Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 9366–9371, August 1998
Developmental Biology

Differential rescue of visceral and cardiac defects in *Drosophila* by vertebrate *tinman*-related genes

MAIJON PARK*, CAROL LEWIS*, DAVID TURBAY†, AMY CHUNG‡, JAU-NIAN CHEN§, SYLVIA EVANS¶, ROGER E. BREITBART‡‖, MARK C. FISHMAN§, SEIGO IZUMO†**, AND ROLF BODMER*††

Departments of *Biology and †Internal Medicine, University of Michigan, 830 N. University, Ann Arbor, MI 48109-1048; ‡Department of Cardiology, Children's Hospital and Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115; §Cardiovascular Research Center, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129; and ¶Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, CA 92093

Communicated by Walter J. Gehring, University of Basel, Basel, Switzerland, May 26, 1998 (received for review December 10, 1997)

ABSTRACT ***tinman*, a mesodermal NK2-type homeobox gene, is absolutely required for the subdivision of the early *Drosophila* mesoderm and for the formation of the heart as well as the visceral muscle primordia. Several vertebrate relatives of *tinman*, many of which are predominately expressed in the very early cardiac progenitors (and pharyngeal endoderm), also seem to promote heart development. Here, we show that most of these vertebrate *tinman*-related genes can readily substitute for *Drosophila tinman* function in promoting visceral mesoderm-specific marker gene expression, but much less in promoting cardiac-specific gene expression indicative of heart development. In addition, another mesodermal NK2-type gene from *Drosophila*, *bagpipe*, which is normally only needed for visceral mesoderm but not heart development, cannot substitute for *tinman* at all. These data indicate that the functional equivalence of the *tinman*-related subclass of NK2-type genes (in activating markers of visceral mesoderm development in *Drosophila*) is specific to this subclass and distinct from other homeobox genes. Despite the apparent overall conservation of heart development between vertebrates and invertebrates, the differential rescue of visceral mesoderm versus heart development suggests that some of the molecular mechanisms of organ formation may have diverged during evolution.**

tinman (*tin*) and *bagpipe* (*bap*) are two mesodermal NK2 class homeobox genes that are closely linked in the *Drosophila* genome (1, 2). *tin* is expressed in the early mesoderm (3), where it appears to confer competence to a field of cells to assume a fate necessary for cardiac and visceral mesoderm development. In contrast, the initial *bap* expression is confined to the gut muscle progenitors, apparently under the control of *tin* (2). In *tin* mutants, the absence of mesodermal subdivision results in a failure of the heart and gut muscle formation, whereas in *bap* mutants only the visceral component is affected (1, 2).

Despite the difference in mature heart morphology, the early embryology of vertebrate heart development is not unlike that of *Drosophila* (4). Moreover, six members of the *tin*-related subclass (5–14) and two members of the *bap*-related subclass (14, 15) of *Nkx* genes (the vertebrate equivalents of the *Drosophila* NK2 genes) have been isolated thus far in various species. There they are also predominantly expressed in the cardiac and/or visceral primordia (reviewed in refs. 4 and 8). The distinction between *tin* versus *bap* relationship of the *Nkx* genes has not been straight forward (thus providing additional motivation for the present study): The homeodomains of the vertebrate *tin*-related genes are very similar to each other (80–90%) and clearly form a distinct subclass from those of the vertebrate *bap*-related genes, to which they are only 55–65% identical. However, the vertebrate *tin*-related homeodomains are equally similar (about 65%) to both *Drosophila tin* and *bap* homeodomains (refs. 4 and 8; see also Fig. 1*A*). By contrast, vertebrate *bap*-related homeodomains are significantly more similar to those of *bap* (70–80%) than to those of *tin* (50–60%) (14, 15). Moreover, each of the *tin*-related genes apparently is expressed in the developing heart, although not exclusively and not in every species (8). The most convincing argument for the *tin* versus *bap* relationship of the vertebrate *Nkx* genes has been the discovery of a closely linked *tin*/*bap*-related pair of genes in the mouse genome (11, 14), which suggests that the common ancestor of vertebrates and invertebrates already had a *tin*- and a *bap*-like gene.

It has been suggested that basic molecular–genetic mechanisms of heart (and perhaps also visceral) mesoderm development may be conserved between vertebrates and invertebrates (4, 8). In particular, it may be that the vertebrate *tin*-related genes, and perhaps even *Drosophila bap*, are functionally interchangeable with *tin* function in *Drosophila*. As a test for this hypothesis, we wanted to determine whether or not the vertebrate *tin*-related genes and/or *Drosophila bap* can substitute for a loss-of-*tin*-function in transgenic flies.

MATERIALS AND METHODS

Constructs and Fly Stocks. Full-length *Nkx2–3* (12, 13), *2–5* (6, 11), *2–7* (13), and *bap* (2) cDNAs were inserted behind the *hsp70* heat shock promoter at the *Kpn*I site of the WH1 vector or the *Xba*I site of hsCasPeR (16) and transgenic flies were generated as for Hs*tin* in ref. 1. At least two independent insertions of each construct were crossed into a *tin* null mutant background (tin^{EC40} and tin^{GC14}, as in ref. 1) and examined for restoration of cardiac and visceral mesodermal marker gene expression indicative of heart and/or visceral mesoderm formation. A *bap* null mutant was generated and kindly provided by M. Frasch (Brookdale Center for Developmental and Molecular Biology, Mt. Sinai School of Medicine, New York, NY): the cytological deficiency $Df(3R)e^{D7}$, which deletes both *tin* and *bap* genes, had been recombined with a transgenic insertion of a 10.7-kb genomic *Bam*HI fragment that reportedly rescues the *tin* mutant phenotype ($Df(3R)e^{D7}$,P[*tin-CasPeRe28*]; see ref. 2).

