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A New *tinman*-Related Gene, *nkx2.7*, Anticipates the Expression of *nkx2.5* and *nkx2.3* in Zebrafish Heart and Pharyngeal Endoderm

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The *Drosophila* homeobox gene *tinman* and its vertebrate homologs *Nkx-2.5* and *Nkx-2.3* are critical determinants of cardiac development. We report here the identification of a new *tinman*-related gene, *nkx2.7*, as well as orthologs of *Nkx-2.5* and *Nkx-2.3* in the zebrafish. Analysis of their expression in the developing zebrafish embryo reveals that *nkx2.7* transcripts are the first to appear in cardiac mesodermal and pharyngeal endodermal precursors of the anterior hypoblast, anticipating both temporally and spatially the later expression of *nkx2.5* and *nkx2.3* in these lineages. The preeminence of *nkx2.7* in these embryonic lineages is consistent with a key role in cell fate determination, perhaps in part through the induction of *nkx2.5* and *nkx2.3*. The findings provide the first molecular clues as to the spatial organization of endodermal and cardiac mesodermal precursors in the zebrafish hypoblast immediately following gastrulation. They suggest a coordinate role for these three *tinman*-related genes in the development of the heart and pharyngeal arches, and reinforce the paradigm of gene duplication and subspecialization between *Drosophila* and vertebrate species. The results provide a framework in which to analyze potential changes in *tinman*-related gene expression during abnormal zebrafish development.

INTRODUCTION

Heart formation in vertebrate embryos occurs via complex processes involving cell lineage specification and structural morphogenesis. These processes must be regulated at a fundamental level by cardiac-specific molecular mechanisms. Significant insight into the molecular regulation of cardiac ontogeny has come with the identification of the homeobox gene *tinman* in *Drosophila*, expressed in the visceral and precardiac mesoderm and then in the definitive heart-like dorsal vessel (Bodmer *et al.*, 1990; Bodmer, 1993). In *tinman* null mutants, the dorsal vessel is absent, indicating that *tinman* is essential for the development of this organ. Vertebrate homologs of *tinman* have been cloned from mouse (*Nkx-2.5* or *Csx;* Komuro and Izumo, 1993; Lints *et al.*, 1993), frog (Tonissen *et al.*, 1994), and chick (Schultheiss *et al.*, 1995). They share with the *Drosophila* gene a *tinman*-like homeobox as well as a conserved amino terminal decapeptide, and they are expressed in the precardiac mesoderm and adjacent pharyngeal endoderm. Potential target DNA binding sequences for murine Nkx-2.5 have been identified that support trans-activation in experimental promoter constructs (Chen and Schwartz, 1995). Mutation of *Nkx-2.5* by homologous recombination in the mouse produced embryos in which cardiac development is not altogether precluded but, rather, is arrested at the looping stage (Lyons *et al.*, 1995). Thus, *Nkx-2.5* is ultimately required for normal murine heart formation, but unlike *tinman*, it is not essential for primary cell lineage determination and early morphogenesis. Recently, a second *tinman* homolog, *Nkx-2.3*, has been shown in *Xenopus* to be expressed in the developing heart as well, and widely in the pharyngeal endoderm (Evans *et al.*, 1995). The relative contributions of these two *tinman*-related genes remain to be determined, as do their positions in the hierarchy of cardiac regulatory factors in vertebrate embryogenesis.

As part of an investigation of cardiac transcription factors in early vertebrate development we sought to identify *tinman*-related genes in the zebrafish, *Danio rerio*. A number of features make the zebrafish particularly well suited to studies of molecular and genetic mechanisms in embryogenesis (Kimmel, 1989), and in heart formation in par-

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ticular (Stainier and Fishman, 1992). Multiple, large, externally fertilized ooctyes develop rapidly and synchronously into transparent embryos in which definitive cardiac progenitors appear as early as 15 hr postfertilization (hpf), the onset of myocardial contraction occurs by 22 hpf, and circulation is initiated by 26 hpf. The relatively simple anatomy of the teleost heart, comprising sinus venosus, atrium, ventricle, and bulbus arteriosus in series, and its formation from the fusion of bilateral mesodermal primordia (Senior, 1909), mirror the earliest stages of the embryonic mammalian heart, making the fish especially attractive for the study of fundamental cardiogenic mechanisms. This can be accomplished in part through the analysis of heritable cardiac phenotypes arising in mutagenesis screens (Driever *et al.*, 1994; Mullins *et al.*, 1994), and from perturbation of cardiac development in wild-type embryos using a variety of molecular manipulations (Stainier and Fishman, 1992; K.-H.L. and R.E.B., unpublished observations). The exceptional clarity of the zebrafish embryo permits early gene expression to be analyzed in exquisite detail.

Here we report the cDNA cloning and characterization of the zebrafish orthologs of *Nkx-2.5* and *Nkx-2.3* and of a new *tinman*-related gene, *nkx2.7*. Analysis of their expression in the early embryo reveals that *nkx2.7* transcripts are the first to appear in endodermal and cardiac mesodermal precursors of the anterior hypoblast, anticipating both temporally and spatially the later expression of *nkx2.3* and *nkx2.5* in these lineages. The findings suggest an important functional role for these genes in the development of the heart and pharyngeal arches and reinforce the paradigm of gene duplication and subspecialization between *Drosophila* and vertebrate species. Further, these results provide a framework in which to analyze potential changes in *tinman*-related gene expression during abnormal zebrafish development.

