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ARTICLE

Cardiac Enhancer Activity of the Homeobox Gene *tinman* Depends on CREB Consensus Binding Sites in *Drosophila*

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Summary: The *Drosophila* homeobox gene *tinman* plays a critical role in subdividing the early mesoderm. In particular, *tinman* is absolutely required for formation of the heart and visceral mesoderm. *tinman* expression is initiated throughout the mesoderm of the trunk region under the control of the bHLH transcription factor encoded by the *twist* gene, a determinant of all mesoderm. Later, *tinman* expression is restricted to the dorsal portion of the mesoderm, a process that is directed by *decapentaplegic* (*dpp*) whose product (a TGF-β-related protein) is secreted by the overlaying ectoderm. Further restriction of *tinman* expression to the cardiac progenitors, in which it will persist throughout development, involves the secreted segmentation gene product encoded by *wingless* (*wg*, a *Drosophila Wnt* gene). Here, we show that strong early expression depends on the synergistic action of an enhancer element at the 5′ end of the gene in conjunction with an element in the first intron. Moreover, two distinct enhancer regions are responsible for *tinman* expression in the heart: one region confers expression in the heat tube associated pericardial cells, the other element drives expression in the contractile, myocardial cells. The latter element contains two CREB consensus binding sites that are essential for cardiac-specific expression. genesis 26:55–66, 2000.

Key words: *Drosophila*; *tinman*; mesoderm; heart; cardiogenesis; patterning; CREB

INTRODUCTION

The molecular processes involved in mesoderm determination and muscle subtype specification have been studied in some detail (for review see Abmayr *et al.*, 1995; Baylies *et al.*, 1998; Bodmer and Frasch, 1999). Specific gene functions have been identified that act during discrete steps of mesoderm differentiation. As a consequence, models of hierarchical networks of genetic interactions have been proposed to govern the sequential embryological events in mesoderm differentiation (for review see Bodmer and Frasch, 1999). At the top of the hierarchy is *twist*, which encodes a basic-helix-loop-helix protein (Thisse *et al.*, 1988) and acts as the first zygotic determinant of mesoderm formation. At blastoderm, *twist* activates the early pan-mesodermal expression of a number of genes, including the homeobox gene *tinman* (Bodmer *et al.*, 1990; Yin *et al.*, 1997), the zinc finger and homeobox gene *zfh-1* (Lai *et al.*, 1991), the MADS-box gene *Dmef2* (Nguyen *et al.*, 1994; Lilly *et al.*, 1994; Cripps *et al.*, 1998; Nguyen and Xu, 1998), and FGF-receptor encoded *heartless* (Shishido *et al.*, 1993). Although each of these genes plays a crucial role in mesoderm differentiation and each is first activated at a similar time point at blastoderm, their mesodermal expression patterns later in development are distinct from one another and their functions affect different processes of differentiation. For example, the FGF receptor encoded by *heartless* is primarily required for dorsal migration of the mesoderm (Beiman *et al.*, 1996; Gisselbrecht *et al.*, 1996; Shishido *et al.*, 1997). By contrast, *tinman* is not required for this process of migration but rather for specifying the differentiation pathway of the dorsal portion of the mesoderm. Without *tinman*, most if not all derivatives of the dorsal mesoderm fail to develop, in particular the heart and visceral muscles (Bodmer, 1993; Azpiazu and Frasch, 1993). *Dmef2*, on the other hand, appears to function in the myogenic-specific differentiation of all muscles (Bour *et al.*, 1995; Lilly *et al.*, 1995; Ranganayakulu *et al.*, 1995), rather than in the earlier specification of mesodermal subdivision or subsets of cells, as *tinman* and *zfh-1* are (Bodmer, 1993; Azpiazu and Frasch, 1993; Lai *et al.*, 1993; Broihier *et al.*, 1998; Su *et al.*, 1999).

In addition to mesoderm endogenous transcription factors, inductive mechanisms involving signals from the ectoderm are important for providing spatial information

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in mesodermal subtype specification. The TGF-β factor encoded by *decapentaplegic* (*dpp*) is expressed in a broad dorsal domain of the ectoderm along the anterior-posterior axis. Dpp is secreted from the dorsal ectoderm and is necessary to maintain *tinman* and *Dmef2* expression in the dorsal mesoderm (Staehling-Hampton *et al*., 1994; Frasch, 1995; Xu *et al*., 1998; Nguyen and Xu, 1998). In addition to the dorsal-ventral patterning role of *dpp*, the secreted segment polarity gene products encoded by *wingless* (*wg*) and *hedgehog* (*hh*) are also crucial for anterior-posterior patterning of the developing mesoderm (Wu et al., 1995; Baylies *et al*., 1995; Lawrence *et al*., 1995; Azpiazu *et al*., 1996; Ranganayakulu *et al*., 1996; Park *et al*., 1996; Riechmann *et al*., 1997; for review see Bodmer and Frasch, 1999). Whereas *dpp* and *tinman* are essential for both heart and visceral mesoderm formation, *wg* is needed primarily for the cardiac component (Wu *et al*., 1995). Although the intersection of *wg* and *dpp* signaling in the context of dorsal mesodermal *tinman* expression appears to be instructive for cardiac cell type specification (W.K. Lockwood and R. Bodmer, unpublished data), other combinations of inductive signals and mesodermal context factors are likely to be involved in specifying other mesodermal subset of cells or contribute to further distinctions between individual cell types (see for example, Su *et al*., 1999).

Here, we show that the dynamic pattern of *tinman* expression in the mesoderm is regulated by a modular set of cis-regulatory elements. High levels of the early pan-mesodermal expression of *tinman* depends on the synergistic interaction between a 350 bp element 5′ to the coding region (Lee *et al*., 1997) and an element in the first intron (Yin *et al*., 1997). *tinman* expression in the heart precursors seems to be regulated by two separate elements in the 3′ region of the gene: One element directs expression to the pericardial cells (as well as in a subset of visceral circular muscles); the other element directs expression primarily in the myocardial cells of the heart. The myocardial-specific expression depends on two CREB consensus binding sites. We discuss the possible function of these sites as potential Dpp response elements.

RESULTS

Cis-Regulatory Elements Reflect Endogenous *tinman* Expression

Endogenous Tinman protein expression in the mesoderm is detected in four progressively restricted phases (Fig. 1B–E): first it is present in all the trunk mesoderm at gastrulation and during germ band extension (stage 8/9), at stage 10 it is restricted to the dorsal mesoderm, at stage 11/12 it is further restricted to the cardiac mesoderm and a subset of visceral mesoderm, and finally after stage 14 is confined to the developing heart (see also Yin *et al*., 1997).

In order to study the regulation of *tinman* expression, we generated transgenic flies with various genomic fragments of the *tinman* gene fused to the *lacZ* reporter gene (Fig. 1A). A large BamHI fragment (2A) that extends from about −7 kb into the first intron shows expression throughout the early mesoderm (Fig. 1F). A fragment 3′ to 2A which contains the second intron (42), the third exon and some 3′ untranslated (3′ UTR) and noncoding regions (34) shows expression in the pericardial cells of the heart and a subset of visceral mesoderm (Fig. 1H; see also Fig. 3). A 1.4 fragment 3′ to 34 contains a myocardial-specific enhancer (73; Fig. 1I). The 3′-most fragment we examined for reporter gene expression contains (at least part of) the dorsal mesodermal enhancer (Fig. 1G; see also Xu *et al*., 1998). The early mesoderm and heart enhancers have been further studied (see below).

