Hhex and Scl function in parallel to regulate early endothelial and blood differentiation in zebrafish

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SUMMARY

During embryogenesis, endothelial and blood precursors are hypothesized to arise from a common progenitor, the hemangioblast. Several genes that affect the differentiation of, or are expressed early in, both the endothelial and blood lineages may in fact function at the level of the hemangioblast. For example, the zebrafish cloche mutation disrupts the differentiation of both endothelial and blood cells. The transcription factor gene scl is expressed in both endothelial and blood lineages from an early stage and can regulate their differentiation. Here we report that in zebrafish the homeobox gene hhex is expressed in both endothelial and blood lineages from an early stage and indicates that hhex functions downstream of cloche.

During embryogenesis, endothelial development is closely associated with endothelial development, both temporally and spatially. For example, the blood islands on the yolk sac of avian and mammalian embryos consist of both endothelial and blood cells (reviewed by Risau and Flamme, 1995; Cleaver and Krieg, 1999). In zebrafish embryos, both endothelial and blood precursors originate from the lateral plate mesoderm and at mid-somitogenesis stages reside in a midline region known as the intermediate cell mass (ICM) (Detrich et al., 1995). Their intimate association and common origin has led to the hypothesis that a progenitor known as the hemangioblast gives rise to both lineages (reviewed by Robb and Elefanty, 1998).

INTRODUCTION

During embryogenesis, blood development is closely associated with endothelial development, both temporally and spatially. For example, the blood islands on the yolk sac of avian and mammalian embryos consist of both endothelial and blood cells (reviewed by Risau and Flamme, 1995; Cleaver and Krieg, 1999). In zebrafish embryos, both endothelial and blood precursors originate from the lateral plate mesoderm and at mid-somitogenesis stages reside in a midline region known as the intermediate cell mass (ICM) (Detrich et al., 1995). Their intimate association and common origin has led to the hypothesis that a progenitor known as the hemangioblast gives rise to both lineages (reviewed by Robb and Elefanty, 1998).

Genes that regulate the differentiation of both endothelial and blood lineages provide additional, albeit circumstantial, evidence for the existence of the hemangioblast. For example, targeted inactivation in mouse of the VEGF receptor gene Flk1 results in the absence of both endothelial and blood cells (Shalaby et al., 1995). The zebrafish cloche mutation also disrupts the early differentiation of endothelial and blood cells, and in fact appears to act upstream of zebrafish flk1 (Stainier et al., 1995; Liao et al., 1997; Thompson et al., 1998). Although these data are consistent with these genes acting at the level of the hemangioblast, cell transplantation studies indicate that they may act at multiple times and in multiple lineages (Shalaby et al., 1997; Parker and Stainier, 1999).

It is reasonable to assume that genes regulating hemangioblast differentiation are expressed at very early developmental stages in both endothelial and blood lineages. To date, only a few genes have been clearly shown to exhibit such an expression pattern. In addition to flk1 and cloche, the transcription factor gene scl also appears to play an important role in hemangioblast differentiation (Robb et al., 1995; Shivdasani et al., 1995; Gering et al., 1998; Liao et al., 1998; Visvader et al., 1998). In zebrafish, scl is first expressed in the anterior and posterior lateral plate mesoderm, regions which give rise to both endothelial and blood cells. Ectopic scl expression in zebrafish embryos results in the expansion of
endothelial and blood precursors at the expense of somitic and pronephric duct tissues (Gering et al., 1998). Targeted inactivation of Scl in mouse disrupts the formation of hematopoietic stem cells and perturbs the angiogenic remodeling of the yolk sac vasculature (reviewed by Barton et al., 1999). In addition, in cloche mutant embryos, scl expression is downregulated and forced expression of scl through injection of a cmv-scl DNA construct restores endothelial and blood differentiation, indicating that scl acts downstream of cloche to regulate both endothelial and blood differentiation (Liao et al., 1998).

