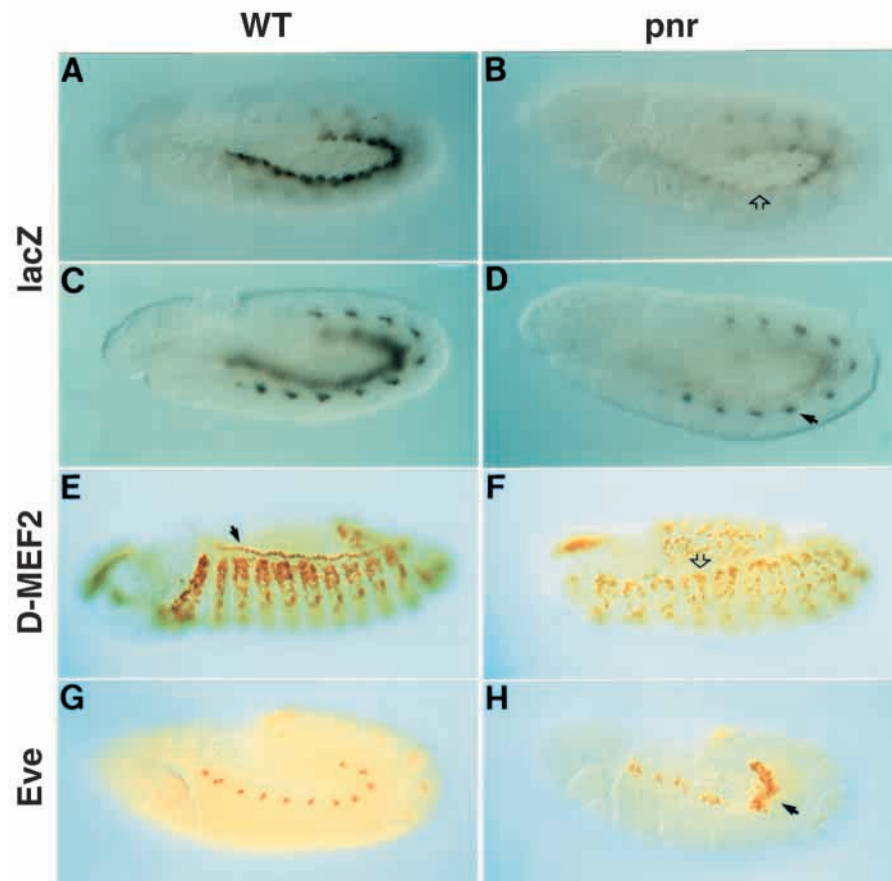
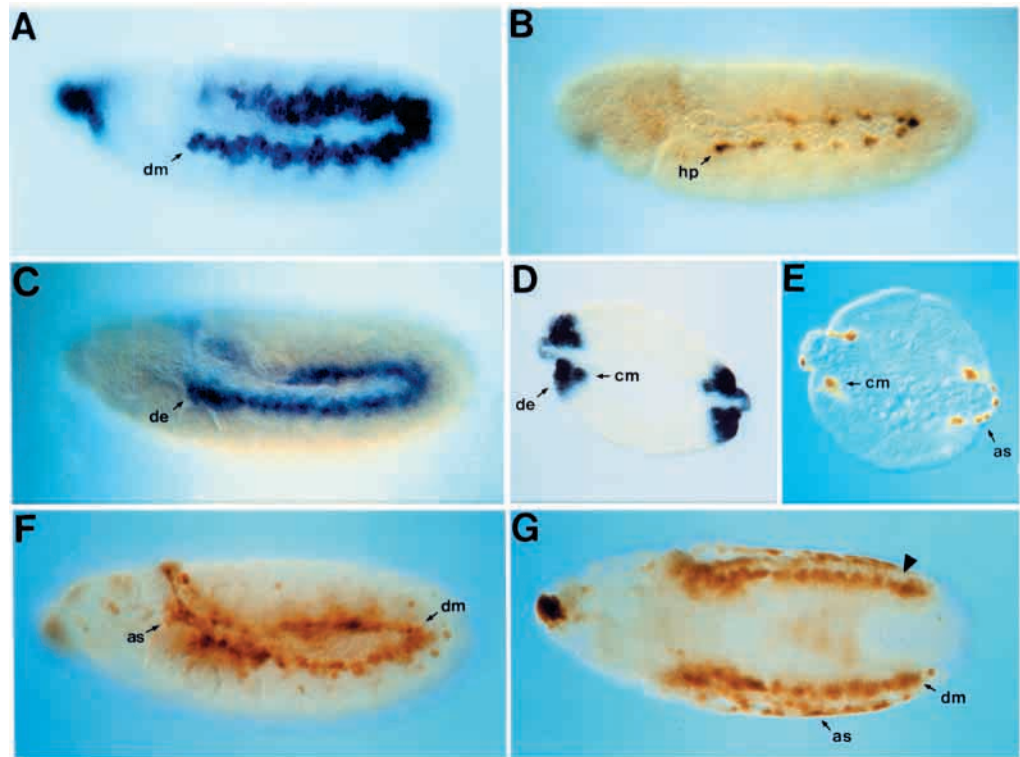


Fig. 2. *pnr* functions during *Drosophila* cardiogenesis. (A-D) Activity of a *D-mef2* heart enhancer is greatly diminished in *pnr* mutant embryos. Early stage 12 embryos stained for *lacZ* expression under the control of a *D-mef2* combinatorial enhancer are shown. (A,C) WT, wild-type embryos. (B,D) *pnr* mutant embryos. (A,B) The focus is on the dorsal mesoderm to illustrate the presence or decrease (open arrow) in reporter gene expression. (C,D) The focus is on the ventral mesoderm to highlight normal *lacZ* expression in ventral muscle founder cells (filled arrow) of both embryo types. (E-H) *pnr* controls cardiac and Eve pericardial cell specification. Wild-type (E,G) and *pnr* mutant (F,H) embryos were stained for D-MEF2 protein as a marker for cardiac cells (E,F) or Eve protein as a marker for pericardial cells (G,H). In the wild-type embryo, the filled arrow points to a row of cardiac cells. In *pnr* mutants, the open arrow indicates the absence of cardiac cells while the filled arrow points to the excess number of Eve-expressing pericardial cells.

related to *Drosophila* Dpp, are produced by the endoderm and interpreted by the precardiac mesoderm (Schultheiss et

al., 1997). Thereafter, vertebrate-specific morphogenetic events occur such as looping of the linear tube and remodeling of the heart into a multichambered organ.