Early Mesodermal Expression Depends On Two Distinct Elements

Although the large fragment 2A confers early mesodermal expression, all fragments 5′ to the coding region without the first intron fail to show significant *lacZ* expression in the mesoderm (Fig. 2A, B). Weak to moderate expression in the early mesoderm is observed when the first intron itself is examined (Fig. 2C; see also Yin *et al*., 1997). However, a significant increase in *lacZ* expression is observed when the first intron is combined with a 350 bp element from the 5′ region (or a larger region containing that element) in the same construct (Fig. 2A, D, E). Interestingly, both of these elements contain a number of putative Twist binding sites (Yin *et al*., 1997; Lee *et al*., 1997). Thus, it appears that an element 5′ to the coding region cooperates with an intronic element to direct the early *twist*-dependent expression of *tinman*.

Pericardial and Visceral Mesoderm Enhancer *tinman*

We wanted to further define the pericardial and visceral mesoderm expression exhibited by fragment 34 in the 3′ region of the *tinman* gene (Fig. 1A, H). Surprisingly, the pericardial nor the visceral mesoderm expression of fragment 34 cannot be localized to a unique non-protein coding region: We tested the second intron (construct 42), the 3′ UTR and the region 3′ to the gene (103A500), but none of them show expression of all or part of the 34 pattern (Fig. 3A; data not shown). Only the subfragment 56 that contains protein coding sequence 3′ to the homeodomain shows 34-like pericardial and visceral mesoderm expression (Fig. 3B,C). Thus, it is likely that this 3′ protein-coding region is necessary for pericardial and visceral mesoderm expression. However, we do not yet know if this is the only element needed or if other regions within fragment 56 are also necessary. Although *tinman* RNA does not persist in the forming visceral mesoderm past stage 11, Tinman protein is present in a segmented pattern until stage 13 (Fig. 4D). Thus, part of Tinman's expression in the visceral mesoderm seems to be encoded by an element that is linked to or overlaps with the pericardial element.

Myocardial Heart Cell Enhancer Maps to an Element 3′ of the Coding Region

Expression of fragment 73 (1.4 kb) is restricted exclusively to the myocardial cells of the forming heart and directs *lacZ* expression from stage 12 on. As Tinman protein, *lacZ* expression is confined to four of the six myocardial cells within each hemisegment (Fig. 1E, I). Unlike Tinman protein, however, no pericardial cell expression is detected with this construct. Thus, it seems that myocardial and pericardial expression is regulated by enhancer elements that act independently from one another.

To determine the minimal size of this myocardial heart enhancer of *tinman*, we examined 600 bp overlapping fragments derived from construct 73 (fragments 103A–C; Fig. 4A). Even though fragment 103A overlaps with 103C for only 22 bp (300bp with 103B), transgenic embryos of all three fragments (103A–C) exhibit *lacZ* reporter gene expression in the myocardial cells (Fig. 4B–D). Interestingly however, the level of expression along the anterior posterior axis varies significantly between the constructs 103A to 103C. Whereas fragment 103B mediate uniformly strong expression (as is fragment 73, Fig. 4C), fragment 103A promotes strong expression only in the anterior myocardial cells, which gradually diminishes posteriorly (Fig. 4B). Conversely, fragment 103C mediates strong expression posteriorly, which gradually lessens anteriorly (Fig. 4D).

In order to characterize the myocardial heart cell enhancer fragment further, we examined the *lacZ* reporter pattern of two slightly out of register fragments that span the small overlap between 103A and 103C (Fig. 4A): a 305bp fragment (Henh1) and a 360bp fragment (Henh2). Two copies of Henh1 (2×) driving *lacZ* confers high levels of cardiac-specific expression beginning at late stage 11 (Fig. 4E–H). However, reporter gene expression is no longer restricted to the Tinman subset of myocardial heart cells that is marked by 103B and 73; rather all myocardial and the pericardial cells are anti-β-galactosidase positive with this construct (Fig. 4E-G), including the two cells per hemisegment that do not normally express *tinman*. Although the expanded portion of expression within the developing heart is not as strong as the 103B pattern (Fig. 4E), this pan-cardiac expansion was never observed in embryos of any of the 103B (or 73) transformant lines (Fig. 4C or 1I). Transformants with one copy of the Henh1 (1×) construct shows a similar pattern of pan-cardiac expression as Henh1(2×), but exhibits weaker "ectopic" expression (data not shown). Cardiac expression seems to be somewhat dosage sensitive, these data suggest that the 305 bp Henh1 fragment is likely to be missing negative control elements that suppress expression in pericardial and a subset of myocardial cells. Transformants of Henh2 driving *lacZ* show a similar cardiac pattern as Henh1, but the ectopic cardiac expression (in Tinman-negative myocardial cells and in pericardial cells) is less pronounced than with Henh2 (Fig. 4A and data not shown). Thus, one of the negative elements necessary for correct myocardial expression is likely to be located in the 100 bp region 3′ of the Henh1 sequence (see Fig. 4A).

Myocardial Heart Enhancer Activity Depends on CREB Consensus Binding Sites

We have identified a 600 bp myocardial heart cell enhancer (103B) 3′ to the *tinman* coding region, and have shown that much of the regulatory information for myocardial expression must be contained within the 255 bp overlap between Henh1 and Henh2 region. Although the restriction of *tinman* expression to the cardiac mesoderm appears to be directed by the changing patterns of *wg* and *dpp* intersects (Wu *et al*., 1995; Frasch 1995; W. Lockwood and R. Bodmer, unpublished data), we did not find any consensus binding sites of known *wg* response element within this genomic region. We did, however, identify two CRE consensus sites within the 255 bp overlap between Henh1 and 2 (Fig. 4A and Fig. 5A). A consensus binding site for CREB has previously been shown to be *dpp* responsive sequence within a visceral mesoderm enhancer of the *Ultrabithorax* (*Ubx*) (Eresh *et al*., 1997; Riese *et al*., 1997). In order to determine if the CRE consensus sites common to Hen1 and 2 are required for cardiac-specific expression, we mutated both CRE sites in Henh1 (CRE-M) and examined *lacZ* expression in transgenic flies (Fig. 5A). In contrast to the wild-type form of Henh1(1x) (Fig. 5B), CRE mutated Henh1(CRE-M) abolishes all heart-specific reporter gene expression in all embryos of the four independent transformants (Fig. 5C), except in one case where very weak pericardial expression is observed at late developmental stages (after the heart tube has formed, data not shown). We conclude that one or both of the CRE consensus sequences in *tinman* myocardial heart cell enhancer are necessary for cardiac-specific expression.

DISCUSSION

tinman expression seems to be regulated by discrete enhancer elements that are distributed 5′ to, 3′ to and within the *tinman* coding region (Yin *et al*., 1997; Lee *et al*., 1997; this study). It has previously been reported that the *twist*-dependent *tinman* expression in the early mesoderm is achieved by an element within the first intron (Yin *et al*., 1997). In addition to confirming this result, we find that the combination of the first intron with an element 5′ to the *tinman* coding region (construct 76) results in higher levels of reporter gene expression are achieved than with the first intron alone (Fig. 2). This suggests that a composite enhancer element is directing high levels of *tinman* expression in the early mesoderm.