MATERIALS AND METHODS

Fish stocks and embryos. Zebrafish, either AB strain (Massachusetts General Hospital, Boston, MA) or wild-type (Ekkwill, Tampa, FL) were raised, handled, and staged according to standard methods (Westerfield, 1995). Embryos prior to 24 hr hpf were staged according to somite number (±1 somite) and converted to hpf for consistency and ease of comparison among stages.

cDNA isolation and sequencing. A 660 nucleotide (nt) *Sph*I fragment of an *XNkx-2.5* cDNA (gift of S. Izumo), encompassing the homeobox and NK2 domains (nt 166–825; Tonissen *et al.*, 1994), was radiolabeled and used to screen 1.2×10^6 recombinants from a 30- to 36-hr whole embryo zebrafish λgt11 cDNA library (gift of K. Zinn) at low stringency, i.e., hybridization in 25% formamide, 5× SSC, at 37°C, and wash in 0.2× SSC/0.2% SDS up to 42°C, according to standard procedures (Benton and Davis, 1977). Among fourteen primary positives, seven were successfully plaque-purified, subcloned into pBluescript II (Stratagene, La Jolla, CA), and partially or completely sequenced and compared to existing gene databases as previously described (Sanger *et al.*, 1977; Yu *et al.*, 1992). One of these cDNA clones corresponded to zebrafish *nkx2.3* (1443 nt, comprising 86 nt 5′ untranslated, 963 nt coding, and 394 nt 3′ untranslated sequences; GenBank accession number U66571), while another corresponded to *nkx2.7* (1080 nt, comprising 186 nt 5′ untranslated, 807 nt coding, and 87 3′ untranslated sequences; GenBank accession number U66573), as described under Results. The remaining isolates derived from more distantly related homeobox genes (not shown).

In addition, a cDNA clone for zebrafish *nkx-2.5* was isolated using a combination of PCR and library screening. Degenerate oligodeoxynucleotide primers corresponding to highly conserved sequences in mouse and frog *Nkx-2.5*, but specifically incapable of amplifying analogous sequences from *nkx2.3* or *nkx2.7*, were synthesized with added 5′ restriction sites (lower case) as follows:

5′-gaagatctggatcc GT(GATC) AA(GA) AT(ACT) TGG TT(CT) CA(GA) AA-3′; homeodomain codons VKIWFQN, sense;

5′-gaagatctggatcc GT(GATC) (CA)G(GATC) GA(CT) GG(GATC) AA(AG) CC(GATC) TG-3′; NK2 domain codons VRDGKPC, sense;

5′-cggaattctaga (CT)TG (GATC)AC (GATC)GT (AG)TT (GATC)A(AG) (AG)TC (GATC)CC-3′; codons GDLNTVQ near the carboxyl terminus, antisense;

5′-cggaattctaga (CT)(CT)A CCA (GATC)GC (GATC)C(GT) (AGT)AT (GATC)CC (AG)TG-3′; codons HGIRAW* at the carboxyl terminus, antisense.

Purified zebrafish genomic DNA (Westerfield, 1995), 100 ng per 100-μl reaction, was amplified using *Taq* DNA polymerase (Perkin–Elmer, Foster City, CA) in standard buffer adjusted to 6 m*M* Mg^{2+} for 35 cycles at 94°C for 30 sec, 54°C for 60 sec, and 72°C for 60 sec. The longest product (352 nt plus primers) was subcloned, sequenced, radiolabeled, and used to screen 10^6 recombinants of a zebrafish adult heart Uni-ZAP XR cDNA library (Short *et al.*, 1988; R.E.B. and B. S. Ticho, unpublished observations; Stratagene, La Jolla, CA) at high stringency, i.e., under conditions modified from above to include 50% formamide at 42°C and a wash temperature of 55°C. Five clones selected at random among multiple primary positives were purified, excised as recommended (Stratagene, La Jolla, CA), and determined by restriction mapping to be identical to each other; one was completely sequenced and proved to be *nkx2.5* (1678 nt, comprising 98 nt 5′ untranslated, 942 nt coding and 638 nt 3′ untranslated sequences; GenBank accession number U66572), as described under Results.

In situ hybridization. Antisense RNA probes were synthesized by *in vitro* transcription of full-length cDNAs in the presence of digoxigenin UTP (Boehringer Mannheim, Indianapolis, IN) as described by Harland (1991). Each of these probes was strictly gene-specific in these assays, showing no cross-hybridization with transcripts of the other two genes: as shown under Results, the *nkx2.3* and *nkx2.5* probes did not detect the earliest expression of *nkx2.7;* similarly, the *nkx2.7* probe did not detect the later expression of *nkx2.5* or *nkx2.3*. This strict specificity was confirmed in each case using probes comprising only nonconserved C-terminal and 3′ untranslated sequences, which yielded results indistinguishable from the full-length probes. No signal above background was detected in control *in situ* hybridization experiments using the corresponding sense transcripts as probes (not shown).

Whole-mount *in situ* hybridization was performed according to Li *et al.* (1994) with the following modifications. Embryos were fixed in 4% paraformaldehyde/0.1% Tween 20 in phosphate-buffered saline for 1 hr at room temperature and then overnight at 4°C prior to rinsing and storage in 90% methanol. After rehydration, embryos were digested with proteinase K, 10–20 μg/ml in phos-

phate-buffered saline/0.1% Tween 20 (PBT) according to developmental stage: 10–20 hpf, 5 min; 24–36 hpf, 15 min; 48–60 hpf, 20 min; 72 hpf, 25 min. Acetylation was repeated three times with 15 μl of acetic anhydride in 5 ml of 0.1 *M* triethanolamine, with the addition of 0.1% Tween 20 and 0.2 *M* triethanolamine during the third round of acetylation, rinsed once in 1 *M* triethanolamine, twice in PBT, once in 100 m*M* glycine, pH 8.0, then three more times in PBT before postfixation in 4% paraformaldehyde/PBT. Hybridization was for 36–40 hr at 58°C. Posthybridization washes and RNAse treatments were modified to include two additional 15-min washes in 0.2× SSC/0.3% CHAPS (Sigma, St. Louis, MO) and one 5-min wash in 0.3% CHAPS/PBT at 53°C. Incubation with anti-digoxigenin F_{ab} fragments (Boehringer Mannheim, Indianapolis, IN), development of alkaline phosphatase color reagent, storage in 90% methanol, and clearing in benzoyl benzoate/benzyl alcohol followed standard protocols (Harland, 1991).

