

A Conserved Role for *H15*-Related T-Box Transcription Factors in Zebrafish and *Drosophila* Heart Formation

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T-box transcription factors are critical regulators of early embryonic development. We have characterized a novel zebrafish T-box transcription factor, *hrT* (*H15*-related T box) that is a close relative of *Drosophila H15* and a recently identified human gene. We show that *Drosophila H15* and zebrafish *hrT* are both expressed early during heart formation, in strong support of previous work postulating that vertebrate and arthropod hearts are homologous structures with conserved regulatory mechanisms. The timing and regulation of zebrafish *hrT* expression in anterior lateral plate mesoderm suggest a very early role for *hrT* in the differentiation of the cardiac precursors. *hrT* is coexpressed with *gata4* and *nkx2.5* not only in anterior lateral plate mesoderm but also in noncardiac mesoderm adjacent to the tail bud, suggesting that a conserved regulatory pathway links expression of these three genes in cardiac and noncardiac tissues. Finally, we analyzed *hrT* expression in *pandora* mutant embryos, since these have defects in many of the tissues that express *hrT*, including the heart. *hrT* expression is much reduced in the early heart fields of *pandora* mutants, whereas it is ectopically expressed subsequently. Using *hrT* expression as a marker, we describe a midline patterning defect in *pandora* affecting the anterior hindbrain and associated midline mesendodermal derivatives. We discuss the possibility that the cardiac ventricular defect previously described in *pandora* and the midline defects described here are related. © 2000 Academic Press

Key Words: zebrafish; *Drosophila*; T box; *H15*; *hrT*; heart formation; *pandora*.

INTRODUCTION

Vertebrates and arthropods both possess specialized vascular structures for the movement of body fluid through the body. Although the multichambered heart of vertebrates is significantly more complex than the pulsatile dorsal vessel of *Drosophila*, both structures are similar in some important aspects of morphology and development. Both contain a similar type of striated muscle and form from bilateral primordia that fuse to form a single structure (Zaffran *et al.*, 1995; Fishman and Chien, 1997). Furthermore, some of the factors that control heart formation in *Drosophila* are also conserved in vertebrates (Bodmer and Venkatesh, 1998). The *Drosophila* homeobox gene *tinman* is required for the

formation of the dorsal vessel (Bodmer, 1993; Evans *et al.*, 1995), and *tinman* homologues in vertebrates, the *Nk-2* genes (Buchberger *et al.*, 1996; Chen and Fishman, 1996; Harvey, 1996; Lee *et al.*, 1996), act redundantly in the formation of the vertebrate heart (Fu *et al.*, 1998; Grow and Krieg, 1998). *MEF2*-related transcription factors are also expressed in both *Drosophila* and vertebrate myocardial precursors, in which they are important in the regulation of downstream genes (reviewed in Fishman and Olson, 1997). Finally, *TGF β* signaling, specifically *dpp* in *Drosophila* and *BMP* family members in chick and zebrafish embryos, is implicated in the induction of cardiac fields in each system (Staehling-Hampton *et al.*, 1994; Frasch, 1995; Kishimoto *et al.*, 1997; Schultheiss *et al.*, 1997; Xu *et al.*, 1998).

Due to these similarities, it has been suggested that vertebrate and arthropod hearts are derived from a primitive structure that was present in their last common ancestor

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(Harvey, 1996; Fishman and Olson, 1997; Bodmer and Venkatesh, 1998). There are, however, also notable differences. In vertebrates, for example, members of the *gata* and bHLH transcription factor families are implicated in various stages of cardiogenesis (reviewed in Fishman and Chien, 1997; Mohun and Sparrow, 1997). At present, no *gata* or bHLH counterparts have been described in *Drosophila* that have comparable roles to the vertebrate genes, although they may yet be found. Finally, whereas *wg* signaling plays a crucial role in the induction of cardiac mesoderm in *Drosophila* (Wu et al., 1995; Park et al., 1996), there is as yet no evidence that *wnt* signaling plays a similarly pivotal role in vertebrate heart formation *in vivo* (Monkley et al., 1996; Eisenberg et al., 1997; Zakin et al., 1998).

Recently, considerable attention has been focused on the important role of T-box transcription factors in the regulation of diverse aspects of early embryogenesis (see Papaioannou and Silver, 1998; Smith, 1999; for reviews). T-box transcription factors are related by a conserved 180-amino acid region, the T domain, responsible for DNA-binding activity (Bollag et al., 1994; Müller and Herrmann, 1997). The T-box transcription factor family so far comprises more than 15 separate genes, identified across several vertebrate and invertebrate species, and many are implicated in the regulation of embryonic cell fate and morphogenesis (Schulte-Merker et al., 1994; Chapman and Papaioannou, 1998; Griffin et al., 1998; Rodriguez-Esteban et al., 1999; Smith, 1999; Takeuchi et al., 1999). In particular, *tbx5* plays a critical role in heart development. Mutations in human *TBX5* result in Holt-Oram syndrome, an autosomal haploinsufficient trait characterized by mild to severe heart malformations combined with forelimb defects (Basson et al., 1997; Li et al., 1997). More recently, Horb and Thomsen (1999) showed that dominant-negative interference of *Xenopus tbx5* leads to near total absence of myocardial morphogenesis and gene expression, confirming the importance of T box transcription factors in vertebrate heart formation. Prior to the study reported here, no *Drosophila* T-box transcription factor that might play a similar function in dorsal vessel formation had been described.

Here we describe a novel vertebrate T-box transcription factor, highly related to *Drosophila* H15 (Brook and Cohen, 1996), that we call *hrT* (H15-related T box). We show that *Drosophila* H15 and zebrafish *hrT* are both expressed during the earliest stages of heart formation. The conserved expression of H15-related genes in early heart formation is a highly significant similarity in the genetic programs utilized in fly and vertebrate heart development and strongly supports previous suggestions that vertebrate and insect hearts were derived from a primitive structure present in the last common ancestor of vertebrates and arthropods (Harvey, 1996; Fishman and Olson, 1997; Bodmer and Venkatesh, 1998). The very early expression of *hrT* in anterior lateral plate mesoderm, and its distribution relative to *nkx2.5* (Lee et al., 1996) and *gata4*, suggests that *hrT* may regulate competence to respond to localized cardio-

genic signals, as suggested for *gata4* (Serbedzija et al., 1998). We have analyzed *hrT* expression in *pandora* (*pan*) mutants, which have defects in many of the tissues that express *hrT*, including the heart (Stainier et al., 1996). In *pan* mutants, *hrT* expression is markedly reduced during early development, but is also ectopically expressed subsequently. Using *hrT* expression as a marker, we have uncovered a significant midline defect in *pan* that is restricted to tissues at the level of the anterior hindbrain. We discuss the potential significance of this midline defect in the cardiac defects found in *pan* embryos.

MATERIALS AND METHODS

Molecular Techniques and Phylogenetic Analysis

hrT was cloned in a previously described PCR-based screen for novel T-box transcription factors (Griffin et al., 1998). A single cDNA clone was obtained from a gastrula-stage library (gift from Thierry Lepage). This clone was sequenced fully on both strands using an ABI automated sequencer and found to encode a single full-length open reading frame. Related sequences were identified using BLAST (Altschul et al., 1990). T-domain amino acid sequences were aligned using ClustalW. Phylogenetic analysis was performed using the PHYLIP package (Felsenfeld, 1989). A distance matrix tree was constructed using ProtDist and Fitch; the frequencies of individual branchpoints were derived from a bootstrapped dataset (100 replicates), obtained using Seqboot, ProtDist, and Consense. AC006379.2 is available through GenBank.

