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D-mef2 is a target for Tinman activation during *Drosophila* heart development

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The NK-type homeobox gene *tinman* and the MADS box gene *D-mef2* encode transcription factors required for the development and differentiation of the *Drosophila* heart, and closely related genes regulate cardiogenesis in vertebrates. Genetic analyses indicate that *tinman* and *D-mef2* act at early and late steps, respectively, in the cardiogenic lineage. However, it is unknown whether regulatory interactions exist between these developmental control genes. We show that *D-mef2* expression in the developing *Drosophila* heart requires a novel upstream enhancer containing two Tinman binding sites, both of which are essential for enhancer function in cardiac muscle cells. Transcriptional activity of this cardiac enhancer is dependent on *tinman* function, and ectopic Tinman expression activates the enhancer outside the cardiac lineage. These results define the only known *in vivo* target for transcriptional activation by Tinman and demonstrate that *D-mef2* lies directly downstream of *tinman* in the genetic cascade controlling heart formation in *Drosophila*.

Keywords: cardiogenic factors/*D-mef2*/heart/*tinman*/transcriptional enhancer

Introduction

Recent studies suggest that the molecular pathways controlling heart formation are evolutionarily ancient and conserved in flies and vertebrates (reviewed in Scott, 1994; Bodmer, 1995; Olson and Srivastava, 1996). The heart is formed in these organisms from a subset of mesodermal precursor cells that become committed to a cardiogenic fate in response to inductive signals from adjacent cells. In *Drosophila melanogaster*, heart precursor cells originate from the dorsal-most region of the mesoderm (Bodmer *et al.*, 1990; Azpiazu and Frasch, 1993; Rugendorff *et al.*, 1994; Dunin Borkowski *et al.*, 1995) and are formed in response to the Wingless (Wg) and Decapentaplegic (Dpp) growth factors (Staehling-Hampton *et al.*, 1994; Frasch, 1995; Lawrence *et al.*, 1995; Wu *et al.*, 1995; Park *et al.*, 1996). These cardiac precursors give rise to a bilaterally symmetric heart-like organ called the dorsal vessel, which is comprised of inner contractile cardial cells and outer pericardial cells (Rugendorff *et al.*, 1994).

NK-type homeobox genes (Kim and Nirenberg, 1989) have been shown to play important roles in cardiogenesis (reviewed in Harvey, 1996). Among this class of genes is *tinman*, which is required for the genesis of cardiac precursor cells and heart formation in *Drosophila* (Azpiazu and Frasch, 1993; Bodmer, 1993). Several *tinman*-related genes have been identified in vertebrates (Komuro and Izumo, 1993; Lints *et al.*, 1993; Tonissen *et al.*, 1994; Evans *et al.*, 1995; Schultheiss *et al.*, 1995; Buchberger *et al.*, 1996; Lee *et al.*, 1996), including *Nkx-2.5* which is expressed throughout the development of the cardiac lineage (Lints *et al.*, 1993) and is required for correct morphogenic and myogenic development of the mouse heart (Lyons *et al.*, 1995). Although the cardiogenic functions of Tinman and Nkx-2.5 have been determined genetically, it is unknown how they exert their effects in the cardiac lineage as no direct target genes for these factors have been identified.

Members of the myocyte enhancer factor-2 (MEF2) family of MADS box transcription factors have also been shown to play important roles in heart development in flies and vertebrates (Bour *et al.*, 1995; Lilly *et al.*, 1995; Ranganayakulu *et al.*, 1995; reviewed in Olson *et al.*, 1995; Q.Lin, J.Schwartz and E.N.Olson, submitted). The single *mef2* gene in *Drosophila* (*D-mef2*) is expressed in the cardiac, somatic and visceral muscle lineages (Lilly *et al.*, 1994; Nguyen *et al.*, 1994; Taylor *et al.*, 1995) where it is required for the differentiation of these different muscle types. In the cardiac lineage, D-MEF2 expression is detected initially in heart precursor cells and later is restricted to the cardial cells of the heart tube. In *D-mef2* mutant embryos, heart precursors are specified properly based on the correct expression of *tinman* (Lilly *et al.*, 1995) and Even-skipped (Bour *et al.*, 1995), but cardial cells are unable to differentiate and fail to express contractile protein genes (Bour *et al.*, 1995; Lilly *et al.*, 1995; Ranganayakulu *et al.*, 1995). Vertebrate species contain four *mef2* genes which are also expressed in the early cardiogenic lineage where they are important for activating muscle structural genes (Chambers *et al.*, 1994; Edmondson *et al.*, 1994; Molkentin *et al.*, 1996; Q.Lin, J.Schwartz and E.N.Olson, submitted).