Later, when *tinman* expression is restricted to the developing heart, expression in the forming cardial and pericardial cells is apparently regulated by separate enhancer elements (Figs. 3 and 4). It is noted, however,

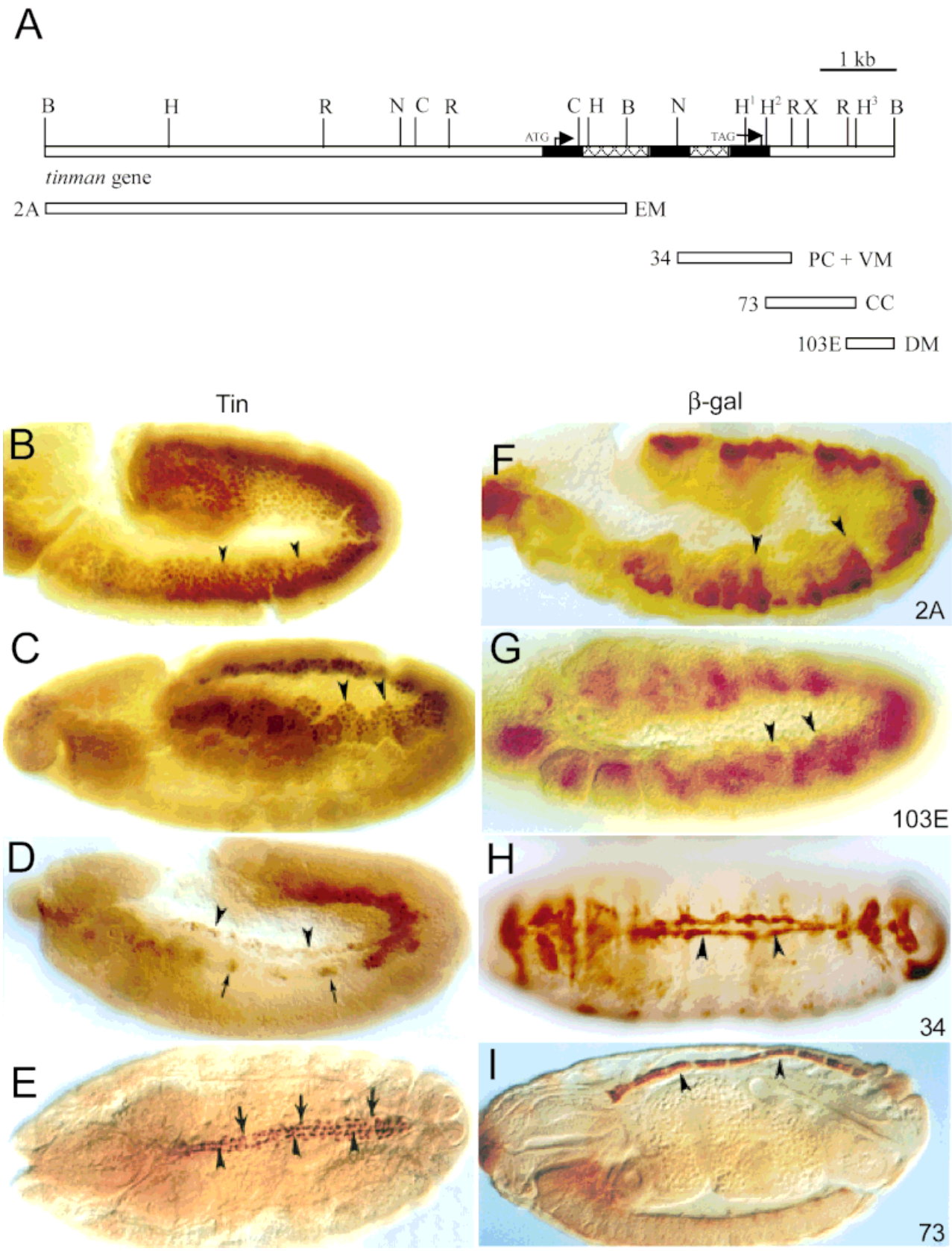
A
1 kb
B H R N C R C H B N H1 H2 R X R H3 B
ATG
TAG
tinman gene
2A EM
34 PC + VM
73 CC
103E DM
Tin
β-gal
B
F
2A
C
G
103E
D
H
34
E
I
73

FIG 1

that pericardial expression observed in fragment 56 transformants does not occur in blocks of four out six cells per hemisegment, as is observed for Tinman protein (Fig. 1E), but seems to be present in all pericardial cells. Pericardial expression of fragment 56 transformants is accompanied by expression in a subset of visceral mesoderm. A 5′ region has also been reported to confer visceral mesoderm expression (Lee *et al*., 1997), although we were unable to detect mesoderm-specific expression with any 5′ fragment by itself (Fig. 2A, B). It is thus possible that the control of unique pericardial and visceral mesoderm expression is dispersed and contributing elements are distributed among various regions of the *tinman* gene. Composite regulatory elements that contribute to overlapping expression patterns have been observed for other genes, for example *even-skipped* (Fujioka *et al*., 1999) and *Dmef2* (Nguyen and Xu, 1998).

The *wg* pathway appears to be specifically required for heart precursor formation and for the final confinement of *tinman* expression to the cardiac mesoderm (Wu *et al*., 1995; Park *et al*., 1996). However, *wg* responsive elements have not been identified, i.e., dTCF consensus sites (Riese et al., 1997; van de Wetering, 1997), neither in the pericardial nor the myocardial elements. Thus, the *wg* pathway responsible for heart formation may act (directly or indirectly) via dTCF-independent binding sites and may employ transcription factor(s) other than dTCF. Moreover, expression of *tinman* in response to *wg* may require additional, as yet unidentified transcription factors, which may influence *tinman* expression via their own elements in order to achieve cardiac specificity.

Although *dpp* is required for maintaining *tinman* expression within the dorsal mesoderm, which in turn is essential for both cardiac and visceral mesoderm formation (Staehling-Hampton *et al*., 1994; Frasch, 1995), it seems that *dpp* is needed again later, along with *wg*, to specify cardiac mesoderm at locations of persistent *wg/dpp* overlap. In support of this hypothesis, overexpression of *dpp* generates cardiac-specific marker gene expression at the *wg/dpp* intersects of the ventrolateral mesodermal cells (W.K. Lockwood and R. Bodmer, unpublished data). Thus, it is possible that normally the *dpp* pathway directly regulates this heart enhancer, in addition to the dorsal mesoderm enhancer (Xu *et al*., 1998). The myocardial heart enhancer apparently does not contain consensus DNA binding sites for Smad proteins, as does the dorsal mesoderm enhancer (Xu *et al*., 1998), but rather depends on two CRE consensus sites. Mutating these CRE sites abolishes reporter gene expression conferred by this heart enhancer (Fig. 5). We do not know if or how the CRE sites of this enhancer are regulated by the *dpp* pathway and/or by CREB proteins. In the case of the midgut enhancer of *Ubx*, it had been shown that CRE sites act as *dpp* response elements (Riese *et al*., 1997; Eresh *et al*., 1997). Moreover, overexpression of a dominant-negative form of the dCREB-B transcription factor suppresses the *dpp* response of this midgut enhancer, suggesting *dpp* signaling may normally modify dCREB activity or that of its partner (Eresh *et al*., 1997). In contrast, a CREB binding protein (dCBP) also binds dTCF in vitro (a *wg*-dependent transcription factor) and dCBP mutants interacts genetically with the *wg* pathway in the midgut enhancer assay (Waltzer and Bienz, 1998). Therefore, it is possible that CREB proteins interact with the *dpp* or the *wg* pathway (or both) in regulating a heart enhancer of *tinman*. It will be interesting to see if mutant version of a dCREB and/or a dCBP affect myocardial *tinman* expression and/or heart formation.