In Situ Hybridization and Antibody Staining

In situ hybridization was performed as previously described (Griffin et al., 1995). Full-length *hrT* cDNA was linearized with *Bam*HI, and DIG-labeled antisense probe was synthesized using T7 polymerase. *ntl* and *gata4* were used as previously described (Schulte-Merker et al., 1992; Serbedzija et al., 1998). *pandora*^{ms313} mutant embryos were obtained from intercrosses of heterozygous adults. The *Drosophila* H15-LacZ enhancer-trap line (gift from Mark Russell) was previously described (Brook et al., 1993; Brook and Cohen, 1996). *Drosophila* H15-LacZ stocks (Brook and Cohen, 1996) were raised at 25°C on standard corn meal agar media. For immunocytochemistry, *Drosophila* embryos were dechorionated in 100% bleach, fixed in 3.7% formaldehyde, and methanol de-vitellinized. Embryos were stained with rabbit anti-β-galactosidase (1:500; Cappel) and mouse anti-Engrailed 4D9 (gift from Tom Kornberg) primary antibodies. Secondary antibodies were goat anti-rabbit Texas red and goat anti-mouse Bodipy (1:200; Molecular Probes). *Drosophila* images were collected with a Bio-Rad MRC 600 confocal microscope system, zebrafish images were collected on a Zeiss Axioplan photo microscope on Ektachrome 64T film, digitized on a Nikon LS 2000 scanner, and assembled into figures in Adobe PhotoShop 5.0 and Adobe Illustrator 8.0.

RESULTS

Identification of H15-Related T-Box Transcription Factors in Drosophila, Zebrafish, and Human

In order to identify potential regulatory factors important in early development, a PCR-based screen was conducted to

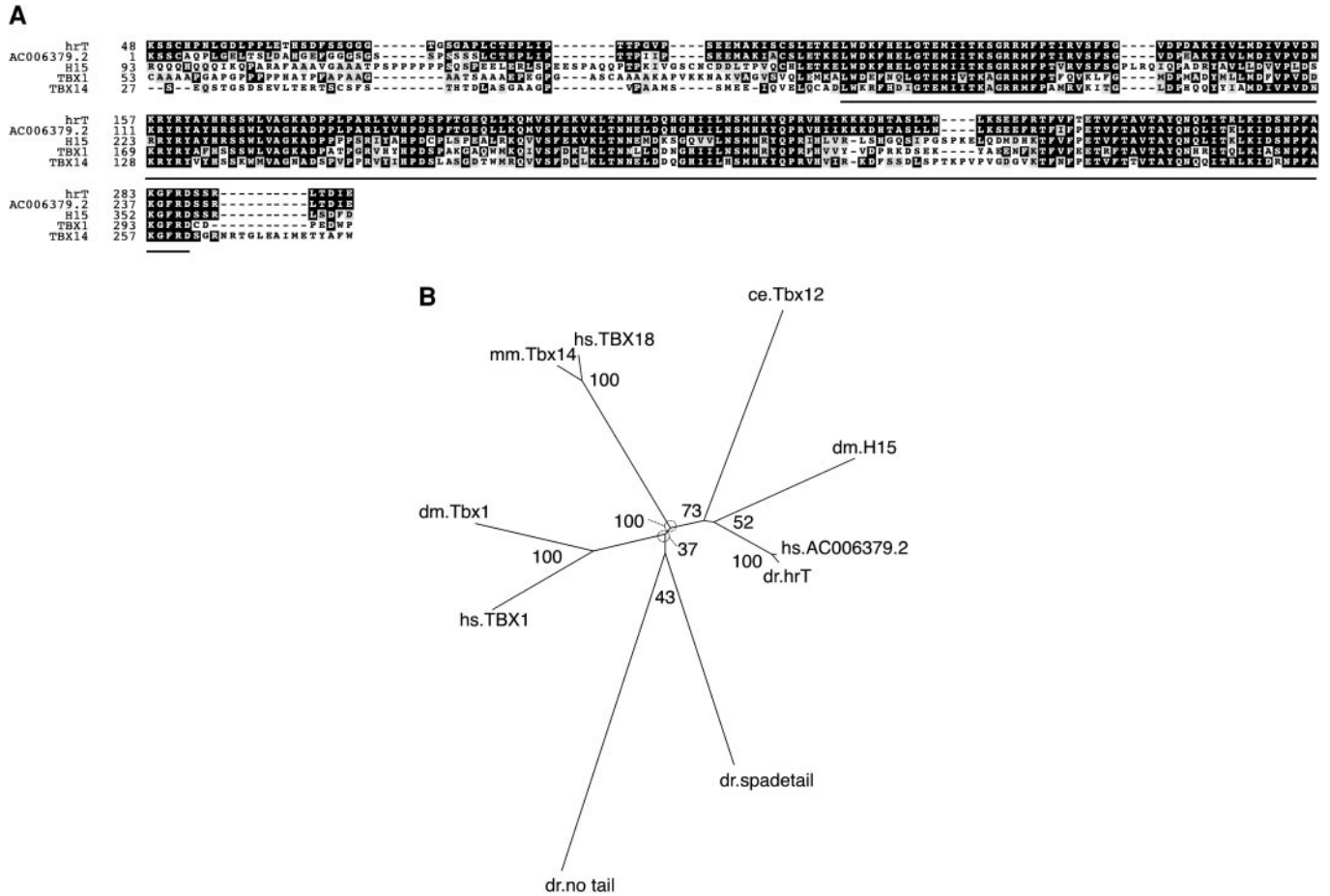


FIG. 1. Identification of an *H15*-related T-box transcription factor subfamily. (A) Amino acid sequence of *hrT* compared to related members of the T-box family. Sequences were aligned using ClustalW and shaded using Boxshade; black shading indicates identity, gray shading indicates similarity, dashes indicate gaps introduced to maximize homology. The T domain is underlined. Numbers before each sequence refer to the positions of the amino acids within each full-length polypeptide; the comparison was limited by the known sequence of AC006379.2. (B) A phylogeny, based upon a distance matrix, was derived from the T-domain amino acid sequences of *tbx1* subfamily members (Papaioannou and Silver, 1998), as well as more distantly related family members. *hrT* is most closely related to a T-box-containing gene found in BAC AC006379.2, followed by *Drosophila H15* and *Caenorhabditis elegans Tbx12*. The tree was derived using the programs Protdist and Fitch in the PHYLIP package; branch lengths are proportional to the number of changes required to derive a putative common ancestor. Numbers at branchpoints refer to the frequency of occurrence of each branchpoint in datasets derived from bootstrap analysis (100 replicates; using Seqboot, Protdist, and Consense). Abbreviations used: dr, *Danio rerio*; dm, *Drosophila melanogaster*; mm, *Mus musculus*; hs, *Homo sapiens*; ce, *C. elegans*.

identify novel T-box transcription factors expressed during early development (Griffin *et al.*, 1998). One of the genes identified in this screen was highly homologous to *Drosophila H15* (Brook *et al.*, 1993; Brook and Cohen, 1996) and which we call *hrT* (*H15*-related T box). *hrT* was also extremely closely related to a sequence identified in a human BAC, AC006379.2 (see Materials and Methods). At the amino acid level, the human and zebrafish sequences were 98.5% identical within the T domain and 89.5% identical over the known sequence of the human gene (Fig. 1A). Phylogenetic analysis of the amino acid sequences of the T domain confirmed the close relatedness of these three

genes within the T-box family as a whole and the *tbx1* subfamily in particular (Papaioannou and Silver, 1998; Fig. 1B). The similarities between *hrT* and *H15* in sequence and expression, described below, suggest that these genes are orthologues, although this is difficult to determine between *Drosophila* and vertebrate sequences. However, we strongly suspect that the human T-box sequence within AC006379.2 is the human orthologue of zebrafish *hrT*, based upon their extremely high similarity at the amino acid level and the fact that their genomic map locations are potentially syntenic. *hrT* is located on LG16 (M. Gates and W. Talbot, pers. comm.), close to the *hoxab* cluster (ze-

brafish have two *hoxa* clusters; Amores *et al.*, 1998), and *AC006379.2* maps to 7p15.1–p13, close to the *HOXA* cluster (map positions of human genes obtained from NCBI). A search of the OMIM database did not identify any obvious candidates for human congenital malformations attributable to human *hrT*.

