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NK-2 Homeobox Genes and Heart Development

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Analysis of the phylogenetically ancient *NK-2* class of homeobox genes has opened up an entirely new approach to molecular, genetic, and biochemical analysis of early heart development. The *Drosophila NK-2* homeobox gene *tinman* plays an essential role in segregating cardiac and visceral muscle potentiality, as well as that of some somatic muscles, in nascent mesoderm of the fly embryo. In its absence, precursor cells for these muscles do not form. *tinman* homologues have now been isolated from vertebrate genomes and at least one of them, *Nkx2-5,* is expressed in heart progenitor cells and is essential for myogenic and morphogenetic differentiation of the mammalian heart. Signaling pathways that establish the *tin* expression domain also appear to be conserved in vertebrates. These findings suggest that heart development in flies and vertebrates utilize similar genetic pathways and engender optimism that the dissection of mammalian heart development will profoundly profit from the rich genetics of *Drosophila.* The findings also prompt the questions: are the hearts of vertebrates and invertebrates actually homologous, and how much can we learn from the comparative approach? In the sections below, the structure, regulation, function, and evolution of *NK* class homeobox genes will be reviewed, emphasizing and contrasting the roles of *tinman* and *Nkx2-5* in heart development. © 1996 Academic Press, Inc.

NK HOMEOBOX GENES—ISOLATION AND CLASSIFICATION

NK homeobox genes were first cloned by screening a *Drosophila* DNA library with degenerate homeodomain oligonucleotides (Kim and Nirenberg, 1989). Four new genes were identified (*NK1–NK4*) and the encoded proteins were classified by Burglin into two new homeodomain protein classes, NK-1 (containing NK1) and NK-2 (containing NK2–NK4) (Burglin, 1993). Additional *NK-1* and *NK-2* genes have now been isolated from diverse phyla (Fig. 1A) and it is clear from homeodomain comparisons that the two classes are distinct and of ancient origin (Fig. 1B). The proteins encoded by two recently isolated mouse genes, *Nkx-5.1* and *Nkx-5.2* (Bober *et al.,* 1994), can be regarded as members of a related but separate class (Figs. 1A and 1B).

NK gene nomenclature is currently nonsystematic. Different given names for individual genes have been listed in Fig. 1A, but for convenience a single name has been chosen for use in this text, in line with trends in the recent literature (see legend to Fig. 1). For example, the original *Drosophila NK-2* isolates have become known by descriptors of their mutant phenotypes: *ventral nervous system defective* (*vnd*) for *NK2, bagpipe* (*bap*) for *NK3,* and *tinman* (*tin*) for *NK4/msh-2.* For vertebrate genes most closely related to *vnd/NK2,* most authors have adopted the *Nkx2* (or related) nomenclature, acknowledging names given to the first vertebrate isolates (Price *et al.,* 1992). Mouse *Nkx-2.3, Nkx-2.5,* and *Nkx-2.6* (Lints *et al.,* 1993) have been renamed *Nkx2-3, Nkx2-5,* and *Nkx2-6,* respectively (Himmelbauer *et al.,* 1995), to satisfy recommendations of the mouse gene nomenclature committee.

Homeodomains of NK-2 proteins have a tyrosine at position 54 (Fig. 1A). Since tyrosine is not found in this position in other homeodomains, it is currently the most unambiguous feature of the NK-2 class and a useful classification tool. At least two distinct families are currently discernible within the NK-2 class (Figs. 1A and 1B; see Burglin, 1993). Referring to the original *Drosophila* isolates, individual proteins tend to be highly related within their homeodomains to either vnd or bap (up to 95% identity), while vnd and bap themselves are only moderately similar (66%). The presence of vertebrate genes within both of these families suggests that this particular split in the *NK-2* homeobox gene class occurred before divergence of the vertebrate and arthropod lines. Among vertebrate genes related to *vnd,* orthologues of *Nkx2-3, 2-5,* and *2-6* seems to represent a distinct phylogenetic group (Fig. 1B).

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A

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                                                                                 tyr54
Organism   Gene Names:                                                             ↓
                                 1        10        20        30        40        50        60
fruitfly   NK2/vnd:              KRKRRVLFTKAQTYELERRFRQQRYLSAPEREHLASLIRLTPTQVKIWFQNHRYKTKRAQ
leech      Lox-10:               R----I--SQ--I----------------------TF-G------------------KSK
nematode   ceh-22:               ---------------------S-K--------A--MQ--------------------KSH
flatworm   Dth-1:                --------S-K-IL----H---KK-----------N--G-S--------------N---H
flatworm   Dth-2:                R----I--SQ--I-------K--K-----------N--N----------------C--S-
rat        Nkx-2.1/TTF-1/T/ebp:  R-------SQ--V-------K--K------------M-H----------------M--QA
frog       XeNK2:                --------S----------------------------------------------M---R
mouse      Nkx-2.2:              --------S----------------------------------------------M---R
fish       nk2.2                 --------S----------------------------------------------M---R
frog       XNkx-2.3:             R--P----SQ--VF------K--------------NSLK--S---------R---C--QR
chicken    cNKx-2.3:             R--P----SQ--VF------K---------------SLK--S---------R---C--QR
mouse      Nkx2-3/Nkx-2.3:       R--P----SQ--VF------K---------------SLK--S---------R---C--QR
frog       XNkx-2.5:             R--P----SQ--V-------K--K-------D---NVLK--S---------R---C--QR
fish       nkx2.5/tinman:        R--P----SQ--V---Q---K--K-------D---NVLK--S---------R---C--QR