Temperature Shift Treatments. Embryos were collected on plates with shallow grape agar at 2-hr intervals at 18°C and aged at 18°C until the embryos reached the desired develop-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

‖Present address: Millennium Pharmaceuticals, Inc., 640 Memorial Drive, Cambridge, MA 02139.
**Present address: Cardiology Division, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215.
††To whom reprint requests should be addressed at: c/o Dr. C. Goridis, Department of Biology, University of Michigan, Ann Arbor, MI 48109-1048. e-mail: rolf@umich.edu.

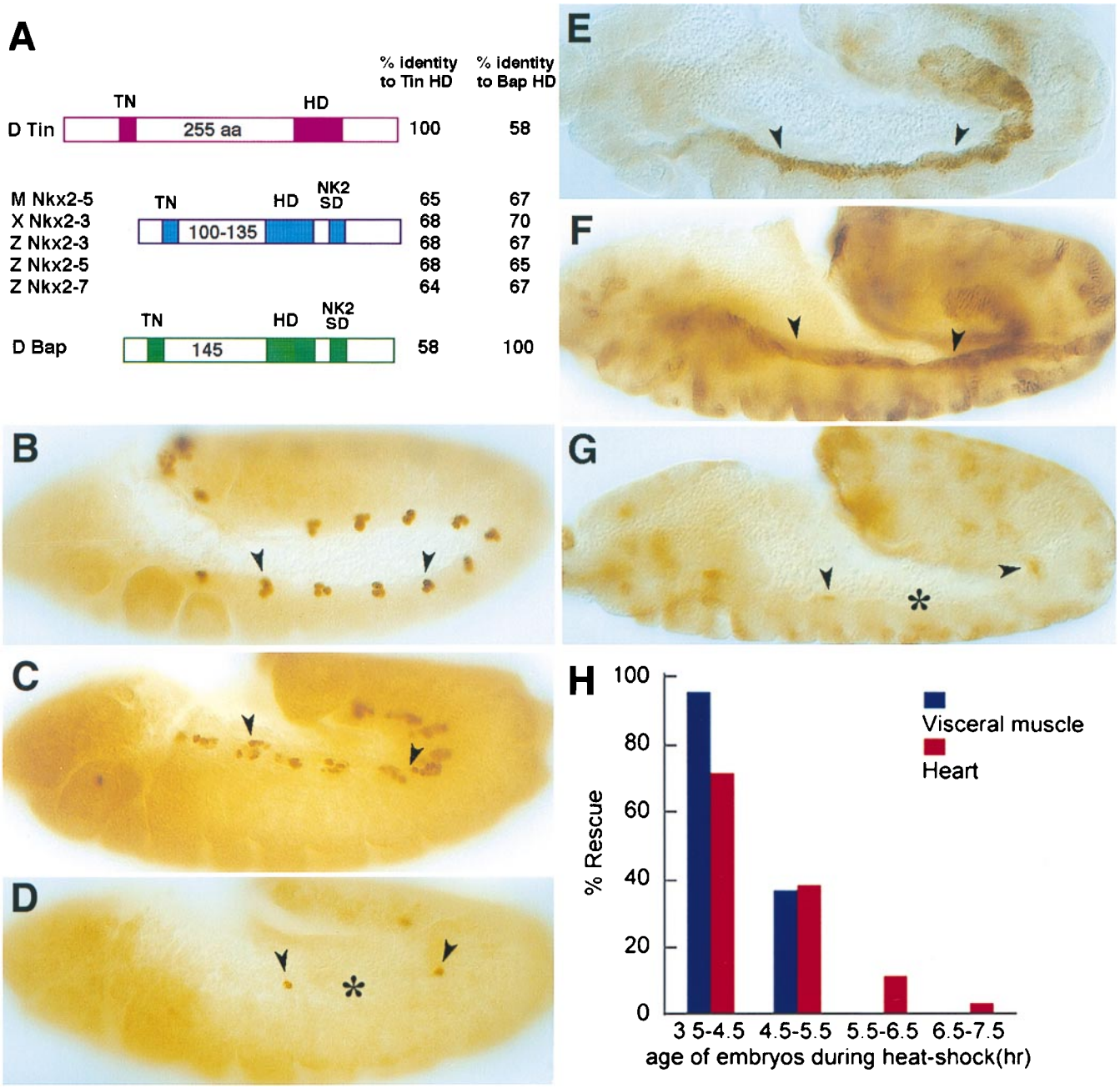

FIG. 1. (*A*) List of the cDNAs used in this study: *Drosophila tin* (D Tin), *Nkx2–5* from mouse (M Nkx2–5/Csx) and zebrafish (Z Nkx2–5), *Nkx2–3* from *Xenopus* (X Nkx2–3) and zebrafish (Z Nkx2–3), and *Nkx2–7* from zebrafish (Z Nkx2–7). Approximate gene structures and sequence identities of the *Nkx* genes to *tin* and *bap* are indicated. TN, Tin/Nkx-specific domain of 11 amino acids (4, 8); HD, homeodomain; NK2-SD, NK2-specific domain (8). (*B–G*) Immunocytochemical staining. Eve expression (*B–D*) in a subset of cardiac progenitors along the dorsal mesodermal border (arrowheads) and FasIII expression (*E–G*) in the visceral mesoderm (arrowheads) of stage 12 wild-type embryos (*B* and *E*), homozygous Hs*tin*,*tin*^{GC14} embryos (*C*, *D*, *F*, and *G*) heat shocked for 30 min between 3.5 and 4.5 hr of development (stage 9, *C* and *F*) or between 5 and 6 hr of development (early stage 11, *D* and *G*). Asterisks indicate the absence of marker gene expression in the presumptive heart (D) and visceral mesoderm (G) when heat shocked later. (*H*) Graph represents the amount of marker gene expression in presumptive cardiac (Eve expression in red) or visceral mesoderm (FasIII expression in blue) as a consequence of heat shock induction of the *tin* transgene. Each column represents the mean of 30 embryos or more. Anterior in all micrographs is to the left and dorsal is up.

mental stage. Staged embryo-containing plates were covered and submerged once in a water bath at 37.5°C for 30–40 min or twice for 20–25 min with 1 hr of incubation at 18°C in between. The embryos were then aged at 18°C until fixation. Ages indicated in the text were adjusted for a standard at 25°C (development was about half as fast at 18°C).

Chimeric Constructs. *tin:Nkx2–5* chimeric constructs were made as follows: a mouse *Nkx2–5/Csx* fragment [310 bp (ref. 11)] containing the homeodomain and the NK2-SD were inserted into the full-length *tin* cDNA in which the homeodomain 38-aa 5′ and 30-aa 3′ to the homeodomain were deleted [bp 1028–1459 (ref. 3)]. The zebrafish TN-homeodomain fragment was made with PCR exactly from the beginning of the TN-domain to the end of the homeodomain [bp 82–591 (ref. 6)] was inserted at the equivalent location in the *tin* cDNA (bp 393-1361). The chimeric cDNAs ware then inserted into the *Xba*I site of hsCasPeRWH1.

Immunocytochemistry. Antibody staining and tissue *in situ* hybridization of whole-mount embryos were performed as described in ref. 1. Anti-Eve (17) was used at 1:10,000 and