The phenotypes of *tinman* and *D-mef2* mutant embryos suggest that *tinman* acts at an early step in the cardiogenic pathway to establish cardiac cell identity, whereas *D-mef2* acts later to control the expression of cardiac structural genes. However, the potential hierarchical relationship between these two genes has not been investigated previously. To define further the roles of MEF2 factors in heart development, we have initiated an analysis of the

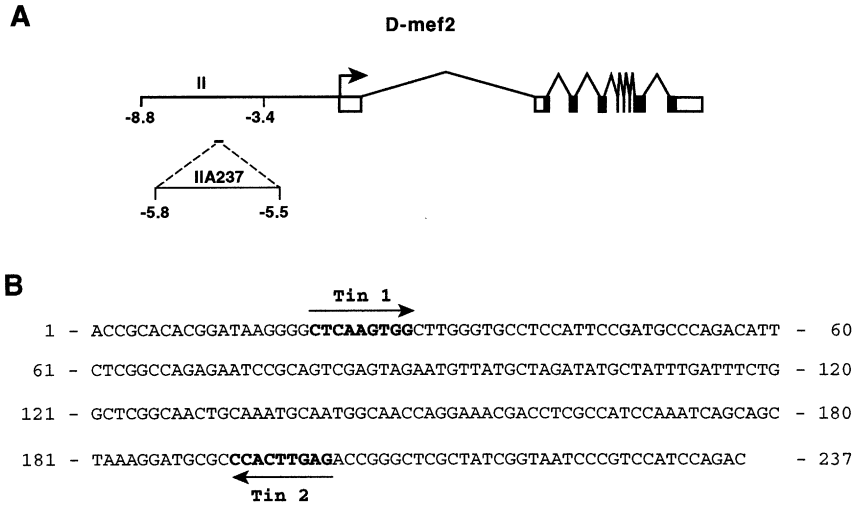

Fig. 1. Location of a minimal *D-mef2* heart enhancer. (**A**) Schematic of the *D-mef2* gene and adjacent 5′-flanking sequence. DNA II is a 5.4 kb region previously shown to contain enhancer elements for cardiac, somatic and visceral muscle cell expression. DNA IIA237 is a 237 bp subregion containing an enhancer element for cardial cell expression. (**B**) Sequence of the IIA237 DNA. Two consensus Tinman binding sites (Tin 1 and Tin 2) are shown in bold.

cis-acting sequences that control the expression of *D-mef2* in the cardiogenic lineage. Here we describe a novel cardiac enhancer that directs *D-mef2* expression in the developing heart and we show that this enhancer is a direct target for transcriptional activation by Tinman.

Results

Tinman binding sites in the regulatory sequences flanking the D-mef2 gene

Sequences controlling *D-mef2* gene expression in embryonic cardiac, somatic and visceral muscle cell lineages have been mapped previously to a 13 kb interval between –12.4 and +0.6 relative to the start of the *D-mef2* transcription unit (Lilly *et al.*, 1995; Schulz *et al.*, 1996). The 5.4 kb region II DNA (between –8.8 and –3.4 kb) contained enhancer sequences for all three cell types and was the only DNA that directed *lacZ* reporter gene expression in the developing heart (Figure 1A). To delimit further the *D-mef2* heart enhancer element, this DNA was sequenced and scanned for potential Tinman binding sites which might be involved in regulating cardial cell expression. Bacterially produced Tinman binds to the sequence T(C/T)AAGTG (Y.Kim, unpublished data) and two identical, convergently oriented CTCAAGTGG sequences that match the Tinman binding consensus were found between –5.8 and –5.5 kb upstream of *D-mef2* in a region we refer to as IIA237 (Figure 1B).

To test whether Tinman can bind to these sites, a DNase I protection assay was performed using a glutathione *S*-transferase (GST)–Tinman fusion protein and IIA237 DNA labeled specifically on either strand. The fusion protein protected both sites on both strands of the IIA237 DNA (Figure 2). These sequences have, therefore, been designated Tin 1 and Tin 2 for Tinman binding sites 1 and 2.

IIA237 DNA functions as a D-mef2 heart enhancer regulated by the tinman gene

To test the IIA237 region for heart enhancer function, the DNA was cloned upstream of a minimal *Hsp43* promoter driving the *lacZ* gene, and germline transformant lines harboring this construct were established. Heart precursor cells first appear at stage 11, and IIA237 transformant embryos expressed the β-galactosidase marker in these progenitors by stage 12 (Figure 3A). In stage 13 embryos, β-galactosidase was detected in heart precursors located along the length of the dorsal-most part of the mesoderm (Figure 3B) and, in late stage embryos, staining was observed in the inner cardial cells of the newly formed dorsal vessel (Figure 3C). Thus, the 237 bp DNA was sufficient for directing reporter gene expression in the cardiac cell lineage and contained a heart enhancer element. Embryos from a total of six IIA237–*lacZ* transformant lines were assayed, and five showed expression in heart precursor and cardial cells. However, β-galactosidase expression was consistently lower in these embryos compared with the levels observed in the 5.4 kb DNA II–*lacZ* transformants, suggesting that an additional sequence(s) in the region II DNA is required for the proper quantitative expression of *D-mef2* in the heart.