The hhex gene (previously called hex, see Materials and Methods) encodes a homebox-containing protein that is highly conserved among vertebrates (Crompton et al., 1992; Bedford et al., 1993; Newman et al., 1997; Ho et al., 1999; Yatskievych et al., 1999). Studies in Xenopus and zebrafish embryos have shown that Hhex can function as a transcriptional repressor and appears to be involved in anterior as well as dorsoventral patterning (Ho et al., 1999; Jones et al., 1999; Brickman et al., 2000). Embryological and genetic studies in mouse have further implicated hhex in forebrain, liver and thyroid formation (Thomas et al., 1998; Martinez Barbera et al., 2000). hhex was first identified as a hematopoietically expressed homeobox gene with expression in various hematopoietic cells and cell lines (Crompton et al., 1992; Bedford et al., 1993). Detailed studies in Xenopus, mouse and chick embryos have revealed that hhex is also expressed in angioblasts (Newman et al., 1997; Thomas et al., 1998; Yatskievych et al., 1999). In addition, overexpression of hhex in Xenopus embryos results in abnormal vascular structures due to an increase in the number of endothelial cells (Newman et al., 1997). These data suggested that hhex might be expressed in both endothelial and blood precursors and play a role in regulating hemangioblast differentiation. In order to test this model and the relationships between Hhex and other regulators of hemangioblast differentiation, we analyzed the expression and function of hhex in the endothelial and blood lineages of the zebrafish embryo.

We show that hhex, like scl, is expressed in the ICM region, which contains both endothelial and blood precursors. hhex expression is significantly reduced in the ICM region of cloche mutant embryos, indicating that hhex acts downstream of cloche. Gain- and loss-of-function analyses indicate that hhex is sufficient but not necessary for endothelial and blood differentiation. We further show that hhex and scl can cross-regulate each other. These and other data (Robb et al., 1995; Shivdasani et al., 1995; Liao et al., 1998) allow us to assemble the initial framework of a molecular pathway controlling hemangioblast differentiation.

MATERIALS AND METHODS

Zebrafish

Adult fish and embryos were maintained and staged as described (Westerfield, 1995). The m378 cloche allele (Stainier et al., 1996) and the cyclops b16 deficiency (Hatta et al., 1991) were used in these studies.

In situ hybridization and histology

Whole-mount in situ hybridization and histology were performed as described (Alexander et al., 1998; Yelon et al., 1999). flk1, fli1, gata1, hhex and scl riboprobes were prepared as described (Stainier et al., 1995; Liao et al., 1997, 1998; Ho et al., 1999).

Renaming and mapping hhex

hhex was renamed hhex at the recommendation of the zebrafish nomenclature committee in order to avoid confusion with hexosaminidase genes which are designated by hex. The use of hhex is consistent with the approved nomenclature for the human and mouse orthologues.

hhex was mapped on the MOP cross (Postlethwait et al., 1998). A pair of primers (F: 5'-CGA TCA AAC CA T CGA GCT GGA GAA GAA GAA 3' and R: 5'-CAT TTC GCC CGG CGG TTC TGG AAC CAT-3') amplifies a 221-base-pair fragment, and the polymorphism was detected by SSCP analysis. To test for the presence of hhex in the b16 allele, the same pair of primers was used for PCR analysis. The PCR conditions and preparation of genomic DNA from individual embryos were described by Postlethwait et al. (1994) and Johnson et al. (1996).

Mapping of cloche

cloche was initially mapped based on its segregation pattern with brass. Crossing a pair of fish heterozygous for both brass and cloche resulted in 55 out of 77 cloche homozygous mutants also being homozygous mutant for brass. As no SSR markers had yet been released in that region (Knapik et al., 1998), we used amplified fragment length polymorphism (AFLP) analysis, as described by Vos et al. (1995), to isolate closely linked polymorphic markers. We analyzed 256 primer combinations (2-base overhang) and isolated five AFLP markers within a useful genetic distance. Subsequent release of SSR markers (Shimoda et al., 1999) led to the identification of markers closer to cloche than our AFLP markers. Analysis of 2112 diploid and 494 haploid embryos indicates that the SSR marker z1496 maps 0.4 centimorgan (cM) proximal to cloche, although this number is likely to be an upper limit since we do not currently have a distal marker to help identify potential misscored embryos. The telomeric location of cloche complicates its isolation, as the existing large insert genomic libraries do not cover this region (W. L. and D. Y. R. S., unpublished observations).

hhex CDNA expression constructs and microinjections

The full-length hhex cDNA was subcloned into the EcoRI site of pCS2+, which contains a CMV promoter. The resulting construct, pCS2cmv-hhex, was used to generate pCS2cmv-hhexPro, which carries a Leu154 to Pro154 mutation. Site-directed mutagenesis was performed as described in Kunkel et al. (1987) and the desired Leu to Pro mutation was verified by DNA sequencing.