anti-FasIII (18) and an antibody that marks the differentiating pericardial cells (obtained from T. Volk, Weizmann Institute, Rehovot, Israel) were used at 1:10. Homozygous mutant embryos were identified by the lack of reporter gene expression present on the balancer chromosomes that were used (as in ref. 1).

RESULTS

We used *hsp70* heat shock promoter constructs to drive expression of the following *Nkx*-type genes in flies (Fig. 1*A*): *Drosophila tin* itself (3), *Xenopus* and zebrafish *Nkx2–3* (12, 13), mouse and zebrafish *Nkx2–5* (6, 11), zebrafish *Nkx2–7* (13), and *Drosophila bap* (2). Transgenic flies harboring these conditional expression constructs were recombined with a *tin* null mutation and assayed for restoration of heart and visceral mesoderm marker gene expression (1). All of the transgenes were expressed ubiquitously and at high levels after induction (data not shown). If *tin* expression is induced after gastrulation, but before the mesoderm subdivides, markers of heart (Fig. 1 *B* and *C*) and visceral mesoderm formation in *tin* mutant embryos (Fig. 1 *E* and *F*) are well restored. Similar results were obtained with an early (Eve; ref. 17) and a late cardiac marker gene (data not shown; see *Materials and Methods*). Similarly, not only the marker gene *FasIII* (18) is activated in the prospective visceral mesoderm, but also the characteristic palisade morphology of the forming visceral mesoderm epithelium seems to be restored (Fig. 1 *E* and *F*). This suggested that appropriate cardiac and visceral mesoderm cell types have, at least in part, been induced as a consequence of *tin* transgene expression during mid-embryogenesis (although a functional heart is not formed with this protocol of transgene induction). In contrast, if *tin* is induced at a later time, the *tin* mutant phenotype as assayed with Eve and FasIII is progressively less rescued (Fig. 1 *D*, *G*, and *H*). Thus, it seems that *tin* function is first required in the early mesoderm to allow the specification of heart and visceral mesoderm progenitor tissues (1, 2).

We used this experimental paradigm to examine the rescue capabilities of the *tin*-related genes of vertebrates (Fig. 1*A*). We first examined the *Nkx2–5* gene, because it is primarily expressed in the early heart (but also in the anterior visceral endoderm) and because it has been shown to be, in part, necessary and sufficient for heart development (5–7, 9). When mouse *Nkx2–5*/*Csx* is induced at the optimal time for rescue (Fig. 1*H*), expression of the visceral mesoderm marker, FasIII, seemed to be significantly restored, but heart markers were not (Fig. 2 *A–D*). The same experiment carried out with *Nkx2–5* derived from zebrafish also gave robust rescue of the visceral mesoderm marker but only minimal rescue of heart markers (data not shown). Moreover, analysis of several independent transgenic insertions or when two (instead of one) heat shocks were applied resulted in essentially the same observations: 60–80% rescue of visceral mesoderm marker gene expression but only minimal rescue of heart markers (<10%). Thus, *Nkx2–5* is capable of efficiently initiating, directly or indirectly,