To verify that the reporter gene was being expressed in progenitor cells of the heart, we generated a fly strain that was homozygous for the *D-mef2–lacZ* fusion gene in a tin^{346}/*TM3* genetic background. By intermating these flies, we could monitor the expression of the *lacZ* gene in *tinman* null embryos that fail to specify cardial cells. β-Galactosidase was not detected in dorsal regions of the mutant embryos, consistent with the conclusion that the 237 bp *D-mef2* enhancer is expressed in heart precursor cells (Figure 4A and C). In comparison, the *D-mef2–lacZ* fusion gene was expressed in the forming and mature heart of tin^{346} heterozygous embryos (Figure 4B and D).

To address further the relationship of *tinman* gene function to *D-mef2* enhancer expression, we introduced the *D-mef2–lacZ* reporter into the $hsTin^{9A}$ strain which contains an inducible *tinman* gene under the control of a heat shock promoter. Ectopic expression of Tinman in heat-shocked embryos resulted in an expanded activation of the enhancer and expression of the β-

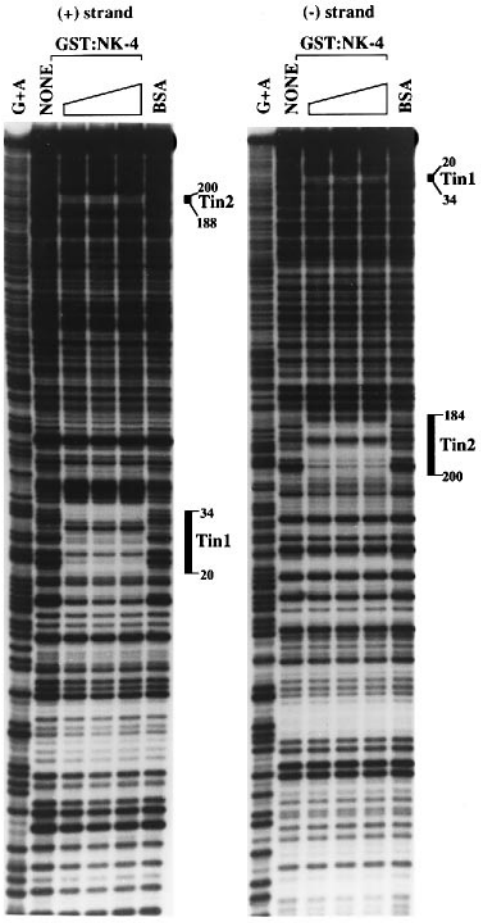

Fig. 2. Tinman binds *in vitro* to the *D-mef2* IIA237 heart enhancer. DNase I protection assay performed with either a ^{32}P-labeled (+) or (–) strand of the IIA237 DNA and increasing amounts of a glutathione *S*-transferase–Tinman fusion protein (GST:NK-4). Lanes: G+A, chemical sequencing reaction of the IIA237 DNA; NONE, reaction without fusion protein added; GST:NK-4, reactions with increasing amounts of fusion protein (50, 100 and 200 ng); BSA, reaction with bovine serum albumin control protein added. Tin1 and Tin2 boxes indicate the two sequences protected from DNase I digestion.

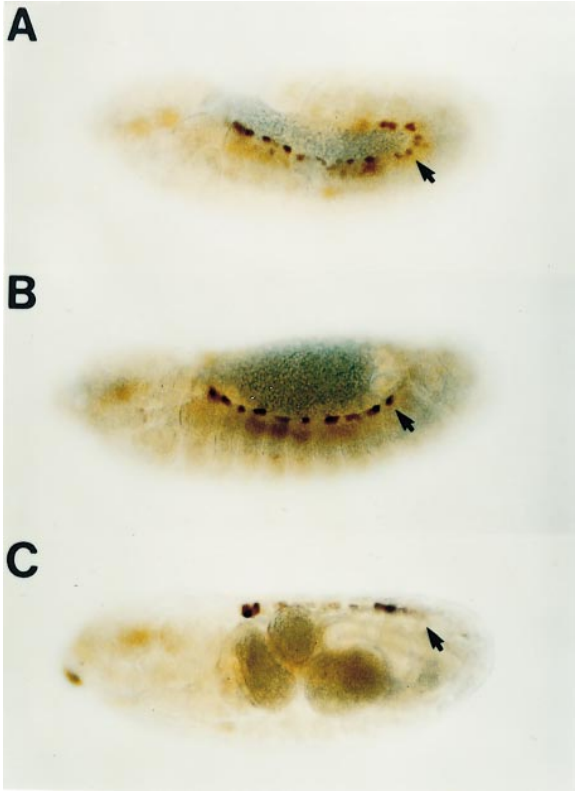

Fig. 3. IIA237 DNA functions as a *D-mef2* heart enhancer. *D-mef2–Hsp43–lacZ* fusion gene expression in transformant embryos. (**A**) Stage 12, (**B**) stage 13, (**C**) stage 17 embryos. Arrows point to β-galactosidase expression in progenitor or differentiated heart cells.