The requirement of *tin* function for cardiogenesis in flies, coupled with the embryological similarities of heart

formation between invertebrates and vertebrates, prompted a search for *tin*-related genes in other species. This led to the

discovery of numerous NK-2 class homeobox genes and several of the homologues are expressed in the cardiac lineage (Harvey, 1996). Recent genetic studies have demonstrated the importance of *tin*-related genes for vertebrate heart development, specifically in the mouse and *Xenopus* systems (Lyons et al., 1995; Grow and Krieg, 1998; Fu et al., 1998). The GATA4, GATA5, and GATA6 zinc finger transcription factors are also expressed in the forming vertebrate heart (Arceci et al., 1993; Kelley et al., 1993; Heikinheimo et al., 1994; Laverriere et al., 1994; Jiang and Evans, 1996; Morrisey et al., 1996, 1997; Jiang et al., 1998). The importance of GATA4 in heart development has been suggested based on its expression in the precardiac mesoderm, its presence in the differentiated myocardium throughout embryogenesis and into adulthood, and its regulation of multiple cardiac structural genes in cell culture studies (Grepin et al., 1994; Ip et al., 1994; Molkentin et al., 1994; Durocher et al., 1997; Murphy et al., 1997; Lee et al., 1998; Sepulveda et al., 1998). However, genetic studies thus far have not demonstrated a definitive role for any GATA gene in cardiomyocyte specification (Kuo et al., 1997; Molkentin et al., 1997; Jiang et al., 1998; Morrisey et al., 1998; Koutsourakis et al., 1999).

The involvement of a GATA factor in *Drosophila* heart development has been hinted at based on the analysis of a cardiac cell enhancer of the *D-mef2* gene. This regulatory DNA is a direct target of Tin as it contains two required factor binding sites and ectopic Tin expression results in enhancer activity outside of the cardiac lineage (Gajewski et al., 1997). It has also been demonstrated that the two Tin sites alone are not sufficient and a GATA sequence element must be present in the enhancer for its normal function. Strikingly, mutation of this GATA element changes the transcriptional specificity of the enhancer from cardiac to pericardial cells (Gajewski et al., 1998). This result led to the hypothesis that Tin and a GATA site-binding protein(s) selectively activate or repress *D-mef2* expression in cells of the dorsal vessel. While three GATA genes, *pannier* (*pnr*), *serpent* (*srp*), and *dGATAc*, have been identified in *Drosophila*, their functions have been verified only in non-cardiac cells and their expression has not been reported in the heart lineage (Abel et al., 1993; Ramain et al., 1993; Winick et al., 1993; Lin et al., 1995; Heitzler et al., 1996; Rehorn et al., 1996; Sam et al., 1996; Riechmann et al., 1998). Thus, a combinatorial function of Tin and GATA transcription factors during *Drosophila* heart development represents a plausible but untested genetic paradigm.

In this report, we demonstrate that *pnr* is expressed in, and functions during, heart development in

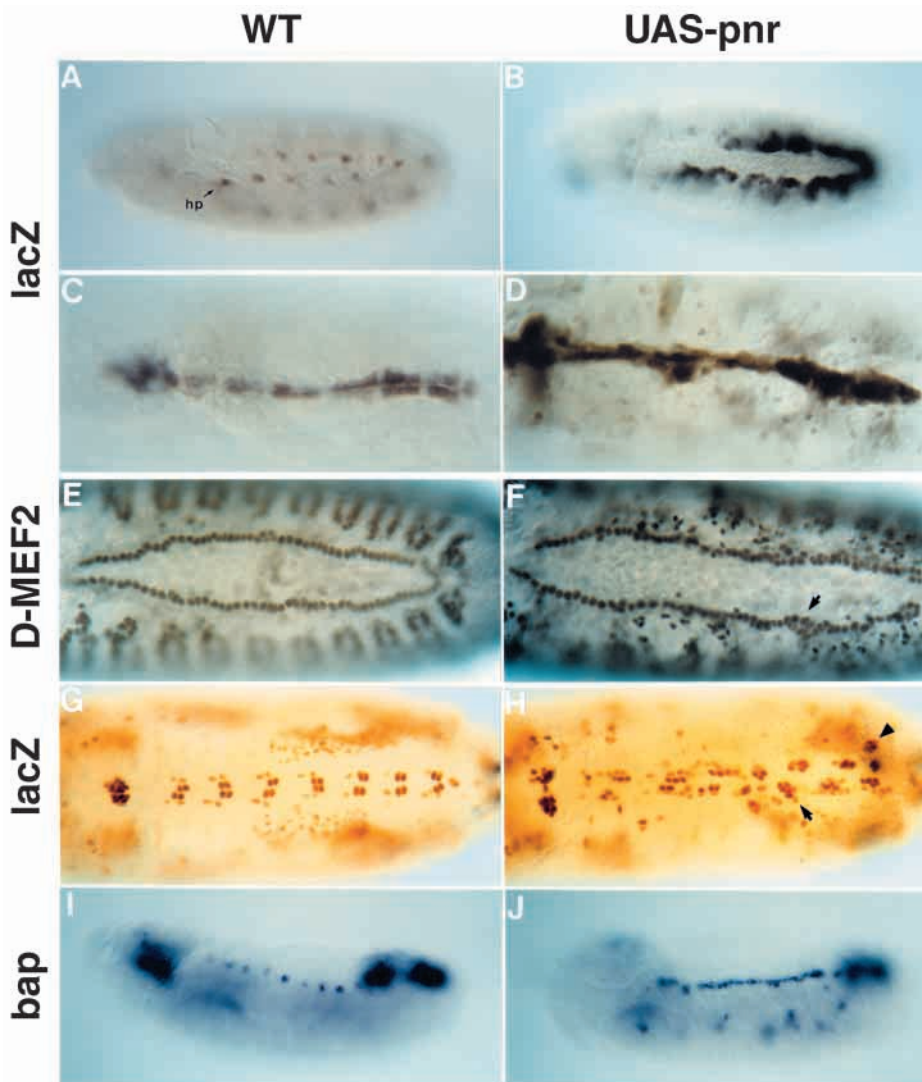


Fig. 3. Forced mesodermal expression of the *pnr* gene affects heart development.