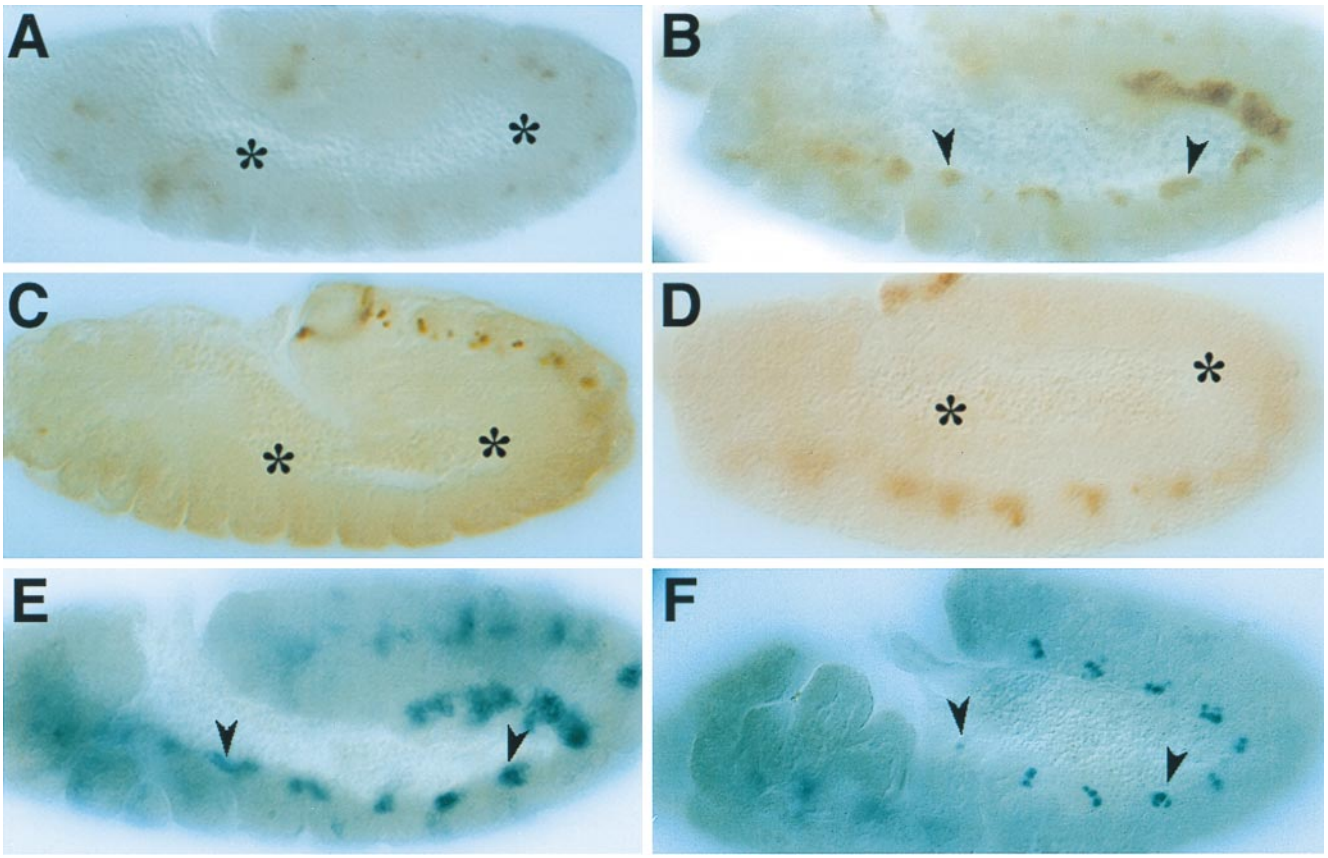

FIG. 2. Stage 12 embryos stained for FasIII (*A*, *B*, and *E*) and Eve (*C*, *D*, and *F*), compare with Fig. 1 *B* and *E* for wild-type patterns. (*A–D*) Induction of full-length *MNkx2–5* in homozygous *tin* mutant background (Hs*MNkx2–5*,*tin*^{EC40}). Without heat shock (*A* and *C*) no visceral mesoderm (*A*, asterisks) nor cardiac progenitors (*C*, asterisks) form, which is typical of *tin* mutants. In contrast, heat shock at 3.5–4.5 hr of development (stage 9) restores marker gene expression for visceral mesoderm considerably (*B*, arrowheads), but does not restore heart development markers (*D*, asterisks, compare with Fig. 1 *B* and *C*). (*E*) Induction of full-length *XNkx2–3* in homozygous *tin* mutant background (Hs*XNkx2–3*,*tin*^{EC40}) restores visceral mesoderm (arrowheads) but not heart marker gene expression (data not shown). The same result was obtained with *ZNkx2–7* (data not shown). When two consecutive heat shocks were given to each of these three transgenes, they did not result in more rescue. (*F*) Induction of full-length *ZNkx2–3* in homozygous *tin* mutant background (Hs*ZNkx2–3*,*tin*^{EC40}) restores not only visceral mesoderm (data not shown) but also cardiac markers (arrowheads).

visceral mesoderm- but not cardiac-specific gene expression. Although unlikely to be the sole reason, it is also possible that quantitative differences in transcriptional activation, mRNA, or protein stability are contributing to this differential activation of visceral mesoderm versus heart markers.