Injection constructs, hhex or scl (Liao et al., 1998), were mixed with Phenol Red to monitor the injection process and approximately 100 pg of DNA was injected per embryo at the 1- to 4-cell stage. At this concentration, we observed less than 5% abnormal embryos. Embryos that needed to be genotyped for cloche were collected from a mapping cross (AB/SJD) and scored with z1496.

RESULTS

hhex is expressed in endothelial and blood precursors

During zebrafish embryogenesis, hhex is expressed in extraembryonic tissues starting at late blastula stages and in embryonic tissues starting at early somitogenesis stages. During gastrulation, hhex is expressed in the dorsal portion of the extraembryonic yolk syncytial layer (YSL), a pattern consistent with its proposed function in dorsoventral patterning (Ho et al., 1999). In this study, we analyze the post-gastrula
expression of hhex and its function in endothelial and blood differentiation.

At the 5-somite stage (12 hours post fertilization, hpf), hhex transcripts are detected in the anterior and posterior lateral plate mesoderm (Fig. 1A,B). hhex expression in the posterior lateral plate mesoderm starts in the bilateral stripes of the nascent ICM, which contains both endothelial and blood precursors (Liao and Zon, 1999; Parker et al., 1999). These bilateral stripes extend both anteriorly and posteriorly as the embryo develops. At the 15-somite stage (16.5 hpf), the posterior ends of these two stripes have merged at the ventral margin of the tailbud (Fig. 1C). At this stage, some of the hhex-expressing cells in the mid-trunk region appear to have migrated to the ventral midline. By 22 hpf (Fig. 1D,F,K), most of the hhex-expressing cells in the trunk and tail have converged to the ventral midline. However, some hhex-expressing cells remain bilateral in the upper trunk region (arrowheads in Fig. 1D,F,J). These cells may later participate in the formation of the Ducts of Cuvier and/or the anterior part of the pronephros. At 22 hpf, scl is also expressed in primary motoneurons (E,G). In H, the white and black asterisks mark the thyroid and liver primordia, respectively. In J and K, the notochord (N) and yolk (Y) are also labeled.

The zebrafish cloche mutation affects the early differentiation of both endothelial and blood cells, suggesting that cloche is a regulator of hemangioblast development (Stainier et al., 1995; Liao et al., 1997, 1998; Thompson et al., 1998). To determine the molecular epistasis between hhex and cloche, we examined hhex expression in cloche mutant embryos. At the 8-somite stage (13 hpf), no hhex expression was detected in the head region and posterior lateral plate mesoderm of cloche mutants (Fig. 2A,B). However, hhex expression in the dorsal portion of the YSL remained unaffected. At the 15-somite stage (16.5 hpf), hhex expression in endothelial and blood precursors diminishes after the onset of circulation (24 hpf) and disappears completely by 30 hpf. At 30 hpf, hhex expression is seen only in the thyroid and liver primordia (Fig. 1H). The temporal and spatial pattern of hhex expression in the endothelial and blood precursors is very similar to that of scl. Transcripts of both genes are detected in the posterior lateral plate mesoderm at early somitogenesis stages and later remain abundant in the ICM region. At 22 hpf, the expression patterns of hhex and scl in the ICM region are essentially indistinguishable (Fig. 1D-G and data not shown). These observations suggest that hhex and scl may function at the same time during endothelial and blood development. hhex expression persists in vascular structures a little longer than does scl expression (data not shown), suggesting that hhex may play additional roles in blood vessel development.
hhex, we injected a DNA construct containing a development, we first performed gain-of-function analyses. In

To analyze the function of hhex in the anterior trunk region (Fig. 2D,F, arrows) expands into a V-shaped pattern (data not shown) and seems to be unique to hhex as it was not observed with scl, flk1 or gata1 (Liao et al., 1997, 1998; Thompson et al., 1998). The residual hhex expression in this region may represent hepatocyte precursors, based on their location and the fact that mouse hhex is expressed in the developing liver (Thomas et al., 1998).