Embryos for sectioning were rehydrated through a graded alcohol series to 25% methanol in PBT and equilibrated with JB-4 or Immunobed glycomethacrylate resin according to manufacturer's instructions (Polysciences, Beaverton, PA) prior to imbedding and sectioning to 5 μm thickness. All specimens were photographed on a Zeiss Axiophot phase contrast microscope with Kodak (Rochester, NY) 160T tungsten color slide film.

RESULTS

Isolation of Zebrafish tinman Homologs

We obtained cDNA clones corresponding to three distinct *tinman*-related genes in the zebrafish using a combination of PCR with degenerate oligonucleotide primers and homology screening of zebrafish cDNA libraries prepared from whole embryos and adult heart (Fig. 1). These genes include the apparent orthologs of *Nkx-2.5* (Komuro and Izumo, 1993; Lints *et al.*, 1993, Tonissen *et al.*, 1994; Schultheiss *et al.*, 1995) and *Nkx-2.3* (Evans *et al.*, 1995), as well as a third gene, here named *nkx2.7* (numbered consecutively as a new gene, per considerations below; conventions for naming zebrafish genes call for lower case letters and no hyphens; Westerfield, 1995). Each of the cDNAs includes full-length protein coding sequences, as evidenced by the presence of in-frame stop codons in the 5′-untranslated regions (not shown; nucleotide sequences submitted to Genbank). Each contains the three highly conserved domains characteristic of the vertebrate *tinman* homologs, i.e., a tinman-like decapeptide near the amino terminus, a 60-amino acid NK-type homeodomain, and a 16-amino acid NK2 domain (Lints *et al.*, 1993; Kim and Nirenberg, 1989). The amino acid sequence encoded by zebrafish *nkx2.5* (314 residues, calculated M_r 35.9 × 10^3) is 67% identical overall (80% similar including conservative substitutions) to *XNkx-2.5* (Tonissen *et al.*, 1994), with marked conservation in the above domains (absolute identity in the homeobox), and at the carboxyl terminus. Zebrafish *nkx2.3* encodes a peptide (321 residues, calculated M_r 36.1 × 10^3) that is 61% identical (74% similar) at the amino acid level to its closest *Xenopus* counterpart, *XNkx-2.3a* (Evans *et al.*, 1995), again with the strongest conservation in the homeobox (identical at all but two residues), NK2 domain, and the amino and carboxyl termini. *nkx2.3* also shares with *XNkx2.3a* a prominent histidine/proline-rich peptide linking the homeodomain and the NK2 domain (Fig. 1).

The third gene, *nkx2.7*, encodes a peptide (269 residues, calculated M_r 30.8 × 10^3; Fig. 1), which, although similar

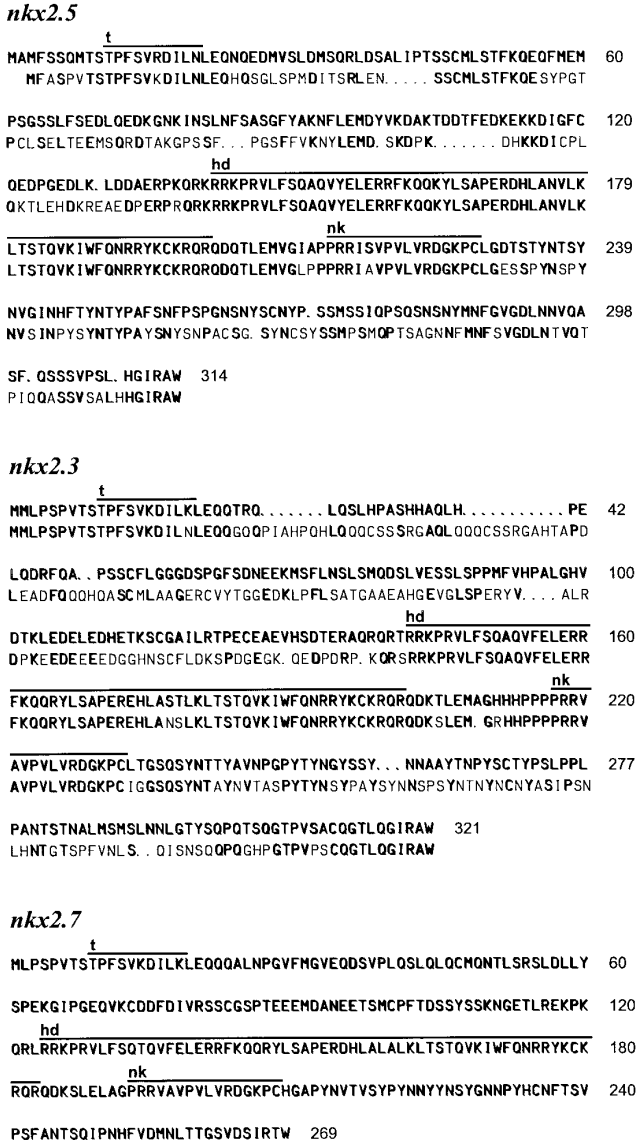

FIG. 1. Predicted amino acid sequences of zebrafish *tinman*-related genes *nkx2.5*, *nkx2.3*, and *nkx2.7*, with residues numbered at the right. Zebrafish *nkx2.5* and *nkx2.3* are shown on the upper lines in comparison to their respective *Xenopus* counterparts (*XNkx-2.5*, Tonissen *et al.*, 1994; *XNkx-2.3a*, Evans *et al.*, 1995) on the lower lines with nonconserved residues shown in lighter typeface. Dots indicate gaps introduced to maximize identities. The *tinman*-like decapeptide (t), homeodomain (hd), and NK2 domain (nk) are overlined in each sequence. The GenBank accession numbers for *nkx2.5*, *nkx2.3*, and *nkx2.7* are U66572, U66571, and U66573, respectively.