galactosidase marker, especially in cells of the anterior procephalic region and the dorsal mesoderm of the extended germ band (Figure 4E). Relatively little induced β-galactosidase expression was detected in ventrally positioned cells of the heat-shocked embryos. Overall, these results indicate that Tinman can activate the expression of the *D-mef2* heart enhancer, but only certain domains of the germ band extended embryo are permissive for transcriptional activation by Tinman. As a control, non heat-shocked embryos showed the normal pattern of β-galactosidase expression in the heart precursor cells (Figure 4F).

Both Tinman binding sites are required for D-mef2 heart enhancer activity in transformant embryos

The functional importance of the Tinman binding sites in the *D-mef2* cardiac enhancer was assessed through the generation of a series of constructs that contained deletions or nucleotide changes in the Tin 1 and Tin 2 sequences (Figure 5). Transformant lines carrying the mutant *D-mef2–lacZ* fusion genes were established and embryos were stained for β-galactosidase. In the IIA166 construct, both ends of the enhancer including the Tin 1 and Tin 2 sites were deleted. This DNA failed to express in cardial cells. Likewise, deletion of only the 27 bp 5′ region (IIA210) or the 44 bp 3′ region (IIA193) inactivated the enhancer. These results suggested that either both Tinman binding sites were required, or other elements located in the 5′ and 3′ sequences flanking the Tin sites must be present for the functional integrity of the *D-mef2* heart enhancer. To discriminate between these possibilities, we created three variant enhancers containing mutations in each Tinman binding site alone or in combination. The mutations in these sites are known to abolish Tinman binding to DNA (Y.Kim, unpublished data). None of the three mutant enhancers were able to direct *lacZ* expression in heart precursor or cardial cells in transformant embryos, indicating that both Tinman binding sites must be present in the IIA237 DNA for the activity of the *D-mef2* enhancer. Together, these results demonstrate that Tinman is a direct activator of *D-mef2* transcription in the developing heart of *Drosophila* and that transcriptional activation by Tinman requires binding to two sites in the cardiac enhancer.

Discussion

Expression patterns and functions of tinman and D-mef2

The results of this and previous studies (Lilly *et al.*, 1995; Schulz *et al.*, 1996) reveal that *D-mef2* transcription in