chicken    cNkx-2.5:             R--P----SQ--V-------K--K-------D---NVLK--S---------R---C--QR
mouse      Nkx2-5/Csx:           R--P----SQ--V-------K----------DQ---VLK--S---------R---C--QR
human      CSX:                  R--P----SQ--V-------K----------DQ---VLK--S---------R---C--QR
mouse      Nkx2-6:               Q--S----SQ--VLA-----K-----T---------ALQ--S---------R---S-SQR
fruitfly   bagpipe/NK3:          -KRS-AA-SH--VF------A------G---SEM-KSL---E---------R------K-
frog       Xbap:                 -KRS-AA-SH--VF------NH-----G---AD--ASLK--E---------R------R-
sponge     prox1:                --RP-A--SH--V-------AV-K--T-H-QSK--TVLH--E---------R---S--Q-
fruitfly   tinman/NK4/msh-2:     ---P----SQ--VL---C---LKK--TGA---II-QKKN-SA---------R---S--GD
flatworm   EgHbx3:               QS------N-F-ISQ--K---K----T-Q--QE--HT-G-------------A--M--LF
flatworm   EgHbx2:               QKRA--S-SSS-VHV--E--DR-K---SA--AEMSRDLG-SE---------R-----KRA

fruitfly   NK1/S59:              P-RA-TA--YE-LVS--NN-KTT----VC--LN--LSLS--E---------R-T-W-KQN
flatworm   EgHbx1:               R-RA-TA--YE-LVT--NK-QST----VY--LN--LSLN--E---------R-T-W-KQN
nematode   ceh-1:                M-RA-TA--YE-LVA--NK-KTS----VV--LN--IQLQ-SE---------R-T-W-KHN
chicken    CHox3:                P-RA-TA--YE-LVA--NK--AT----VC--LN--LSLS--E---------R-T-W-KQH
mouse      Nkx-1.1:              P-RA-TA--YE-LVA--NK-KAT----VC--LN-GLSLS--E---------R-T-W-KQN

mouse      Nkx-5.1:              -K-T-TV-SRS-VFQ--ST-DMK----SS--AG--ASLH--E---------R-N-W--QL
mouse      Nkx-5.2:              -K-T-TV-SRS-V-Q--ST-DMK----SS--AC---SLQ--E----T----R-N-W--QL
```

B

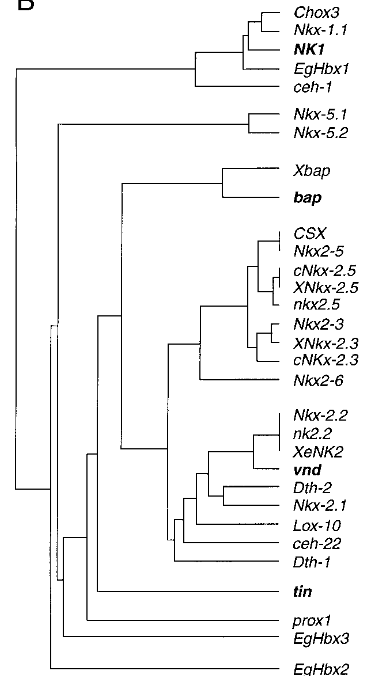

FIG. 1. Conserved features of NK-2 homeodomain proteins. (A) Compilation of homeodomain sequences from NK proteins. The 60-amino-acid homeodomain is represented along with gene names and species of origin. The tyrosine at homeodomain position 54 (tyr54, arrowed), unique to NK-2 homeodomain proteins, is shaded. To highlight the divergence between classes, amino acids conserved within isolates related to NK-1 and Nkx-5 (bottom panels) that never occur in the NK-2 class (top panel) are shaded. (B) Dendrogrammatic representation of the sequence relationships between NK homeodomains created by progressive pairwise alignment of sequences using the Pile-up program of the Wisconsin/GCG suite of programs. (C) Domain structure of NK-2 homeodomain proteins. Only members whose full structure is know have been listed. (D) Compilation of TN-Domain sequences from different isolates. The distance in amino acids from the predicted N-terminal methionine (NH_2-METHIONINE) is given in parentheses. (E) Compilation of NK2-Specific Domain (NK2-SD) and linker region sequences from different isolates. The linker region refers to those amino acids between the homeodomain and the NK2-SD. Where conservation of the linker sequence is observed between isolates, the full amino acid sequence is given. Where no conservation is observed, the number of amino acids separating the homeodomain and the NK2-SD is given in parentheses. Dots represent gaps in the sequence. The Nkx2-6 sequence is incomplete by one amino acid. Relative to other isolates, a single amino acid insertion (E or D) is observed in the bap and Xbap sequences, represented above the line to preserve maximum homology. In A–E, the original *Drosophila* isolates are represented in bold. References for individual genes are as follows: *NK1-NK4* (Kim and Nirenberg, 1989); *vnd* (Jiminez *et al.*, 1995); *Lox-10* (Nardelli-Haefliger and Shankland, 1993); *ceh-22* (Okkema and Fire, 1994); *Dth-1/Dth-2* (Garcia-Fernandez *et al.*, 1991); *EgHbx1/3* (Rangini *et al.*, 1989); *Nkx-2.1/Nkx-2.2/Nkx-2.3* (Price *et al.*, 1992); *TTF-1* (Guazzi *et al.*, 1990); *T/ebp* (Mizuno *et al.*, 1991); *XeNK2* (Saha *et al.*, 1993); *nk2.2* (Anukampa and Wilson, 1995); *XNkx-2.3* (Evans *et al.*, 1995); *cNKx-2.3* (Buchberger *et al.*, 1996); *XNkx-2.5* (Tonissen *et al.*, 1994); *nkx2.5* (R. Breitbart, personal communication); zebrafish *tinman* (M. Fishman, personal communication); *cNkx-2.5* (Schultheiss *et al.*, 1995); *Nkx2-3/Nkx2-5/Nkx2-6* (Lints *et al.*, 1993); *Csx* (Komuro and Izumo, 1993); *CSX* (I. Komuro, personal communication); *tinman* (*tin*) (Bodmer, 1993); *msh-2* (Bodmer *et al.*, 1990); *bagpipe* (*bap*) (Azpiazu and Frasch, 1993); *prox1* (Seimiya *et al.*, 1994); *Xbap* (P. Krieg, T. Mohun, personal communications); *S59* (Dohrmann *et al.*, 1990); *ceh-1* (Hawkins and McGhee, 1990); *CHox3* (Hawkins and McGhee, 1990); *Nkx-1.1/Nkx-5.1/Nkx-5.2* (Bober *et al.*, 1994).

The position occupied by *tin* is somewhat uncertain. None of the existing gene isolates is significantly more homologous to *tin* than to *vnd* or *bap* (Fig.1B). At present it is not clear whether the sequence difference between *NK-2* members or families reflects unique functional properties or merely the idiosyncratic path of their evolution (see below).

FUNCTIONAL ANALYSIS OF NK-2 PROTEINS

The NK-2 Homeodomain

NK-2 genes encode sequence-specific DNA-binding transcriptional activators. The most characterized member is

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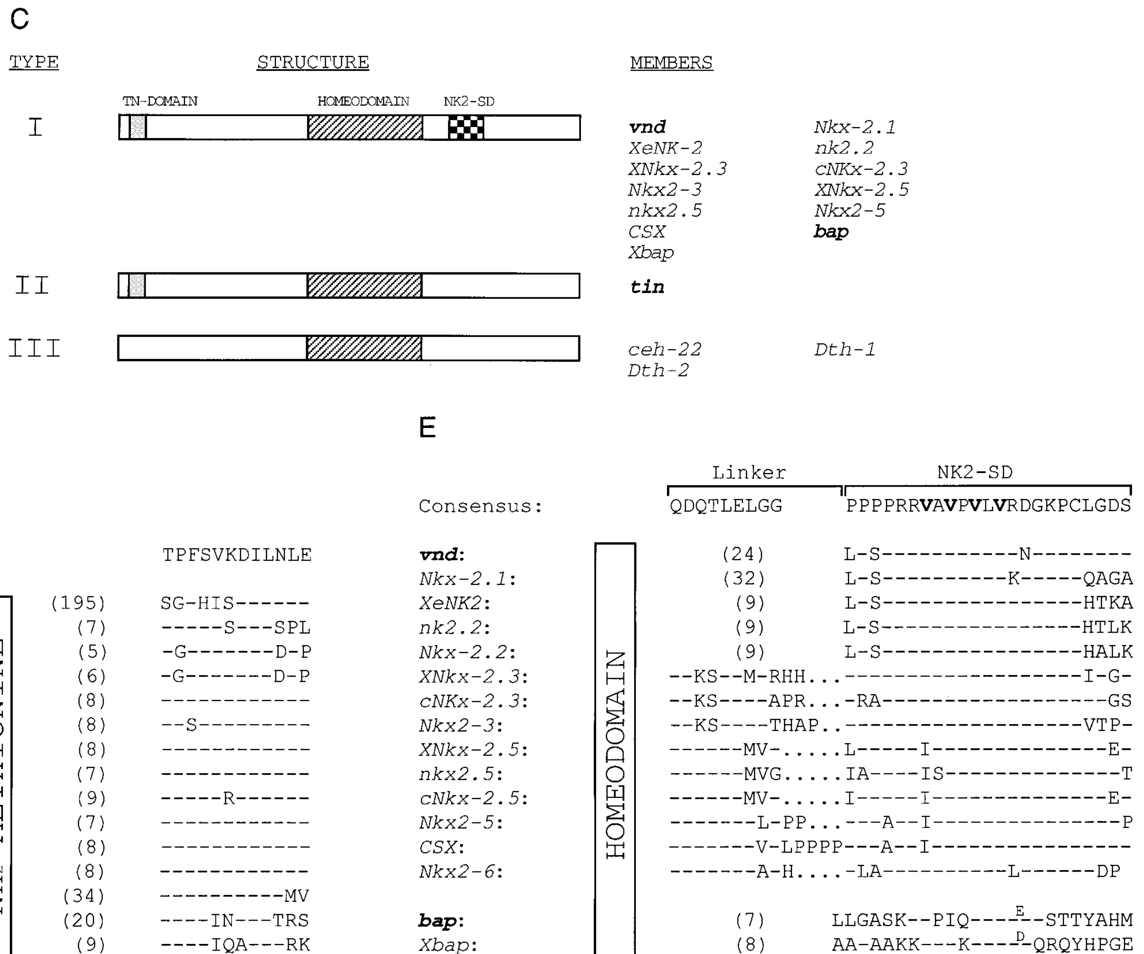

FIG. 1—*Continued*

Nkx-2.1 (*TTF-1; T/ebp*), the first vertebrate *NK-2* member cloned (Guazzi *et al.,* 1990; Mizuno *et al.,* 1991). *Nkx-2.1* is expressed in the developing thyroid, lung, and brain and a number of thyroid and lung target genes are already known (Guazzi *et al.,* 1990; Kikkawa *et al.,* 1990; Mizuno *et al.,* 1991; Francis-Lang *et al.,* 1992; Civitareale *et al.,* 1993; Bohinski *et al.,* 1994; Ray *et al.,* 1996). The sequences to which Nkx-2.1 binds within the cis-elements of these target genes differ considerably from those recognized by other homeodomain proteins (Laughon, 1991) and this specificity is determined wholly by the Nkx-2.1 homeodomain (Guazzi *et al.,* 1990).