Conserved Expression of Zebrafish *hrT* and *Drosophila H15* during Heart Formation

hrT is a potential orthologue of *Drosophila H15* (Brook and Cohen, 1996). Since *H15* has previously been characterized only during imaginal disc development (Brook and Cohen, 1996), we compared the expression patterns of *hrT* and *H15* during embryogenesis. We found that *H15* and *hrT* were expressed in diverse tissues during embryogenesis but, remarkably, both were expressed in cardiac progenitors. In *Drosophila*, as in vertebrates, a single cardiovascular structure, the dorsal vessel, is formed by fusion from bilateral primordia. The bilateral primordia consist of a single row of cardioblasts, the myocardial cells, as well as nonmyogenic pericardial cells. The cardial cells are at the leading edge of the mesoderm during the movements leading to dorsal closure, and the pericardial cells are lateral to them. We analyzed *Drosophila H15* expression using an enhancer-trap line, *H15-lacZ*. *H15-lacZ* expression was detected bilaterally in the cardial cells from stage 12 and during movement of these cells to form a double row of cardial cells at the dorsal midline (Figs. 2A and 2D). In addition, *H15-lacZ* was also expressed in a segment polarity pattern in the anteriormost cells of each segment of the lateral abdominal epidermis (Fig. 2B), as well as in the head and peripheral and central nervous systems (Figs. 2C and 2D).

In zebrafish, as in other vertebrates, the heart develops from bilateral primordia derived from lateral plate mesoderm adjacent to the hindbrain (Stainier *et al.*, 1993; Fishman and Chien, 1997). These primordia migrate toward the midline underneath the anterior hindbrain, where extensive morphogenetic remodeling occurs and a single cardiac structure is formed (Stainier *et al.*, 1993; Yelon *et al.*, 1999). The venous end of the differentiated cardiac tube moves anteriorly and to the left and traverses underneath the left eye and eventually onto the anterior surface of the yolk cell. Zygotic expression of zebrafish *hrT*, which was also maternally expressed (data not shown), was detected in diverse tissues, but was most prominently associated with heart formation. *hrT* expression, which is described in more detail below, was first detected in anterior lateral plate mesoderm, as cardiac differentiation became detectable (Fig. 3A), and continued to be expressed throughout the heart during the movements described above. *hrT* expression in lateral plate mesoderm became restricted to a subset of the lateral plate including the myocardial precursors, as identified by *nkx2.5* expression (Figs. 3F and 4). Subsequent to this, *hrT* continued to be expressed in the heart tube as it forms (Figs. 3H, 3K, and 3L) and came to be located underneath the left eye (Figs. 3M and 3N).

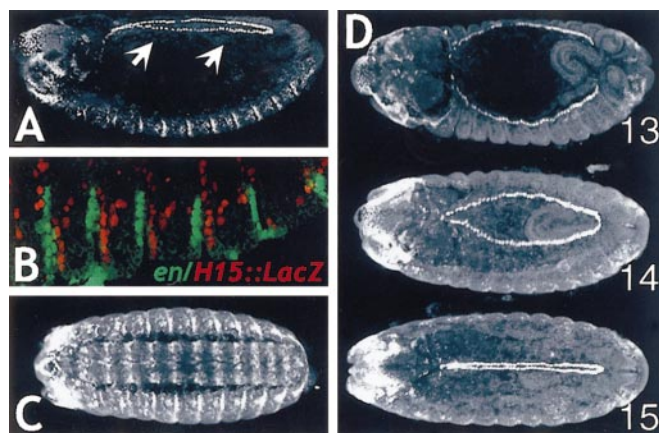


FIG. 2. *Drosophila H15* is expressed in the primordia of the dorsal vessel. Embryos are viewed laterally (A and B), ventrally (C), or dorsally (D); anterior is on the left in all cases. (A) *H15* is expressed ventrally in segmentally reiterated stripes in the CNS, dorsally in the cardial cells of the heart (arrows), and in numerous cells in the head. (B) Double labeling of *H15-lacZ* (red) with *Engrailed* (green). *Engrailed* expression marks the posterior row in each segment of the lateral abdominal epidermis, and *H15-lacZ* is strongly expressed in the anteriormost cells of each segment. In addition to ectodermal cells, *H15-lacZ* is also expressed in cells of the visceral mesoderm and peripheral nervous system. (C) Ventral view showing segmentally reiterated expression of *H15-lacZ* in the central nervous system. (D) Dorsal views of embryos at the stages indicated, showing migration and fusion of the bilateral cardiac primordia. *H15-lacZ* was expressed in the leading row of cells, the cardial cells, in each dorsal vessel primordium as they migrate toward each other at dorsal midline.

These data show that zebrafish *hrT* and its putative *Drosophila* orthologue *H15* are expressed during differentiation of cardiac precursors and the formation of a single cardiovascular structure. The conservation of cardiovascular expression of *H15*-like genes in these two distantly related species adds strong support to the hypothesis that vertebrate and arthropod hearts are homologous structures with a common evolutionary origin (Harvey, 1996; Fishman and Olson, 1997; Bodmer and Venkatesh, 1998) and suggests that the ancestral *H15* gene was involved in the formation of this structure.

Expression of *hrT* Suggests an Early Role in Specification of the Heart Fields

As mentioned above, zygotic expression of *hrT* was mostly associated with cardiac tissue. *hrT* expression was first detected in anterior lateral plate mesoderm at the bud stage (data not shown), which is 2 h earlier than *tbx5* is detected (G. Begemann, pers. comm.). By the 5- to 10-somite stages, *hrT* was strongly expressed in anterior lateral plate mesoderm, as well as in two bilateral groups of