We then examined the rescue abilities of *Nkx2–7* from zebrafish, because its expression pattern appears to prefigure the expression of *Nkx2–5* in the heart and also that of *Nkx2–3* in the anterior endoderm (13). As it is the case with the *Nkx2–5* genes, *Nkx2–7* induction in fly embryos only rescues efficiently marker gene expression in the presumptive visceral mesoderm (data not shown). Thus, *Nkx2–5* and *Nkx2–7* can substitute for *tin* with respect to activating visceral mesoderm markers but not (or only minimally) with respect to markers of heart development. This suggests that the direct target genes of *tin* required for cardiac development are likely to be distinct from those required for visceral mesoderm development, and perhaps the regulation of cardiac targets has diverged more extensively between vertebrates and invertebrates.

Next, we examined the rescue activities of *Nkx2–3*, which in *Xenopus* has an expression pattern similar to that of *Nkx2–5* (12) and also produces enlarged hearts when overexpressed (5). As with *Nkx2–5*, expression of *Xenopus Nkx2–3* (*Xnkx2–3*) activates visceral mesoderm- (Fig. 2*E*) but not cardiac-specific gene expression (data not shown). In contrast, however, *Nkx2–3* from zebrafish (*ZNkx2–3*), whose normal expression domain includes primarily the presumptive anterior endoderm and does not seem to overlap extensively with the heart progenitors after gastrulation (13), can rescue not only visceral mesoderm markers (data not shown) but also cardiac-specific gene expression in *Drosophila* (Fig. 2*F*). Thus, it seems that all three of these *tin*-related *Nkx* genes have similar activities in promoting *Drosophila* gene expression specific for visceral mesoderm in place of *tin*, but only *ZNkx2–3* has significant levels of both *tin* activities. Sequence comparisons between *tin* and *ZNkx2–3* or all other known *Nkx* genes, however, did not reveal particularly striking similarities outside the TN and the homeodomain between *tin* and *ZNkx2–3* (see *Discussion*).

Since all of the NK2-type genes examined thus far were capable of restoring the activation of visceral mesoderm markers, we wondered if NK2-type genes in general are functionally promiscuous with respect to visceral mesoderm-specific gene expression when present in the mesoderm at the time when *tin* is normally required. We chose to examine the rescue ability of *bap* because it is the closest relative of *tin* in *Drosophila*. When tested in a *bap* null mutant background, in which no visceral mesoderm forms (as in Fig. 3*A*; M. Frasch, personal communication), ubiquitous induction of transgenic *bap* activity by heat shock rescues visceral mesoderm marker gene expression considerably (Fig. 3 *A* and *B*). However, induction of *bap* expression in *tin* mutant embryos does not activate heart or visceral mesoderm-specific gene expression (Fig. 3 *C* and *D*). Thus, in contrast to the *tin*-related *Nkx* genes, *bap* is clearly not able to substitute for any of the *tin* functions during mesodermal subdivision. This is particularly remarkable, since *tin*, but not *bap*, seems to have lost its NK2-specific domain, and the homeodomains of the *tin*-related *Nkx* genes are as similar to those of *bap* as to those of *tin* (Fig. 1*A*). Thus, because only *Nkx2–3*, *2–5*, and *2–7* but not *bap* exhibit (partial) *tin*-like functions in our *Drosophila* assay, it is suggested that this group of *Nkx* genes is evolutionarily more closely related to *tin* than to *bap*. This suggestion of a *tin*-related subclass of NK2-type genes is further supported by the fact that the vertebrate *bap*-related homeodomains are considerably more similar to that of *bap* than to that of *tin* (11, 14).

Since most of the *tin*-related *Nkx* genes do not rescue heart but do rescue visceral mesoderm markers, we wondered which structural differences between *tin* and these genes could account for this lack of cardiogenic activity. Obvious candidates are the NK2-specific domain (not present *tin*), the difference in length and sequence of the region from TN to homeodomain, and the difference in homeodomain sequence (see Fig. 1*A*). In a first attempt to distinguish between these possibilities, we made chimeric *tin*/*Nkx2–5* cDNA constructs (Fig. 4*A*) and examined their ability to rescue cardiac markers in *tin* mutant embryos.

When the *tin* homeodomain (including some flanking sequences) was replaced by the *Nkx2–5* homeo- and NK2-specific domain (Fig. 4*A*), heart-specific markers were significantly restored (Fig. 4 *B* and *C*). These data suggest that the