Random oligonucleotide selection has been used to determine binding sites for a number of NK-2 proteins: *Drosophila* vnd (Tsao *et al.,* 1994), mouse Nkx2-5 (Chen *et al.,* 1995; T. Mohun, personal communication), and *Xenopus* Nkx-2.2 and 2.3 (T. Mohun, personal communication). In all cases, the high-affinity sites conform to the consensus 5′ T(C/T)AAGTG 3′, in which the 5′ (C/T)AAG 3′ core is a unique variant of the 5′ TAAT 3′ core recognized by the Antennapedia homeodomain class (see Damante *et al.,* 1994). Some (but not all) natural target sites recognized by Nkx-2.1 bear close similarity to the *in vitro*-derived consensus, as do those of another NK-2 member, the *C. elegans* protein ceh-22 (Kikkawa *et al.,* 1990; Mizuno *et al.,* 1991; Francis-Lang *et al.,* 1992; Civitareale *et al.,* 1993; Damante *et al.,* 1994; Okkema and Fire, 1994; Ray *et al.,* 1996).

These binding site determinations suggest that NK-2 proteins have the same or very similar binding specificities *in vitro.* However, specificities may be modified *in vivo* through association with other factors (Popperl *et al.,* 1995), and the homeodomain itself is known to serve as a protein:protein interface (Pomerantz *et al.,* 1992; Kutoh *et al.,* 1992; Lai *et al.,* 1992). An elegant experiment addresses whether the divergent homeodomain of tin has, during the course of its evolution, acquired a unique specificity. When a chimaeric form of *tin* in which the homeobox has been substituted for that of murine *Nkx2-5* is introduced into flies lacking *tin* function, formation of heart and visceral muscle progenitor cells is restored, as they are with wild-

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type *tin* (R. Bodmer, personal communication). This suggests that the homeodomains of Nkx2-5 and tin are interchangeable and that their sequence differences relate solely to ancestry.

The specificity of DNA binding by homeodomains is thought to be controlled primarily by helix 3 (the recognition helix), which becomes positioned within the major groove of DNA upon binding. For some homeodomains, amino acid 50 (within the recognition helix) makes critical base pair contacts (Triesman *et al.,* 1989; Hanes and Brent, 1989). Other amino acids within the homeodomain also contact DNA and contribute to affinity or specificity (Percival-Smith *et al.,* 1990; Kissinger *et al.,* 1990; Otting *et al.,* 1990). Mutagenesis of the Nkx-2.1 homeodomain has shown that amino acids outside of the recognition helix are indeed critical for binding (Damante and Di Lauro, 1991). Although these have not been mapped in detail, the tyrosine 54 that is unique to NK-2 proteins is likely to be involved (Damante *et al.,* 1994). NMR structures of the Nkx-2.1 and NK2 homeodomains (Viglino *et al.,* 1993; Tsao *et al.,* 1994) suggest a working model in which tyrosine 54, normally lying outside of the recognition helix in the unbound state, becomes incorporated into this helix as it lengthens upon DNA binding. It is then positioned to make crucial contacts with the 5′AAG3′ core of the binding site (Damante *et al.,* 1994).

Other Domains

Two peptide domains, in addition to the homeodomain, are conserved within NK-2 class proteins (Figs. 1C–1E). Currently, most members (referred to here as Type I) contain both the conserved *TN-Domain* near the amino terminus (Lints *et al.,* 1993; Bodmer, 1995) and the *NK2-Specific Domain* (NK2-SD) carboxy terminal to the homeodomain (Price *et al.,* 1992; Lints *et al.,* 1993). Not all NK-2 proteins bear these homology domains. Those from the most primitive organisms analyzed (flatworms and *C. elegans*) lack both domains (Type III, Fig. 1C), as do isolates related to NK1 and Nkx-5.1. This suggests that the ancestral *NK* gene possessed neither domain. tin is again notable in that it carries the TN-Domain but lacks the NK2-SD (Type II, Fig. 1C). This arrangement could be degenerate or represent a transitional form.

The functions of the TN-Domain and NK2-SD are not known. The TN-Domain actually has weak similarity (consensus: FS(I/V)—(I/L)(L/M)) to a conserved peptide present in a variety of transcription factors (B. Hensch, personal communication), for example, the *octapeptide* in paired box proteins (Burri *et al.,* 1989; Allen *et al.,* 1991) and the *Hep motif* in homeodomain proteins related to Hlx and engrailed (Allen *et al.,* 1991).

The NK2-SD, on the other hand, is unique to NK-2 class proteins (Fig. 1E). It is separated from the homeodomain by a linker of 9–32 amino acids which, among vertebrate members, shows some conservation. The NK2-SD itself contains a proline-rich region, a hydrophobic core with valine or isoleucine in every second position ((V/I)n(V/I)nVnV), possibly a protein:protein interface (Price *et al.,* 1992), and flanking basic amino acids (Fig. 1E). The NK2-SDs of the *bagpipe* family show less conservation, although some of the features mentioned above are still in evidence (Fig. 1E). So far, a specific assay has not been found which describes NK2-SD function. It is not required for high-affinity sequence-specific DNA binding *in vitro* (Guazzi *et al.,* 1990; Damante *et al.,* 1994), nor for transactivation of synthetic or natural promoters in transient transfection assays (Chen and Schwartz, 1995; De Felice *et al.,* 1995). The domain may dock with factors that modulate transcriptional activity in its natural setting.

Preliminary studies hint that Nkx-2.1 and Nkx2-5 carry both transcriptional activation and inhibitory domains (De Felice *et al.,* 1995; Chen and Schwartz, 1995). The transcriptional activity of these two proteins is low when analyzed on reporter genes carrying multimerized binding sites (Chen and Schwartz, 1995; De Felice *et al.,* 1995). However, activity of Nkx2-5 is stimulated 50× when the C-terminal region of the protein is deleted (Chen and Schwartz, 1995). These assays are, of course, highly artificial and may not accurately reflect how the protein is regulated. For example, while limited C-terminal deletions of Nkx-2.1 also result in a dramatic (85×) activation when assayed in a heterologous context (fused to a GAL4 DNA binding domain) in fibroblasts, this is much weaker in thyroid cells (14×) and not observed at all in the context of the Nkx-2.1 homeodomain (De Felice *et al.,* 1995). Nevertheless, the studies do point to possible associations with other factors that activate, repress, or de-repress transcriptional activity (see below). The data so far do not implicate the NK2-SD directly in this particular activity.

