Uncoupling heart cell specification and migration in the simple chordate Ciona intestinalis

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Summary

The bHLH transcription factor Mesp has an essential but ambiguous role in early chordate heart development. Here, we employ the genetic and morphological simplicity of the basal chordate Ciona intestinalis to elucidate Mesp regulation and function. Characterization of a minimal cardiac enhancer for the Ciona Mesp gene demonstrated direct activation by the T-box transcription factor Tbx6c. The Mesp enhancer was fused to GFP, permitting high-resolution visualization of heart cells as they migrate and divide. The enhancer was also used to drive targeted expression of an activator form of Mesp, which induces heart formation without migration. We discuss the implications of Tbx6-Mesp interactions for the evolution of cardiac mesoderm in invertebrates and vertebrates.

Key words: Cardiac specification, Migration, Chordate, Mesp

Introduction

The Ciona heart field can be traced to a single pair of cells in 110-cell embryos, the B7.5 blastomeres (Davidson and Levine, 2003; Hirano and Nishida, 1997). Mesp is the only regulatory gene expressed exclusively in these cells (Imai et al., 2004; Satou et al., 2004). During gastrulation, the B7.5 blastomeres divide into two distinct lineages. The rostral daughters (trunk ventral cells or TVCs) migrate to form the heart, while the caudal daughters remain in the tail and differentiate into anterior tail muscle cells (Fig. 1A). Homology to vertebrate heart cells becomes evident after neurulation, when TVCs express orthologs of the core cardiac regulatory genes Nkx2.5, Hand (Davidson and Levine, 2003; Satou et al., 2004) and Gata4 (B.D., unpublished). The TVCs then migrate anteriorly and ventrally to fuse along the ventral midline in a manner reminiscent of vertebrate heart cell migration (Davidson and Levine, 2003). After metamorphosis, the Ciona heart rudiment differentiates into a contractile linear tube, which expresses orthologs of vertebrate heart structural genes including a cardiac-specific splice variant of Troponin I (MacLean et al., 1997).

Mesp is expressed in the emerging heart field of mouse embryos prior to expression of the core cardiac regulatory genes (Saga et al., 2000). There are two Mesp paralogs, and chimeric cells lacking both (Mesp1 and Mesp2) display cell autonomous defects in heart formation (Kitajima et al., 2000). Ciona contains a single Mesp gene. Morpholino-based suppression of Mesp function in Ciona savignyi causes a block in heart cell migration and specification, leading to the formation of supernumerary tail muscle cells (Satou et al., 2004). Despite the central importance of Mesp function in early chordate heart development, the factors that direct Mesp expression in the emerging heart field have not been defined (Haraguchi et al., 2001). Furthermore, it remains to be determined whether Mesp functions primarily as a migration factor, as inferred from vertebrate analyses, or as a specification factor, as proposed in the Ciona study.

Here, we present evidence that Ciona Mesp is directly activated by the T-box transcription factor Tbx6c. There are three Tbx6 paralogs in Ciona, Tbx6a, Tbx6b and Tbx6c. Tbx6b and Tbx6c are activated by the maternal muscle determinant Macho 1, and initiate muscle gene expression (Yagi et al., 2005). While Tbx6b has a predominant role in muscle specification, Tbx6c independently regulates gene expression in the anterior tail muscle lineage (Yagi et al., 2005).

The Mesp enhancer was used to selectively express an activator form of Mesp in the early heart field. Heart cell migration is inhibited, but beating heart tissue nonetheless differentiates at an ectopic location in the anterior tail. These results demonstrate that heart specification and migration can be uncoupled, and implicate Mesp as a crucial cardiac determinant.