Six vertebrate homologs of *tinman* have been identified thus far (Bodmer, 1995; Harvey, 1996; Bodmer and Venkatesh, 1998), some of which exhibit an expression pattern that is remarkably similar to the cardiac restricted expression pattern of *tinman*. Of these, *Nkx2-5* has been most extensively studied from *Amphioxus* to humans (Komuro and Izumo, 1993; Lints *et al*., 1993; Lyons *et al*., 1995; Lee *et al*., 1996; Schott *et al*., 1998; Tanaka *et al*., 1999; T.V.Venkatesh and R. Bodmer, unpublished data). *Nkx2-5* is the first of the *tinman* homologs to be expressed in the bilaterally symmetrical cardiogenic regions of the anterior lateral plate mesoderm. The regulation of both *tinman* and *Nkx2-5* appears to be encoded in a modular fashion in that spatially separate enhancers direct expression to different parts of the heart and its precursors (Yin *et al*., 1997; Xu *et al*., 1998; Searcy *et al*., 1998; Lien *et al*., 1999; Reecy *et al*., 1999; Tanaka *et al*., 1999; this study). The genomic distribution of the enhancers, however, seems to be different between *tinman* and *Nkx2-5*: *tinman* expression primarily depends on enhancer elements that are located either within or 3′ to the coding region, whereas the regulatory elements of *Nkx2-5* that have been identified so far appear to be located in the 5′ region, except

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FIG 1. Tinman protein and regulation of *tinman* expression. **(A)** Map of fragments from the of the *tinman* gene that regulate four discrete patterns of expression. B: BamHI, H: HindIII, R: EcoRI, N: NheI, C: Cla I, X: Xho I, EM: early mesoderm, DM: dorsal mesoderm, VM: visceral mesoderm, PC: pericardial heart cells, CC: myocardial heart cells. Black bars indicate exons. **(B–E)** Tinman protein. **(B)** Stage 9 embryo showing Tinman localization in all trunk mesoderm nuclei (arrowheads). **(C)** Stage 10 embryo showing restriction of Tinman to the dorsal mesoderm (arrowheads). **(D)** Stage 11/12 embryo showing Tinman in the cardiac (arrowheads) and visceral (arrows) mesoderm. **(E)** Stage 15/16 embryo showing Tinman in myocardial (arrowheads) and pericardial (arrows) cells of the newly assembled heart tube. Note that only four out of six cells per hemisegment are positive. (F–I) *lacZ* reporter gene expression visualized with anti-??-gal antibodies. Due to the perdurance of β-gal, anti-β-gal staining is observed beyond the presence of endogenous Tinman protein. **(F)** Construct 2A driving *lacZ* reporter gene expression in the early mesoderm (arrowheads, stage 10 embryo). **(G)** Construct 103E driving *lacZ* in the dorsal mesoderm (arrowheads, stage 11 embryo). **(H)** Construct 34 driving *lacZ* in pericardial cells (arrowheads) and visceral mesoderm (not shown in this focal plane, stage 15/16 embryo). **(I)** Construct 73 driving *lacZ* in the myocardial cells of heart (arrowheads, stage 15/16 embryo). Note the pattern of expression is similar to the myocardial expression of Tinman **(E)**.