to *nkx2.3* and *XNkx-2.3* in the tinman domain, homeobox, and NK2 domain, diverges from them substantially elsewhere, including the carboxyl terminus, and lacks the histidine/proline-rich peptide sequence between the homeobox and NK2 domains (56% identical, 70% similar overall, compared to XNkx-2.3a; Evans *et al.*, 1995). Furthermore, *nkx2.7* is even less similar to sequences reported for *Nkx-2.1* (TTF-1; 42% identical, 60% similar), *Nkx-2.2* (43% identical, 61% similar), and the partial sequences available for *Nkx-2.4*, and *Nkx-2.6* (Guazzi *et al.*, 1990; Price *et al.*, 1992; Lints *et al.*, 1993; Rudnick *et al.*, 1994). On this basis, and on the basis of its expression patterns (see below), *nkx2.7* is a new member of the *tinman*-related subfamily of homeodomain genes. We found no evidence for the existence of still other closely related *tinman* family members in the zebrafish, either from genomic Southern blotting or from the examination of multiple additional cloned cDNAs, which included only more distant homeobox sequences from other subfamilies (data not shown).

nkx2.5 Marks the Paired Cardiac Primordia and Fusing Heart Tube

We undertook a detailed study of the embryonic expression of these three genes by *in situ* hybridization with gene-specific probes in order to better understand their roles in vertebrate embryogenesis; adult stages were not examined. Throughout these Results, apparent cell movements are inferred with some confidence from examination of multiple stained embryos at very closely spaced developmental stages; however, confirmation of these movements will require cell fate mapping using injected lineage tracers.

nkx2.5 transcripts first appear at the 5 somite stage (12 hpf) in bilateral cords of cells in the ventral-most hypoblast, lying in close proximity to the yolk, adjacent to the future hindbrain, and just rostral to the future otic placodes (Fig. 2A and data not shown). These paired structures, initially parallel to each other, move toward the midline, first at their posterior ends forming a ''Y'' configuration (Figs. 2B and 2C), and then anteriorly to form a ring at the 20 somite stage (19–20 hpf; Figs. 2D and 2E). On cross section (Fig. 3A), the cells of the paired primordia do not appear to be organized around a lumen, in contrast to results reported for immunostained sections showing tubular primordia (Stainier *et al.*, 1993; this discrepancy may derive from technical differences in fixation and embedding techniques). These cells also begin to express α-tropomyosin, indicating phenotypic differentiation of the myocardium (data not shown; Stainier *et al.*, 1993). The ring encircles the *portion moyenne*, a cluster of mesodermally derived cells that will give rise to the endocardium (Senior, 1909; Stainier *et al.*, 1993), and which do not express this gene (Fig. 2E; also see Figs. 3A, 3B, and 4). The *portion moyenne* is surrounded in the horizontal plane by *nkx2.5*-expressing cells at this stage but is contiguous ventrally with the yolk syncytial layer and dorsally with noncardiac mesoderm just beneath the hindbrain (section data not shown). By 24 hpf, the nascent myocardium forms a cone with its base on the yolk at the future confluence of the vitelline veins, and its apex at the developing junction with the ventral aorta (Fig. 2F; Stainier *et al.*, 1993; Senior, 1909). Thereafter, *nkx2.5*-expressing cells form paired ventrorostral and dorsocaudal extensions of the elongating heart tube (Figs. 2G and 2H), comprising the future inflow (sinus venosus) and outflow (bulbus arteriosus), respectively, prior to looping and anatomic chamber differentiation. *nkx2.5* transcripts persist in the embryonic heart at least through 48 hpf and are also present in adult heart (data not shown). Extracardiac expression of *nkx2.5* was detected at a low level after prolonged staining in loosely clustered cells lying caudal to the cardiac primordia bilaterally, at 18–20 hpf (Figs. 2C–2E). This pattern of expression appears similar to that for *nkx2.3* and *nkx2.7*, which go on to mark pharyngeal arch endoderm (see below and Figs. 2K–2N and 2Q). However, the extra-cardiac expression of *nkx2.5* is transient and does not appear in definitive endoderm in the zebrafish, in contrast to its homologs in other vertebrates (Komuro and Izumo, 1993; and Lints *et al.*, 1993). Thus, in the zebrafish, *nkx2.5* expression reveals the entire progression of heart formation and is relatively cardiac specific.

nkx2.3 Is a Marker of Early Endoderm in the Developing Pharyngeal Arches and Gut

Zebrafish *nkx2.3* is not expressed in the early heart, unlike its closest structural orthologs *XNkx-2.3a* and *-2.3b* in the frog (Evans *et al.*, 1995). Instead, the earliest *nkx2.3* expression is detected at the 22 somite stage (20 hpf) in bilateral, loosely arrayed clusters of hypoblast cells in the ventrolateral extremes of the embryo, initially caudal to the fusing cardiac progenitors (Fig. 3C and data not shown). This expression becomes more robust by 24 hpf, comprising bilaterally symmetric clusters of ventrolateral cells (Fig. 2Q). Over the ensuing 12 hr, *nkx2.3*-expressing cells become consolidated into paired arrays of transverse stripes (Fig. 2R). By 48 hpf these appear in whole mount embryos to represent the five clefts between the six pharyngeal arches, i.e., the mandibular, hyoid, and first through fifth branchial arches (Fig. 2S; Kimmel *et al.*, 1995). Examination in several planes of section shows that *nkx2.3* expression is confined to the endoderm and, indeed, marks the entire epithelial lining of the pharynx, including the floor overlying the forming heart, the convexities of the pharyngeal arches, and the pharyngeal pouches where staining is particularly intense (Figs. 3G, 3I–3K, and data not shown). This pattern of foregut endoderm expression persists at least through 72 hpf (data not shown). In addition, *nkx2.3* marks the mid- and hindgut epithelium beginning at 36 hpf (Figs. 2S and 3L, and data not shown). Thus the expression of *nkx2.3* in the zebrafish is similar to that of its *Xenopus* counterparts in endoderm but is not present in the heart, despite the strong sequence conservation between them.