NK-2 HOMEOBOX GENES AND THEIR ROLE IN CARDIOGENESIS

tinman and Drosophila Heart Development

Interest in the role of *NK-2* genes in heart development began with isolation of the *Drosophila* gene *tin* and characterization of its role in formation of the *Drosophila* dorsal vessel or heart (Kim and Nirenberg, 1989; Bodmer *et al.,* 1990). *Drosophila* has an open circulation with a pulsatory muscular vessel that pumps cellular haemolymph around the body cavity. The heart is a linear dorsal midline structure containing muscular *cardial* and nephrocytic *pericardial* cells, as well as a lymph gland (derived from cardiac mesoderm), ring gland, and radiating attachment (alary) muscles (Rizki, 1978; Rugendorff *et al.,* 1994). The cardial cells resemble vertebrate cardiac muscle in that myofilaments insert head on into adherens type junctions, similar to intercalated discs (Rugendorff *et al.,* 1994).

The *tin* gene is first expressed in presumptive mesoderm before gastrulation (Bodmer *et al.,* 1990), just minutes after activation of *twist,* a basic helix-loop-helix gene situated at

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the top of the genetic cascade for mesodermal specification (Nusslein-Volhard, 1991). Before gastrulation, the fate of mesodermal cells is undecided (Beer *et al.,* 1987), so *tin* is likely to be involved in the earliest stages of mesodermal patterning. In fact, *twist* may regulate *tin* directly since binding sites for the twist protein can be found within the *tin* promoter (Bodmer, 1995). *tin* remains transcriptionally active in mesodermal progenitors throughout gastrulation and during subsequent spreading and organisation of the mesoderm into a bilayer. The gene is then turned off in all mesoderm except that in paired dorsal regions of the trunk that contain precursors for muscles of the heart, midgut (Bodmer *et al.,* 1990), and dorsal body wall (M. Frasch, personal communication). *tin* is not expressed in gut endoderm. Expression in visceral progenitors is transient, but transcripts are detected in both cardial and pericardial cells of the heart throughout larval development.

Fly embryos in which *tin* function has been inactivated do not form midgut or heart muscles, nor their progenitors (Bodmer *et al.,* 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Some body wall muscles are also disrupted. Dorsal muscles are missing and others are abnormally patterned or have too many nuclei (Azpiazu and Frasch, 1993). These latter effects may reflect the ability of some cardiac or visceral progenitors to incorporate into the somatic lineage in the absence of *tin* function (Azpiazu and Frasch, 1993).

Ubiquitous expression of *tin* in fly embryos using a heat shock promoter partially rescues formation of heart, midgut muscle, and body wall muscles, but does not induce ectopic heart formation (Bodmer, 1993). Similarly, induction of *tin* expression in ventral mesoderm expands the visceral progenitor population, but not that of the heart (Azpiazu and Frasch, 1993). Thus, *tin* does not appear to be a heart master regulatory gene in the strictest sense—heart formation clearly requires signals other than *tin* (Wu *et al.,* 1995). The earliest role of *tin* is therefore in embryonic patterning, acting to define the dorsal domain of mesoderm in which developmental potential is restricted to the cardiac, visceral and some body wall muscle lineages (Bodmer, 1993; Azpiazu and Frasch, 1993).

bap, also an *NK-2* class homeobox gene, appears to lie downstream of *tin* in the visceral lineage (Azpiazu and Frasch, 1993). *bap* is transiently expressed in segmentally reiterated patches of dorsal mesodermal cells that contain progenitors of the midgut muscle. *bap* is also expressed in foregut and hindgut muscle progenitors and from a late stage in the heart. Only 30–40% of bap-expressing cells develop into visceral mesoderm, the rest most likely forming somatic muscle. In a partial loss of function *bap* mutant, midgut muscle is reduced by ~70% and some of its progenitors are transformed into body wall muscle or gonadal mesoderm (Azpiazu and Frasch, 1993). In a null mutant, no midgut muscle forms, although the heart is normal (M. Frasch, personal communication).

Genetic experiments suggest that *bap* expression in the midgut muscle is directly dependent upon *tin.* In *tin* null embryos, no midgut mesodermal progenitors form and *bap* is not activated. In embryos carrying a weak *tin* allele, some muscle progenitors do form, but *bap* mRNA does not accumulate and visceral development is disrupted as in the partial loss of function *bap* mutant (Azpiazu and Frasch, 1993). Ectopic ventral expression of Decapentaplegic, a TGFβ-related factor thought to be involved in specifying dorsal embryonic domains, induces ectopic expression of *tin* and *bap,* as well a marker of the visceral mesodermal lineage, fasciclin III (Staehling-Hampton *et al.,* 1994; Frasch, 1995).

In summary, *tin* appears to sit at or close to the head of a pathway that restricts developmental potency within mesoderm to dorsal derivatives. Although specification of cardiac, visceral and dorsal body wall muscles depends on *tin,* multiple regulatory signals are required for formation of these lineages.

tinman Homologues and the Vertebrate Heart

The *myoD*-related myogenic factors that are master regulators of skeletal muscle development are not expressed in vertebrate heart muscle (Olson, 1993). In order to identify heart regulatory genes, two groups searched for murine homologues of *tin.* New members of the vertebrate *NK-2* class were discovered and one of these, *Nkx2-5/Csx,* was found to be expressed at high levels in the developing and adult heart (Komuro and Izumo, 1993; Lints *et al.,* 1993). As expected of a *tin* homologue, *Nkx2-5* expression was first detected in early cardiac progenitors (Lints *et al.,* 1993), present in vertebrates as paired bilaterally symmetrical cell populations at the anterior-lateral aspect of the mesodermal plate (Rawles, 1943; DeRuiter *et al.,* 1992). *Nkx2-5* expression continues as paired progenitors fuse into a crescent and undergo further morphogenesis.

Nkx2-5 cognate genes have now been isolated from human, chicken, quail, frog, and fish (Fig. 1A), indicating strong conservation among vertebrates. Figure 2 shows the *in situ* hybridization patterns of *tin* in fly embryos and that of *Nkx2-5* or its cognates in the cardiogenic region of frog, mouse, chick, and zebrafish embryos. The patterns in these species are strikingly similar, with expression in paired myocardial progenitor cells derived from splanchnic mesoderm, continuing as progenitors begin to differentiate and coalesce at the midline to form a heart tube.

Vertebrate *Nkx2-5* genes do not appear to be expressed in nascent mesoderm, with the possible exception of the zebrafish homologue (M. Fishman, personal communication). Murine *Nkx2-5* is expressed in cardiac mesoderm around the time it undergoes a transformation from mesenchyme to a cuboidal epithelium, the first physical sign of the committed state (Lints *et al.,* 1993). However, the timing of *Nkx2-5* activation relative to heart muscle commitment can be more accurately assessed in the chicken and frog systems. In the chicken, Bader and colleagues have shown that individual cells or explants isolated from stage 4 (mid-gastrulation) embryos can differentiate as cardiac muscle when cultured (Montgomery *et al.,* 1994). The process is sensitive to inhibitors if explants are taken before

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stages 7–8, the beginning of differentiation (Gonzalez-Sanchez and Bader, 1990; Montgomery *et al.,* 1994). *cNkx2-5* expression is first detected by *in situ* hybridization at stage 5, early enough to be considered an early response to heart induction or an early marker of heart commitment (Schultheiss *et al.,* 1995). A similar conclusion was reached in the frog, where *XNkx-2.5* transcripts accumulate from midgastrulation (Sater and Jacobson, 1989; Evans *et al.,* 1995; Nascone and Mercola, 1995).