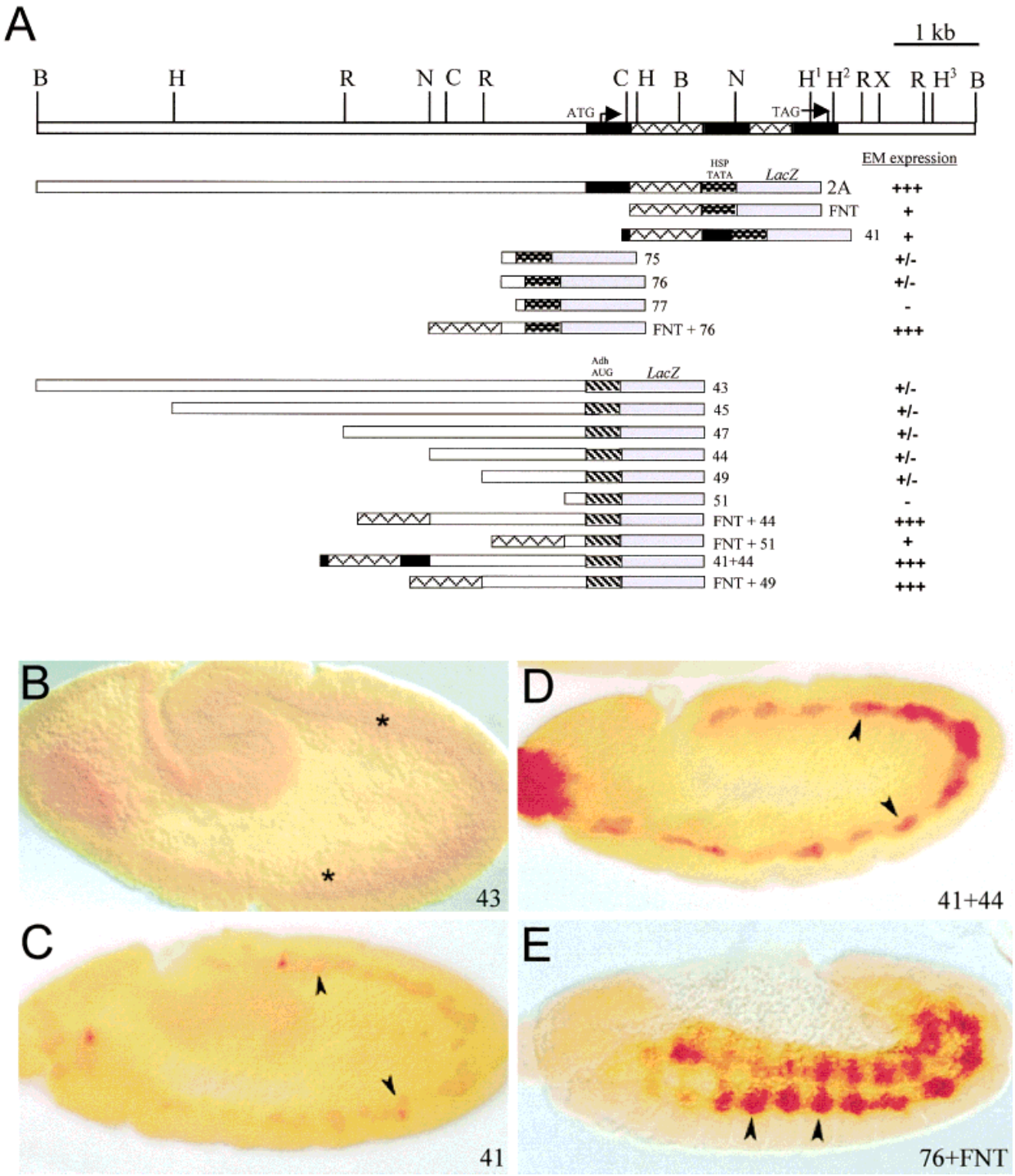

FIG 2. Localization of the early mesoderm *tinman* enhancer to the first intron (Yin *et al*., 1997) and −1 kb region. **(A)** Map and expression summary of constructs used. **(B)** Construct 43 driving *lacZ* (stage 11 embryo). Note absence of expression in the mesoderm (asterisks). **(C)** Intron I containing construct 41 driving *lacZ* in the mesoderm (arrowheads, stage 11 embryo). Construct 41 + 44 driving *lacZ* in the mesoderm (arrowheads, stage 11 embryo). Construct 76 + FNT driving *lacZ* (arrowheads, stage 12/13 embryo).

for one element in the 3′ region that causes expression in the right ventricle of the heart (Tanaka *et al*., 1999).

Interestingly, both *tinman* and *Nkx2-5* expression in the precardiac mesoderm are strongly responsive to Dpp-like TGF-β signals, which originate from adjacent germ layers (ectoderm and endoderm, respectively; Staehling-Hampton *et al*., 1994; Frasch, 1995; Schultheiss *et al*., 1995; 1997; see also review by Nascone and

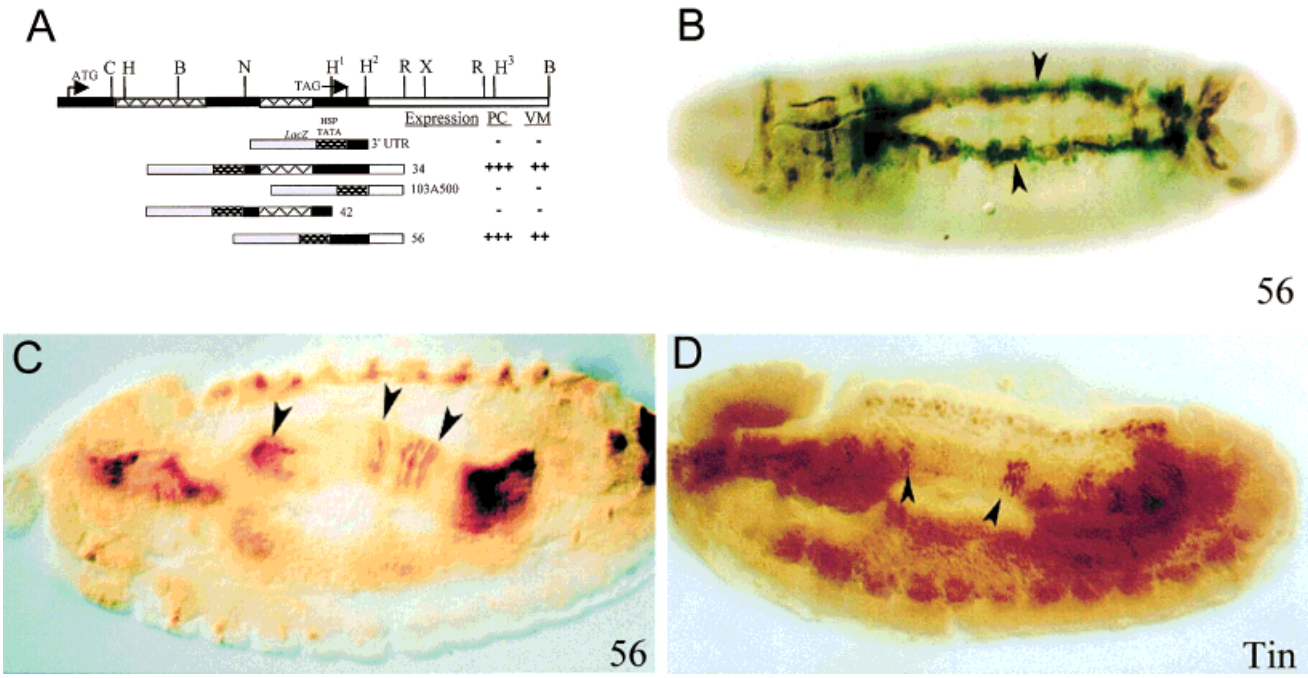

FIG 3. Analysis of the pericardial/visceral mesoderm *tinman* enhancer. **(A)** Map and expression summary of constructs used. **(B)** Construct 56 driving *lacZ* double labeled with anti-PC antibodies (dorsal view of stage 15 embryo). Note the coincidence of *lacZ* expression with pericardial cells (arrowheads). **(C)** Construct 56 driving *lacZ* in a subset of the visceral mesoderm (arrowheads, lateral view of stage 13 embryo). **(D)** Same stage embryos as (C) showing Tinman expression in visceral mesoderm (arrowheads).

Mercola, 1995). Since in *Drosophila* dorsal mesodermal and cardiac *tinman* expression appears to be mediated by Smad and CREB sites, respectively, it will be interesting to see if *Nkx2-5* expression also depends on Smad and/or CREB consensus sites.

MATERIALS AND METHODS

Tinman Antibody Production

In order to generate Tinman protein products in vitro, DNA fragments to be inserted into expression vectors were prepared by PCR using the full-length *tinman* cDNA as a template. One DNA fragment contained the full length *tinman* protein coding region minus the stop codon (1245 bp, forward primer: 5′- CCC GCG GAT CCA TGT TGC AGC ACC ATC AGC AGC AGG-3′, reverse primer 5′-CGA CCC GGG CAT GTG CTG CAT CTG TTG CTG CTG-3′). The other fragment contained a smaller portion of the *tinman* coding region that included the homeodomain-containing 3′ end of the gene, also without the stop codon (508 bp, forward primer: 5′-CGC GGA TCC GCG ATG AAG CGA AAG CCT-3′, reverse primer same a for full-length). These constructs were subcloned into the BamHI and XmaI sites of the pQE30 vector (Qiagen; Chatsworth, CA), which contains an inducible promoter and a 3′ sequence coding for six histidine residues (6×His tag) followed by a stop codon. Proteins produced by this expression construct contain this 6×His tag which permits their rapid purification using Ni-NTA chromatography (see below). Both *tinman* inserts were engineered with a 5′ BamHI site and a 3′ XmaI site for unidirectional cloning. Polymerase chain reaction (PCR) fragments and the pQE30 vector were digested with these two restriction enzymes followed by ligation and transformation into competent JM107 cells. Transformants were identified by their ability to produce an appropriately sized protein in response to induction by isopropyl-β-D-thiogalactopyranoside (IPTG, see below).