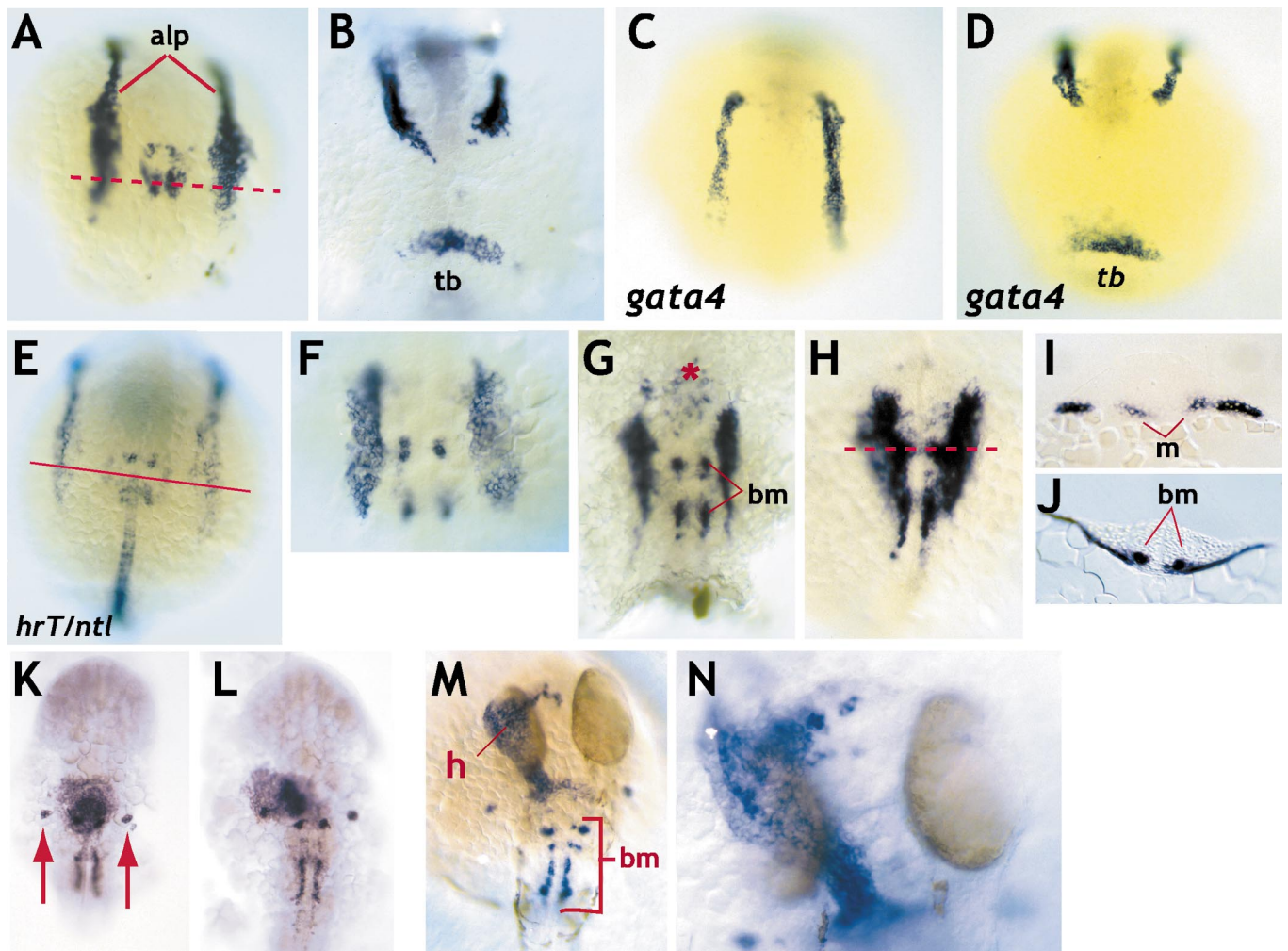


FIG. 3. Expression of *hrT* during heart formation. All views are of the dorsal hindbrain region, anterior at the top, unless otherwise indicated. (A) 10-somite embryo. *hrT* is strongly expressed in anterior lateral plate mesoderm (alp) and also in two groups of mesenchymal cells close to the midline (dotted line indicates plane of section in J). Note the posterior limit of expression in the lateral plate relative to the mesenchymal expression. (B) Vegetal view of embryo in (A) showing expression in anterior lateral plate and in a crescent of mesenchyme adjacent to the tail bud (tb). (C and D) Expression of *gata4* at 8–10 somites. (C) Anterior view, hindbrain region is rotated slightly out of view relative to embryo in (A). *gata4* expression is throughout anterior lateral plate mesoderm, as described (Serbedzija *et al.*, 1998). (D) Vegetal view, showing the anteriormost expression in the lateral plate, as well as *gata4* expression in mesodermal cells adjacent to the tail bud. Compare with *hrT* expression (A and B). (E) 10-somite embryo double labeled with *hrT* and *no tail*, which is expressed in the notochord. The parasagittal mesenchymal expression and anterior lateral plate expression of *hrT* overlaps with the anterior limit of the notochord, as indicated by the solid line. (F) 15 somites. The anterior limit of *hrT* expression in lateral plate is now approximately at the anterior hindbrain, and the posterior limit now coincides with the mesenchymal cells that express *hrT*. (G) 18 somites. *hrT* is detected in the mesenchymal cells (*) at the midline. Expression is also observed for the first time in hindbrain branchiomotor neurons (bm) situated between the cardiac expression sites. (H) 20 somites. The bilateral cardiac primordia begin to fuse anteriorly and posteriorly and appear to surround the midline mesenchymal cells (dotted line indicates plane of section in J). (I) Transverse section as indicated in (A), showing expression in the cardiac primordia situated laterally, as well as in mesenchyme (m) closer to the midline. (J) Transverse section as indicated in (H). Note expression of *hrT* in hindbrain branchiomotor neurons (bm). (K) 24 hpf. The cardiac cone begins to coalesce and tilt. Note expression of *hrT* in clusters of cells lateral to the hindbrain (arrows). (L) 26 hpf. The forming heart tube has moved anteriorly and to the left. *hrT* expression is strongest in the ventricle, the *hrT*-expressing atrial progenitors have yet to coalesce. (M) 31 hpf. *hrT* expression in the heart (h) is located ventral to the left eye, expression in branchiomotor neurons is clearly distinguished (bm). (N) Detail of *hrT* expression in the heart at 31 hpf. *hrT* expression appears weaker near the atrioventricular junction.