**hhex can induce the premature and ectopic expression of endothelial and blood genes in wild-type embryos**

To analyze the function of hhex in endothelial and blood development, we first performed gain-of-function analyses. In these experiments, we injected a DNA construct containing a full-length hhex cDNA under the control of a CMV promoter (cmv-hhex) into wild-type zebrafish embryos at the 1- to 4-cell stage and subsequently examined the expression of endothelial and erythroid genes. Controlled by the CMV promoter, a mosaic and constitutive expression of exogenous hhex is initiated after the mid-blastula transition. Such expression limits its influence on dorsoventral patterning while still allowing its effect on endothelial and blood development to be analyzed. The injected embryos were collected at tailbud stage (10 hpf) and 24-30 hpf to assess flk1 and gata1 expression. At tailbud stage, flk1 and gata1 are normally not yet expressed (Fig. 3A,D). Overexpression of cmv-hhex caused premature expression of flk1 in 47% and gata1 in 81% of the injected embryos at tailbud stage (Fig. 3B-E; Table 1). At 24-30 hpf, we observed ectopic expression of flk1 in 57% and gata1 in 28% of the injected embryos (Fig. 3G-J; Table 2).

The presence of a homeodomain in Hhex suggests that it functions to regulate transcription by binding DNA. To determine whether the DNA binding activity of Hhex is required for the observed premature and ectopic expression of flk1 and gata1, we constructed and injected a form of Hhex (cmv-hhexPro), which contains a leucine to proline mutation between Helix 2 and Helix 3 of the homeodomain. The replacement of this highly conserved leucine disrupts the ability of the homeodomain protein to bind DNA (Le Roux et al., 1993) and has been previously used to analyze the function of Mix.1 (Mead et al., 1996) and Nkx2.3 and 2.5 (Grow and Krieg, 1998). Overexpression of cmv-hhexPro did not result in significant premature or ectopic expression of flk1 or gata1 in the injected embryos (see Table 1). These data suggest that the DNA binding activity of Hhex is necessary to induce the premature or ectopic expression of flk1 and gata1.

Because hhex is also expressed in the YSL at tailbud stage, it was necessary to determine whether hhex expression in the YSL could contribute to the induction of flk1 and gata1 expression. When cmv-hhex was injected into the yolks of 1000-cell- to sphere-stage embryos to confine its expression to the YSL, no premature expression of flk1 was observed (data not shown). Altogether, these data show that ectopic expression of hhex in embryonic tissues leads to the premature or ectopic expression of endothelial and erythroid genes, suggesting that hhex functions in the development of these cell types.

**hhex can restore the expression of endothelial and blood genes in cloche mutants**

To further test whether hhex functions downstream of cloche as suggested by the hhex expression defects in cloche mutants, we injected cmv-hhex into 1- to 4-cell stage embryos collected from a cloche mapping cross. The injected embryos were fixed at the 5- to 7-somite stage (12-13 hpf) to examine the expression of various endothelial and blood genes (flk1, fli1, tie1, gata1 and scl). Embryos with ectopic expression were genotyped with the SSR marker z1496, which is very tightly

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Fig. 2. **hhex expression is disrupted in the endothelial and blood lineages of cloche mutants.** hhex expression in wild-type (left) and cloche mutant (right) embryos at the 8-somite stage (13 hpf) (A,B), the 15-somite stage (16.5 hpf) (C,D,E), and 22 hpf (F). (A,C) Dorsoanterior views. (B,D,F) Dorsomedial views. (E) Dorsoanterior view. Black arrows (D,F) mark the hhex-expressing cells in the mid-trunk region of cloche mutants that on a lateral view appear to lie most ventrally (data not shown) and are thought to contribute to the liver. Black arrowheads (E,F) point to the residual hhex expression in the ventral region of the tail in cloche mutants. hhex continues to be expressed in the dorsal YSL during early somitogenesis stages (A,B).
linked to cloche (see below). A relatively equal number of homozygous wild-type and homozygous cloche mutant embryos were found with ectopic expression of endothelial and blood genes (see Table 2), indicating that hhex overexpression can induce the expression of endothelial and blood genes in the absence of cloche function.