Acquisition of cardiac potential within the mesoderm of frog and chick embryos can be perceived as a progressive process (Gonzalez-Sanchez and Bader, 1990; Montgomery *et al.,* 1994; Nascone and Mercola, 1995). Individual steps include receipt of inductive signals from an axis organising centre (Sater and Jacobson, 1990) and from anterior endoderm (Schultheiss *et al.,* 1995; Nascone and Mercola, 1995). In considering the possible stepwise nature of commitment, it has been useful to adopt the terms *specification* and *determination* (Jacobson and Sater, 1988), proposed by Slack to describe the progressive increase in stability of the committed state (Slack, 1984). A key point is whether *Nkx2-5* is expressed only in fully determined cardiac cells. This issue is somewhat complicated by the fact that *Nkx2-5* is initially expressed in anterior endoderm (the proposed heart inducing tissue) and ectoderm, as well as in cardiogenic mesoderm (Lints *et al.,* 1993; Schultheiss *et al.,* 1995; Evans *et al.,* 1995). It is not clear if *Nkx2-5* expression in endoderm has anything to do with heart induction, but it is likely to have some consequence for pharyngeal patterning. For example, *Nkx2-5* expression continues in anterior endoderm after formation of the pharynx. Expression is restricted to the pharyngeal floor and then continues in a derivative structure, the thyroid. Expression in thyroid overlaps that of *Nkx-2.1,* shown by gene targeting to be essential for thyroid development and differentiation (Kimura *et al.,* 1996). *Nkx2-6* (Lints *et al.,* 1993) is also expressed from an early time in the pharynx, but only in its pouches (C. Biben and R. P. Harvey, unpublished data). In zebrafish, a new *NK-2* gene, *nkx2.7,* and its *Nkx2-3* cognate (*nkx2.3*) are expressed in the pharynx (R. Breitbart, personal communication). These data suggest that *NK-2* homeobox genes have been recruited to the task of pharyngeal patterning during vertebrate evolution. It is interesting that the *tin* gene is expressed in epithelial cells of the stomatodeum, a nonmesodermal structure in the head that may be involved in pharyngeal patterning (Bodmer, 1993).

Despite the complexities of endodermal expression, studies in the frog allow us to make a reasonably accurate assessment of the stage of heart commitment at which *Nkx2-5* is activated in mesoderm. *XNkx-2.5* mesodermal expression is more extensive than the region actually fated to the myogenic heart (Tonissen *et al.,* 1994). The pattern appears similar to the extent of the *heart morphogenetic field,* or region of heart potency from which the heart is formed by both positive and negative interactions (Sater and Jacobson, 1990). Since explants from the field have heart potency when cultured in a neutral environment, according to Slack we can say that the cells are *specified* (Slack, 1984). Although not rigorously proven, expression of *XNkx-2.5* most likely contributes to heart potency within the field as well as its regulative characteristics (Jacobson and Sater, 1988). However, since only a portion of these cells become fully determined and differentiate into heart muscle, we can also say that *XNkx-2.5* expression does not necessarily reflect the fully *determined* state. Although it is not yet clear if the morphogenetic field concept can be applied to heart development in all vertebrates (Jacobson and Sater, 1988), these observations in *Xenopus* and those in chick embryos described above reinforce the notion that *Nkx-2.5,* like *tin,* participates in patterning the region of mesoderm from which cardiac progenitors are derived through multiple interactions.

Are there *Nkx2* genes that perform the visceral function of *tin? Nkx2-5* itself is only expressed in the developing spleen, pancreas, and a small portion of the stomach (Lints *et al.,* 1993; Evans *et al.,* 1995). However, preliminary expression data shows that *Nkx2-3,* a close relative of *Nkx2-5,* is expressed broadly in visceral mesoderm in frog, chick, and mouse embryos (Evans *et al.,* 1995; Buchberger *et al.,* 1996; R. P. Harvey, unpublished data), although not those of zebrafish (R. Breitbart, personal communication). For these former cases at least, it is attractive to propose that *Nkx2-3* performs the visceral function of *tin* and therefore that *tin* function in vertebrates is distributed between multiple *Nkx2* genes.

Gain of Function Effects of Nkx2-5

Direct injection of *XNkx-2.5* mRNA into cleaving *Xenopus* embryos leads to enlarged hearts at the tadpole stage

FIG. 2. Expression of *tin* and its vertebrate relatives in heart progenitor cells during embryogenesis. All panels depict the results of wholemount *in situ* hybridizations using digoxygenin-labeled cRNA probes. (A) Lateral view of a *Drosophila* embryo hybridized to a *tin* probe. At this stage, heart progenitors have migrated to the dorsal midline. (B) *Xenopus* embryo at stage 20 (ventroanterior view) hybridized with a *XNkx-2.5* probe. Note hybridization signal in paired progenitors that have almost fused at the ventral midline and the narrower patch that has migrated more anteriorly corresponding to expression in pharyngeal floor (P. Krieg, personal communication). (C, D) Mouse embryos at E7.5 (anterior aspect) and E8.75 (ventral aspect), respectively, hybridized to an *Nkx2-5* probe. Note the clear separation of the heart progenitors at E7.5. (E, F) Ventral aspect of chick embryos at stages 6 and 10, respectively, hybridized to a *cNkx-2.5* probe. (G, H) Zebrafish embryos at 19.5 (ventroanterior aspect) and 24 (lateral aspect) hr of development, respectively, hybridized to an *nkx2.5* probe. Note expression in paired progenitor populations at 19.5 hr. Signal along the midline is background hybridization due to probe trapping. Images have been kindly contributed from the following sources: A, R. Bodmer; B, K. Patterson, P. Krieg; C, D, C. Biben, T. Lints, R. P. Harvey; E, F, T. Schultheiss, A. Lassar; G, J. Alexander, D. Stainier; H, Q. Xu, R. Breitbart.

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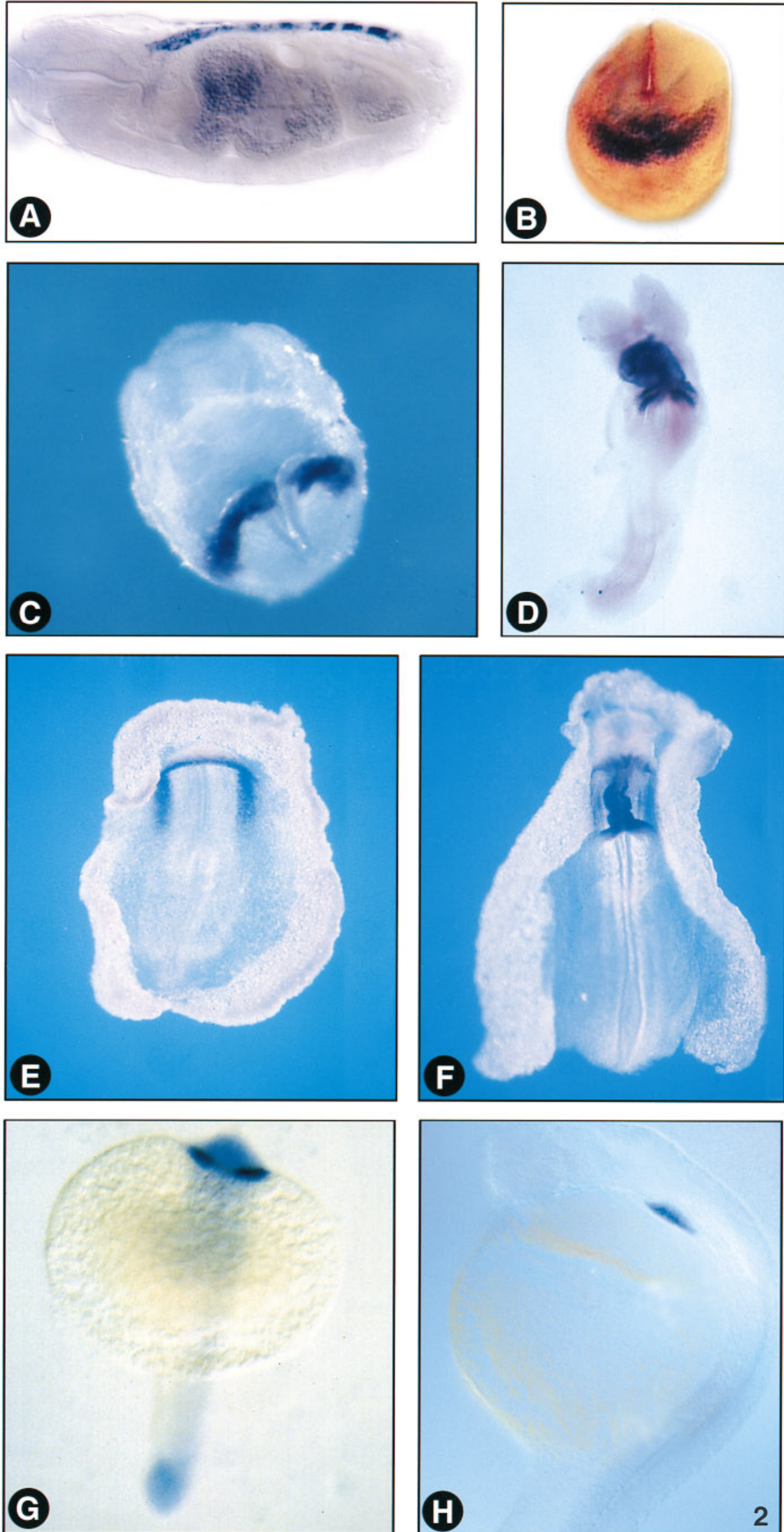
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(P. Krieg, personal communication). Similar results have been obtained in zebrafish embryos with its *Nkx2-5* cognate, although in this case ectopic myosin-positive tissue was seen in rare cases (M. Fishman, personal communication). While it is not known whether this phenotype occurs through recruitment of more cells into the heart progenitor pool from the heart field, proliferation of committed heart progenitors, or by some other mechanism, the observation strengthens the conclusion drawn above that *Nkx2-5* has a role in patterning the anterior of the embryo and that it must, like *tin,* work in collaboration with other regulators to specify the heart lineage.