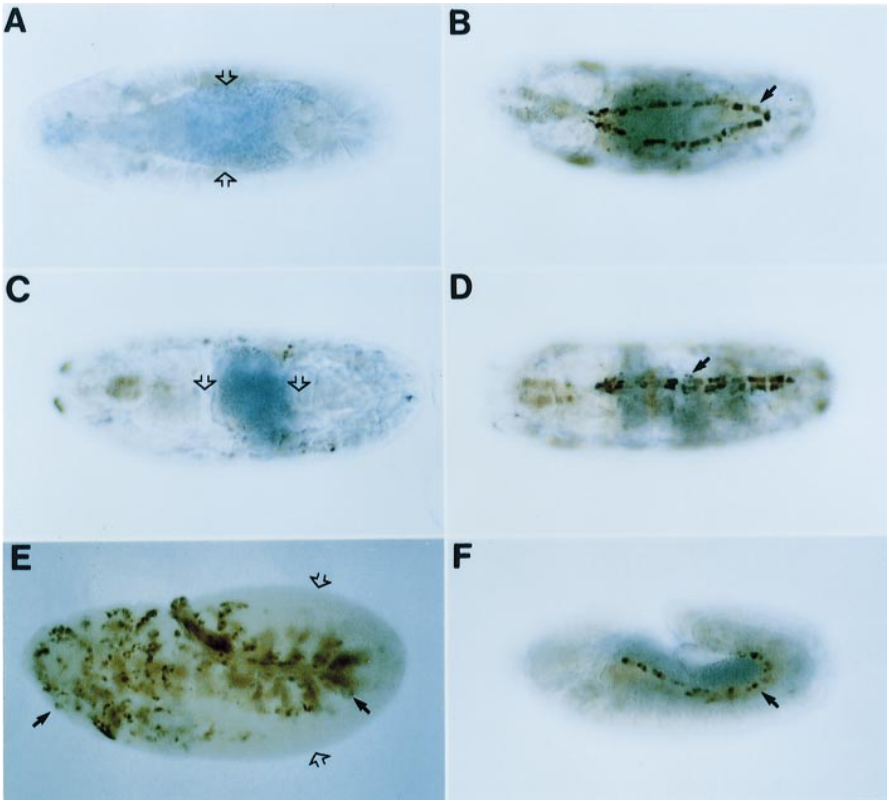

Fig. 4. Regulation of the *D-mef2* heart enhancer by *tinman*. *D-mef2–Hsp43–lacZ* fusion gene expression in stage 13 (**A**) and stage 17 (**C**) tin^{346} homozygous mutant embryos, stage 14 (**B**) and stage 17 (**D**) tin^{346}/*TM3,ftz-lacZ* heterozygous embryos, and stage 12 heat-shocked (**E**) and non-heat-shocked (**F**) embryos. Open arrows indicate the absence of β-galactosidase staining in heart precursor cells (A), cardial cells (C) or the ventral region of the extended germ band in heat-shocked Tinman embryos (E). Solid arrows point out β-galactosidase activity in cardial cells of the forming (B and F) or mature heart (D) and in expanded domains of heat-shocked Tinman embryos (E).

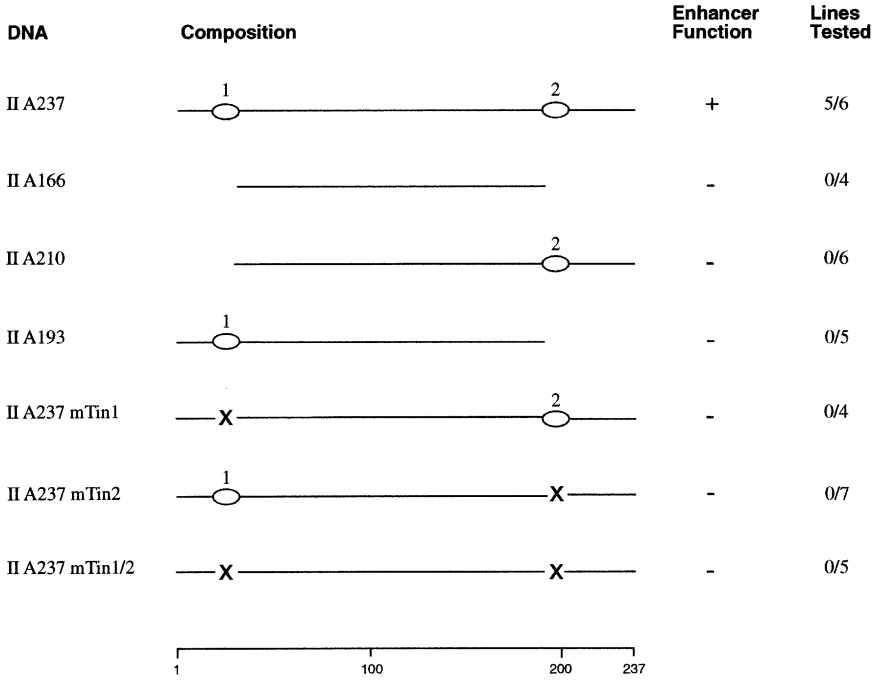

Fig. 5. Effect of the mutagenesis of Tinman binding sites on *D-mef2* enhancer activity in transformant embryos. The names of the different *D-mef2*–Chab constructs are listed on the left. Composition indicates the starting 237 bp IIA237 DNA and its deleted or mutated derivatives. Tinman binding sites are indicated by the numbers 1 and 2, and an X designates a mutated Tin site. These sites were changed from CTCAAGTGG to CTgtAGacG in the mutant constructs. Cardial cell and heart enhancer function of the tested DNAs and the number of lines assayed for each construct are shown on the right.

the early mesoderm and in developing muscle cell lineages is regulated by a complex set of independent control regions located upstream of the gene. In the early *Drosophila* embryo, the expression pattern of *D-mef2* is very similar to that of *tinman*. Both genes are expressed initially throughout the mesoderm within the ventral furrow and in the germ band extended embryo. *D-mef2* transcripts accumulate normally in these cells in *tinman* mutant embryos (Lilly *et al.*, 1994; Nguyen *et al.*, 1994) and *tinman* is expressed normally in *D-mef2* mutant embryos (Lilly *et al.*, 1995), suggesting that the two genes are activated independently in the early mesoderm. The most likely regulator of *tinman* and *D-mef2* is the basic helix–loop–helix transcription factor Twist, as neither gene is expressed in *twist* mutant embryos (Bodmer *et al.*, 1990; Lilly *et al.*, 1994; Nguyen *et al.*, 1994; Taylor *et al.*, 1995) and ectopic expression of Twist expands the expression pattern of both genes (Taylor *et al.*, 1995).