**hhex and cloche map to the lower telomeric regions of LG 12 and LG 13, respectively**

hhex expression is downregulated in cloche mutants. In addition, hhex overexpression can restore endothelial and blood gene expression in cloche mutants. These data indicate that hhex functions downstream of cloche, or in fact that cloche encodes Hhex. In order to test this latter possibility, we mapped both cloche and hhex. Using half-tetrad analysis (Johnson et al., 1995), we estimated the Gene to Centromere Distance for cloche to be 65.8 cM (179 wild-type and 24 mutant embryos were generated after blocking the second meiotic division of gynogenotes), suggesting that cloche is located in a very distal region. We subsequently found that cloche is linked to the pigmentation mutation brass, which was previously mapped to LG 13. The estimated genetic distance between brass and cloche is 28.5 cM (see Materials and Methods). These data place cloche to the lower telomeric region of LG 13. A bulk segregant analysis screening for AFLP markers identified several closely linked markers, including one (ctgt) that maps 1.1 cM proximal to cloche. Upon release, we also tested a cluster of SSR markers that map at the distal end of LG13. From the 1999 version of the MGH map (Shimoda et al., 1999), the closest marker to cloche is z1496 (Fig. 4A). Analysis of 4718 meioses indicates that z1496 lies 0.4 cM proximal to cloche.

hhex was mapped on the MOP cross (Postlethwait et al., 1998) to the lower end of LG12 near z1141 (Fig. 4B), placing it at about position 77.5 cM on the MGH map. Table 3 shows the mapping statistics.

### Table 1. *hhex* gain-of-function analysis

<table>
<thead>
<tr>
<th>DNA injected</th>
<th>Stage analyzed</th>
<th>In situ probe</th>
<th>Number of injected embryos</th>
<th>Number of embryos with premature or ectopic expression</th>
<th>% of embryos with premature or ectopic expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjected</td>
<td>Tailbud</td>
<td>flk1</td>
<td>61</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cmv-hhex</td>
<td>Tailbud</td>
<td>flk1</td>
<td>60</td>
<td>28</td>
<td>46.6</td>
</tr>
<tr>
<td>cmv-hhexPro</td>
<td>Tailbud</td>
<td>flk1</td>
<td>65</td>
<td>6*</td>
<td>9.2*</td>
</tr>
<tr>
<td>Uninjected</td>
<td>Tailbud</td>
<td>gata1</td>
<td>47</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cmv-hhex</td>
<td>Tailbud</td>
<td>gata1</td>
<td>112</td>
<td>91</td>
<td>81.2</td>
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<tr>
<td>cmv-hhexPro</td>
<td>Tailbud</td>
<td>gata1</td>
<td>83</td>
<td>8*</td>
<td>9.6*</td>
</tr>
<tr>
<td>Uninjected</td>
<td>24 hpf</td>
<td>flk1</td>
<td>45</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cmv-hhex</td>
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<td>flk1</td>
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<td>67</td>
<td>56.8</td>
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<tr>
<td>cmv-hhexPro</td>
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<td>flk1</td>
<td>110</td>
<td>6*</td>
<td>5.5*</td>
</tr>
<tr>
<td>Uninjected</td>
<td>24 hpf</td>
<td>gata1</td>
<td>52</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cmv-hhex</td>
<td>24 hpf</td>
<td>gata1</td>
<td>89</td>
<td>25</td>
<td>28.1</td>
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<td>cmv-hhexPro</td>
<td>24 hpf</td>
<td>gata1</td>
<td>107</td>
<td>9*</td>
<td>8.4*</td>
</tr>
</tbody>
</table>