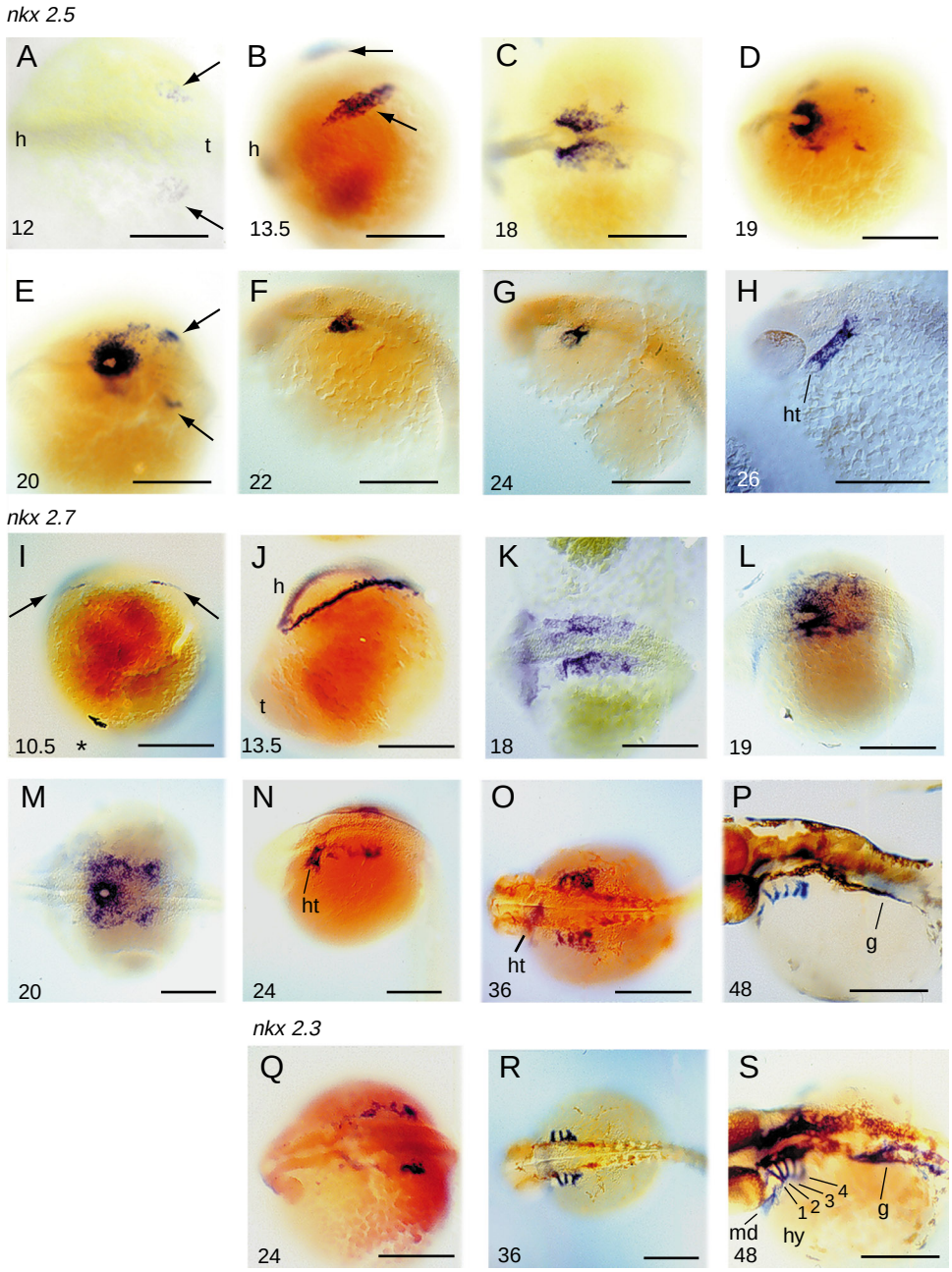

FIG. 2. Whole mount zebrafish embryos stained by *in situ* hybridization using gene-specific antisense RNA probes. Blue–violet staining indicates the presence of the cognate gene transcripts. (A–H) *nkx2.5* staining at 12 (A), 13.5 (B), 18 (C), 19 (D), 20 (E), 22 (F), 24 (G), and 26 (H) hpf showing the progression from paired cardiac primordia (white arrows) to fused heart tube (ht). Noncardiac regions of staining are indicated (black arrows). (I-P) *nkx2.7* at 10.5 (I), 13.5 (J), 18 (K), 19 (L), 20 (M), 24 (N), 36 (O), and 48 (P) hpf showing the earliest staining in the postgastrulation hypoblast (black arrows); white arrows indicate consolidations of apparent precardiac mesoderm. (Q–S) *nkx2.3* at 24 (Q), 36 (R), and 48 (S) hpf. Specimens are viewed dorsolaterally with the rostral end of the embryo to the left and the dorsal axis up, except I, which is a head-on view (dorsal up), and A, C, M, O, and R, which are true dorsal projections. Brown pigment is endogenous melanin that appears after 24 hpf. Faint staining of neural structures was not reproducible. Labels: h, head; t, tailbud; y, yolk; ht, heart; g, gut; md, mandibular; hy, hyoid, and 1, 2, 3, 4, first through fourth branchial clefts; *, artifact. Numbers in lower left corner indicate hpf. Scale bars indicate 250 μm.