Materials and methods

Ascidians: collection, handling and experimental techniques

Ciona adults were collected from Half Moon Bay, Oyster Point and San Diego (M-Rep, CA). No significant experimental discrepancies were observed among animals from different sources. Rearing, fertilization, decortonation, in situ hybridization, electroporation and lacZ staining were conducted as described previously (Corbo et al., 1997). For double in situ hybridization, two techniques were used (the second protocol is a modified version of the TSA Plus Fluorescence Systems protocol, Perkin Elmer, USA). (1) lacZ was stained with Fast Red (Davidson and Levine, 2003) using a fluorescein-labeled probe, while Tbx6c was hybridized to a digoxigenin-labeled probe and
stained using AP/NBT/BCIP as described previously (Corbo et al., 1997). (2) Tbx6c or Tbx6b were stained with Fast Red using a DIG-labelled probe, the first antibody was then stripped by incubation for 10 minutes in 100 mM glycine-HCl (pH 2.2), 0.1% Tween-20 and then taken through four short rinses in PBT. The second antibody (anti-ITCC-POD, Roche, USA) was subsequently applied at a 1:1500 dilution overnight at 4°C. Embryos were then quenched for 20 minutes in 0.3-3% hydrogen peroxide (in PBT), rinsed twice in PBT and then rinsed three times for 5 minutes in TNT buffer [0.1 M TRIS-HCl (pH 7.5), 0.15 M NaCl, 0.05% Tween-20]. Finally, the embryos were incubated in 300 μl freshly diluted (1:50) × 1× Plus Fluorescein Tyramide Stock Solution for 15 minutes and mounted in Prolong Gold antifade reagent (Invitrogen, USA).

Construction of transgenic DNAs

Genomic DNA was isolated from the pooled sperm of 3-4 adults, using the PureGene DNA Isolation kit (Gentra Systems), and used as a template for PCR-based isolation of the required genomic fragments. These fragments were then cloned into either the pCES vector (Harafuji et al., 2002) or modified versions of this vector, as described below.

**Mesp reporter constructs**

The 5' flanking DNAs from C. intestinalis Mesp were initially isolated using the following primers [numbers indicate the base-pair (bp) distance 5' of the EST predicted transcript]:

**Cs-Mesp1916**, ggcTCTAGAGGGTTCACAGTGACGTTCCATGC;

**Ci-Mesp121123**, ggcTCTAGATCTGTAAGTGAGAGC; and

**Cs-Mesp121123**, aaagCGGCGGCGCATATATATAGTTCATAATC-AACCTG.

The use of lowercase letters indicates padding on the primer that is not incorporated into the construct.

These fragments were fused in-frame with lacZ in the pCES vector using the PCR-generated XbaI and NotI sites, replacing the Ci-forkhead minimal promoter. These constructs were then used as templates for further 5' deletions using the appropriate forward primers. Mesp reporter constructs containing ~200 bp or less 5' DNA began to drive ectopic expression of lacZ in the tail muscles and sometimes in the notochord. However, it was determined that this ectopic expression was due to a vector artefact that was eliminated by removing an ~320 bp fragment of the vector in between the XbaI site and an EcoO1091 site upstream of the polylinker. In all subsequent constructs this area of the vector was removed. Single nucleotide mutations were generated by PCR amplification using primers with appropriately altered sequences. Mesp-GFP was constructed by replacing the lacZ-coding region from the Mesp1916-lacZ construct with the enhanced-GFP (eGFP)-coding region.

**Recombinant Mesp, MyoD constructs**

The Mesp and MyoD bHLH DNA-binding domains were amplified from the Mesp and MyoD EST clones (Yagi et al., 1997) or the MyoD [Ci-MDF (Meedel et al., 1997)] EST clone (GC42d13), respectively, which were obtained from the Ciona intestinalis Gene Collection Release1 (Satou et al., 2002), using the following primers:

**Mesp-HLHf**, aaaaCTAGCAGCGGCAAAACCCGCTATGGAAA-GAGAACG;

**Mesp-HLHb**, aaaaCTAGCTTGCAGCGACGTGGTCTTCCTCTTG;

**Myo-DLHf**, aaaaCTAGCGGACGAGTGAACATGGAACGAC; and

**Myo-DLHb**, aaaaCTAGTGTCATCAGTGTGGTGGC.

They were then sub-cloned using the PCR-generated Nhel and SpeI sites into a modified pCES vector in which the lacZ-coding region was replaced by a small fragment containing Nhel and SpeI sites. (This vector was generated by using the primer CCGCGATATT-GAGCTAGCTTCAACTAGTGGAATTTGACGCTAGCGCC-GGTGCG along with its reverse complement.) A VP16 fragment was generated from the Ci-SnaVP16 construct (Fujiiwara et al., 1998) using the primers VP16f (aaaCTAGGCACTAACCCGACC) and VP16b (ggaaATTCCATTACACACCTGACTCGTCAAATG).