Nkx2-5 Knockout Mice

A knockout of the murine *Nkx2-5* gene has recently been reported (Lyons *et al.,* 1995). Targeted interruption of the homeodomain caused abnormal heart morphogenesis at E8.5 and early embryonic death due to hemodynamic insufficiency. A linear heart tube formed apparently normally, but looping morphogenesis was severely disrupted and the subsequent events of trabeculation and endocardial cushion formation were also blocked.

Several aspects of this phenotype are worthy of further consideration. First, while it establishes *Nkx2-5* as essential for early heart development in a mammal, it has not confirmed a *tin*-like role for the gene. If this were true, no cardiac lineage would form (Bodmer, 1993). The heart tube in *Nkx2-5* mutants contains beating myocytes which express several myofilament genes at normal levels. Commitment to the cardiac muscle lineage could in principle be partially compromised yet lead to a relatively normal heart due to regulation. However, the normal propensity for beating cardiac muscle foci to form in ES cell-derived embryoid bodies that are mutant for *Nkx2-5* suggests that heart commitment is not compromised in the absence of the *Nkx2-5* gene (Lyons *et al.,* 1995).

There are two broad ways in which the disparity between the *tin* and *Nkx2-5* mutant phenotypes could be reconciled. First, the phenotype may reflect only one facet of *Nkx2-5* function. This could occur if the targeted mutation was not null for some aspects of *Nkx2-5* function (see below) or if other *Nkx2* genes were also expressed in the early cardiogenic region and partially compensated for loss of *Nkx2-5.* Implicit in both of these possibilities is the suggestion that the Nkx2-5 protein would have two levels of function: those that are revealed by the mutant phenotype and those that are not. There is a strong suggestion that individual members of the *Nkx2* gene family substantially overlap in their expression domains, where they may be functionally redundant. For example, expression of *Nkx-2.1* and *Nkx2-5* overlap in the developing thyroid (Lazzaro *et al.,* 1991; Lints *et al.,* 1993), *Nkx-2.1* and *Nkx-2.2* in the developing brain (Price *et al.,* 1992), *Nkx2-5* and *Nkx2-6* in the tongue (R. Harvey, unpublished data), and *Nkx-2.1* and *Nkx2-6* in the lung (Price *et al.,* 1993; C. Biben and R. Harvey, unpublished data). In *Xenopus, XNkx-2.5* and *XNkx-2.3* are expressed in very similar if not identical patterns in the early cardiogenic region (Tonissen *et al.,* 1994; Evans *et al.,* 1995). Chicken *NKx-2.3* is also expressed in the developing and adult heart, although not as early as *cNkx-2.5* (Buchberger *et al.,* 1996). Further gene characterization and genetic experiments will be required to find out which mouse genes, if any, act redundantly with *Nkx2-5.*

Redundancy, however, could work on another level. For example, even though *Nkx2-5* may indeed play a *tin*-like role, the activity of other classes of regulators, perhaps the MEF2 proteins (Olson *et al.,* 1995), may be sufficient to initiate heart development in vertebrates, at least to the point where *Nkx2-5* mutant hearts fail. Alternatively, mesodermal patterning and commitment to the heart lineage in vertebrates may not require *Nkx2* genes at all, even though they are the nearest relatives of *tin.* In this case, *Nkx2-5* would be subservient to a higher regulatory order, yet to be discovered. Redundancy presents intriguing mechanistic and evolutionary problems (Tautz, 1992; Thomas, 1993) and it is necessary to deal with them so that we can fully understand the regulatory circuits that underpin heart development and exploit the opportunities offered by cross-species comparisons.

Nkx2-5 and Myogenesis

Irrespective of the precise evolutionary relationship between *tin* and *Nkx2-5,* analysis of the vertebrate gene has revealed valuable insights into genetic control of heart development. In mutant hearts, expression of the ventricle-specific *myosin light chain 2* gene (*MLC2V*) is inhibited (Lyons *et al.,* 1995), demonstrating a role in at least one branch of the heart myogenic program. It is not at all clear why this particular myofilament gene, and not others, is affected. The related atrial *myosin light chain 2* gene, for example, is expressed at normal levels.

The narrow myogenic phenotype displayed in *Nkx2-5* mutants may relate to the issues of redundancy discussed above, or *Nkx2-5* may indeed be required uniquely for *MLC2V* regulation, or both. *MLC2V* is the only known myofilament gene in the mouse that is activated in a restricted region of the developing cardiac crescent, probably the ventricular progenitors (O'Brien *et al.,* 1993; Lyons *et al.,* 1995). There is, however, no evidence to date that *Nkx2-5* confers regional information onto its downstream target genes or to heart progenitors in general. First, analysis of mutant hearts with regional markers suggests that other aspects of regional myogenic specialization can be accomplished in the absence of *Nkx2-5* (Lyons *et al.,* 1995). Furthermore, mice carrying a β-galactosidase transgene driven by the proximal *MLC2V* promoter show transgene expression with temporal and spatial characteristics very similar to that of the endogenous *MLC2V* gene, even when crossed into the mutant *Nkx2-5* background (Ross *et al.,* 1996). This result must be interpreted cautiously since it is not at all clear why an *MLC2V* transgene, but not the endogenous gene, should be ex-

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pressed in *Nkx2-5* mutant embryos. Nevertheless, it does suggest that *Nkx2-5* is not required for the restriction of *MLC2V* expression to the ventricles. Recent studies have identified a cardiac muscle-enriched ankyrin-repeat protein (CARP) that may be a transcriptional coregulator of the *MLC2V* gene (Y. Zou, K. Chien, personal communication). Expression of the *CARP* gene is down-regulated in *Nkx2-5* mutant hearts, demonstrating that at least one axis through which *Nkx2-5* regulates *MLC2V* is indirect.