(A-D) Expression of *pnr* in over-expression of a *D-mef2* heart enhancer and cardiac hyperplasia. Stage 11 (A,B) and 16 (C,D) embryos stained for *lacZ* expression under the control of a *D-mef2* cardiac cell enhancer are shown. (A,C) WT, wild-type embryos; (B,D) UAS-*pnr*, embryos expressing *pnr* in the mesoderm under the control of a *twi-Gal4* driver. Abbreviation: hp, heart precursors. (E-J) Overspecification of cardiac cells in embryos expressing *pnr* throughout the mesoderm. Stage 14 (E,F) embryos stained for D-MEF2 protein, stage 16 (G,H) embryos stained for *lacZ* expression due to the *E2-3-9* enhancer trap marker, and stage 13 (I,J) embryos stained for *bap* RNA are shown. (E,G,I) Wild-type embryos; (F,H,J) embryos expressing *pnr* in the mesoderm under the control of a *twi-Gal4* driver. While 104 cardiac cell nuclei are present in normal embryos stained for D-MEF2 (E), an average of 153 nuclei are observed within the dorsal vessel of *pnr*-expressing embryos (F; $n=20$). As the latter counts did not include D-MEF2-positive nuclei present outside of the heart tube, the average increase is most likely an underestimate of the total number of cardiac cells induced. Arrows point to clusters of extra cardiac cell nuclei within the dorsal vessel (F,H), while the arrowhead indicates extra cells located outside of the heart tube (H).

Drosophila. *pnr* acts as a cardiac cell identity gene and works synergistically with *tin* in the specification of the cardiac cell fate. Likewise, the homologous GATA4 protein of mice can serve as a cardiogenic factor in *Drosophila*. These results demonstrate a combinatorial activity of Tin and GATA proteins during heart development and identify a conserved function for two GATA factors in cardiac cell specification and gene regulation.

MATERIALS AND METHODS

Fly strains

$y^{w^{67c23}}$ served as our wild-type stock. The Gal4 strain used to direct gene expression in the mesoderm and mesectoderm was *twi-Gal4* (Baylies and Bate, 1996). The UAS lines included *UAS-pnr* (Haenlin et al., 1997), *UAS-tin* (Yin and Frasch, 1998) and *UAS-GATA4* generated in this study. The latter was obtained by isolating a 1.7 kb mouse GATA4 cDNA, containing the complete protein open reading frame, from the pMT2-GATA4 expression vector (Arceci et al., 1993) and cloning it into the *EcoRI* site of pUAST (Brand and Perrimon, 1993). Insert orientation and integrity were confirmed by DNA sequencing. The pUAST-GATA4 DNA was injected into $y^{w^{67c23}}$ embryos and 11 transgenic lines were established using standard transformation procedures as described in Gajewski et al. (1997). Ten of these showed moderate to strong function when tested in the fly cardiogenic assays. The *pnr* mutant strain used was *pnr¹ ru st e ca/TM3, Sb* (Heitzler et al., 1996). *pnr¹* results in embryonic lethality when homozygous and may be considered a null allele based on the severe dorsal hole observed in mutant embryos. The *D-mef2* enhancer-*lacZ* reporter genes used were *IIA237* and *IIA341* (Gajewski et al., 1997, 1998). Several *pnr* mesodermal enhancer-*lacZ* lines, containing 457 bp of DNA from immediately upstream of the gene (Winick et al., 1993), were generated in this study. Six showed a reproducible pattern of *lacZ* gene expression in the dorsal mesoderm and amnioserosa of transformant embryos. The *E2-3-9* enhancer trap line has been described in Hartenstein and Jan (1992).

RNA in situ hybridization and embryo sectioning

A *pnr* cDNA cloned in pNB40 (Winick et al., 1993) was excised and inserted into pBluescript (Stratagene, La Jolla, CA). *tin* (NK4) and *bap* (NK3) cDNAs (Kim and Nirenberg, 1989) were also cloned into the same plasmid and linearized DNAs served as templates to generate antisense cRNA probes using the DIG RNA labeling kit (Roche, Mannheim, Germany). RNA expression in whole-mount embryos was detected using the protocol of Ingham and Jowett (1997), except that the prehybridization and hybridization steps were at 60°C. To investigate *pnr* expression in the mesoderm, stained embryos were postfixed for 5 minutes in 2% formaldehyde, rinsed in PBT and taken through a series of sucrose/PBS treatments (10%, 15%, 20%, 25%, 30% sucrose). Embryos were then saturated for 2 hours in a 1:1 mixture of 30% sucrose in PBS/Tissue-Tek OCT compound (VWR, West Chester, PA), transferred to a plastic OCT block and frozen on dry ice. 12 µm sections were obtained using a Reichert-Jung 2800 Frigocut Cryosectioner. Embryo sections or whole-mount embryos were mounted in 50% glycerol under coverslips for photography using a Zeiss Axioskop photomicroscope and Kodak Ektachrome 64T film.

Immunohistochemical staining of embryos

Embryos obtained from the mating of *twi-Gal4* and the various *UAS* lines were collected at 29°C, while all other embryos were collected at 23°C. Processing and immunostaining of whole-mount embryos was as described in Patel et al. (1987) using the Vectastain Elite ABC Kit (Vector, Burlingame, CA). Primary antibody dilutions were as follows: mouse anti-β-galactosidase (Promega, Madison, WI), 1:1000; rabbit anti-D-MEF2 (Lilly et al., 1995), 1:1000; mouse anti-

Even-skipped (Eve; Developmental Studies Hybridoma Bank, University of Iowa), 1:100; mouse mAb3 (Yarnitzky and Volk, 1995), 1:50; mouse anti-Fasciclin III (Developmental Studies Hybridoma Bank), 1:50; rat anti-Sim (Nambu et al., 1990), 1:200. Biotin-conjugated secondary antibodies (Vector) were as follows: horse anti-mouse, 3:1000; goat anti-rabbit, 1:1000; rabbit anti-rat, 1:300. Immunostained embryos were photographed as described for RNA processed embryos.

To quantitate the relative numbers of cardiac cells present in wild-type, *twi-Gal4;UAS-pnr* and *twi-Gal4;UAS-GATA4* embryos, D-MEF2-positive nuclei present within the dorsal vessel were counted in 20 embryos of the specific genotype. Similarly, the relative numbers of the Eve-expressing cells in wild-type versus *twi-Gal4;UAS-pnr* embryos were determined from counts of Eve-positive nuclei present in the dorsal mesoderm of 20 embryos of the two genotypes.