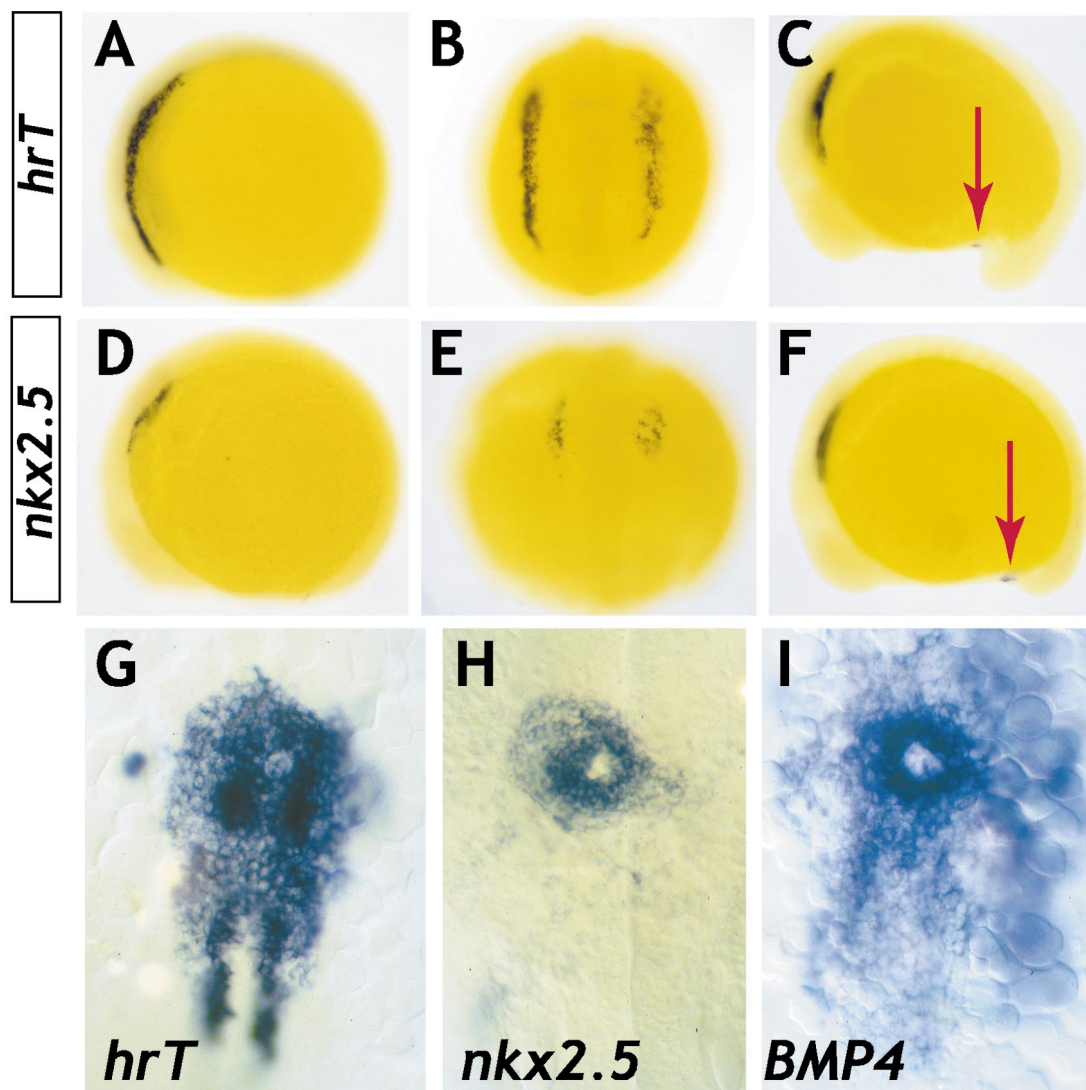


FIG. 4. Comparison of *hrT* expression with *nkx2.5* and *BMP4*. Comparison of *hrT* (A–C) and *nkx2.5* (D–F) at 6- to 8- and 12- to 14-somite stages. At 6–8 somites *hrT* expression extends to the anterior limit of the lateral plate mesoderm (A and B), whereas *nkx2.5* is restricted to lateral plate mesoderm adjacent to the hindbrain only (D and E). At 12–14 somites, *hrT* expression (C) in lateral plate mesoderm is in the vicinity of the hindbrain only and coincides approximately with *nkx2.5* (F). Note that *nkx2.5* is also expressed in cells on the ventral yolk cell (arrow in F), where *hrT* (arrow in C; Fig. 3B) and *gata4* (Fig. 3D) are also expressed. (G–I) Expression of cardiac markers during formation of the cardiac cone (20 somites). (G) *hrT* is expressed in the cone and more posteriorly; (H) *nkx2.5* expression is restricted to the cardiac cone; (I) *BMP4* is expressed in the cardiac cone and more posteriorly, similar to *hrT*.

mesenchymal cells close to the anterior notochord (Figs. 3A and 3I) and in a crescent of lateral plate tissue adjacent to the tail bud (Fig. 3B). With the exception of the mesenchymal cells near the notochord, *hrT* expression was highly reminiscent of expression of *gata4*, an important regulator of early heart formation and myocardial gene expression in vertebrates (Figs. 3C and 3D; Mohun and Sparrow, 1997; Durocher et al., 1997; Lee et al., 1998; Searcy et al., 1998; Sepulveda et al., 1998; Serbedzija et al., 1998; Lien et al., 1999). Double labeling with *hrT* and a notochord marker, *no*

tail (Schulte-Merker et al., 1992), showed that the lateral plate expression of *hrT* extended posteriorly beyond the anterior limit of the notochord (Fig. 3E). Between the 10- and the 15-somite stage, *hrT* expression was lost in the most anterior and posterior regions of the lateral plate but was maintained in the vicinity of the hindbrain (Fig. 3F). Analysis of sectioned embryos showed that *hrT* was expressed throughout the myocardium at these and later stages (Fig. 3I and data not shown). Thus, *hrT* expression resembled expression of *gata4* in many respects: both genes

are expressed throughout anterior lateral plate mesoderm as well as in a group of cells adjacent to the tail bud.

Vertebrate *Nk-2* genes are homologous to *Drosophila tinman* and collectively play a crucial role in early heart formation (Harvey, 1996; Fu *et al.*, 1998; Grow and Krieg, 1998). We compared *hrT* expression with *nkx2.5*, which of all the *Nk-2* genes is the most specific for myocardial tissue (Lee *et al.*, 1996). At the 6- to 8-somite stage, *nkx2.5* was expressed in lateral plate mesoderm adjacent to the hindbrain (Figs. 4D and 4E), whereas *hrT* was expressed throughout anterior lateral plate mesoderm (Figs. 4A and 4B). In contrast to the early expression of *hrT* throughout anterior lateral plate mesoderm, *tbx5* is first expressed approximately 2 h later than *hrT*, at 6–8 somites, and is restricted to lateral plate mesoderm in the vicinity of the hindbrain and extending posteriorly to the level of the pectoral fin bud (G. Begemann, pers. comm.); this distribution is comparable to *Xenopus tbx5* expression (Horb and Thomsen, 1999). At the 12- to 14-somite stage, *hrT* expression in lateral plate mesoderm resembled expression of *nkx2.5*, although *hrT* was still more extensively expressed (Figs. 4C and 4F). Remarkably, we found that *nkx2.5* was also coexpressed with *hrT* and *gata4* in mesodermal cells near the tail bud (Figs. 4C and 4F). The coexpression of *hrT*, *gata4*, and *nkx2.5* in two separate locations suggests that a conserved epistatic regulatory relationship operates in both tissues.

During late somitogenesis, *hrT* expression became more complex and expression in the heart was dynamic, consistent with the previously described morphogenetic movements of the cardiac primordia (Stainier *et al.*, 1993; Yelon *et al.*, 1999). At 18 h postfertilization (hpf; 18 somites), *hrT* expression was observed in loosely packed mesenchymal cells in the midline immediately anterior to the heart fields (Fig. 3G). These cells appear to become surrounded by the heart fields as they fuse (19 hpf; Fig. 3H) and may be the endocardial progenitors (Stainier *et al.*, 1993).

When the heart fields had fused anteriorly and posteriorly, *hrT* expression was expressed in the cardiac cone, but expression also extended posteriorly and was similar to *BMP4* expression (20 hpf; Figs. 4G and 4I; Chin *et al.*, 1997). This contrasts with *nkx2.5* expression, which is detected only in the cardiac cone (Fig. 4H). Subsequently, *hrT* was expressed in the cardiac tube as it formed and appeared strongest in the ventricle, which coalesces prior to the atrium (Yelon *et al.*, 1999; Figs. 3K and 3L). Subsequently, *hrT* was localized underneath the left eye and appeared slightly weaker in the region of the atrioventricular junction (Figs. 3M and 3N). *hrT* was still detectable in the heart at 72 hpf, the oldest stage examined (data not shown). Thus, *hrT* was continuously expressed in the myocardial lineage from the initiation of myocardial differentiation through to formation of the differentiated, two-chambered heart. This expression pattern suggests an early role for *hrT* in the early differentiation of myocardial cells, as well as a later role in organ growth and remodeling.

***hrT* Is Expressed in Diverse Tissues throughout the Embryo**

At 20–22 hpf, when fusion of the heart is occurring, *hrT* expression is seen in bilateral clusters of 8–10 cells situated lateral to the anterior hindbrain (Fig. 3J). These clusters continue to express *hrT* through to at least 72 hpf (data not shown) and are likely to be arch-associated catecholaminergic neurons described by Guo *et al.* (1999). *hrT*-expressing cells adjacent to the tail bud became localized to the ventral surface of the yolk extension, adjacent to the anal opening (Figs. 5A and 5B). Between 24 and 48 hpf this expression spread over the entire ventral surface of the yolk tube and occasionally into the anal opening (data not shown). Transient *hrT* expression was seen in the aorta around 24 hpf, possibly in endothelial progenitors (Figs. 5A and 5C). *hrT* was also expressed in a variety of neuroectodermal derivatives, such as hindbrain branchiomotor neurons from 18 hpf (Figs. 3H, 3J, and 3M; Chandrasekhar *et al.*, 1997, 1999), a bilateral pair of nuclei medial to the eye, and retinal cells adjacent to the lens (Fig. 5D).