Expression of the cardiac α-actin gene, an early marker of myogenic differentiation in the embryo, does not appear to be disturbed in *Nkx2-5* mutant embryos. However, recent *in vitro* experiments by R. Schwartz and colleagues suggest that Nkx2-5 collaborates with Serum Response Factor (SRF) in activating this gene in cardiac muscle cells (R. Schwartz, personal communication). Each of these factors can dramatically facilitate the ability of the other to activate the actin promoter in transiently transfected fibroblasts. Nkx2-5 protein can bind to essential Serum Response Elements (SREs) in the actin promoter that are also sites for SRF interaction. In doing so, it facilitates the binding of SRF to these sites, in a way that resembles the ability of another homeobox protein, Phox, to facilitate binding of SRF to the c-fos SRE (Grueneberg *et al.,* 1992). Although an Nkx2-5/SRF complex over the SRE has not yet been rigorously demonstrated, SRF and Nkx2-5 can associate in solution and can be coimmunoprecipitated from transfected muscle cell extracts.

These experiments suggest an important collaborative interface between two transcription factor families in heart development and support the genetic evidence described above that directly implicate *Nkx2-5* in regulation of cardiac myogenesis. The data also fit the increasing evidence from a variety of sources that homeodomain and MADS box factors such as SRF and MEF2 exert their function through collaboration with other proteins.

If the proposed Nkx2-5/SRF interaction is meaningful *in vivo,* why then is expression of *cardiac α-actin* not perturbed in *Nkx2-5* mutant embryos? Once again, it is convenient to invoke genetic redundancy, but another possible explanation is that the documented homeodomain disruption is not null for all aspects of *Nkx2-5* function. This suggestion is given weight by the fact that in the *in vitro* assays performed by Schwartz *et al.,* the DNA-binding function of Nkx2-5 is not required for association with SRF and coactivation of the actin gene. The interaction requires only the N-terminal regions of the Nkx2-5 homeodomain. The mutation introduced into the *Nkx2-5* locus by gene targeting disrupted helix three of the homeodomain (essential for DNA binding), but left the N-terminal homeodomain sequences that apparently interact with SRF intact (Lyons *et al.,* 1995). If a truncated protein was produced in the mutants, it might still perform cofactor-dependent functions. A similar DNA-binding-independent activity for the pair ruled homeodomain protein ftz has recently been reported (Copeland *et al.,* 1996). A complete knockout of the *Nkx2-5* gene will present an opportunity to distinguish genetically between the two possible levels of its function.

Nkx2-5 and Heart Morphogenesis

Nkx2-5 also appears to regulate heart morphogenesis. Mutant hearts rarely develop beyond their primitive state, a linear tube with an open atrioventricular chamber (Lyons *et al.,* 1995). Looping morphogenesis, a key early step in achieving the correct spatiotemporal organization of the heart chambers and great vessels, is dramatically inhibited. Trabeculation and endocardial cushion formation are also blocked. Although the genetic basis of heart looping is not understood, defects in looping are thought to underlie many human congenital heart malformations, particularly those that are associated with abnormalities of laterality (Merklin and Verano, 1963; Campbell and Deuchar, 1966; Layton *et al.,* 1980; Burn, 1991). Trabeculation and endocardial cushion formation occur at later stages of heart development as a result of local inductive interactions (Meyer and Birchmeier, 1995; Huang *et al.,* 1995). In *Nkx2-5* mutants, myocardial functions directly or indirectly essential for these processes may not be expressed.

A note of caution about overzealous interpretation of cardiac morphogenetic defects is warranted here. It is entirely possible that morphogenetic progression in the developing heart is dependent upon normal function. The key question is whether the morphogenetic defects in *Nkx2-5* mutant hearts are a direct consequence of the mutation or an indirect result of poor myogenic performance. This concern is heightened by the fact that defective hearts are observed at similar stages in mouse embryos carrying mutations in genes encoding p120rasGAP, affecting vascular organization (Henkemeyer *et al.,* 1995), and the basic helix-loop-helix (bHLH) factor Scl/tal-1, affecting formation of blood cells (Robb *et al.,* 1995; Shivdasani *et al.,* 1995) However, at least one of the morphogenetic defects observed in *Nkx2-5* mutants appears to be direct. Recent results from this laboratory show that the gene encoding the bHLH transcription factor eHAND, normally expressed in several lineages within the embryo including the heart (Cserjesi *et al.,* 1995) and implicated in heart morphogenesis (Srivastava *et al.,* 1995), is not expressed in the myocardium of *Nkx2-5* mutants (C. Biben and R. P. Harvey, unpublished observations). These findings lead to the remarkable conclusion that *Nkx2-5* controls both myogenic and morphogenetic progression in heart development. For the first time, the regulatory logic that integrates these two processes is accessible at a genetic and biochemical level.

UPSTREAM OF *TINMAN* AND *NKX2-5*

Two signaling molecules have recently been shown to be required for formation of heart progenitors in *Drosophila.* One is Decapentaplegic (dpp), a member of the TGFβ superfamily, normally expressed in the dorsal ectoderm exactly overlying the mesoderm to which *tin* expression becomes restricted (Azpiazu and Frasch, 1993). dpp acts through an inductive interaction to maintain *tin* expression in dorsal

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mesoderm and is therefore essential for formation of cardiac and visceral progenitors (Staehling-Hampton *et al.,* 1994; Frasch, 1995). Ectopic expression of dpp in ventral ectoderm expands the expression domain of *tin* and *bap* into ventral mesoderm (Staehling-Hampton *et al.,* 1994; Frasch, 1995). Interestingly, expression of *tin* in ventral mesoderm increases the number of visceral muscle progenitors, but not those of the cardiac lineage. Formation of cardiac cells apparently requires other dorsal signals.

A close vertebrate relative of *dpp,* the *bone morphogenetic protein 2* gene (*BMP-2*), is expressed in definitive endoderm in the cardiogenic region of frog (Clement *et al.,* 1995) as well as mouse and chick embryos (R. Arkell, R. Beddington; T. Schultheiss, A. Lassar, personal communications). Chicken *BMP-2* is expressed in endoderm in a crescent precisely underlying the zone of *cNkx-2.5* expression in mesoderm. Remarkably, ectopic placement of BMP-2 protein in the anterior part of the chick embryo induces ectopic *cNkx-2.5* expression (T. Schultheiss, A. Lassar, personal communication). Thus, like *dpp, BMP-2* acts on mesoderm through an inductive interaction and the spatial restriction of its expression serves to define the area of cardiac potential.

The *Drosophila* segment polarity gene *wingless* (*wg*), encoding a signaling molecule of the wnt superfamily, is also required for formation of cardiac muscle precursors in the fly (Wu *et al.,* 1995). *wg* is expressed at the posterior boundaries of parasegments in the ectoderm, where it is required for correct segmentation (Klingensmith and Nusse, 1994). A number of other segment polarity genes—those that support *wg* expression—as well as genes downstream in the wg signaling pathway, are also essential for heart formation (Park *et al.,* 1996). wg signaling appears to play some role in restricting cardiac potential within the *tin* domain, since ectopic expression of *wg* leads to an overabundance of heart progenitors at the expense of visceral progenitors (Lawrence *et al.,* 1995; Park *et al.,* 1996). While the highest level of *wg* expression is in ectoderm, low expression has also been detected in mesoderm itself (Baker, 1987; Wu *et al.,* 1995) and this alone may suffice for specification of heart forming cells (Lawrence *et al.,* 1995). Thus, heart formation may involve a combination of inductive, planar, and autocrine wg signaling.

The *wg* signaling pathway appears to be conserved in vertebrates. Not only are there a large number of vertebrate wg-related proteins (the wnt family), but vertebrate homologues of its downstream signaling molecules have also been isolated (Klingensmith and Nusse, 1994). At least one wnt protein, *Wnt-2,* is expressed in the cardiac crescent of mouse embryos (S. Monkley, B. Wainwright, personal communication). Although few details are known, this suggests that *wg* signaling is also part of cardiogenesis in mammals.