Gene expression analyses in mutant and Gal4/UAS embryos

To analyze the function of the *D-mef2* heart enhancer in *pnr* mutant embryos, the *IIA341 D-mef2* enhancer-*lacZ* fusion gene (integrated on chromosome III) was recombined onto the *pnr¹* chromosome and a stock of the genotype $y^{w}; IIA341 pnr^1/TM3, Sb, ftz-lacZ$ was generated and designated *P11*. Expression of the *D-mef2-lacZ* gene was assayed in *pnr* homozygous and heterozygous embryos, with the latter identified based on the expression of the marker linked to the balancer chromosome. Identical results were obtained when the *P11* flies were out-crossed to wild-type flies, followed by mating of F₁ $w^+ Sb^+$ siblings and staining of embryos for β-galactosidase activity. Likewise, the expression of the cardiac and pericardial cell markers were analyzed in embryos produced from matings of *pnr¹/TM3* or *P11/+* flies, with comparable results obtained in either of the genetic backgrounds.

To analyze mesodermal and neural markers in embryos with forced expression of *pnr*, *mGATA4* and/or *tin*, protein or RNA expression was assayed in embryos of the following genotypes: *twi-Gal4;UAS-pnr* and *twi-Gal4;UAS-GATA4*, obtained from crosses of homozygous *twi-Gal4* females to homozygous *UAS-pnr* or *UAS-GATA4* males and *twi-Gal4;UAS-pnr/IIA341* and *twi-Gal4;UAS-GATA4;IIA341*, obtained from crosses of homozygous *twi-Gal4;IIA341* females to homozygous *UAS-pnr* or *UAS-GATA4* males. For the analysis of the *IIA237* cardiac cell-specific enhancer, D-MEF2 protein and Sim protein in the mesectoderm, *twi-Gal4;IIA237* homozygous females were mated to *UAS-pnr*, *UAS-GATA4* or *UAS-tin* homozygous males and *UAS-tin/+;UAS-pnr/+* or *UAS-GATA4/+;UAS-tin/+* trans-heterozygous males.

RESULTS

pnr expression and function in the cardiac lineage

tin gene function is required for heart development in *Drosophila* (Azpiazu and Frasch, 1993; Bodmer, 1993). The initial programming of the cardiac lineage occurs at a time when *tin* is broadly expressed in the dorsal mesoderm (Fig. 1A). A subset of the *tin*-expressing cells will become heart precursors, appearing in 11 clusters along the dorsalmost part of the mesoderm (Fig. 1B). The *D-mef2* enhancer-*lacZ* fusion gene marks heart progenitors at stage 11 and will eventually be expressed in four pairs of cardiac cells per segment of the dorsal vessel (Gajewski et al., 1997, 1998; Nguyen and Xu, 1998). Thus, the *tin* expression domain is significantly larger than the territory of heart precursor specification, suggesting the involvement of additional factors in the formation of these cells.

The *D-mef2* heart enhancer requires the presence of at least

three elements for its activity, including two Tin binding sites and one GATA sequence (Gajewski et al., 1997, 1998). It was previously shown that the GATA gene *pnr* is expressed in cells of the dorsal ectoderm around the time of heart cell specification (Winick et al., 1993; Heitzler et al., 1996; Fig. 1C). However, there was no report of *pnr* transcription in the mesoderm. To investigate this possibility, we stained embryos for *pnr* RNA and examined embryo cross-sections. At late stage 10, gene expression is observed in the dorsal ectoderm of the germband-extended embryo (Fig. 1D). We also detected *pnr* RNA in four clusters of cells located in the dorsalmost part of the mesoderm that corresponds to the cardiogenic region. Additionally, we identified a *pnr* mesodermal enhancer that directs *lacZ* expression in the heart-forming region, but not in the overlying ectoderm (Fig. 1E-G). Therefore, *pnr* is expressed in the cardiogenic mesoderm where it could function in cardiac cell specification and the regulation of *D-mef2* transcription.

The *D-mef2* IIA341 enhancer is active in both cardiac and ventral muscle founder cells (Fig. 2A,C) and a GATA site is needed for the cardiac cell expression (Gajewski et al., 1998). To determine if *pnr* function is important for enhancer activity in either of these cell types, we monitored the expression of the enhancer-*lacZ* fusion in *pnr* mutant embryos. We observed a strong reduction of reporter gene expression in the dorsal mesoderm of the mutants (Fig. 2B), while normal *lacZ* expression was detected in the ventral muscle precursors (Fig. 2D). These results suggest Pnr is a transcriptional regulator of the *D-mef2* heart enhancer, consistent with the observation that the protein can bind the essential GATA sequence in an electrophoretic mobility shift assay (data not shown). Alternatively, or in addition, these results could be indicative of a requirement of *pnr* function for the formation of the *D-mef2*-expressing cardiac cells.

To determine if *pnr* function was essential for the specification of cardiac cells, wild-type and mutant embryos were stained for D-MEF2 protein that serves as a marker for these cells. A distinct row of cardiac cells is observed in a lateral view of normal embryos at stage 13 as they migrate dorsally along the overlying ectoderm during the process of dorsal vessel formation (Fig. 2E). In contrast, these cells are greatly diminished or completely absent from the dorsal mesoderm of mutant embryos (Fig. 2F). To assess the formation of the pericardial cells in the same genetic background, embryos were stained for Eve protein, which is a marker for a subset of these cells. Eleven clusters of Eve-positive cells, each comprising three or four cells, are detected in wild-type embryos at stage 12 (Fig. 2G). In contrast, an overabundance of Eve-expressing pericardial cells is observed in the *pnr* embryos (Fig. 2H). These results suggest that *pnr* function is vital to the formation of cardiac cells, while simultaneously playing an important role in controlling the production of at least one pericardial cell type.

Forced expression of *pnr* results in supernumerary cardiac cells

To test the possibility that Pnr is an activator of the *D-mef2* heart enhancer, we used the Gal4/UAS system (Brand and Perrimon, 1993) to force the expression of the GATA factor throughout the mesoderm, while monitoring the activity of the *lacZ* reporter gene under the control of the regulatory sequence.

The driver line used was *twi-Gal4*, which directs Gal4 expression in the mesoderm throughout embryogenesis (Baylies and Bate, 1996). As noted, the enhancer is active in 11 clusters of heart precursor cells in wild-type embryos by stage 11 (Fig. 3A). In comparably staged embryos expressing *pnr* throughout the mesoderm, we observed a strong overactivation of the fusion gene in the dorsal mesoderm with both the intensity and cellular realm of β -galactosidase activity increased (Fig. 3B). In normal embryos at stage 16, the enhancer is active in four pairs of cardiac cells in most segments (Fig. 3C). In contrast, an enlarged heart was seen in embryos with ectopic *pnr* expression (Fig. 3D). These results demonstrate that Pnr can activate the *D-mef2* heart enhancer and forced expression of the protein culminates in increased numbers of cells in the dorsal vessel.