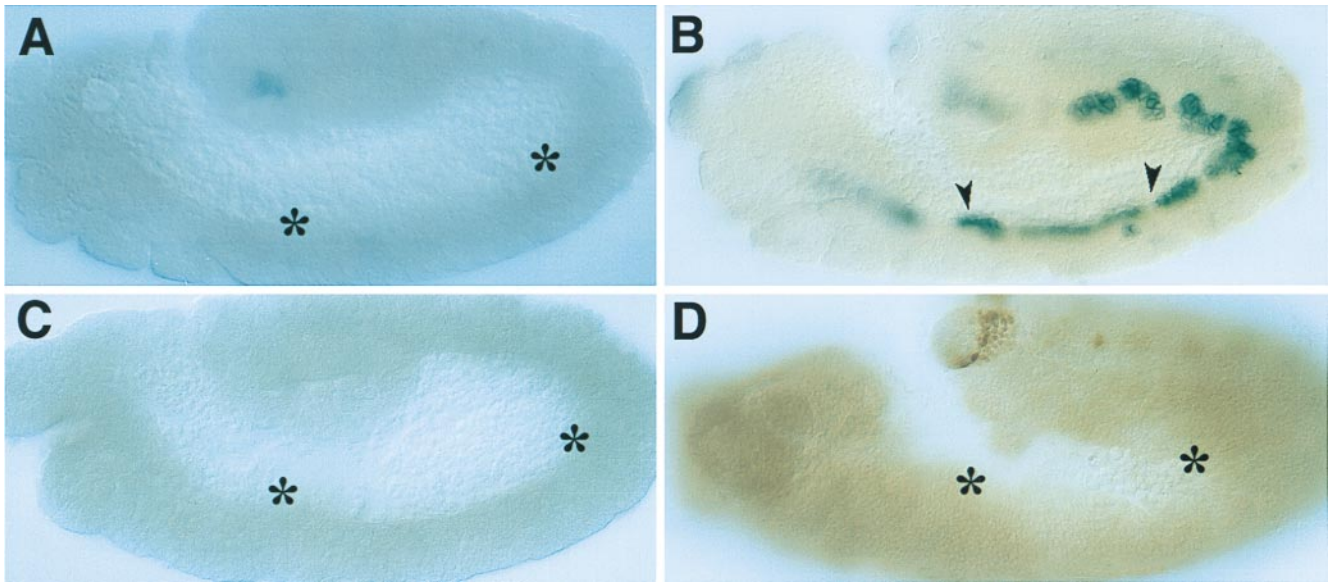

FIG. 3. Stage 12 embryos stained for FasIII (*A–C*) and Eve (*D*), compare with Fig. 1 *B* and *E* for wild-type patterns. (*A* and *B*) Induction of full-length *bap* in homozygous *bap* mutant background (Hs*bap,Df(3R)e*^{D7},P[*tin-CasPeRe28*]; see ref. 2). Without heat shock no visceral mesoderm forms (*A*, asterisks), which is typical of *bap* mutants. In contrast, heat shock at 3.5–4.5 hr of development (stage 9) restores visceral mesoderm marker considerably (*B*, arrowheads). (*C* and *D*) Induction of full-length *bap* (same transformant as in *A* and *B*) in homozygous *tin* mutant background (Hs*bap,tin*^{EC40}) with a heat shock at stage 9 does not restore marker gene expression for either visceral mesoderm (*C*) or cardiac progenitors (*D*). The same results were obtained with other transgene insertions or when two heat shocks were applied. Tissue *in situ* hybridization after heat shock with a *bap* antisense RNA probe shows high and ubiquitous levels of *bap* expression (data not shown).