**Gel shift assays**

Binding assays were conducted as described previously (Fujiiwara et al., 1998). Labeled and competitor DNAs were prepared by annealing the following oligonucleotides with their complementary fragments (bold underlined bases indicate putative T-box binding sites):

**Ci-B**, gatcCTTAAAGGCGATAATGACCT;

**Ci-C**, gatcTCTATGCGCGGATAAAAGCAAC;

**Ci-D**, gatcACTAAATTGACACCTTCCAC;

**Ci-B Mut-1**, gatcCTTAAAGGCGATAATGACCT.

The GST-Tbx6c fusion protein was expressed using a partial Tbx6c cDNA (containing the full T-box DNA-binding domain) obtained from the Ciona intestinalis Gene Collection Release1 (Satou et al., 2002) (GGC43g03). This coding region was fused into the pGEX-5x-1 expression vector and purified from bacterial extracts using glutathione agarose beads.

**Confocal microscopy**

Transgenic embryos with GFP-expressing cells were fixed for 1 hour in 0.3% formaldehyde in seawater, mounted in Vectashield mounting medium (Vector labs, CA) and stored at −20°C. Confocal images were obtained on a Leica TCS SL1 laser scanning confocal microscope. Images were processed using the BitPlane Imaris 3.2 software package.

**Results**

Tbx6-binding sites are essential elements of the Mesp minimal enhancers

To investigate how Mesp expression is restricted to the emerging heart field, we isolated minimal enhancers in the 5' flanking regions of the Ciona intestinalis and Ciona savignyi Mesp genes (Ci-Mesp and Cs-Mesp). A 110-bp region of the Ci-Mesp 5' flanking sequence and a 105-bp region of Cs-Mesp are sufficient to drive reporter gene expression in the B7.5 lineage of electroporated embryos (Fig. 1B,C,F; Ci-110, Cs-105). Short 3- to 13-bp distal deletions eliminate the activity of both enhancers (Fig. 1D-F; Ci-107, Cs-92). Comparison of these distal sequences identified a putative motif matching the consensus-binding site for Ciona Tbx6 transcription factors (Yagi et al., 2005). The Ci-Mesp distal motif is highly degenerate, matching only six of the nine base pairs comprising the Tbx6-binding consensus (including one mismatch in the core CACC; Fig. 1E). Therefore, the Cs-Mesp distal sequence was required to identify a putative Tbx6-binding site. There are two additional putative Tbx6-binding sites contained in each of the minimal Mesp enhancers (Fig. 1E). Single nucleotide changes that disrupt the distal motif abolish Mesp-lacZ reporter gene expression (Fig. 1F; Ci-B Mut-1, Cs-B Mut-1), whereas single nucleotide changes that enhance this motif have no effect (Fig. 1F; Ci-B Mut-T). In Ciona intestinalis, there is an additional putative T-box binding site (Fig. 1E, site A) upstream of the required distal site (site B). We performed mutational analysis in the context of a longer construct that includes site A (Fig. 1F; Ci-138). Inclusion of the upstream T-box binding site A is sufficient for limited reporter gene expression despite the disruption of site B (Fig. 1F; Ci-B Mut-2 138). Mutations in the third putative T-box binding site also diminished reporter gene expression (Fig. 1F; Ci-C Mut-2 138).
Tbx6c binds to the Mesp enhancer and participates directly in Mesp activation

In a comprehensive survey of Ciona regulatory genes, Tbx6c is the only T-box factor specifically expressed in the B7.5 blastomeres prior to Mesp activation (Imai et al., 2004). In early cleavage stages, Tbx6c is expressed in adjoining tail muscle blastomeres (Takatori et al., 2004) (Fig. 2A), and Mesp-lacZ is transiently expressed in these cells (Fig. 2B). By the 110-cell stage, Tbx6c expression has become restricted to the B7.5 blastomeres (Fig. 2C). At this stage, Mesp-lacZ staining is initiated in the B7.5 blastomeres and co-hybridization assays demonstrate the overlapping expression of Mesp-lacZ and Tbx6c (Fig. 2D). Thus, Mesp-lacZ reporter expression mirrors the Tbx6c expression pattern. Later, as gastrulation proceeds, Tbx6c expression expands to include the tail muscle lineages (Fig. 2E). However, Mesp expression (and Mesp-lacZ reporter expression) remains confined to the B7.5 cells as they divide (Fig. 2F) and invaginate (Fig. 2G,H).