*The staining intensity of the premature and ectopic expression in these embryos was significantly weaker than that seen in embryos injected with cmv-*hhex* (data not shown).
Hhex shares very high sequence identity with its Xenopus, chick, mouse and human homologues (Ho et al., 1999). Comparative mapping provides additional evidence that *hhex* is the orthologue of human HHEX and mouse *Hhex*. Table 4 shows conserved syntenies with human and mouse chromosomes. The lower end of zebrafish LG12 contains several genes that are apparent orthologues of human genes lying in the middle of the long arm of human chromosome 10, between 10q23 and 10q24, and others from human chromosome 17. This group of genes occupies a single chromosome in zebrafish, two in humans and five in mouse, suggesting that the mouse genome (which separated from the human lineage about 100 million years ago; Kumar and Hedges, 1998) has suffered more chromosome rearrangements in this region than either the human or zebrafish genomes in the 430 million years since their divergence. In summary, these data show that *cloche* does not encode zebrafish Hhex.

### The expression of endothelial and blood genes is not affected by a loss of Hhex function

Previous studies have shown that the gamma-ray induced mutation *b16* is a deletion in the lower telomeric region of LG12 (Talbot et al., 1998). We found that *hhex* is also deleted in the *b16* allele (Fig. 5A), allowing us to examine the consequences of the loss of Hhex function during embryogenesis. The expression patterns of several blood (*scl* and *gata1*) and endothelial (*flk1*, *tie1*, and *tie2*) genes were analyzed in *b16* mutant embryos. Since the cyclops gene is also deleted in *b16*, we were able to identify *b16* homozygotes by their cyclopia. We did not observe any significant difference in the expression pattern of endothelial and blood genes between wild-type and *b16* mutant embryos (Fig. 5B). These results suggest that Hhex function is not essential for early endothelial and blood development. Loss of *hhex* function may be compensated in this process by another gene such as *scl*, whose expression pattern and function upon overexpression appears to be similar to that of *hhex* (Gering et al., 1998; Liao et al., 1998).

### hhex and scl can regulate each other

*hhex* and *scl* can both regulate endothelial and blood gene expression and exhibit similar expression patterns in endothelial and blood precursors, suggesting that both genes function at the same time during hemangioblast development. To investigate the relationship between *hhex* and *scl*, we overexpressed one of the two genes and examined the expression of the other at the 5- to 7-somite stage (12-13 hpf).

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**Table 2. Genotype of cmv-*hhex* injected embryos showing ectopic expression**

<table>
<thead>
<tr>
<th>In situ probe</th>
<th>genotyping results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wt/wt</td>
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<tr>
<td><em>scl</em></td>
<td>14</td>
</tr>
<tr>
<td><em>gata1</em></td>
<td>18</td>
</tr>
<tr>
<td><em>flk1</em></td>
<td>18</td>
</tr>
<tr>
<td><em>flk1</em></td>
<td>8</td>
</tr>
<tr>
<td><em>tie1</em></td>
<td>13</td>
</tr>
</tbody>
</table>

*cmv-*hhex* was injected into 1- to 4-cell stage embryos collected from a *cloche* (*clo*) mapping cross. The expression of endothelial and blood genes was examined in the injected embryos at the 5- to 7-somite stage (12-13 hpf) or at tailbud stage (10 hpf (*flk1*). The embryos showing ectopic (*scl*, *gata1*, *flk1*, *flk1*) or premature (*tie1*) expression were collected and genotyped using the closely linked SSR marker *z1496*.

Wt, wild type.