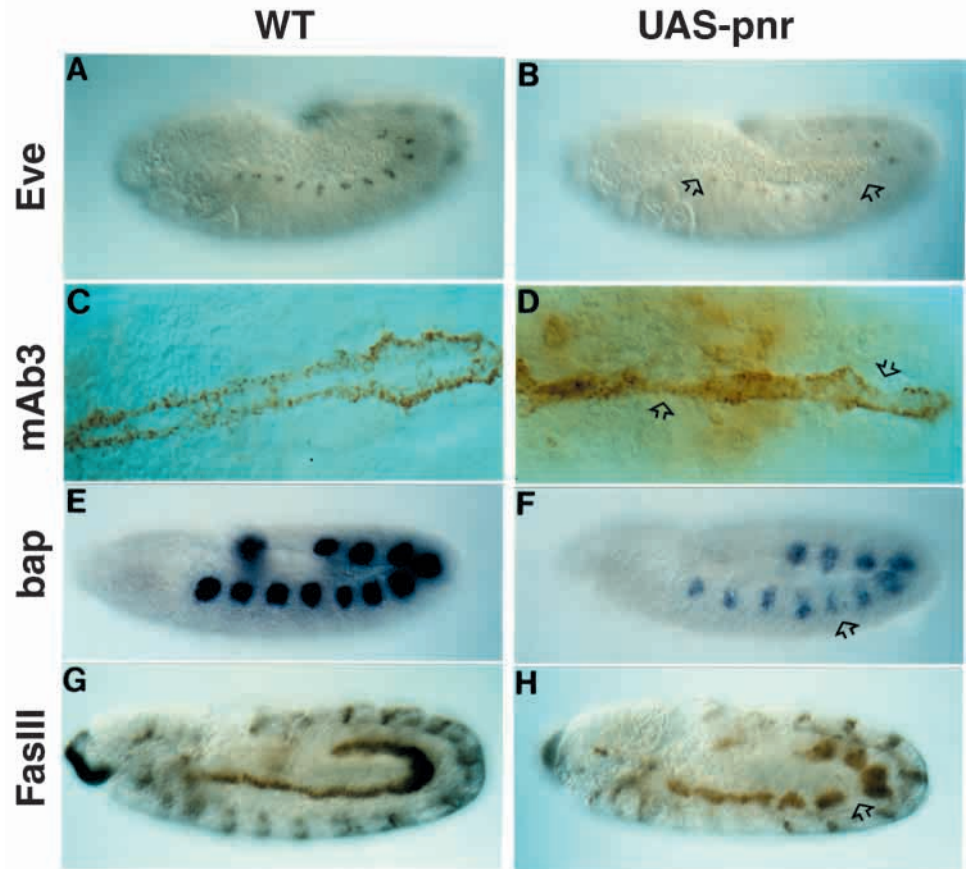
With an enlargement of the heart in embryos with ectopic *pnr* expression, it was of interest to determine the identity of the additional cells. D-MEF2 protein, *bagpipe* (*bap*) RNA (Azpiazu and Frasch, 1993) and the *E2-3-9* enhancer trap insertion (Hartenstein and Jan, 1992) detect cardiac cells in the heart. These markers were used to assess the relative numbers of cells in *pnr*-expressing embryos as compared to wild type. All markers showed a considerable increase in cardiac cell count within the heart tube and throughout dorsal regions of the embryos (Fig. 3E-J). For example, an average 47% increase was observed in the number of D-MEF2-positive nuclei present within the dorsal vessel of *pnr*-expressing embryos. Thus, forced mesodermal expression of Pnr leads to an overproduction of cardiac cells.

With the generation of supernumerary cardiac cells, we wanted to ascertain if this increased population occurred at the expense of other derivatives of the dorsal mesoderm. The use of diagnostic markers indicated the gain in cardiac cells was paralleled by reduced levels of staining for the other cell types. As D-MEF2 is expressed in all muscles in the embryo, it was used to assess the formation of dorsal body wall muscles. In the *pnr*-expressing embryos, a decrease and abnormal organization of these muscles was observed (Fig. 3F). Likewise, there was an average 40% diminution in the population of Eve pericardial cells based on the absence or reduction in the Eve-expressing cell clusters (Fig. 4B) and the pericardial cell-associated antigen detected by mAb3 also exhibited an abnormal expression pattern (Fig. 4D). Finally, there was an apparent loss of cells within the visceral mesoderm lineage based on the decreased levels of *bap* RNA (Fig. 4F) and the patchy expression of FasIII in visceral muscles (Fig. 4H). These results suggest there may be a recruitment of cells into the cardiac lineage at the expense of other dorsal mesodermal cell types. Also, since there is an increase of cardiac but not Eve-expressing pericardial cells in the hearts of ectopic *pnr*-expressing embryos, we propose that Pnr is functioning as a cell-specific cardiogenic factor within this genetic context.

Mouse GATA4 can function as a cardiogenic factor in *Drosophila*

Pnr and the GATA4 protein of mice share an 85% sequence identity in regions that include their two zinc finger domains that are involved in protein-protein interactions and DNA binding (Fig. 5A). Since GATA4 is expressed in the cardiac lineage and regulates several heart-expressed genes in