Transformants were cultured overnight at 37°C in the presence of ampicillin and kanamycin. Cells were then induced with 1 mM IPTG and cultured for an additional 4 h at 37°C. After centrifugation the pelleted cells were solubilized in guanidine buffer (6 M guanidine HCl, 0.1 M NaH_2PO_4, 0.01 M Tris HCl, pH 8). Solubilized fusion proteins were placed on columns containing Ni-NTA resin that binds the 6×His affinity tag. Columns were washed with 20 volumes guanidine buffer, 20 volumes buffer B (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris HCl, pH 8) and the 20 volumes buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris HCl, pH 6.3). The bound fusion protein was eluted with an imidazole/urea buffer (250 mM imidazole, 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris HCl, pH 7.5. Eluted fusion protein was dialyzed using a step-wise dilution of urea (6 M, 4 M, 2 M, 0 M urea, in 0.5 M NaCl, 0.01% Tween, 0.1% NP40 and 1 mM DTT). The

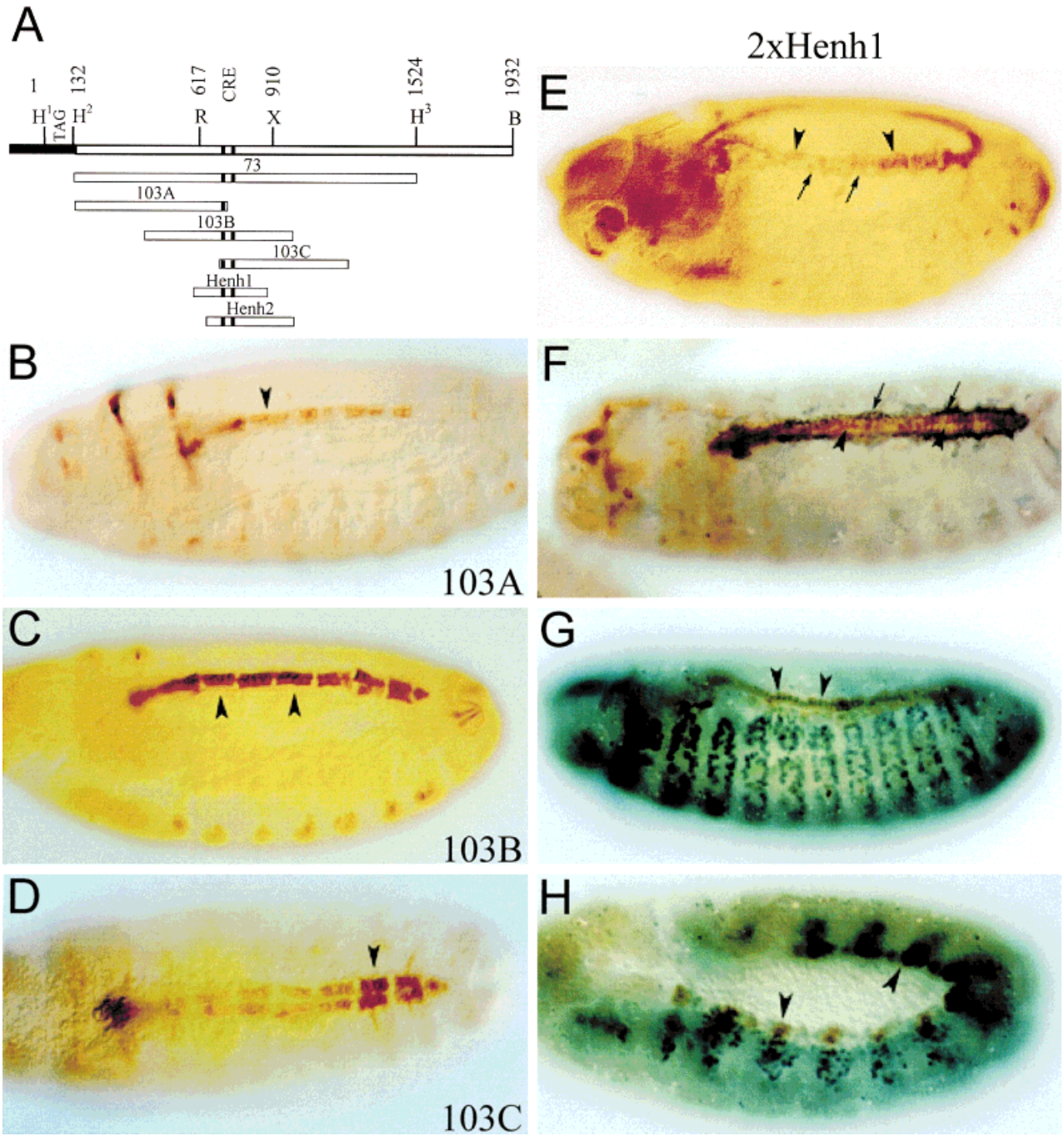

FIG 4. Analysis of the myocardial *tinman* enhancer. **(A)** Map and expression summary of constructs used. **(B–F)** Dorsal view of stage 15/16 embryos. **(B)** Construct 103A driving *lacZ* preferentially in the anterior myocardial cells (arrowhead). **(C)** Construct 103B driving *lacZ* strongly in myocardial cells along the entire heart and in the identical pattern as construct 73 (Fig. 1I). **(D)** Construct 103C driving *lacZ* preferentially in the posterior myocardial cells (arrowhead). **(E–H)** Two copies of Henh1 construct driving *lacZ*. **(E)** *lacZ* expression in all myocardial (arrowheads) and more weakly in all pericardial cells (arrows) of the heart. Note that the myocardial cells that normally do not express *tinman* express *lacZ* more weakly. **(F)** Embryo double-labeled with anti-PC (blue) and anti-β-gal showing the juxtaposition anti-PC labeled pericardial cells next to strongly *lacZ* expressing myocardial cells. **(G)** Note the coincidence of *lacZ* (brown) and *Dmef2* expression (blue) in the myocardial cells (arrowheads, stage 14 embryo). **(H)** Note the coincidence of *lacZ* and *Dmef2* expression in the forming cardiac mesoderm (arrowheads, stage 11 embryo).