Later, as the mesoderm differentiates, *tinman* expression becomes regulated by Dpp and is restricted to dorsal mesodermal cells located adjacent to Dpp-expressing ectodermal cells (St Johnston and Gelbart, 1987; Bodmer, 1990; Azpiazu and Frasch, 1993; Frasch, 1995). The function of *tinman* in the dorsal mesoderm is to determine the formation of heart precursor cells and to activate the expression of *bagpipe*, another NK-type homeobox gene required for specification of visceral muscle precursor cells (Azpiazu and Frasch, 1993; Bodmer, 1993). During this refinement of *tinman* gene expression, D-MEF2 is expressed in cardiac, somatic and visceral muscle cell precursors and later is expressed in the differentiated heart, body wall, and gut musculature (Bour *et al.*, 1995; Lilly *et al.*, 1995). Despite the expression of *D-mef2* throughout the course of mesoderm formation and differentiation, gene function is required only for the differentiation of heart, somatic and visceral muscle cells (Bour *et al.*, 1995; Lilly *et al.*, 1995; Ranganayakulu *et al.*, 1995). Thus, genetic studies have demonstrated an early function for *tinman*, and a late function for *D-mef2*, in the development of specific derivatives of the mesoderm.

Tinman regulation of D-mef2 in the cardial cell lineage

D-MEF2 is expressed in all of the contractile cardial cells of the heart tube where one of its functions is to activate the transcription of the three myosin subunit genes (Bour *et al.*, 1995; Lilly *et al.*, 1995; Ranganaykulu *et al.*, 1995). The IIA237 region upstream of *D-mef2* functions as a transcriptional enhancer in most, but not all of these D-MEF2-positive cells as enhancer activity was not observed in short, segmentally repeated sections of the heart tube (Figures 3C and 4D). However, this result is consistent with the regulation of the IIA237 heart enhancer by Tinman, as *tinman* is expressed in this pattern, i.e. in most but not all of the cardial cells of the mature heart (Bodmer, 1993). Thus, *D-mef2* requires Tinman and at least one other regulatory input for its expression in the complete complement of cardial cells.

The requirement for two Tinman binding sites for the activity of the *D-mef2* heart enhancer suggests some manner of cooperativity between Tinman proteins bound to these sites, perhaps mediated by the interaction of Tinman molecules with each other. Other NK-type homeodomain proteins can also bind this consensus sequence. For example, the NK-2 protein, which is the product of the *ventral nervous system defective* (*vnd*) locus of *Drosophila* (Jimenez *et al.*, 1995), has a consensus binding site of T(T/C)AAGT(G/A)G (Tsao *et al.*, 1994) which matches the Tinman sites in the *D-mef2* cardiac enhancer. *vnd* is expressed in cells of the developing nervous system and midgut of the embryo (Jimenez *et al.*, 1995; Mellerick and Nirenberg, 1995) and it is conceivable that NK-2 could bind to the IIA237 DNA in these cells. However, since the IIA237 enhancer is not expressed in CNS or midgut cells, it seems likely that additional factors must work with the NK proteins to regulate target genes in the appropriate cell types. Thus, the selective activation of the *D-mef2* enhancer by Tinman may require a co-activator protein that is expressed in the cardial cells. Because the IIA237 region contains all the information necessary for transcriptional activation by Tinman, it will be of particular interest to screen for other potential regulatory elements within this enhancer to identify the transcription factors that may cooperate with Tinman to control cardial cell gene expression.

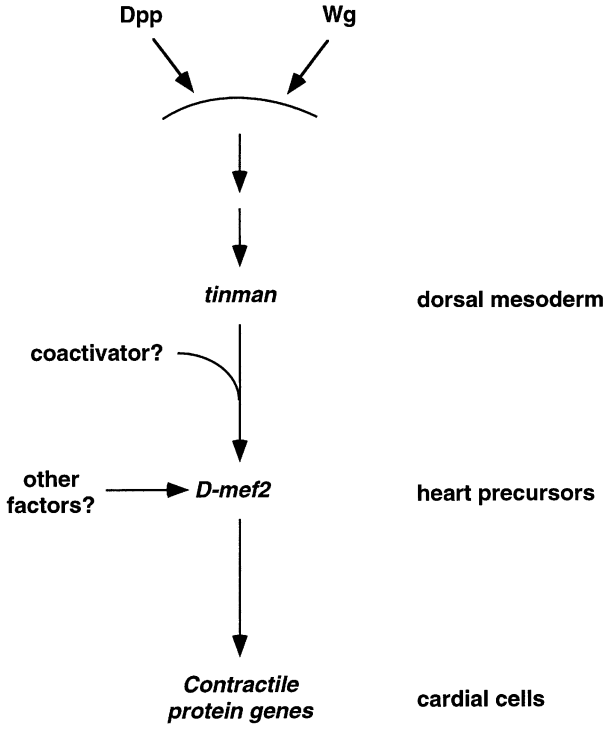

Fig. 6. Schematic of a regulatory hierarchy controlling cardiac gene expression during *Drosophila* heart development.