Aberrant Expression of *hrT* in *pandora* Reveals a Midline Patterning Defect

Pandora (*pan*) mutant embryos have a highly pleiotropic phenotype affecting many tissues including the eye, heart, and tail (Stainier *et al.*, 1996). The cardiac defect, analyzed in detail by Yelon *et al.* (1999), consists of delayed and dramatically reduced expression of cardiac-specific myosin isoforms during somitogenesis. At later stages, atrial-specific gene expression and development recover to a degree but ventricular-specific gene expression and development remain markedly defective.

Since *hrT* is expressed in many of the tissues affected in *pan* embryos, and is likely to be a regulator of cell fate, we examined *hrT* expression in *pan* mutant embryos. In *pan* mutant embryos up to 24 hpf, *hrT* expression was dramatically reduced in all tissues in which it is expressed, including the heart (Figs. 6A and 6B). The reduction in *hrT* expression in cardiac tissue correlates with the reduction in cardiac myosin expression (Yelon *et al.*, 1999) and is consistent with a requirement for *hrT* in the differentiation of myocardial precursors. Since we have excluded *hrT* as a candidate for *pan* based upon linkage analysis (data not shown), this suggests that *pan* function may be important in the regulation of *hrT* expression. By 31 hpf, however, *hrT* expression in *pan* mutants had recovered to near normal levels, but was spatially disrupted in the heart (Fig. 6C; see also Yelon *et al.*, 1999) and tail (data not shown). In the heart, *hrT*-expressing cells were poorly coalesced and were not localized to the left, as in wild-type embryos (Fig. 6C, compare with Fig. 3L). However, since *pan* embryos are significantly delayed in their development relative to wild-type (Yelon *et al.*, 1999), the distribution of *hrT*-expressing cells in the *pan* heart may simply reflect this delay. In the tail, *hrT*-expressing cells on the yolk tube were more abundant and, in some embryos, were scattered over the

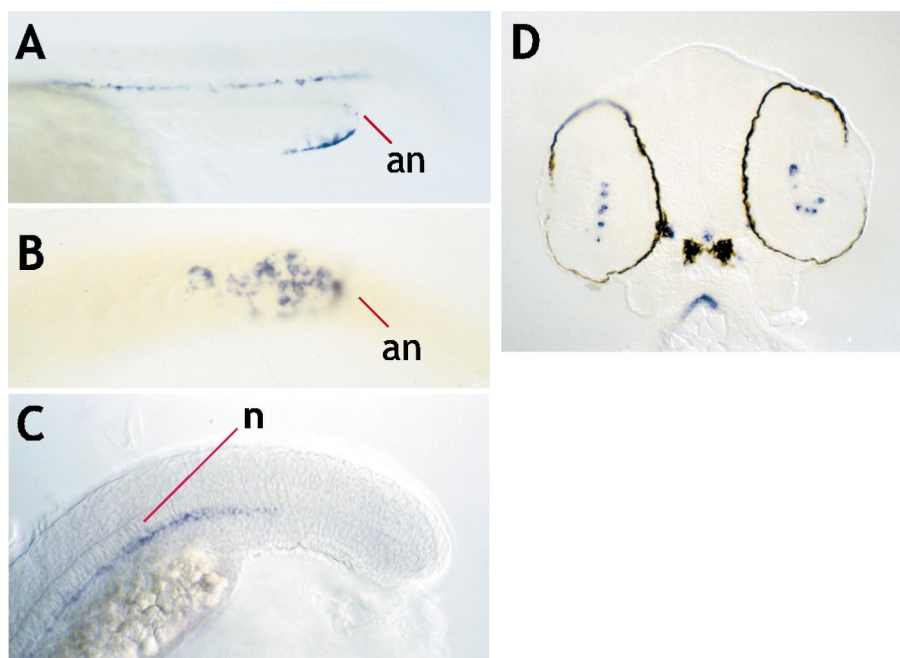


FIG. 5. Expression of *hrT* in retina and posterior structures. (A) Lateral view of trunk and tail region of 24-hpf embryo (anterior to left) showing *hrT* expression in aorta (uppermost expression) and in cells on the ventral yolk tube anterior to the anal opening (an). (B) Ventral view of trunk of embryo in (A), anterior to left, showing *hrT*-expressing cells on the yolk tube. (C) Expression of *hrT* in aorta at 24 hpf. Embryo has been cleared in glycerol and viewed with DIC to show *hrT* staining relative to notochord (n). (D) Coronal section, dorsal uppermost, through 48-hpf embryo at the level of the eye, showing *hrT* expression in cells of the outer retina, adjacent to the lens.

posterior yolk cell (data not shown). Since *hrT* and *BMP4* expression frequently coincide, we analyzed *BMP4* expression in *pan* mutants. In the heart field at 31 hpf, *BMP4* expression was affected in a manner similar to that of *hrT*; however, in the tail, *BMP4* was dramatically upregulated in ventral cells in comparison with wild-type (Figs. 6H and 6I; Chin *et al.*, 1997). The changes in *hrT* and *BMP4* expression in the tail may be significant for posterior morphogenesis since the yolk tube extension fails to form in *pan* mutant embryos. Finally, *hrT* is also ectopically expressed in the dorsal retina and/or the adjacent diencephalon of *pan* mutants at this stage (data not shown).

In *pan* embryos at 48 hpf, we observed striking ectopic expression of *hrT* in the region of the anterior hindbrain. *hrT* was ectopically expressed in tissue underlying the anterior hindbrain, presumably pharyngeal tissue (Fig. 6F). This ectopic *hrT* expression coincided with a severe neural patterning defect in the overlying hindbrain (Fig. 6G). In *pan* mutant embryos, the branchiomotor neurons in the anterior hindbrain, detected by *hrT* staining, were disorganized and met at the midline of the neural tube, whereas they were clearly separated in wild-type embryos (Figs. 6E and 6G; Chandrasekhar *et al.*, 1997, 1999). The branchiomotor neuron defects were localized to the anterior hindbrain and did not affect either the nuclei of the vagus nerve, located more posteriorly (Fig. 6G), or the paired nuclei

medial to the eyes, which also express *hrT* (data not shown). Thus, in young *pan* embryos, *hrT* is expressed at reduced levels, whereas in older *pan* embryos, *hrT* was ectopically expressed and revealed a severe midline patterning defect at the level of the anterior hindbrain. This midline defect may provide insight into the origin of the ventricular defect found in *pan* embryos.

DISCUSSION

In this paper, we have characterized a subfamily of T-box transcription factors that are closely related to *Drosophila* *H15* (Brook and Cohen, 1996). We have identified a novel zebrafish T-box transcription factor, *hrT* (*H15*-related T box) and a closely related human sequence, *AC006379.2*. The sequence conservation and similarity in expression of *hrT* and *H15*, discussed below, strongly suggest that these genes are orthologues, although it is difficult to assign orthologous relationships between arthropod and zebrafish genes with certainty. Similarly, *AC006379.2* is an extremely good candidate for the human *hrT* orthologue. *hrT* and *AC006379.2* are 98.5% identical within the T domain and were almost inseparable by phylogenetic analysis. Furthermore, both genes are physically located close to *hoxa* clusters; *HOXA* in human and *hoxab* in zebrafish. It will be