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**Table 3. Map statistics for *hhex***

<table>
<thead>
<tr>
<th>Marker</th>
<th>Pat</th>
<th>Mat</th>
<th>R</th>
<th>N</th>
<th>Distance (cM±s.e.m.)</th>
<th>95% LOD</th>
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<tbody>
<tr>
<td><em>z1400</em></td>
<td>14</td>
<td>13</td>
<td>1</td>
<td>22</td>
<td>4.54±4.44</td>
<td>0.1, 22.8</td>
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<tr>
<td>STS.90.79</td>
<td>45</td>
<td>25</td>
<td>3</td>
<td>30</td>
<td>10.00±5.48</td>
<td>2.1, 26.5</td>
</tr>
<tr>
<td><em>z1141</em></td>
<td>18</td>
<td>19</td>
<td>0</td>
<td>35</td>
<td>0</td>
<td>0, 10.0</td>
</tr>
<tr>
<td><em>hhex</em></td>
<td>37</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Marker, marker name; Pat, number of segregants with paternal genotype; Mat, number of segregants with maternal genotype; R, recombinants; N, total segregants scored for both markers; Distance, distance between the two markers; 95%, 95% confidence interval in cM; LOD, logarithms of the odds score.

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**Fig. 4. cloche and hhex map to different linkage groups.** (A) Genetic map of the lower half of LG13, based on the map published by Postlethwait et al. (1998). The grey arrow points to the region where *cloche* maps. The SSR marker *z1496* used for genotyping the injected embryos maps 0.4 cM proximal to *cloche*. (B) Genetic map of the lower half of LG12 showing the relative position of the genes listed in Table 4. *hhex* (grey arrow) maps near *cyclops* and the SSR marker *z1411*. 

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**Table 3. Map statistics for hhex**

<table>
<thead>
<tr>
<th>Marker, marker name</th>
<th>Pat</th>
<th>Mat</th>
<th>R</th>
<th>N</th>
<th>Distance (cM±s.e.m.)</th>
<th>95% LOD</th>
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<tr>
<td><em>z1400</em></td>
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<td><em>hhex</em></td>
<td>37</td>
<td>54</td>
<td></td>
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</tr>
</tbody>
</table>
Fig. 5. b16 mutant embryos do not contain hhex but show apparently normal endothelial and blood gene expression. (A) PCR amplification of an intron in hhex shows that it is present in wild-type embryos (lanes 1 to 5) but absent in b16 mutant embryos (lanes 6-10), indicating that hhex is deleted in the b16 deficiency. (B) scl expression in wild-type and b16 mutant embryos at 24 hpf. scl expression appears unaffected in b16 mutants, which were identified by their cyclopia. The increase in scl expression in the trunk of b16 mutants (arrow) is at least partly due to the reduced circulation, which leads to the accumulation of blood cells in this region.
heterozygous fish (Fig. 7A,B). At the 10-somite stage, some fli1 expressing cells in the lateral plate mesoderm appear to migrate toward the ventral midline in wild-type embryos. This expression is absent in a quarter of the embryos from a cloche cross (Fig. 7C,D), and genotyping with z1496 showed that all the embryos with defective fli1 expression were cloche mutants (data not shown). At the 20-somite stage (Fig. 7E-H), fli1 expression is normal in the cranial neural crest, pharyngeal pouches and pronephric primordia but absent in the endothelial lineage of cloche mutants, except at the ventral region of the tail as previously reported (Thompson et al., 1998; Brown et al., 2000). These data suggest that fli1 expression in the endothelial lineage is defective in cloche mutants, a defect obscured at early stages by fli1 expression in the adjacent intermediate mesoderm (Fig. 7B).

Overexpression of hhex can induce ectopic fli1 expression

To position fli1 relative to hhex and scl, we injected cmv-hhex or cmv-scl into embryos collected from a cloche mapping cross at the 1- to 4-cell stage. Ectopic expression of fli1 was observed at tailbud stage in approximately 20% of the hhex injected embryos but, at the dose used, not in the scl injected embryos (Fig. 7I and data not shown). (Although scl overexpression can induce ectopic hhex expression, it is possible that the level of ectopic hhex expression following scl overexpression is not sufficient to induce ectopic fli1 expression. In addition, it is possible that higher effective concentrations of Scl would lead to ectopic fli1 expression.) Genotyping revealed that a quarter of the cmv-hhex injected embryos showing ectopic fli1 expression were cloche mutants (Table 2). These data indicate that Hhex can also positively regulate the expression of fli1 in the absence of cloche function.