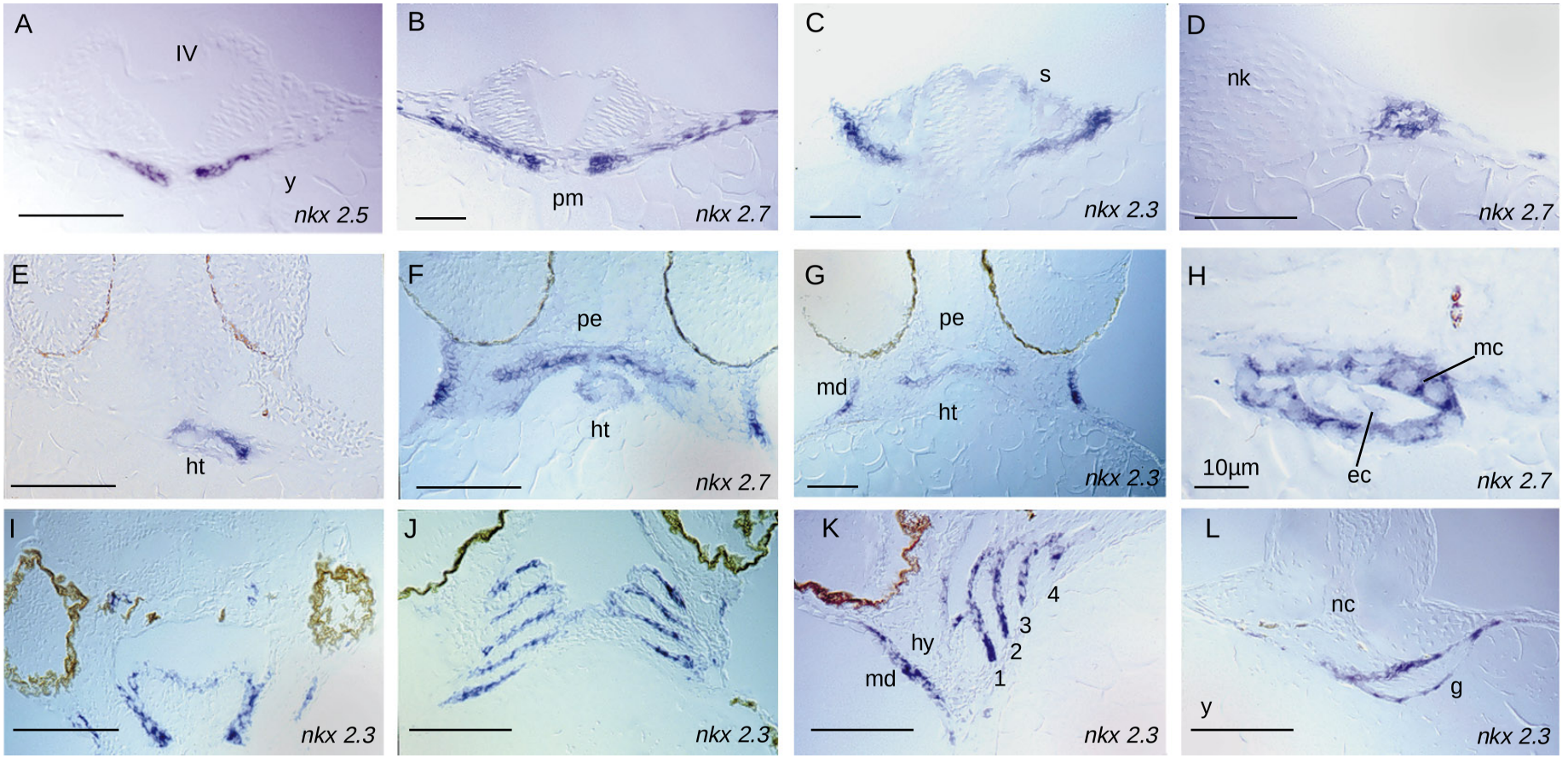

FIG. 3. Sections of embryos stained by *in situ* hybridization using gene-specific antisense RNA probes for *nkx2.5*, *nkx2.7*, and *nkx2.3* as labeled on each panel. Blue–violet staining indicates the presence of the cognate gene transcripts. (A–C) Transverse sections with *nkx2.5* (A), *nkx2.7* (B), and *nkx2.3* (C) staining at 18, 19, and again 19 hpf, respectively, showing the relationship of the expressing cells to the fourth ventricle (IV) of the hindbrain, the yolk (y), the *portion moyenne* (pm), and the first somite (s). (D) Detail from a transverse section (right side only) of an 8 somite (13 hpf) embryo showing the position of very early *nkx2.7*-expressing hypoblast cells laterally between the neural keel (nk) and yolk (y). (E–G) Horizontal sections at 48 hpf with *nkx2.5* (E), *nkx2.7* (F), and *nkx2.3* (G) staining, showing heart (ht) and/or overlying pharyngeal epithelium (pe) and mandibular arch epithelium (md). Brown pigment is melanin in the retina. (H) Detail of the cardiac tube from a horizontal section of a 36 hpf embryo stained with *nkx2.7*. The single layer of myocardial cells (mc) that express this gene surround poorly preserved endocardial cells (ec) that do not; the space between these layers is occupied *in vivo* by the cardiac jelly. (I–K) Transverse (I), angled (J, 45° to horizontal plane), and parasagittal (K) sections of 48 hpf embryos with *nkx2.3*, showing staining of pharyngeal epithelium both in the pouches and over the convexities of the arches, including the mandibular (md), hyoid (hy), and first through fourth gill (1,2,3,4) arches. Brown pigment as above. (L) Detail of an axial section of a 48 hpf embryo showing *nkx2.3* staining of the gut epithelium (g) caudal to the pharynx; notochord (nc) and yolk (y) are indicated for purposes of orientation. Scale bars indicate 250 μm, except in H where the bar is 100 μm.