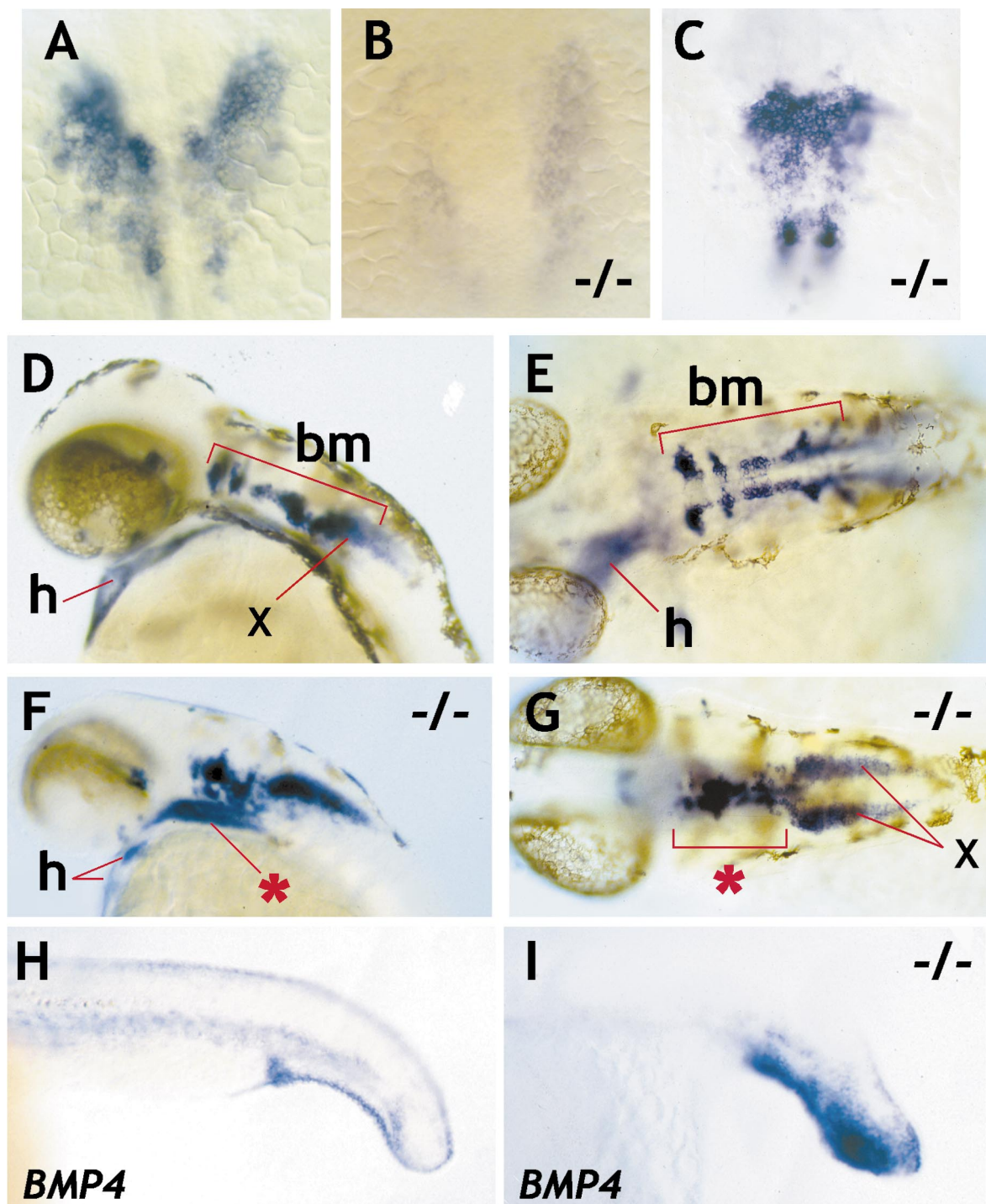


FIG. 6. Aberrant expression of *hrT* in *pandora*. Dorsal view of anterior hindbrain at 17 (A and B) and 31 hpf (C), anterior uppermost. *hrT* expression is dramatically reduced in *pan*^{-/-} embryos (B) relative to WT (A). (C) At 31 hpf, *hrT* expression in the heart is at a relatively normal level, but is not localized to the left and appears disorganized. Lateral (D) and dorsal (E) views of wild-type embryos at 48 hpf showing expression of *hrT* in the heart (h) and branchiomotor neurons of the hindbrain (bm). Note that the nuclei of the vagus nerve are not visible in the focal plane of (E). In *pan*^{-/-} embryos at 48 hpf (F and G), ectopic expression of *hrT* is detected in a large group of cells, possibly pharyngeal, located ventral to the anterior hindbrain (* in F). The heart is correctly localized on the anterior yolk cell; a gap is visible in *hrT* expression in the heart, presumably indicative of the ventricular defect. The organization of the branchiomotor neurons in the anterior hindbrain is disrupted (* in G) and they meet at the midline. Paired nuclei medial to the eyes and the nuclei of the vagus nerve (X) appear normal. (H and I) Lateral views of *BMP4* expression in WT and *pan*^{-/-} tail at 31 hpf, showing greatly increased *BMP4* expression in ventral tail cells of the *pan*^{-/-} embryo.

extremely interesting to analyze the expression pattern and function of the mammalian *hrT* orthologue and determine if *AC006379.2* is associated with any as yet unmapped human congenital malformations, as described for *TBX1* (Chieffo et al., 1997), *TBX3* (Bamshad et al., 1997, 1999), and *TBX5* (Basson et al., 1997; Li et al., 1997) and suggested for *TBX15* (Agulnik et al., 1998).

An Ancestral Role for *H15*-Related T-Box Genes in Cardiac Tissue Formation

hrT is closely related to *Drosophila H15* (Brook and Cohen, 1996). It was very exciting to discover that expression of these potential orthologues in cardiac precursors was conserved in the fly and the fish. Both *hrT* and *H15* are expressed in myocardial precursors; zebrafish *hrT* may also be expressed in endocardial precursors (see Fig. 3H and Stainier et al., 1993). Prior to this study the evidence that the hearts of vertebrates and arthropods had a common evolutionary origin was the conserved expression of *tinman*-related homeobox genes and *MEF2*-related factors, and the possible involvement of *dpp/BMP* signaling, in heart formation in both *Drosophila* and vertebrates (reviewed in Harvey, 1996; Fishman and Olson, 1997; Bodmer and Venkatesh, 1998). Our finding that *H15*-related T-box transcription factors have conserved expression in the heart from the earliest phase of cardiogenesis significantly strengthens this view. Moreover, the conservation of *H15*-like gene expression in the developing hearts suggests that these genes perform an important and essential function in heart development. *H15* is a well-characterized target of *Wg* signaling in the leg imaginal disc (Brook and Cohen, 1996), and *wg*, in concert with *dpp*, is required in *Drosophila* for the initial specification of cardiac mesoderm (Wu et al., 1995; Park et al., 1996). Whether *wnt* signals play a similarly pivotal role in vertebrate heart formation has not yet been demonstrated. Although several *wnt* ligands are expressed in the cardiac crescent of mouse and chick embryos (Monkley et al., 1996; Eisenberg et al., 1997; Zakin et al., 1998), their function in the heart has not been determined, and targeted mutagenesis of one of them did not cause any heart defects (Monkley et al., 1996). The analysis of *H15* and *hrT* regulation in the heart fields may shed light on whether *wnt* signals play a similar role in the vertebrate heart as *wg* signaling does in *Drosophila*.

Regulation and Definition of the Heart Field in Zebrafish

T-box transcription factors are increasingly recognized as critical regulators of diverse developmental processes, and much attention has been focused recently on the important role of *tbx5* in heart development. Mutations in human *TBX5* are the cause of Holt-Oram syndrome, an haploinsufficient syndrome characterized by heart and forelimb defects (Basson et al., 1997; Li et al., 1997), and dominant-negative interference of *Xenopus tbx5* leads to near-total

absence of differentiated heart tissue and early cardiac markers (Horb and Thomsen, 1999). However, *Xtbx5* is expressed relatively late in the heart fields in *Xenopus*, and *hrT* expression in zebrafish precedes *tbx5* expression by approximately 2 h (G. Begemann, pers. comm.). Thus, *tbx5* is a relatively late marker of cardiogenic tissue in comparison to *hrT*, and *hrT* may be genetically upstream of *tbx5*. It remains to be seen whether the *Xtbx5* dominant-negative construct might also interfere with the function of *Xenopus hrT*.