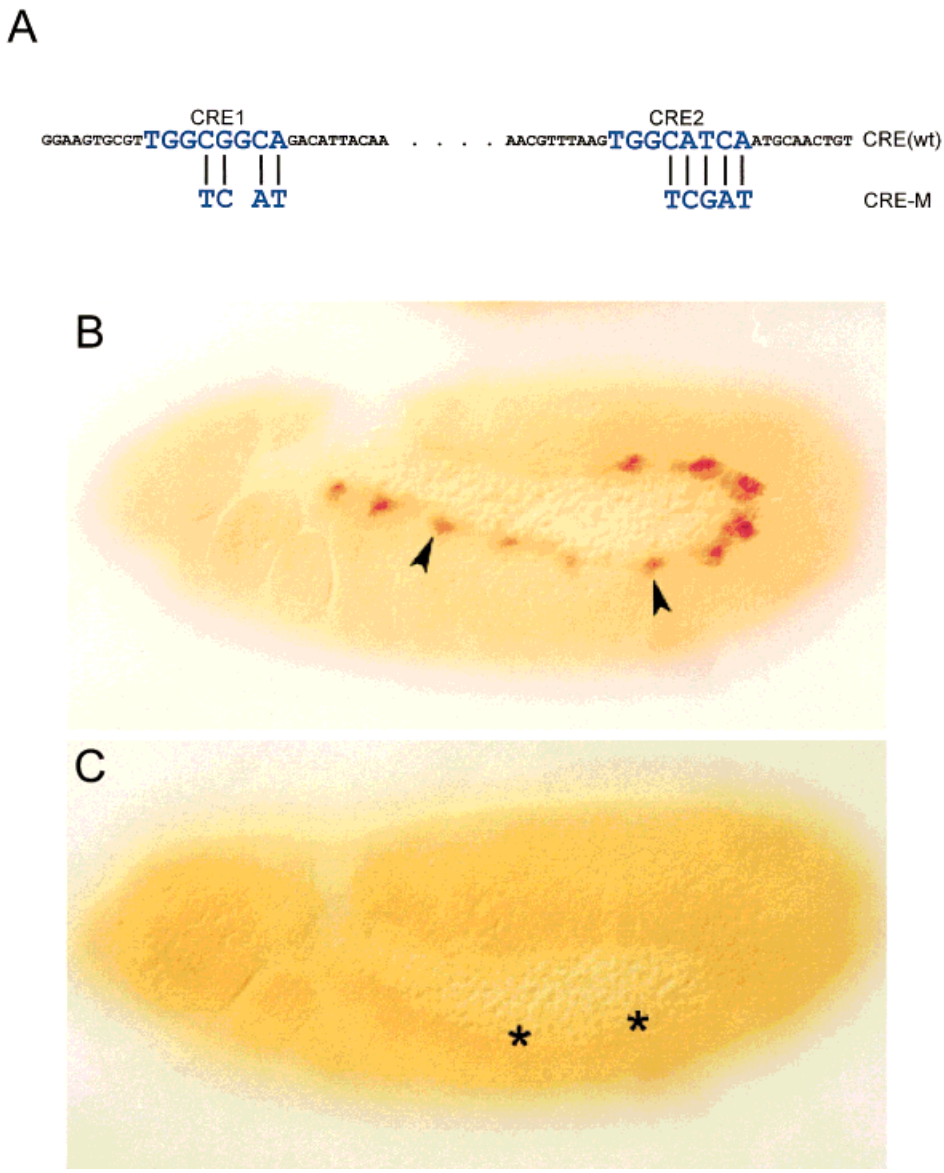

FIG 5. Mutated CREB consensus binding sites abolishes cardiac expression of myocardial heart enhancer. **(A)** Wild-type and mutant sequence of CRE1/2-containing Henh1. **(B)** Stage 11/12 embryo containing one copy of wild-type Henh1 driving *lacZ*. Note expression in the cardiac mesoderm (arrowheads). All five independent insertions of Henh1 (1× and 2×) showed strong expression in the cardiac mesoderm. **(C)** Stage 11/12 embryo containing one copy of CRE1/2-mutated Henh1 driving *lacZ*. Note the lack *lacZ* expression in the cardiac mesoderm (asterisks). All four independent insertions of the mutated Henh1 construct lacked expression in the cardiac mesoderm.

final solution, which lacked urea, included 20 mM phosphate-buffered saline (PBS) (pH 7.5) and 20% glycerol. Most of the fusion protein remained soluble under these conditions. Sodium dodecyl sulfate (SDS) gel electrophoresis of the solution showed that the majority of the solubilized protein in all samples migrated as single band (data not shown). There were a few additional bands containing relatively insignificant amounts of protein. (These were later determined to be degradative products of the Tinman fusion proteins.) Consequently, the proteins used for antibody production were obtained by preparative SDS gel electrophoresis of the solutions containing the eluted fusion proteins. Regions of polyacrylamide gels that contained the primary fusion protein band were excised and frozen.

Anti-Tinman antibodies were prepared by Immuno-Dynamics Inc. (La Jolla, CA.). A combination of both the full-length Tinman fusion protein as well as the smaller homeodomain-containing protein were used as antigens in each of two rabbit. Excised gel bands containing Tinman fusion proteins were injected into rabbits following standard procedures. Animal were boosted four times and the sera obtained following the final boost was purified with CM Affi-Gel blue (Bio-Rad; Richmond, CA) affinity chromatography to produce the protease free globulin fraction used in these studies.

Generation of Promoter-*lacZ* Constructs and Transgenic Flies

Cloning of genomic region of *tinman* is described in Bodmer (1993) and Azpiazu and Frasch (1993). Position −1 of the genomic DNA sequence is defined as the base before the first ATG codon of the open reading frame of the *tinman* cDNA (Bodmer *et al.*, 1990). Position +1 is

defined as first base after the TAG stop codon of the *tinman* cDNA. Intron sequences are numbered separately.

Construct 2A was made with the 8.0 kb *Bam*HI upstream fragment that extends into intron I (see Fig. 1A). 2A was blunted and subcloned into the StuI cloning site of the vector pWHL, which contains the hsp70-TATA-box (providing a core promoter) in front of the *lacZ* coding region (Ip *et al.*, 1992). Three restriction fragments, 1.4 kb ClaI-NheI (intron I containing construct 41), 0.7 kb NheI-HindIII[1] (intron II containing construct 42) and 1.64 kb NheI-EcoRI (construct 34, containing intron II and 3′ coding and noncoding sequences), were subcloned the same way into pWHL. A 1.0 kb HindIII[1]-EcoRI fragment (construct 56) having part of the third exon, the 3′ UTR and some 3′ noncoding region was generated by partially digesting the NheI-EcoRI fragment with HindIII before subcloning into pWHL. Intron I (807 bp) was also amplified by PCR using plaque-forming units (pfu) polymerase and primers pairs which had an overhang of sequences for SphI site. The forward primer was 5′-CCG GCA TGC GTA AGC TTG GAA GTT TTG AA-3′ and the reverse primer was 5′-GGC GCA TGC GCA TGC GCG CTG CGG GAA AGC AG-3′. The PCR product was digested with SphI and cloned into pWHL. The identity of the constructs was confirmed by sequencing. Short regions of 5′ upstream region were cloned in a similar way. Construct 75 (−1159 to −959, 202 bp) was amplified with forward primer 5′-GCC GCA TGC TAT GTA TAT GTG TGT ACT GT-3′ and reverse primer 5′-GGC GCA TGC CAC TTG AAA CTG TTA TGA TT-3′. Construct 76 (−1159 to −807, 352 bp) was amplified using forward primer 5′-GCC GCA TGC TAT GTA TAT GTG TGT ACT GT-3′ and reverse primer 5′-CGG GCA TGC GCG TCT TGT GTA TTA AAC GG-3′. Construct 77 (−928 to −801, 120 bp) was amplified by forward primer 5′-GCC GCA TGC TTT AAA CCA AAC ATA TTC CC-3′ and reverse primer 5′-CGG GCA TGC GCG TCT TGT GTA TTA AAC GG-3′.