Gel shift assays confirm specific binding of the Tbx6c protein to the three T-box sites contained in the minimal Ci-Mesp enhancer (Fig. 2I). The same single nucleotide substitutions in the distal Tbx6-binding motif that disrupt reporter gene expression also inhibit competition by unlabeled oligonucleotides (Fig. 2I; Ci-B-Mut-1). Alignment of orthologous sequences upstream of vertebrate Mesp genes reveals an abundance of conserved putative Tbx6-binding sites (Fig. 2J, see Discussion).

Although Tbx6c is the best candidate for the endogenous Mesp activator, it is possible that the Mesp enhancer may also respond to the more broadly expressed Ciona Tbx6 paralogs, Tbx6a or Tbx6b. Both genes are expressed in the B7.5 lineages, as well as in the tail muscle lineages, throughout early embryogenesis (Fig. 2H) (Takatori et al., 2004). Tbx6c and Tbx6 recognize nearly identical consensus sequences (Yagi et al., 2005). Thus, an additional activator may be required to mediate a selective response to Tbx6c (or to Tbx6b in the B7.5 blastomeres, see Discussion). Determination of the precise roles of these Tbx6 factors in Mesp regulation will require further testing.

Mesp-GFP expression visualizes heart cell migration

We employed the Ci-Mesp enhancer fused to GFP for visualization of embryonic heart cell migration (Fig. 3). By the end of neurulation, each B7.5 blastomere has divided twice. The four descendants have a similar morphology (although the rostral TVCs are smaller) and display close membrane adhesion (Fig. 3A). During tail extension, the rostral TVCs separate from their caudal sisters, adhere to the head endoderm and migrate anteriorly along the ventral surface of this rudimentary tissue (Fig. 3B-D). Later, as the
TVCs meet along the ventral midline, they are closely apposed to the underlying epidermis and extend filopodia (see bottom inset, Fig. 3D-F). Thus, TVCs exhibit two phases of directed cell migration: anterior movements along the ventral endoderm followed by midline positioning associated with filopodial extensions.

After meeting along the midline, the TVCs initiate asymmetric cell divisions. The first round leads to a bi-linear cluster of eight cells, four large outer cells and four small inner cells (Fig. 3G,H). After hatching, the four outer cells undergo another round of asymmetric division leading to a total of 12 cells, four larger outer cells and eight smaller inner cells (data not shown). During this same time period, the caudal B7.5 lineage differentiates into four tail muscle cells (Fig. 3H). These cells remain in their initial position, undergoing no further cell divisions or migration.

Fig. 2. Direct activation of Ciona Mesp by Tbx6c. (A-H) In situ hybridizations, all embryos are shown from the vegetal (future dorsal) side except for the one in G, which is shown from the ventral side; arrows in E-G indicate B7.5 lineage cells. (A) 32- to 64-cell stage embryo hybridized with a probe against Tbx6c (blue). (B) Mesp-lacZ transgenic embryo at the same stage hybridized with probe against lacZ (blue). (C) 110-cell embryo hybridized with probe against Tbx6c (blue). (D) Mesp-lacZ transgenic 110-cell embryo co-hybridized with probes against Tbx6c (blue) and lacZ mRNAs (red). Nuclear staining by the lacZ probe indicates nascent transcripts, whereas Tbx6c transcripts are detected in the cytoplasm owing to an earlier onset of expression. (E) Gastrulating embryo hybridized with a probe against Tbx6c (blue). (F) Mesp-lacZ transgenic embryo at the same stage hybridized with a probe against lacZ (blue). (G) Embryo following gastrulation hybridized with probe against Mesp (blue). The B7.5 lineage cells have now involuted to the ventral side, soon after this stage Mesp expression becomes undetectable. (H) Mesp-lacZ transgenic 110-cell embryo co-hybridized with probes against Tbx6b (red) and lacZ mRNA (green), such asymmetric left- or right-sided incorporation of the Mesp-lacZ reporter gene was a common occurrence. (I) Gel shift assays. The GST-Tbx6c fusion protein was incubated with radiolabeled sequences containing each of the putative Tbx6-binding sites from the Ci-Mesp 110-bp enhancer. The first lane for each probe demonstrates binding to the GST-Tbx6c fusion protein. In the second lane, unlabeled competitor inhibited binding. For site B, a fragment containing a mutated binding site (Ci-B Mut-1) fails to inhibit binding (lane 3). (J) Diagrams of conserved blocks of sequence from the S' flanking regions of vertebrate Mesp2 and Mesp1 genes. The characterized mouse Mesp2 enhancer region (Haraguchi et al., 2001) is highly enriched for probable T-box binding motifs in two out of the three conserved sequence blocks. Alignment of zebrafish and Fugu Mesp1 flanking DNA reveals a small block of conserved proximal sequence highly enriched with putative T-box binding motifs. The characterized mouse Mesp1 enhancer region also contains numerous probable T-box binding sites (Haraguchi et al., 2001) (data not shown), but mouse and zebrafish sequences do not align. Conservation of the putative T-box binding sites is shown in green; in the motif sequence shown below, capital letters indicate conservation and green indicates a match to the consensus. Numbers indicate the distance in base pairs from the zebrafish translation start site.