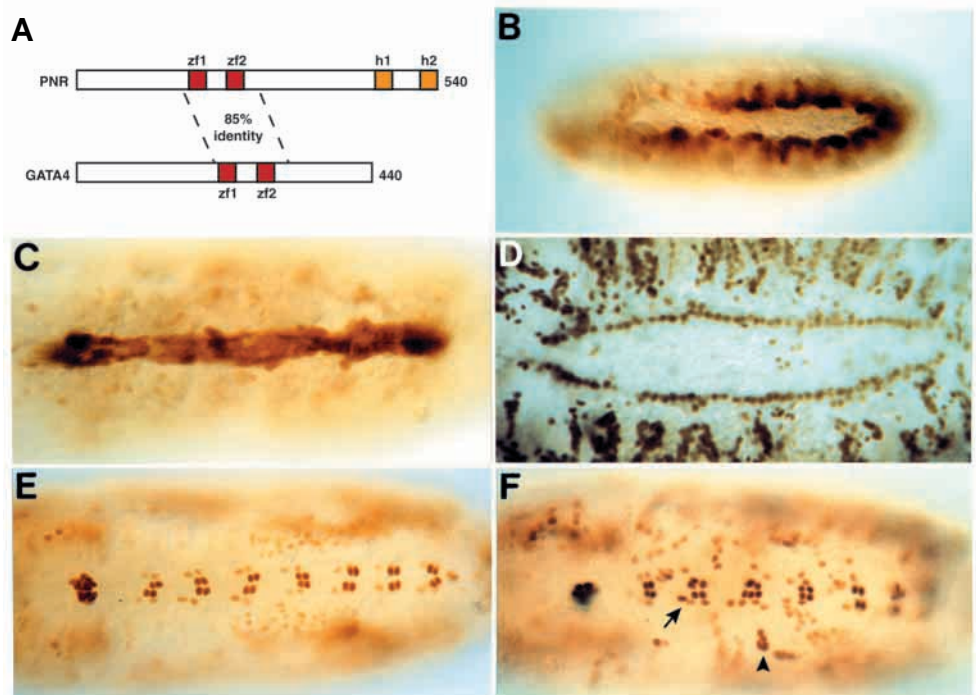
Fig. 4. Reduction of non-cardial dorsal mesodermal cell types in embryos with forced expression of *pnr*. (A,C,E,G) WT, wild-type embryos; (B,D,F,H) UAS-Pnr, embryos expressing *pnr* in the mesoderm under the control of a *twi-Gal4* driver. Several probes were used to assess the status of various derivatives of the dorsal mesoderm. (A,B) anti-Eve, precursors of pericardial cells and body wall muscle DA1; (C,D) mAb3, pericardial cells of the mature dorsal vessel; (E,F) *bap* RNA, precursors of the visceral mesoderm; (G,H) anti-FasIII, visceral muscle cells. Open arrows indicate an absence of cells or reduced staining of markers.



vertebrates, we wanted to test its ability to function in *Drosophila* embryos when expressed in the mesoderm. Using the *twi-Gal4* driver, we directed GATA4 expression in the mesoderm and monitored the activation of the *D-mef2* heart enhancer and the formation of cardiac cells. As was observed with Pnr, forced expression of GATA4 results in a robust activation of the *D-mef2-lacZ* fusion gene in the dorsal mesoderm (Fig. 5B; compare to Fig. 3A). Additionally, GATA4 expression programs an overproduction of cardiac cells in the dorsal vessel based on several diagnostic markers including the *D-mef2-lacZ* fusion

gene (Fig. 5C; compare to Fig. 3C), D-MEF2 protein (Fig. 5D; compare to Fig. 3E) and the *E2-3-9* enhancer trap insertion (Fig. 5F). Based on the D-MEF2 staining, an average 17% increase in the number of cardiac cells was observed in the

Fig. 5. Cardiogenic function of the mouse GATA4 transcription factor in *Drosophila* embryos. (A) Schematic diagram of Pnr and GATA4 illustrating an 85% sequence identity over 114 and 111 amino acids of the proteins, respectively. zf1 and zf2 represent the conserved zinc finger domains of the two factors while h1 and h2 denote the two putative amphipathic helices of Pnr. (B) Stage 11 and (C) stage 16 embryos of the genotype *twi-Gal4;UAS-GATA4* stained for *lacZ* expression under the control of a *D-mef2* cardiac cell enhancer. (D) Stage 14 embryo of the same genotype stained for D-MEF2 protein. An average of 122 D-MEF2-positive nuclei are observed within the dorsal vessel of GATA4-expressing embryos ($n=20$). (E,F) Stage 16 wild-type and *twi-Gal4;UAS-GATA4* embryos, both carrying the *E2-3-9* enhancer trap insertion chromosome that results in β -galactosidase activity in subsets of cardiac cell nuclei. The arrow points to a cluster with extra cardiac cell nuclei within a segment of the dorsal vessel, while the arrowhead indicates extra cardiac cells located outside of the heart tube.