Regulatory network controlling cardiac gene expression in Drosophila

Our analysis of *D-mef2* expression and function in the heart, coupled with the findings of others, suggests a genetic hierarchy controlling gene expression during *Drosophila* cardiogenesis (Figure 6). Wg is required for the specification of cardiac progenitors (Wu *et al.*, 1995), while Dpp produced in the ectoderm determines the expression domain of *tinman* in the dorsal mesoderm (Staehling-Hampton *et al.*, 1994; Frasch, 1995). Tinman is a direct activator of *D-mef2* in heart precursor cells and this regulation probably requires a Tinman co-activator

protein, since Tinman expression in the dorsal mesoderm is much broader than *D-mef2*, and ectopic Tinman expression can activate the *D-mef2* cardiac enhancer in some but not all regions of the embryo. Additional factor(s) are required for the expression of *D-mef2* in the full complement of cardial cells because the Tinman-dependent enhancer is active in only a subset of cells of the dorsal vessel. Late-expressed contractile and structural protein genes can be divided into *D-mef2*-dependent and *D-mef2*-independent subgroups. The cardial cell transcription of the three myosin subunit genes requires *D-mef2* function (Ranganayakulu *et al.*, 1995) while the β*Tub60D* and *TmI* genes are expressed normally in the heart of *D-mef2* mutant embryos (Bour *et al.*, 1995; Ranganayakulu *et al.*, 1995; Lin *et al.*, 1996). Whether these latter genes are activated directly by Tinman or require other as yet unidentified factor(s) for their cardial cell expression remains to be determined.

Conservation of genes and mechanisms controlling heart formation

The NK-type homeobox genes *Nkx-2.3*, *Nkx-2.5* and *Nkx-2.7* are closely related to *tinman* and their expression during cardiac development in chick, fish, frog and mouse embryos suggests that these genes may perform functions related to those of *tinman* (Komuro and Izumo, 1993; Lints *et al.*, 1993; Tonissen *et al.*, 1994; Evans *et al.*, 1995; Schultheiss *et al.*, 1995; Buchberger *et al.*, 1996; Lee *et al.*, 1996). Moreover, the preferred DNA binding site for Nkx-2.5 (Chen and Schwartz, 1995; Durocher *et al.*, 1996) is identical to the Tinman sites in the *D-mef2* cardiac enhancer, and inactivation of the *Nkx-2.5* gene in mice results in abnormal heart development (Lyons *et al.*, 1995). Likewise, the four vertebrate *mef2* genes are expressed in the developing heart (Chambers *et al.*, 1994; Edmondson *et al.*, 1994; Molkentin *et al.*, 1996) like their *Drosophila* homolog, and recent studies indicate that *mef2C* is required for proper heart development in mice (Q.Lin, J.Schwartz and E.N.Olson, submitted). In light of our results on Tinman regulation of *D-mef2* in cardial cells, it will be interesting to determine whether the *Nkx-2.5* genes activate *mef2* gene expression during vertebrate cardiac morphogenesis. The coincident expression of the *Nkx-2.5* and *mef2C* genes in the primordial heart of the mouse embryo is consistent with this type of regulatory hierarchy (Lints *et al.*, 1993; Edmondson *et al.*, 1994; Molkentin *et al.*, 1996). Parallel studies of such genetic interactions in flies and vertebrates should provide important insights into evolutionarily conserved mechanisms controlling heart development and cardiac gene expression.

Materials and methods

DNA sequence determination

DNAs were sequenced using the Sanger dideoxy method combined with automated fluorescence detection (Spurgeon *et al.*, 1995). The 5.4 kb *D-mef2* genomic DNA II (Schulz *et al.*, 1996) was sequenced using a series of internal primers. Overlapping sequences were linked together and scanned for potential Tinman binding sites using the GCG DNA sequence analysis program. Wild-type and mutated *D-mef2* DNAs to be tested for enhancer function in the CaSpeR-*Hsp43*-AUG-βgal (Chab) transformation vector were sequenced using a primer from the *Hsp43* promoter region. Primers were obtained from Genosys Biotechnologies (Woodlands, TX).

DNase I protection assay

Footprinting assays were performed as described (Hoey *et al.*, 1988). The IIA237 *D-mef2* heart enhancer in pCRII (Invitrogen, San Diego, CA) was digested with either *Hin*dIII (minus-strand labeling) or *Xba*I (plus-strand labeling) and digested DNAs were treated with alkaline phosphatase. Dephosphorylated DNAs were redigested with either *Xba*I (minus-strand labeling) or *Hin*dIII (plus-strand labeling), eluted from a gel, and ^{32}P-end labeled with T4 kinase. Binding reactions were then performed with a GST–NK-4 fusion protein and 12.5 fmol of labeled DNA in 10 μl of binding buffer containing 25 mM HEPES (pH 7.5), 3 mM $MgCl_2$, 1 mM EDTA, 0.5% NP-40, 10% glycerol, 1 μg of poly[d(I–C)]. After incubation for 15 min at room temperature, $CaCl_2$ (final 0.5 mM) and DNase I (0.05 U) were added to the binding reactions for DNase I digestion. Reactions were terminated after 1 min incubation and analyzed on an 8% denaturing polyacrylamide gel.