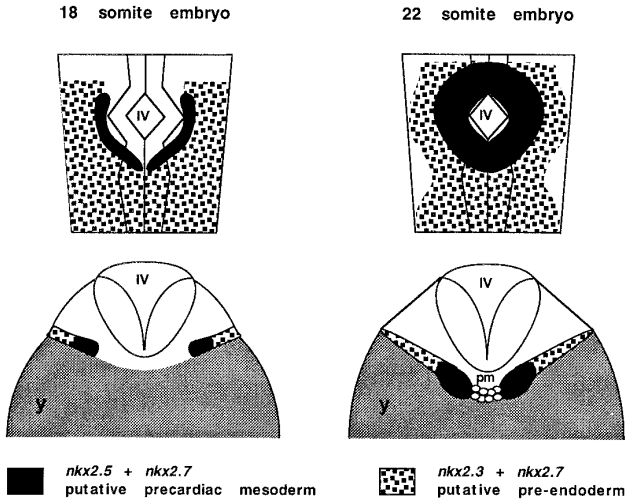

FIG. 4. Schematic representation of the position of cardiac mesodermal and pharyngeal endodermal progenitors in the zebrafish anterior hypoblast at 18 somites (18 hpf) and 22 somites (20 hpf). The upper diagrams are idealized ventral views, while those below represent transverse sections. Shading marks the domains of gene expression, as indicated in the key. The fourth ventricle in the hindbrain (IV) is labeled to help with orientation, as is the *portion moyenne* (pm). See text for details.

Expression of the Novel Gene nkx2.7 Precedes and Overlaps nkx2.5 and nkx2.3

The onset of *nkx2.7* expression soon after gastrulation at the 0–1 somite stage (10.5 hpf) is the earliest among the three *tinman*-related genes in the zebrafish (Fig. 2I). It appears first in cells in a V-shaped band, apex anterior, lying ventrolaterally in the anterior hypoblast. Initially two to three cells wide, this band broadens by the 8–9 somite stage (13 hpf; Figs. 2J and 3D). As development proceeds further, the rostral-most expression of *nkx2.7* wanes while the lateral segments of the band on either side extend medially within the mesendoderm (Figs. 2K and 3B). The cells in these bands are more densely consolidated anteromedially, and these consolidations migrate further medially to form a Y-shaped structure at the 20 somite stage (19 hpf), a ring at 22 somites (20 hpf), and a rudimentary fused heart tube at 24 hpf, identical to the patterns seen with *nkx2.5* (compare Figs. 2D and 2L, 2E and 2M, 2G and 2N). Double staining with *nkx2.5* and *nkx2.7* probes indicates that both genes are expressed in the same cells in these cardiac primordia (data not shown). Cardiac expression of *nkx2.7* persists at 36 hpf in the single cell layer of myocardium (Figs. 2O, 2P, 3F, and 3H; the endocardial cell layer is not well preserved in sections of whole mount embryos). The more lateral extracardiac *nkx2.7*-expressing cells appear to be the same as those that later express *nkx2.3* and go on to form the pharyngeal arch and gut endoderm (compare Figs. 2N–2S and 3F, 3G), as confirmed by double staining with *nkx2.3* and *nkx2.7* probes (not shown). Thereafter, the expression of *nkx2.7* in definitive endoderm diminishes and, in contrast to *nkx2.3*, is undetectable at 60 hpf.

The expression of *nkx2.7*, therefore, precedes and then overlaps that of *nkx2.5* in the myocardial lineage and that of *nkx2.3* in the endoderm (Fig. 4). This is particularly well illustrated by comparing stained sections showing that, just prior to heart tube fusion, the ventral hypoblast comprises *nkx2.5*-expressing cells of the cardiac primordia that lie relatively medial (Fig. 3A) and *nkx2.3*-expressing cells that are lateral (Fig. 3C), while both groups of cells express *nkx2.7* (Fig. 3B). At 36 hpf, *nkx2.7* expression encompasses both the heart and overlying pharyngeal endoderm (Fig. 3F) which are marked individually by *nkx2.5* (Fig. 3E) and *nkx2.3* (Fig. 3G), respectively. While individual cells were not monitored continuously, the precise positional information obtained at very frequent developmental intervals argues strongly that the earliest *nkx2.7*-expressing cells and their descendants go on to form both heart and definitive endoderm.

DISCUSSION

Evolution of Vertebrate Genes Related to tinman

nkx2.7 is a new member of the *tinman*-related subfamily of NK homeodomain genes. It contains the hallmark domains—the *tinman*-like amino terminal decapeptide, homeobox, and NK2 domain—that are characteristic of its vertebrate relatives. Outside these regions, *nkx2.7* diverges substantially from the other members, although it is somewhat more similar to *nkx2.3* than to *nkx2.5*. Zebrafish *nkx2.5* and *nkx2.3*, on the other hand, appear on the basis of sequence conservation to be the structural orthologs of their namesakes in other species. As noted (Evans *et al.*, 1995), the evolution of these genes fits an increasingly well-established paradigm—recognized also in the myogenic bHLH and MEF2 transcription factor families, among others—in which a single *Drosophila* gene is represented in vertebrates by a group of related genes with subspecialized and often diversified function (Abmayr *et al.*, 1995).