Table 1. Heart cell migration in transgenic embryos

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>% Normal</th>
<th>% Enhanced migration</th>
<th>% Reduced migration</th>
<th>% Normal migration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesp-lacZ or GFP</td>
<td>95</td>
<td>84</td>
<td>1.1</td>
<td>0</td>
<td>1.1</td>
</tr>
<tr>
<td>Mesp-VP16</td>
<td>104</td>
<td>7.6</td>
<td>3.4</td>
<td>0</td>
<td>5.2</td>
</tr>
</tbody>
</table>

Embryos were only scored if they displayed normal morphology. Embryos often displayed altered migration on one side as a result of mosaic incorporation of the transgenes, so each side of the embryo was scored independently. The ‘Enhanced/Reduced migration’ categories include embryos in which the majority of marked cells were found in the head/tail region, respectively. The ‘+/-’ categories denote increased or normal number of GFP expressing cells, respectively. Each row represents the combination of two trials. For Mesp-VP16 the ‘Other’ category consists of normal cell number and migration patterns, but abnormal anterior tail muscle morphology.

Numbers in bold indicate the most highly represented category for each treatment.
Constitutively active Mesp drives heart differentiation in the anterior tail

A constitutively active form of Mesp (Mesp-VP16) was selectively expressed in the B7.5 lineage using the Mesp enhancer (Fig. 4). The VP16 moiety is a potent acidic activator (e.g. Rusch and Levine, 1997). Targeted expression of Mesp-VP16 inhibits migration while promoting ectopic heart differentiation (Fig. 4). In tailbud embryos, the TVCs fail to separate from their caudal siblings, forming a continuous cell mass in the anterior tail region (compare Fig. 4D with 4A, see Table 1). Despite the lack of migration, transgenic cells are able to divide and differentiate as heart precursor cells (Fig. 4E-H, Tables 1, 2). When these transgenic tadpoles were reared through metamorphosis, many failed to form viable juveniles. The majority of the remaining transgenic juveniles had aberrant heart morphology (see Fig. 4G,H, Table 2). Most dramatically, some of the Mesp-VP16 transgenic juveniles (13%) contain ectopic beating heart tissue at the site where the tail is normally resorbed and histolyzed. Some of these juveniles had no other heart tissue (Fig. 4G, see also Movie 1 in the supplementary material). In other cases, two hearts form. Apparently, mosaic incorporation of the Mesp-VP16 fusion gene in the caudal lineage causes the transformation of the anterior tail muscles into beating heart tissue, whereas the rostral TVCs – lacking Mesp-VP16 – migrate to form a completely normal heart (Fig. 4H, see also Movies 2 and 3 in the supplementary material). As a control, we also expressed an activator form of the Ciona ortholog of MyoD (MyoD-VP16) in the B7.5 lineage. This construct did not lead to ectopic heart formation, instead it disrupted heart differentiation (see Table 2).

To further characterize the phenotype of the non-migrating cells, Mesp-VP16 transformants were stained with lineage-
Fig. 5. Activator and repressor Mesp fusion proteins alter the expression of B7.5 lineage markers. (A-C) Control, (D-F) Mesp-VP16. (A,D) Transgenic Mesp-lacZ embryos. (B,E) Embryos stained using a probe for Hand-like. (C,F) Embryos stained using a probe for Raldh2. All embryos are shown laterally, anterior to the left.

specific marker genes (Fig. 5). The two B7.5 lineages can be distinguished by the expression of Hand-like (Ci-Notrlc) in the TVCs, and retinaldehyde dehydrogenase 2 (Ci-Raldh2) in the anterior tail muscles (Fig. 5B,C) (Nagatomo and Fujiwara, 2003; Satou et al., 2004). Transgenic Mesp-VP16 embryos display Hand-like expression in the non-migrating cluster of B7.5 lineage cells (Fig. 5E), whereas Raldh2 expression was reduced or eliminated (Fig. 5F). These observations suggest that Mesp-VP16 causes the transformation of the caudal B7.5 lineage, so that these cells differentiate into heart cells rather than anterior tail muscles.