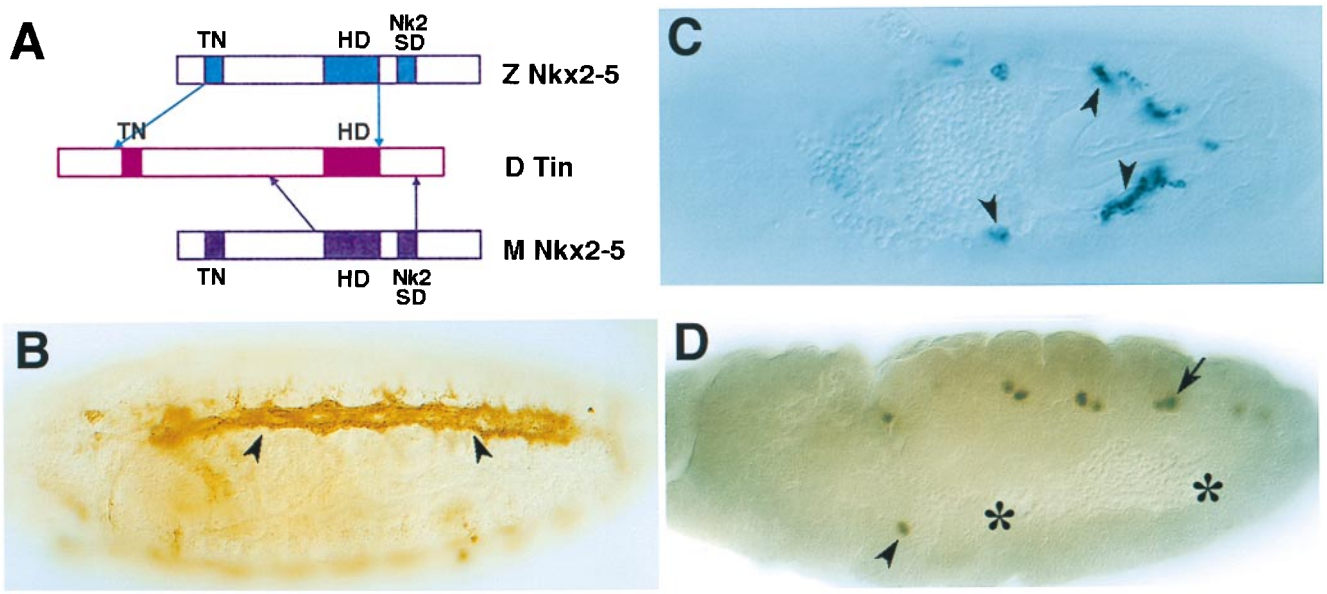

FIG. 4. (*A*) Schematic of the chimeric constructs between *tin* and *Nkx2–5*. In one construct, the *tin* homeodomain was replaced by the *MNkx2–5* homeodomain and NK2-specific domain (*tin:2.5*HD-NK2SD). In the other construct, the region from the TN domain to the homeodomain of *tin* was replaced by the same region from *ZNkx2–5* (*tin:2.5*TN-HD). (*B* and *C*) Immunocytochemical staining of a monoclonal antibody specific for pericardial cells of the heart at late stages of development (obtained from T. Volk). Wild-type (*B*) and Hs*tin:2.5*HD-NK2SD,*tin*^{EC40} embryos heat shocked at 3.5–4.5 hr of development (stage 9, *C*). Note the presence of many pericardial cells (arrowheads) after induction of this chimeric *tin* gene, although in a disorganized pattern. Without induction, pericardial cells do not form at all (data not shown). The same result was obtained with antibodies against Eve. Visceral mesoderm marker is also restored in heat-shocked embryos of this genotype (data not shown). (*D*) Early heat shock (3.5–4.5 hr of development) of Hs*tin:2.5*TN-HD,*tin*^{EC40} embryos restores visceral mesoderm marker (data not shown), but as with the full-length *Nkx2–5* genes, heart markers are absent (asterisks), except for an occasional cell (arrowhead indicates Eve expressing cell). Eve expression in the central nervous system is not affected (arrow).

homeodomains of *tin* and *Nkx2–5* are functionally interchangeable in this *Drosophila* assay. This implies that the target specificity of the *tin* gene product for cardiac-specific gene expression is unlikely to be encoded exclusively by the homeodomain and flanking sequences (in contrast what seems to be the case for some of the *hox* genes). Consistent with such an interpretation is the finding that *in vitro* both *tin* and *Nkx2–5* recognize the same consensus binding site (19; T. V. Venkatesh and R.B., unpublished data). In addition, the presence of the NK2-specific domain (in conjunction with the *Nkx2–5* homeodomain) does not appear to be interfering with the cardiac rescue ability of the *tin* transgene.

We also replaced the region between (and including) the TN domain and the homeodomain of *tin* with the equivalent region of *Nkx2–5* (Fig. 4*A*). When examined in transgenic *tin* mutant embryos, this chimeric protein rescues FasIII expression in the presumptive visceral mesoderm (data not shown), as the full-length *Nkx2–5* genes do, but heart development markers were not appreciably restored (Fig. 4*D*). The cardiogenic activity of *tin* is likely to involve the region between the TN domain and the homeodomain. This region is much larger (255 amino acids) in *tin* than in the *tin*-related *Nkx* genes (100–135 amino acids) or in *bap* (145 amino acids; see Fig. 1*A*). Either *tin* contains a *Drosophila* heart-specific domain (not present or conserved in the other genes) or these vertebrate *Nkx* genes have less overall cardiogenic activity, simply because their TN to homeodomains are shorter than those of *tin*. These results support the idea that *tin* in insects or its ancestor has diverged more than the vertebrate *tin*-related genes from their postulated ancestor. It will be interesting to find out whether or not, in a converse experiment, *Drosophila tin* is capable of substituting for *Nkx2–5* and restoring cardiac differentiation of *Nkx2–5* mutant hearts (7).

DISCUSSION

The data we present here provide strong evidence that a subset of the NK2-type genes, the postulated *tin*-related *Nkx* genes, can substitute for *tin* function with respect to the restoration of markers of visceral mesoderm development but not with respect to those of heart development (with the exception of zebrafish *Nkx2–3*). This rescue activity seems to be specific to the *tin*-related subclass of *Nkx* genes, which excludes the *Drosophila* NK2-type gene *bap* and probably also its vertebrate counterparts. We propose that *tin* in flies has diverged significantly from its ancestor after the vertebrate/invertebrate split during evolution (thereby losing its NK2-SD). During this process, *tin* may have adopted a new fly- or insect-specific cardiac function, thereby changing its spectrum of interactions and targets. Alternatively, or in addition, the ancestor of the vertebrate *tin*-related genes may have lost some of its old cardiogenic functions and perhaps adopted additional functions during evolution. Consistent with this view is the finding that the different *Nkx* genes have little similarities between the TN and the homeodomain (e.g., ref. 13; the case of *Nkx2–3* is discussed, see below). We presently do not know whether the *tin*-specific cardiogenic activity is encoded in a discrete domain N-terminal to homeodomain or whether this activity is distributed along much of the coding region.