INSECT AND VERTEBRATE HEARTS—ARE THEY HOMOLOGOUS?

As detailed above, striking parallels are emerging between flies and vertebrates in both the signaling molecules that act to segregate their heart muscle lineages from mesoderm and in the genetic interpretation of those signals through *NK-2* homeobox gene pathways. While there are a host of interesting and unanswered questions, the findings raise the possibility that the hearts of flies and vertebrates are actually homologous (Bodmer, 1995) or, more precisely, independent adaptations of a common ancestral structure. Similar debate is ongoing regarding the evolution of photosensitive organs and eyes, previously thought to have appeared independently in the animal kingdom as many as 40 times (Halder *et al.,* 1995). Based on the findings that all visual systems so far analyzed utilize homologous pigment proteins and that development of the dissimilar *Drosophila* and mammalian eyes are regulated by functionally identical genes (Halder *et al.,* 1995), it is now supposed that there was a monophyletic origin for photoreceptor cells in evolution.

Metazoans (see Fig. 3) have evolved an incredible diversity of hearts and heart-like structures (reviewed in Withers, 1992), with multiple or accessory hearts being commonplace, even in lower vertebrates. However, there is a trend toward a cardiovascular plan with unidirectional circulation, closed vascular system and dominant multichambered valvular heart under neuronal control. Remarkably, the hearts of the most active cephalopod molluscs, cuttlefish, and squid, achieve outputs approaching that of the human heart. This shared solution to cardiovascular efficiency has long been considered a classical example of convergent evolution, or parallelism (Martin, 1980).

Is there evidence for an ancestral structure from which vertebrate and invertebrate hearts evolved? Comparative anatomy and embryology suggest that hearts developed from pulsatory muscular vessels, components of a vascular system (reviewed in Martin, 1980; Randall and Davie, 1980). The origin of the vertebrate heart can be traced back to the ancestral cephalochordate level, represented today by amphioxus (Randall and Davie, 1980). Although amphioxus does not have a true heart, it has a number of pulsatory muscular vessels which pump blood unidirectionally through an ''in parallel'' vascular bed, as seen in vertebrates. Tunicates (a more primitive chordate) do have hearts, but they are probably not homologous to the vertebrate heart since they develop atypically from an invagination of a pericardial vesicle and exhibit bidirectional pumping through an ''in series'' vascular bed (Nunzi *et al.,* 1979; Randall and Davie, 1980). Thus, the vertebrate heart probably arose from one or more muscular vessels similar to the ones seen in amphioxus (Randall and Davie, 1980), although it remains possible that the common ancestor of amphioxus and vertebrates possessed a more advanced structure.

In invertebrates, evidence also suggest that hearts evolved from muscular vessels. A possible evolutionary progression is presented in one class of annelid worms, the oligochaetes (Stephenson, 1930; Martin, 1980). In primitive forms, there are one or more muscular dorsal vessels that are structurally and functionally an extension of the gut sinus—the vascular-like cavity between the endodermal gut epithelium and its muscular coat. In more advanced forms, a completely

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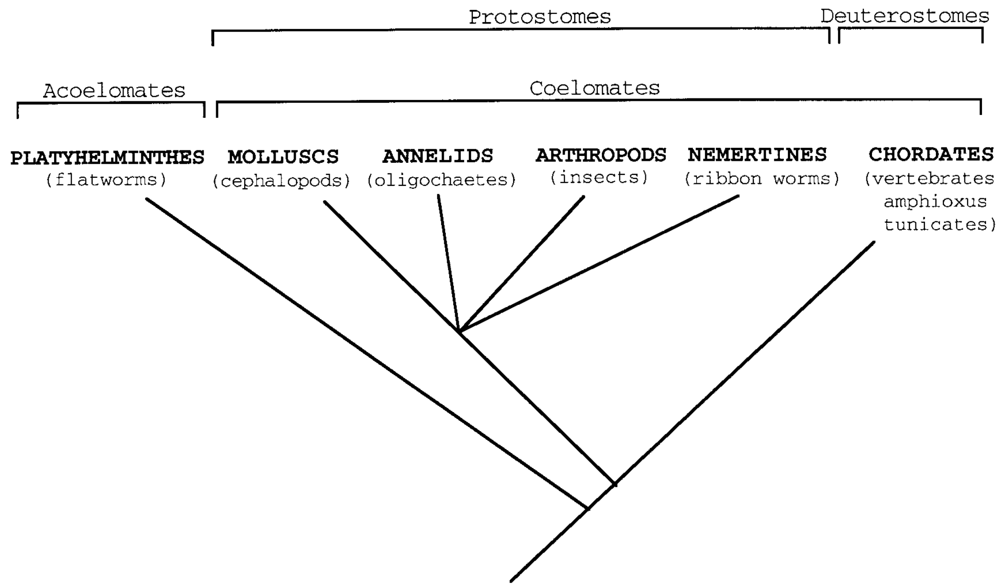

FIG. 3. Simplified, partial evolutionary tree depicting metazoan evolution. This figure has been adapted from Field *et al.* (1988) and Turbeville *et al.* (1992) to represent the relative phylogenetic relationship of organisms discussed in the text.

separate dorsal muscular vessel has formed, which is often valved and capable of autonomous peristaltic contraction. In yet others, specialized valvular hearts that lead directly from the dorsal vessel or gut sinus are present. With respect to ontogeny, muscular vessels appear to arise from the same progenitors as muscles of the gut (Stephenson, 1930; Anderson, 1973; Martin, 1980) The paradoxical discrepancy in position of invertebrate heart structures (dorsal) compared to those of vertebrates (ventral) can be accounted for by the apparent inversion of this whole axis in one group after divergence of their common ancestor (Holley *et al.,* 1995).

Was a vascular system already in place in the common ancestor of protostomes and deuterostomes? The nermertean (ribbon) worms, long regarded as acoelomates and a sister group to flatworms, appear to have a closed vascular system. However, recent morphogenetic and 18S rRNA sequence data strongly support a minority hypothesis that they are actually coelomates (Fig. 3) and that the coelom was previously misconstrued as vessels (Turbeville *et al.,* 1992). While there is a suggestion of channels in flatworms, it is not totally clear what these structures represent (Martin, 1980). Thus, a demonstration that muscular vessels were present in the common vertebrate/invertebrate ancestor is lacking.

The definition of the term "homology" in reference to morphology is under constant scrutiny and jealously guarded by comparative morphologists (Bolker and Raff, 1996). The definition is in fact fundamental to their discipline. New definitions of homology which include the criteria of conserved genetic pathways have been formulated (Roth, 1984), but must be used cautiously since homology between genes or genetic pathways is not sufficient for establishing morphological homology (Bolker and Raff, 1996). Clearly, use of the term "homology" in relation to the phylogenetic origins of hearts needs careful qualification. Since no ancestral heart or even progenitor tissue has been identified, we certainly cannot yet say that the hearts of *Drosophila* and vertebrates are homologous. However, homology between the underlying genetic pathways seems likely and we can be optimistic that experimental dissection of vertebrate heart development will continue to profoundly profit from the rich genetics of *Drosophila,* at least up to a point.

There would, however, seem to be a limit to the amount of information on genetic control of vertebrate heart development that can be gleaned from *Drosophila* genetics. Genetic homology should be strictly limited to the earliest stages of heart development, not extending beyond the linear tube structure. Complex morphogenesis in vertebrate (or even invertebrate) hearts presumably requires other genetic pathways. However, these limitations may not be absolute. *Nkx2-5* appears to be involved in heart morphogenesis beyond the linear tube stage. Intriguingly, this may reflect opportunistic recruitment of an existing regulatory pathway in the generation of further morphogenetic complexity. This is truly an exciting bonus, not only because we stand to learn more than expected about genetic control of mammalian heart development from cross-species comparisons, but also for the valuable insights into molecular and regulatory evolution that will follow.

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