Discussion

Tbx6-Mesp interactions during chordate heart development

The detailed characterization of Mesp enhancers in Ciona intestinalis and Ciona savignyi led to the identification of Tbx6 as a crucial activator of the emerging cardiac field. Because of their degeneracy, it was not possible to identify Tbx6 sites through the use of phylogenetic footprinting or clustering of binding motifs. Instead, identification depended on the combination of functional assays and a comparison of orthologous regulatory elements from the two Ciona species. The ability of transcription factors to bind degenerate sites is an important caveat when attempting to identify regulatory elements using computational methods.

Tbx6c is probably necessary, but not sufficient, for the activation of Mesp in the B7.5 lineage. Mesp expression relies on two maternal factors, Macho 1 and β-catenin (Satou et al., 2004) (Fig. 6A). Macho 1 is upstream of both Tbx6b and Tbx6c, and thus presumably regulates Mesp expression through these genes. By contrast, β-catenin is likely to direct Mesp expression through a Tbx6-independent pathway. It is conceivable that BMP signaling works in concert with Tbx6c to activate Mesp, as point mutations in a putative SMAD-binding site nearly abolished the activity of an otherwise normal Mesp-lacZ fusion gene (B.D., unpublished).

A second activator would also explain the restriction of Mesp to the B7.5 lineage during gastrulation, despite expansion of the Tbx6c expression pattern into the developing tail muscles (see Fig. 2E). In principle, a localized repressor could delimit Mesp expression; however, extensive mutagenesis and internal deletions within the Mesp minimal enhancer did not cause expansion of the reporter gene expression (data not shown).

Tbx6-Mesp interactions in Ciona raise the possibility that Tbx6 activated Mesp in the ancestral chordates. Vertebrates contain two closely linked Mesp paralogs, one primarily involved in heart development (Mesp1) and the other in

Table 2. Transgenic juvenile heart phenotypes

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>% Normal</th>
<th>% Mild disruption</th>
<th>% Severe disruption</th>
<th>% No heart</th>
<th>% Ectopic heart</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesp-lacZ or GFP</td>
<td>230</td>
<td>74</td>
<td>10</td>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mesp-VP16</td>
<td>120</td>
<td>49</td>
<td>2</td>
<td>29</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>MysD-VP16</td>
<td>202</td>
<td>19</td>
<td>15</td>
<td>34</td>
<td>32</td>
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</tr>
</tbody>
</table>

‘Mild disruption’ indicates improper beat indicative of minor myocardial abnormalities. ‘Severe disruption’ generally indicates major to nearly complete loss of beating myocardial tissue. The ‘No heart’ category includes some juveniles in which a pericardial chamber was present but no beating myocardial tissue was observed.

Data for the Mesp-VP16 and MysD-VP16 juveniles represent two trials, whereas the Mesp-lacZ/GFP control data is from four trials. Many of the transgenic Mesp-VP16 juveniles died soon after metamorphosis or displayed general morphological disruptions. This may be due to the loss of Raldh expression (and the subsequent interference with retinoic acid synthesis) in fully penetrant Mesp-VP16 juveniles (see Table 3).
somitogenesis (Mesp2) (Kitajima et al., 2000; Takahashi et al., 2005). There are indications that both vertebrate Mesp genes maintain the ancestral requirement for Tbx6 activation. Alignment of vertebrate Mesp1 and Mesp2 5’ flanking sequences identify numerous potential T-box binding motifs (Fig. 2F). Moreover, the Tbx6 and Mesp1 expression domains overlap in the early primitive streak of mice (Chapman et al., 1996; Saga et al., 1996), and Mesp2 expression is lost in Tbx6–/– mutant mice (White et al., 2003). It is conceivable that the ancestral gene was also regulated by Notch signaling. Mesp2 is one of the first read-outs of the periodic expression of Notch signaling genes (the somitogenic clock). In chick, the somitogenesis clock is active in the emerging heart field at the time when Mesp1 may be activated (Jouve et al., 2002). The Ciona Mesp minimal enhancers lack obvious Su(H)-binding sites. However, preliminary studies suggest that the inhibition of Notch signaling diminishes Mesp expression (B.D., unpublished). Further studies will be required to determine whether Tbx6 and Notch regulate Mesp in the heart field of Ciona and vertebrates.