Despite the conserved coding sequences among the vertebrate *nkx2.5* orthologs and *nkx2.3* orthologs, the data reported here indicate that the transcriptional regulation of these genes differs in different species. In the mouse, frog, and chick, *Nkx-2.5* is expressed both in heart and in pharyngeal endoderm (Lints *et al.*, 1993; Tonissen *et al.*, 1994; Schultheiss *et al.*, 1995). In the zebrafish, however, *nkx2.5* transcripts do not appear in definitive endoderm, at least through 48 hpf, although they are transiently expressed at a low level in cells that may be endodermal precursors. The regulation of *nkx2.3*, expressed in zebrafish pharyngeal arch and gut endoderm but not in the cardiac lineage, also differs from that of its orthologs in

the frog, which are expressed both in endoderm and heart (Evans *et al.*, 1995). Thus, the early embryonic expression patterns of *nkx2.5* and *nkx2.3* are relatively lineage-specific in the zebrafish as compared to other vertebrates. These genes have apparently come to be regulated under different developmental programs in different species following their emergence by gene duplication in ancestral vertebrates, as also noted for the MEF2 transcription factor family (Ticho *et al.*, 1996).

To understand more completely the developmental role of the *tinman*-related gene family, it will be important to identify putative *nkx2.7* orthologs in other vertebrates. The potential for functional redundancy among the members of this family has been previously noted; thus, a murine *nkx2.7* ortholog might substitute, in part, for the lack of *Nkx-2.5* in the *Nkx-2.5*$^{-/-}$ mouse (Evans *et al.*, 1995; Lyons *et al.*, 1995), supporting normal cardiac development in the earliest stages prior to heart tube looping. It has not escaped notice, however, that *nkx2.7* may instead be unique to the fish and subserve functions which, in other vertebrates, are met by *Nkx-2.5* and *Nkx-2.3* alone.

Role of nkx2.7, nkx2.5, and nkx2.3 in Partitioning the Embryonic Hypoblast

The zebrafish hypoblast comprises cells that involute from the blastoderm margin beneath the epiblast (future ectoderm) during gastrulation and go on to differentiate into mesoderm and endoderm (Warga and Kimmel, 1990). Prior to involution, both cardiac and endodermal progenitors reside near the marginal extremes of the blastoderm (Kimmel *et al.*, 1990; Solnica-Krezel *et al.*, 1995). Intercalating cell movements result in rearrangement of the hypoblast in the gastrula, such that the precise positional organization of mesodermal versus endodermal progenitors for the period between gastrulation and phenotypic differentiation has not been fate mapped (Warga and Kimmel, 1990; Kimmel *et al.*, 1990; Ho and Kimmel, 1993; Solnica-Krezel *et al.*, 1995). *nkx2.7* appears to be expressed in cells of both the mesodermal and endodermal lineages. Our findings suggest that the former lie relatively medially in the ventral hypoblast and give rise to the myocardium while the latter reside more laterally and give rise to endoderm derivatives, including the epithelial lining of the pharyngeal arches and pouches (Fig. 4). *nkx2.7* anticipates positionally as well as temporally the expression of *nkx2.5* and *nkx2.3* in these lineages: precardiac mesoderm is marked by *nkx2.7* followed 4.5 hr later by *nkx2.5*, while endodermal precursors are marked by *nkx2.7* followed 9.5 hr later by *nkx2.3*. Confirmation of these relationships must await experiments in which cell fate and specific gene expression can be determined simultaneously. The findings, however, provide the first molecular clues as to the spatial organization of endodermal and cardiac mesodermal precursors in the zebrafish hypoblast immediately following gastrulation (Fig. 4). They bridge the gap between the earliest localization of lineage-restricted progenitors in the blastula and the later emergence of differentiated phenotypes (Kimmel *et al.*, 1990; Stainier *et al.*, 1993).

Gene Regulatory Mechanisms in the Ontogeny of the Definitive Heart and Endoderm

nkx2.7 is likely to be a principal element in the molecular hierarchy underlying cardiac and endoderm development. The sequential activation of the *tinman*-related genes raises the possibility that the *nkx2.7* product might interact with or regulate *nkx2.5* and *nkx2.3* in their respective lineages. In the heart, mechanisms of myocardial-specific gene regulation must also involve other cell type-restricted transcription factors—particularly the MADS box factor MEF2C (Edmondson *et al.*, 1994) and the zinc finger factor GATA-4 (Kelley *et al.*, 1993; Heikinheimo *et al.*, 1994)—that appear simultaneously, or nearly simultaneously, with *Nkx-2.5* in the precardiac mesoderm of vertebrate species, including the zebrafish (Ticho *et al.*, 1996). Analogous mechanisms, involving *nkx2.7*, *nkx2.3*, and other endodermal factors are also likely to operate in endoderm development.

The inductive role of anterior endoderm upon precardiac mesoderm is well established in vertebrates (Jacobson and Sater, 1988; Muslin and Williams, 1991; Nascone and Mercola, 1995; Schultheiss *et al.*, 1995; and references therein). The expression of *nkx2.7* in both these embryonic tissues begs the question as to whether *tinman*-related genes might have a central role in that induction, as posed by others (Lints *et al.*, 1993; Evans *et al.*, 1995). For example, *nkx2.7* might control the expression of inductive mediators such as cell surface or soluble ligands and cognate receptors; however, there is no reason to expect *a priori* that genes in both tissues would have to be regulated by members of the same transcription factor family.

A better understanding of heart and endoderm ontogeny, and the relationship between them, will come in part from the elucidation of signals that induce *nkx2.7*, *nkx2.5*, and *nkx2.3*, and of the target genes that are in turn activated by them. *nkx2.7* is the earliest molecular marker of zebrafish precardiac mesoderm and anterior endoderm found to date. *nkx2.5* is also likely to be a key regulator of cardiac development, while *nkx2.3* is the only endoderm-specific gene identified at present in the zebrafish. Taken together, the findings reported here provide an essential framework and important molecular tools for the investigation of abnormal developmental phenotypes arising in mutagenesis screens and through molecular perturbation of the embryo.

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