An upstream 2.1 kb genomic ClaI fragment that also contains part of exon I including position −1 was subcloned into pBSK+. From this subclone, sequences upstream of −1 bp (deleting the native ATG) was amplified using the T7 primer and an internal reverse primer (from −20 to −1, primer sequence 5′-TTC GGT ACC CCT CGC TGT GCG ATC ACT TG-3′) with a restriction site overhang of KpnI. The PCR product was cut with KpnI and ClaI and subcloned into pBSK+ (construct 35). The identity of the PCR product was confirmed by cycle sequencing. Construct 35 was then digested with KpnI and BamHI and subcloned into the KpnI-BamHI site of pCasper-AUG-βgal (Thummel *et al.*, 1988) (construct 44). 5′ deletions of construct 35 were made by digesting with EcoRI and KpnI (construct 49, −1085 to −1) or PvuII and KpnI (construct 51, −372 to −1) and ligating into pCasper-AUG-βgal similar to construct 44. Larger fragments that included region upstream of the ClaI site were generated by fusing the different length genomic fragments into the ClaI site. Constructs 43, 45, and 47 were generated by fusing the BamHI-ClaI, HindIII-ClaI, and EcoRI-ClaI fragments, respectively, with construct 35 at the ClaI site. All deletions were subcloned into pCasper-AUG-βgal as KpnI–BamHI or KpnI-EcoRI fragments.

To check the interaction of intron I and 5′ upstream region, intron I containing construct 41) was subcloned into ClaI site of construct 35 to fuse with upstream region (construct 41 + 44). To further test the results from construct 41 + 44, intron I sequences from construct FNT were also fused with construct 51 and 44 at the PvuII and ClaI sites, respectively, to generate constructs FNT + 51 and FNT + 44, respectively. Fusions of construct 76 and the first intron sequences were also generated. This was achieved by PCR amplification of region 76 using same forward primer (described above) but with an overhang of sequences for XbaI and same reverse primer sequence (described above) with an overhang of sequences for SphI site. Similarly I intron sequences were generated by PCR using forward and reverse primer with an overhang of SphI and XbaI sites. Both PCR products were purified, digested with SphI and XbaI, mixed and ligated into SphI site of pWHL, to generate FNT + 76. Orientation and the sequence in the final insert were verified by sequencing.

A 1.4 kb HindIII (+132 to +1524) fragment (H^2-H^3, see Fig.1A) of the 3′ region was subcloned into StuI site of pWHL after blunting the ends (construct 73). Primers (listed below) were made with SphI overhangs and fragments were amplified using pfu polymerase. SphI digested PCR fragments were subcloned into SphI site of the pWHL vector. Fragment 103A (+132 to +744, 612 bp) was amplified with 5′-GCC GCA TGC AAG CTT AAG ACC AAT CAG TT-3′ (forward) and 5′-GGC GCA TGC CCA ACG CAC TTC CCC TTT CC-3′ (reverse), fragment 103A500 (+132 to +633, 501 bp) with 5′-GCC GCA TGC AAG CTT AAG ACC AAT CAG TT-3′ (forward) and 5′- GGC GCA TGC GAA TTC GTT TTT CAT AAA AC-3′ (reverse), fragment 103B (+402 to +1022, 611 bp) with 5′-GCC GCA TGC TCA GCT AAA GAT CAA TCA GA-3′ (forward) and 5′-GGC GCA TGC CTG CAA CCC CCC GTT TAC CC-3′ (reverse), fragment 103C (+722 to +1232, 530 bp) with 5′-GCC GCA TGC AGT GCG TTG GCG GCA GAC AT-3′ (forward) and 5′-GGC GCA TGC TCC AGT AAC TAT TTG ATG TC-3′ ′ (reverse), fragment 103D (+1232 to +1832, 650 bp) with 5′-GCC GCA TGC CGG GTA AAC GGG GGG TTG CA-3′ (forward) and 5′-GGC GCA TGC TAT AAA ATG TCT TGG CTA AA-3′ (reverse), fragment 103E (+1332 to +1932, 701 bp) with 5′-CGG GCA TGC GAG ACA TCA AAT AGT TAC TG-3′ (forward) and 5′-GGC GCA TGC GGA TCC TCG ACG GAG GGG CA-3′ (reverse), Henh1 (+616bp to +921bp, 305 bp) with 5′-GCC GCA TGC GAA TTC GAA GGC GAA CGG CA-3′ (forward) and 5′-GGC GCA TGC CTC GAG CCC CCA ATG AAA TA-3′ (reverse), and Henh2 (+661 to +913, 362 bp) with 5′-GCC GCA TGC AAT TGA AAT GTT TTC CCT TT-3′ (forward) and 5′-GGC GCA TGC GCT GCA ACC CCC CGT TTA CC-3′ (reverse). Two copies of Henh1 (2×) were made using

the same forward and reverse primers: in one reaction forward primers with SphI overhang and reverse primer with XbaI overhang were used, in the other reaction forward primers with XbaI overhang and reverse primers with SphI overhang were used. PCR products from both reactions were purified from gel, digested with XbaI and SphI, and ligated into SphI site of pWHL (in head to tail orientation). Mutations in CRE sites of Henh1 were created in two PCR steps using overlapping mutant oligos and the forward and reverse primers described above for Henh1. Sequence of the mutant CRE1 oligo was 5′-AGG TTG CAT TTA TGA AAC GTT TAA GTG GTC GAT ATG C-3′ and sequence of the mutant CRE2 oligo was 5′- TCA TAA ATG CAA CCT TTG TAA TGT CAT CGA CCA ACG CAC T-3′ (see Fig. 5A). The first two PCR products were generated using mutant CRE1 oligo and reverse Henh1 primer in one PCR reaction and mutant CRE2 oligo and forward Henh1 primer in another reaction. Both PCR products were separated on an agarose gel, purified, mixed together, denatured and reannealed. From this mixture, DNA carrying mutations in both CREB sites was amplified by the Henh1 forward and reverse primers. The final PCR product was purified, sequenced and then subcloned into the SphI site of pWHL.

Transgenic flies were generated by injecting early blastoderm embryos of the genotype *w*; +/ +; +/ + with the *tinman* enhancer-*lacZ* reporter constructs (100 μg/ml) along with transposase-containing helper plasmid (50 μg/ml) according to standard procedures (Spradling, 1986; Pirrotta, 1988). Three to six independent transformants were analyzed for each promoter or enhancer construct.

Immunocytochemistry

Immunohistochemical staining of fixed embryos was performed as described (Bodmer, 1993; Su *et al*., 1998). Antibodies and dilutions were as follows: anti-Dmef-2 antibodies were used at 1:1,000 (Lilly *et al*., 1995), antibodies against a pericardial-specific cell surface antigen were used at 1:10 (Wu *et al*., 1995; Yarnitzky and Volk, 1995), and affinity-purified mouse or rabbit anti-β-galactosidase antibodies (anti-β-gal) were used at 1:1,000 and 1:4,000, respectively (Cappell; Malverne, PA). For double antibody staining the protocol by (Su *et al*., 1998) was followed, except that during the color reaction the substrate for horseradish peroxidase, diaminobenzidine (Sigma; St. Louis, MO), was used first (brown staining), followed by the Chromogen (SG substrate kit, Vector Laboratories; Burlingame, CA) color reaction (blue-gray staining). For some antibody staining, the ABC Elite kit (Vector Laboratories) was used to enhance the signal according to the manufacturer's instructions.

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