**Constitutively active Mesp uncouples heart cell specification and migration**

The detailed analysis of Mesp-GFP reporter expression in early Ciona embryos provided single-cell resolution of the directed migration of heart progenitors. Confocal imaging identified two phases in the directed movement of heart cells: anterior migration and ventral fusion. Intriguingly, a comparable, possibly conserved, bi-phasic mode of heart cell migration has recently been characterized in zebrafish (Natalia Glickman, personal communication). The simplicity of Ciona cell lineages, and the ability to independently manipulate cardiac migration and specification programs (see below), should permit the systematic identification of the signals and networks underlying the early migration of heart cells.

Previous studies of Mesp function have been interpreted as indicating primary roles in either heart cell migration (Kitajima et al., 2000) or specification (Satou et al., 2004). In mice, chimeric Mesp1/Mesp2 knockout cells fail to migrate into the forming heart, but this might be secondary to a disruption of early specification events. In Ciona, Mesp morpholinos block the expression of heart markers, but this might be secondary to a disruption of early migration. The present study indicates that the primary function of Mesp is cardiac specification. The demonstration that an activator form of Mesp can drive the differentiation of ectopic beating heart tissue suggests that Mesp acts as a cardiac determinant independently of any role in migration. The manipulation of Mesp function led to the uncoupling of heart cell migration and specification. It is not clear why the activator form of Mesp interferes with migration. However, Mesp is transiently expressed in the B7.5 lineage, and is lost from the heart progenitors prior to the onset of migration. Perhaps this downregulation is essential for migration and the prolonged expression of Mesp-VP16 blocks migration. Regardless of the mechanism, the uncoupling of heart cell migration and specification sets the stage for the detailed investigation of each process.

**Determination of the heart field within the Mesp expression domain**

In both vertebrates and Ciona, the Mesp expression domain extends beyond the definitive heart field into neighboring mesodermal precursor populations. Thus, Mesp expression alone is not sufficient to drive cardiac specification. Preliminary studies indicate that an inductive event determines the definitive heart field within the Mesp expression domain (B.D., unpublished). According to our current model (Fig. 6A), Mesp specifies a field of potential heart cells. Subsequently, inductive signals release this latent cardiac potential in a subset of the Mesp expression domain. This model is consistent with the ability of a constitutively active form of Mesp to drive heart specification in the entire Mesp expression domain, bypassing the inductive signal (Fig. 6B). This model is also consistent with recent findings regarding the broad cardiac potential of the early chick mesoderm (Eisenberg and Eisenberg, 2004).

A role for Mesp in heart development may have first evolved in the chordates. Despite conservation of the core cardiac gene network (Nkx2.5-Gata4-Hand) in Drosophila and vertebrates (Zaffran and Frasch, 2002), there is no ortholog of Mesp expressed in the Drosophila heart field (Moore et al., 2000). In both mice and Ciona, Mesp is expressed in the emerging cardiac mesoderm prior to the initial expression of the core heart transcription factors (Saga et al., 2000; Satou et al., 2004). Thus it appears that Mesp was recruited during chordate evolution to act upstream of these conserved regulatory genes in setting up the initial heart field. Identification of Mesp downstream targets in Ciona will clarify the link between Mesp and the established heart gene network.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/21/4811/DC1

**References**


**Table 3. Marker gene expression in transgenic embryos**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Normal</th>
<th>Reduced</th>
<th>No stain</th>
<th>Ectopic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control-Raldh</td>
<td>40</td>
<td>83</td>
<td>15</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Mesp-VP16-Raldh</td>
<td>33</td>
<td>36</td>
<td>42</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Control-Hand-like</td>
<td>161</td>
<td>94</td>
<td>1.2</td>
<td>0</td>
<td>4.3</td>
</tr>
<tr>
<td>Mesp-VP16-Hand-like</td>
<td>71</td>
<td>39</td>
<td>0</td>
<td>0</td>
<td>61</td>
</tr>
</tbody>
</table>

Embryos were only scored if they displayed normal morphology. *Reduced* indicates very low levels of staining in comparison with controls (for example, see Fig. 5F). For Raldh, ‘Ectopic’ indicates staining in the head region, this staining was always very weak. For Hand-like, ‘Ectopic’ indicates staining in or near the tail region (see Fig. 5E).