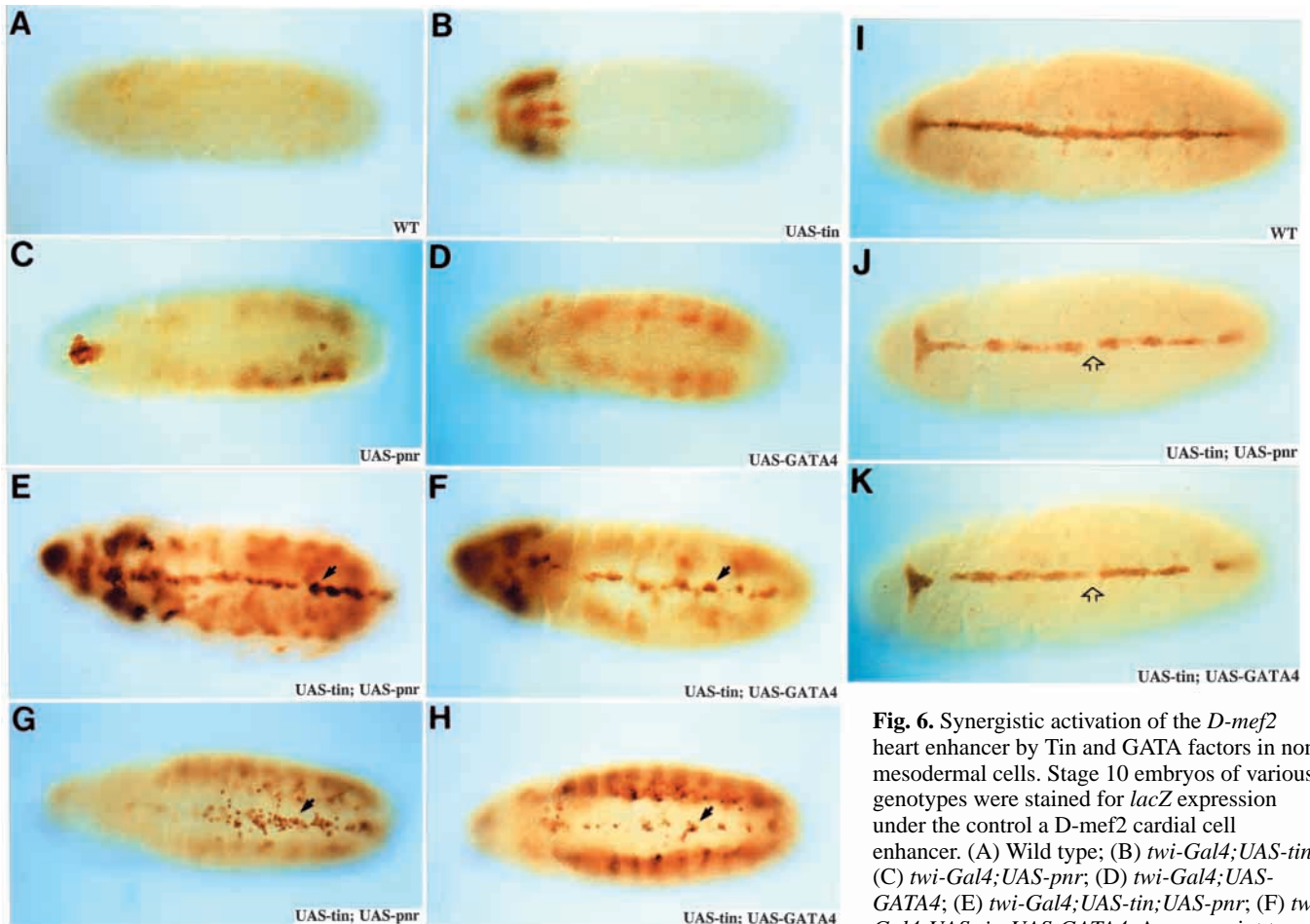


Fig. 6. Synergistic activation of the *D-mef2* heart enhancer by Tin and GATA factors in non-mesodermal cells. Stage 10 embryos of various genotypes were stained for *lacZ* expression under the control a *D-mef2* cardiac cell enhancer. (A) Wild type; (B) *twi-Gal4;UAS-tin*; (C) *twi-Gal4;UAS-pnr*; (D) *twi-Gal4;UAS-GATA4*; (E) *twi-Gal4;UAS-tin;UAS-pnr*; (F) *twi-Gal4;UAS-tin;UAS-GATA4*. Arrows point to

new expression of the heart reporter gene along the CNS ventral midline in the latter two genotypes. (G,H) Presence of cardiac cells in the CNS based on D-MEF2 protein staining in *twi-Gal4;UAS-tin;UAS-pnr* and *twi-Gal4;UAS-tin;UAS-GATA4* embryos, respectively. (I-K) Co-expression of Tin and GATA factors in the mesectoderm results in reduced expression of the CNS midline cell regulator Sim. Stage 10 embryos of differing genotypes were stained for Sim protein in ventral midline cells: (I) wild type; (J) *twi-Gal4;UAS-tin;UAS-pnr*; (K) *twi-Gal4;UAS-tin;UAS-GATA4*. Arrows indicate the significant loss of Sim expression and midline cells within the CNS.

dorsal vessel of GATA4-expressing embryos. Overall, such enhancer regulation and cardiac cell induction demonstrate that the mouse GATA4 protein can function as a cardiogenic factor in *Drosophila*. Since mouse GATA1 (data not shown) and *Drosophila* Serpent (D. Hoshizaki, personal communication) fail to function comparably in heart induction assays, specificity exists in these cardiogenic properties of Pnr and GATA4.

Synergistic activity of the Tin and GATA factors in cardiac cell specification

Certain NK-2 class homeodomain and GATA family proteins have been shown to physically interact in their cooperative activation of gene expression in cell culture systems (Durocher et al., 1997; Lee et al., 1998; Sepulveda et al., 1998). To test the possibility that Tin and GATA factors could functionally interact in an embryological context, we expressed the *tin*, *pnr* and *mGATA4* genes independently or in combination in *Drosophila* embryos. The driver line used was again *twi-Gal4*, which directs Gal4 expression in the mesoderm throughout embryogenesis and also transiently in cells of the mesectoderm through stage 10 (Baylies and Bate, 1996). This latter region

will give rise to midline cells of the central nervous system (CNS). To monitor aspects of cardiogenesis, we included in the various genetic backgrounds the IIA237 cardiac cell-specific enhancer of *D-mef2* linked to *lacZ* (Gajewski et al., 1997).

The results of such experiments are presented in Figure 6, with a focus on the expression of the cardiac marker in the non-mesodermal midline cells. When *tin*, *pnr* or *mGATA4* are expressed alone in the *twi* enhancer-expressing cells, the *D-mef2* heart enhancer is activated ectopically in the cephalic (*tin*; Fig. 6B) or dorsal (*pnr* and *mGATA4*; Fig. 6C,D) mesoderm. As Tin is a known regulator of this enhancer, it could be activating the *D-mef2* sequence in the head region through its fortuitous interaction with a co-factor normally expressed in these cells. The results are striking when both Tin and either of the GATA factors are co-expressed under the control of the *twi-Gal4* driver (Fig. 6E,F). The cardiac cell marker is now activated in both the cephalic region and throughout the dorsal and ventral trunk mesoderm. Likewise, a strong ectopic expression of the *D-mef2* heart enhancer is detected in ventral midline cells of the developing CNS. The data point to a combinatorial interaction of Tin and the two GATA factors in the de novo activation of the cardiac cell marker in both

mesodermal and non-mesodermal cells. They also suggest these genetic combinations are inducing a cardiac cell fate along the ventral midline of the CNS. In support, we observed the activation of the endogenous *D-mef2* gene in ventral midline cells as well (Fig. 6G,H).

If cells are being directed into the cardiac lineage and away from a neural fate, genes normally transcribed along the ventral midline should be reduced in their expression. To test this prediction, we monitored the expression of the Single-minded (Sim) protein in wild-type embryos relative to those expressing the Tin-Pnr or Tin-GATA4 combinations. Sim is a bHLH class transcription factor that functions as a master regulator of CNS midline cell development (Nambu et al., 1990). In wild-type embryos, Sim is expressed in all midline cells (Fig. 6I). In contrast, an interrupted pattern of Sim was observed in embryos expressing Tin-Pnr (Fig. 6J) or Tin-GATA4 (Fig. 6K). The absence of this key regulator from cells along the ventral midline is consistent with such cells adopting a cardiac fate.