D-mef2–Chab enhancer test constructs

The *D-mef2* IIA237, IIA166, IIA210 and IIA193 DNAs were generated by PCR amplification using the DNA II template, the terminal 20 nucleotide 5′ and 3′ primers, and dNTP solutions, LA PCR buffer and LA *Taq* enzyme provided in the TaKaRa LA PCR kit (PanVera, Madison, WI). PCR products were cloned into the pCRII vector using the TA cloning kit from Invitrogen (San Diego, CA). To generate the *D-mef2* IIA237mTin1, IIA237mTin2 and IIA237mTin1/2 mutant DNAs, a PCR-based method was also used. For IIA237mTin1, two separate DNA fragments (A1 and A2) were amplified by PCR with specific primers (A1 DNA: SP6 and mT2R; A2 DNA: mT25 and T7) and IIA237 template DNA. The A1 and A2 DNAs were mixed, denatured and reannealed. DNAs were gap-filled and reamplified by PCR using 30 amplification cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) with SP6 and T7 primers. The amplified DNA fragments were then cloned into pCRII. For the IIA237mTin2 construct, the same method was employed using different primers (A3 DNA: SP6 and mT1R; A4 DNA: mT15 and T7). For the IIA237mTin1/2 DNA, an A5 DNA was generated by PCR using the mT15 and T7 primers and IIA237mTin1 template DNA. The A5 and A3 DNAs were then mixed, reamplified by PCR and subcloned. Primers used were as follows: SP6, 5′-ATTTAGGTGACACTATA-3′; mT1R, 5′-GCAGCTAAAGGATGCGCCgtCTacAGACCGGGCTCGC-TATC-3′; mT15, 5′-GATAGCGAGCCCGGTCTgtAGacGGCGCATC-CTTTAGCTGC-3′; mT2R, 5′-AATGGAGGCACCCAAGCgtCTacA-GCCCCTTATCCGTGTC-3′; mT25, 5′-GACACGGATAAGGGGC-TgtAGacGCTTGGGTGCCTCCATT-3′; T7, 5′-TAATACGACTCACT-ATAGGG-3′. Lower case letters correspond to the introduced base changes. The wild-type or mutant *D-mef2* sequences were isolated from the pCRII vector and cloned into Chab.

Drosophila germline transformation

The *D-mef2*–Chab DNAs were stably introduced into the *Drosophila* genome by P element-mediated germline transformation (Rubin and Spradling, 1982). The *y* w^{67c23} strain was used for embryo injections and transformed flies were identified by w^+ eye color selection. A minimum of four lines were established for each construct. The insertion of the IIA237 *D-mef2*–Chab DNA on chromosome II in the *IIA237-9* line was determined using standard linkage analysis. *IIA237-9* flies were crossed with *tin*^{346}/*TM3,ftz-lacZ* flies to generate a *IIA237-9*/*IIA237-9*; *tin*^{346}/*TM3,ftz-lacZ* stock. Upon intermating of these flies, the expression of the IIA237–*lacZ* DNA was assayed in *tin*^{346} heterozygous or homozygous embryos, with the former identified by the expression of the marker linked to the balancer chromosome. *tin*^{346} has been classified as a null allele of the *tinman* gene (Azpiazu and Frasch, 1993). *IIA237-9* flies were also crossed to *hsTin*^{9A}/*TM3,ftz-lacZ* flies to generate embryos that could be heat treated to induce ectopic expression of the Tinman protein. The heat shock regimen was as follows. Embryos were collected for 2 h at 25°C on yeasted grape-agar plates and then aged for 4 h at 25°C. Embryos were heat-shocked for 30 min by placing the plates in a water bath maintained at 37°C, followed by recovery for 30 min at 25°C. The heat shock/recovery cycle was repeated twice and the embryos were aged an additional 3 h at 25°C. Under these conditions, only the *IIA237-9*;*hsTin*^{9A} embryos showed an altered distribution of the β-galactosidase expression marker.

Embryo β-galactosidase assays

Processing and immunostaining of whole mount embryos for β-galactosidase activity was as described (Patel *et al.*, 1987) using the Vectastain Elite ABC Kit (Vector, Burlingame, CA). The anti-β-galactosidase primary antibody (Promega, Madison, WI) was used at a 1:1000 dilution while the horse anti-mouse–biotin conjugate secondary antibody (Vector)

was used at a 3:1000 dilution. Stained embryos were mounted in 50% glycerol under cover slips for photography using a Nikon Optiphot photomicroscope and Kodak Ektachrome 64T film.

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