Despite the proven role of *tbx5* in heart formation and the potential importance of *hrT*, implied by the conserved expression described here, expression of neither factor is limited to cardiac precursors during differentiation of precardiac mesoderm. What then are the mechanisms that define the cardiac precursors? Recent evidence suggests it is a complex process involving multiple levels of control, one of which is likely to involve *hrT*. Serbedzija et al. (1998) used fate mapping and cell ablation to analyze the positions of the cardiac progenitors in the zebrafish. Intriguingly, they found that the heart was derived from only a subset of cells in the putative heart field, as defined by expression of *nkx2.5*. Cells adjacent to the anterior notochord that express *nkx2.5* are prevented from adopting a myocardial fate by a repressive influence from the notochord, even after physical ablation of the actual myocardial progenitors (Goldstein and Fishman, 1998). Intriguingly, cells that replenish the heart field after its ablation are derived from anterior lateral plate mesoderm that does not express *nkx2.5* but, at the time the ablations were performed (10 somites), does express *gata4* and *hrT*.

These studies show that at the time that *hrT* and *gata4* are widely expressed in anterior lateral plate mesoderm, the fates of cells in this region are plastic and can adopt a cardiac fate under experimental conditions. We favor the possibility that *gata4/hrT* expression confers precardiac potential within the lateral plate mesoderm (Fig. 7; Serbedzija et al. (1998). *gata4/hrT*-expressing cells may be competent to express *nkx2.5* and *tbx5*, which promote the full myocardial fate, either by being responsive to signals localized to the vicinity of the hindbrain or by acting in a combinatorial manner with additional myocardial factors expressed in lateral plate mesoderm. In support of the idea that *gata4/hrT*-expressing cells are competent to express *nkx2.5*, we observed coexpression of *nkx2.5* with *gata4* and *hrT* not only in the heart fields, but also in noncardiac mesoderm adjacent to the tail bud. It will be interesting to determine the requirement for *hrT* and/or *gata4* in the regulation of *nkx2.5* expression and in cardiac fates in general.

We attempted to address the role of *hrT* in the regulation of myocardial-specific genes by overexpression of *hrT* mRNA in zebrafish embryos. Although we observed upregulation of *nkx2.5* expression in these experiments, we also observed a dramatic upregulation of a neural crest marker (*fkf6*) and melanocytes (S.C. and K.J.P.G., unpublished observations). In addition, *hrT* overexpression also

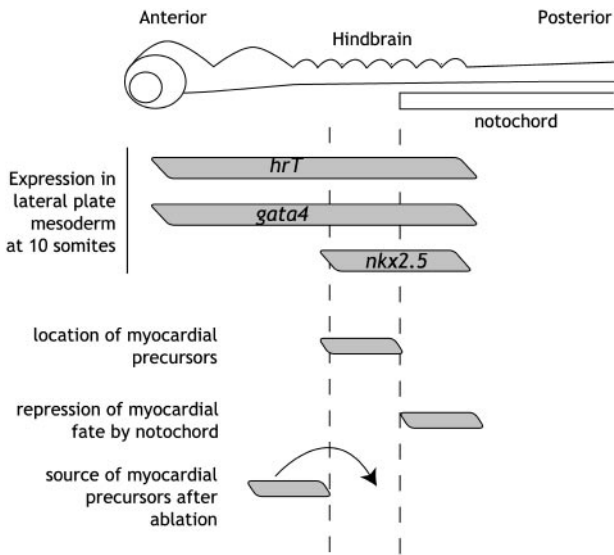


FIG. 7. Cartoon demonstrating origin of cardiac progenitors relative to early expression of *hrT*, *gata4*, and *nkx2.5* in lateral plate mesoderm. Expression of all three genes overlaps in the anteroposterior axis with the anterior notochord, but *hrT* and *gata4* expression extends into the most anterior lateral plate. Fate-mapping studies show that cardiac progenitors are localized to the anterior of the *nkx2.5* expression domain (Serbedzija *et al.*, 1998). After ablation of the cardiac progenitors, regulation of cardiac progenitors occurs but the source of new progenitors is from lateral plate mesoderm that expresses *hrT* and *gata4* but did not previously express *nkx2.5* (the so-called “regulatory compartment”). Cells that express *nkx2.5* and are adjacent to the notochord contribute to the heart only after ablation of the notochord (Goldstein and Fishman, 1998).

caused severe morphological defects that, in our opinion, preclude any meaningful interpretation of these phenotypes. Similarly, Horb and Thomsen (1999) found that overexpression or dominant-negative interference with *Xtbx5* caused defects that could not be attributed to the *in vivo* function of *Xtbx5*. It is hoped that the development of targeted misexpression vectors utilizing myocardial-specific promoters, and the phenotypic analysis of mutant alleles of *hrT* or its mammalian orthologue, will permit a more informative analysis of *hrT* function in the regulation of *nkx2.5* and the myocardial fate.

Are the Ventricular and Midline Defects in *pan* Linked?

pan mutant embryos have a variety of defects, including developmental delay and a large reduction in ventricular tissue of the heart (Stainier *et al.*, 1996; Yelon *et al.*, 1999). *hrT* is expressed in many if not all of the tissues that require *pan* function, and *hrT* expression is aberrant in *pan* mutant embryos. Since we have excluded *hrT* as a candidate for *pan* based upon linkage analysis (data not shown), *pan* function

appears to be important in the regulation of *hrT* expression, and the altered *hrT* expression that we have observed in *pan* embryos is likely to be an important aspect of the *pan* phenotype. The effects of *pan* on *hrT* expression are complex: *hrT* expression is much reduced at early times, recovers to normal levels later, and is also ectopically expressed. Indeed, ectopic *hrT* expression has revealed a previously unrecognized defect in *pan* that may be relevant to our understanding of the ventricular defect in this mutant. We found that *pan* mutant embryos have a severe midline defect at the level of the anterior hindbrain, consisting of a large domain of ectopic mesodermal or endodermal *hrT* expression, probably in pharyngeal tissue, and a concomitant derangement of anterior hindbrain patterning. The hindbrain defect is consistent with a loss of ventral midline fates, leading to ventrolateral cell types (the branchiomotor neurons) meeting at the midline (Fig. 6G).

The anterior hindbrain region is a critical one for heart development since this is the anteroposterior level at which cardiac progenitors arise and where the bilateral cardiac primordia fuse (Stainier *et al.*, 1993; Yelon *et al.*, 1999). Ventricular progenitors arise closer to the midline than atrial progenitors, and their formation may be regulated in part by midline signaling (Yelon *et al.*, 1999). It is possible therefore that the ventricular and midline neuroectodermal defects observed in *pan* embryos may be causally related by a midline patterning signal. *pan* function might be required either for release of the putative signal or in the responding tissues. It will be interesting to determine, using mosaic analysis, whether *pan* functions cell-autonomously in the myocardial precursors and whether the heart and hindbrain defects are linked as suggested above. Our observation that *BMP4* expression is greatly upregulated in the ventral tail of *pan* embryos suggests that the *pan* phenotype may involve non-cell-autonomous effects, at least in some tissues. Undoubtedly, full understanding of the complex phenotype of *pan* must await molecular cloning of this locus.

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Note added in proof. The zebrafish *hrT* gene described in this study is unrelated to the HRT gene family recently described by Nakagawa *et al.*, (1999) *Dev. Biol.* **216**, 72–84, which are bHLH transcription factors related to *Drosophila* *Hairy*.

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