DISCUSSION

In this report, we demonstrate the *Drosophila* GATA gene *pnr* is expressed in the cardiogenic mesoderm where it has a crucial function in heart development based on its role in promoting the cardiac cell lineage while preventing the over-production of Eve-expressing pericardial cells. With the non-overlapping expression patterns of the Ladybird early and Eve proteins in cardiac and pericardial precursors, respectively, these lineages appear to arise from neighboring, yet distinct primordia within repeated parasegmental domains of the dorsal mesoderm (Azpiazu et al., 1996; Jagla et al., 1997). Possibly through interactions with different co-factors in the distinct progenitor types, the Pnr protein plays an instructive role in the formation of heart muscle cells and a repressive function in the production of Eve non-muscle cells. Thus, through combinatorial interactions, Pnr could carry out dual functions during the early stages of cardiogenesis. The ability of *pnr* to function as a transcriptional regulator of *achaete* and *scute* in the establishment of thoracic bristle pattern, mediated through genetic interactions with the *u-shaped* modulator gene, has been demonstrated previously (Haenlin et al., 1997; Garcia-Garcia et al., 1999).

tin gene function is also required for the specification of different cardiac cell types, but the expression domain of the gene is much broader than the limited regions of heart precursor formation (Azpiazu and Frasch, 1993; Bodmer, 1993). It has been proposed that *tin* endows the dorsal mesoderm with the competence to form the heart (and other derivatives), yet additional factors must work with the gene since ectopic *tin* expression does not result in the production of ectopic heart tissues (Yin and Frasch, 1998). The expression patterns and cardiac phenotypes of the *wg* and *sloppy paired* genes are consistent with one or both of the genes being centrally involved in the spatially restricted production of heart precursors along the embryonic anteroposterior axis (Lawrence et al., 1995; Wu et al., 1995; Azpiazu et al., 1996; Park et al., 1996; Jagla et al., 1997; Riechmann et al., 1997; Gajewski et al., 1998). However, the function of at least one additional component must be invoked for the formation of heart cells in the dorsalmost part of the mesoderm. Based on genetic results

presented in this study, Pnr can function as a cardiogenic factor that works combinatorially with Tin to specify the cardiac cell fate.

Our prior analysis of a cardiac cell enhancer of the *D-mef2* gene showed that both Tin and GATA sequence elements were required for proper cardiac cell expression (Gajewski et al., 1997, 1998). This work predicted the existence of a heart-expressed GATA factor in flies and the robust activation of *D-mef2-lacZ* reporters in embryos with forced expression of *pnr* argues strongly for this protein being a direct activator of the enhancer. In the ectopic Pnr-expressing embryos, enhancer activation and cardiac cell formation are not restricted to the dorsalmost part of the mesoderm, but appear to coincide with the broader domain of *tin* gene expression. It is possible that, in wild-type embryos, Pnr activity is limiting while Tin is in excess, leading to normal levels of enhancer expression and cardiac cell formation in the dorsalmost mesoderm. However, when Pnr is induced and maintained at high levels throughout the mesoderm, it could then act coordinately with Tin to turn on shared target genes and induce supernumerary cardiac cells in an expanded cardiogenic domain. When both factors are expressed simultaneously, the *D-mef2* enhancer is activated and the cardiac cell specification program is induced in mesodermal and neural cell types. Taken together, these results indicate that, in the presence of *tin* function and other genetic inputs, *pnr* can function as a cardiac cell identity gene.

In vertebrates, the GATA4, GATA5, and GATA6 transcription factors are expressed in overlapping patterns during heart development with transcripts from all three genes detected in the early precardiac mesoderm (Arceci et al., 1993; Kelley et al., 1993; Heikinheimo et al., 1994; Laverriere et al., 1994; Jiang and Evans, 1996; Morrissey et al., 1996, 1997; Jiang et al., 1998). Considering also the extensive studies on the ability of these proteins to activate cardiac gene expression in cultured cells (Grepin et al., 1994; Ip et al., 1994; Molkenkin et al., 1994; Durocher et al., 1997; Murphy et al., 1997; Lee et al., 1998; Sepulveda et al., 1998), it is likely that one or more of these factors play a pivotal role in controlling cardiomyocyte specification. However, all three genes have been individually mutated in mice and phenotypic studies to date have failed to demonstrate a requirement for any of the transcriptional regulators in cardiac lineage commitment. *GATA4* null mice form bilateral heart tubes that fail to fuse at the ventral midline, yet these tubes contain differentiated cardiomyocytes that express numerous cardiac structural genes (Kuo et al., 1997; Molkenkin et al., 1997). *GATA5* null mice are viable (Molkenkin et al., 1997) and *GATA6* mutant embryos die shortly after implantation, a result that precludes the analysis of a potential heart phenotype (Morrissey et al., 1998; Koutsourakis et al., 1999). It is possible that functional redundancies exist among the genes and the unmasking of a cardiac specification defect may only occur through the analysis of animals harboring combinations of null and conditional mutant alleles.

The use of the Gal4/UAS expression system (Brand and Perrimon, 1993) has allowed us to develop an assay to test the ability of genes to promote cardiac cell formation. Pnr and mouse GATA4 share an 85% amino acid identity within their zinc finger dimerization and DNA-binding domains. These regions have also been shown to mediate protein-protein interactions with heterologous transcription factors (Durocher

et al., 1997; Haenlin et al., 1997; Tsang et al., 1997; Lee et al., 1998; Molkentin et al., 1998; Sepulveda et al., 1998; Svensson et al., 1999; Tevosian et al., 1999). Based on the ability of *pnr* to activate the *D-mef2* heart enhancer, cause cardiac hyperplasia and synergize with *tin* in inducing cardiac cell markers, we tested the capacity of *mGATA4* to function similarly. The GATA4 protein can perform like Pnr in all of these processes, indicating it can function as a cardiogenic factor in the *Drosophila* system. Given these results, it will be of interest to test the ability of GATA4 to rescue phenotypes associated with *pnr* loss-of-function, including heart defects and other non-mesodermal developmental abnormalities. In contrast to GATA4, the non-heart expressed GATA1 protein is inactive in heart induction assays even though it shares a 76% amino acid identity with Pnr throughout their conserved domains. The molecular basis of this specificity is currently unknown, but the analysis of systematically altered forms of the GATA factors should provide insights into their functional characteristics.

In summary, the discovery of early heart phenotypes in *pnr* mutant embryos, coupled with the demonstration of uniquely conserved cardiogenic abilities of Pnr and GATA4, provide novel evidence for the function of GATA family members in the specification of a heart cell type. In an embryological context, these proteins can work with the Tin homeodomain factor to program cells into an apparent cardiac fate in both mesodermal and non-mesodermal cell types. This genetic combination appears to be essential, but not necessarily sufficient, for cellular commitment to the cardiac lineage as other factors may contribute to the specification process. Additional studies using the *Drosophila* cardiogenic assay should prove instrumental in revealing other key members of this genetic program.

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