Nkx2–3 of zebrafish (as *tin* of *Drosophila*) is capable of initiating cardiac-specific gene expression, but the other *tin*-related *Nkx* genes including *Nkx2–3* from *Xenopus* are not. A possible reason for this observation could be that *ZNkx2–3* has low levels of sequence similarities to *tin* that have initially not been obvious (13) and that are not present in the other genes. Indeed, there is a 54-aa stretch 5′ to the homeodomain of *ZNkx2–3* (amino acids 83–136; ref. 13) that is 23% identical (with only one 2-aa gap) to a 56-aa region in between the TN and the homeodomain of *tin* (amino acids 200–255; ref. 3). The same region in any of the other *Nkx* genes does not show any similarity to *tin*. Further domain-swap experiments are needed to determine whether this region is of functional significance for heart-specific gene expression in the *Drosophila* assay. It is possible that the postulated common ancestor of vertebrate

and insect *tin*-related genes already contained this 54/56-aa region, but because of the extremely low level of sequence identity, this remains speculative.

The fact that most vertebrate *tin*-related *Nkx* are expressed predominantly in the developing heart but rescue only visceral mesoderm markers in flies, and that *ZNkx2–3* is not expressed in heart tissue but does rescue heart markers suggests that molecular mechanisms of organ development are interchangeable, within limits, between different organs during the course of evolution. It may be that *tin* in flies acts in heart and visceral organ development whereas its relatives in vertebrates have adopted distinct and regionally more localized functions in the development of either one of these organs. For example, *XNkx2–3* may have adopted a function in heart development during recent vertebrate evolution, whereas its counterpart in zebrafish may have assumed a function in pharyngeal endoderm development.

With these considerations in mind, we propose that the spectrum of developmental functions that a *tin*-related gene may be able to assume is likely to be restricted to a limited set (e.g., heart and visceral mesoderm and endoderm), delineated by the spectrum of functions of the postulated ancestor. The fact that in *Drosophila tin* is still required for both heart and visceral mesoderm is consistent with this hypothesis: Although *tin* in *Drosophila* may have retained much of its developmental requirement for the spectrum of organs specified by the postulated ancestor, its amino acid sequence may have diverged significantly and adopted fly heart-specific functions that may be distinct from those of most of the *tin* relatives in vertebrate heart development.

Note Added in Proof. The ability of *Nkx2–5* to rescue mutant defects in *Caenorhabditis elegans* and *Drosophila* has recently been reported by Haun *et al.* (20) and Ranganayakulu *et al.* (21), respectively.

We thank M. Frasch for the *bap* mutant fly stocks prior to publication, the *bap* cDNA and Even-skipped antibodies. We thank T. Volk for the antibody against against pericardial cells. M. Park was supported by a fellowship from the Organogenesis Center at the University of Michigan. This work was supported by grants from the National Institutes of Health (to R.B. and S.I.), and a grant from the American Heart Association (to R.B.). R.E.B. was supported by grants from the Boston Children's Heart Foundation, the National Institutes of Health, and by an Established Investigatorship from the American Heart Association.

1. Bodmer, R. (1993) *Development* **118,** 719–729.
2. Azpiazu, N. & Frasch, M. (1993) *Genes Dev.* **7,** 1325–1340.
3. Bodmer, R., Jan, L. Y. & Jan, Y.-N. (1990) *Development* **110,** 661–669.
4. Bodmer, R. (1995) *Medicine (Baltimore)* **5,** 21–27.
5. Cleaver, O. B., Patterson, K. P. & Krieg, P. A. (1996) *Development* **122,** 3549–3556.
6. Chen, J.-N. & Fishman, M. C. (1996) *Development* **122,** 3809–3816.
7. Lyons, I., Parson, L. M., Hartley, L., Li, R., Andrews, J. E., Robb, L. & Harvey, R. (1995) *Genes Dev.* **9,** 1654–1666.
8. Harvey, R. P. (1996) *Dev. Biol.* **178,** 203–216.
9. Fu, Y. & Izumo, I. (1995) *Roux's Arch. Dev. Biol.* **205,** 198–202.
10. Newman, C. S. & Krieg, P. A. *Dev. Genet. (Amsterdam)* **22,** 230–239.
11. Komuro, I. & Izumo, S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8145–1949.
12. Evans, S. M., Yan, W., Murillo, M. P., Ponce, J. & Papalopulu, N. (1995) *Development* **121,** 3889–3899 .
13. Lee, K.-H., Xu, Q. & Breitbart, R. E. (1996) *Dev. Biol.* **180,** 722–731.
14. Tanaka, M., Kasahara, H., Bartunkova, S., Schinke, M., Komuro, I., Inagaki, H. Lee, Y., Lyons, G. E. & Izumo, S. (1998) *Dev. Genet. (Amsterdam)* **22,** 239–249.
15. Newman, C. S., Grow, M. W., Cleaver, O. B., Chia, F. & Krieg, P. A. (1997) *Dev. Biol.* **181,** 223–233.
16. Thummel, C. S., Boulet, A. M. & Lipshitz, H. D. (1988) *Gene* **74,** 445–456.
17. Frasch, M., Hoey, T., Rushlow, C., Doyle, H. & Levine, M. (1987) *EMBO J.* **6,** 749–759.
18. Patel, N., Snow, P. M. & Goodman, C. S. (1987) *Cell* **48,** 975–988.
19. Chen, C. Y. & Schwartz, R. J. (1995) *J. Biol. Chem.* **27,** 15628–15633.
20. Haun, C., Alexander, J., Stainier, D. & Okkema, P. G. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 5072–5075.
21. Ranganayakulu, G., Elliott, D. A., Harvey, R. P. & Olson, E. N. (1998) *Development,* in press.