**DISCUSSION**

**hhex expression in endothelial and blood precursors**

Zebrafish Hhex shares very high sequence identity with its homologues in other vertebrates both inside and outside the homeodomain (Ho et al., 1999). Our comparative mapping data indicate that zebrafish hhex is the orthologue of human HHEX and mouse Hhex (Table 4). hhex is expressed in the developing cardiovascular system in a broadly conserved temporal and spatial pattern. In *Xenopus*, hhex expression in the angioblasts has been reported to initiate several hours after the onset of flk1 expression and does not appear to be associated with the developing hematopoietic system (Newman et al., 1997). In chick embryos, hhex is expressed in blood islands starting at mid-gastrulation stages and in
extraembryonic and intraembryonic vascular endothelial cells as vessels form (Yatskievych et al., 1999). The most detailed expression information is available in mouse: hhex is first expressed in the nascent blood islands of the visceral yolk sac at the same stage that flk1 is first expressed in this population of cells (Thomas et al., 1998). hhex is subsequently expressed in the embryonic angioblasts and endocardium and then rapidly turned down as these endothelial cells differentiate. Similarly, in zebrafish, hhex expression parallels that of flk1 (Fouquet et al., 1997; Liao et al., 1997; Sumoy et al., 1997). It first appears anteriorly and posteriorly by the 3-somite stage. Posteriorely, it is expressed in the nascent ICM, which contains both endothelial and blood precursors. This expression pattern is reminiscent of that exhibited by flk1, gata2 and scl (Liao et al., 1998; Thompson et al., 1998; Brown et al., 2000). Subsequently, hhex expression is observed in the developing endothelial cells including the endocardium until about the onset of circulation. This evolutionarily conserved expression pattern suggests that hhex plays an important role in regulating the differentiation of both endothelial and blood cells.

hhex and scl expression in the ICM region parallel each other at early stages. At later stages, hhex is transiently expressed in the forming blood vessels while scl is expressed in circulating blood cells (Liao et al., 1998). These data suggest that hhex and scl both function in controlling early endothelial and blood differentiation and that hhex and scl play additional roles in later stages of endothelial and blood formation, respectively.

**Hhex can function as a positive regulator of endothelial and blood gene expression**

Gain-of-function analyses show that hhex is capable of inducing premature and ectopic expression of flk1 and gata1 (Fig. 3). It is unclear whether the cells expressing premature or ectopic flk1 or gata1 would eventually differentiate into endothelial or blood cells. Even though we did observe ectopic expression of late endothelial genes such as tie1 and tie2 upon hhex overexpression, the cells expressing these genes did not appear elongated as most differentiated endothelial cells do. Moreover, the ectopic expression of flk1 and gata1 did not appear to be limited to the mesodermal germ layer, as some of the flk1 and gata1 expressing cells were observed in dorsal (i.e. ectodermal) regions of the embryo (data not shown). This situation is reminiscent of that seen with gata5 overexpression where ectopic expression of myocardial genes is observed much more frequently than the formation of ectopic beating tissue (Reiter et al., 1999). Thus, while some of the cells ectopically expressing flk1 or gata1 may be ectopic endothelial or blood cells, most of them probably represent other cell types in which only part of the endothelial or blood transcription program has been activated.

Extra-embryonic tissues have been implicated in endothelial and blood formation in mouse (Belaoussouf et al., 1998). Zebrafish hhex, like mouse hhex, is expressed in extraembryonic tissues, but this expression does not appear to play a direct role in endothelial or blood formation: overexpression of hhex in the YSL did not cause ectopic flk1 or gata1 expression. Thus, by virtue of its expression in endothelial and blood precursors, we propose that Hhex functions cell-autonomously in the differentiation of these cell types.

Overexpression of hhex mRNA in Xenopus embryos led to vascular malformations but no ectopic expression of the endothelial marker Xmsr was observed (Newman et al., 1997). These results and the fact that flk1 expression appears to precede hhex expression in Xenopus embryos led Newman et al. (1997) to propose a role for Hhex in regulating endothelial cell proliferation. Thus, Hhex may have both an inductive and proliferative role in the endothelial lineage.

In various settings, Hhex has been shown to function as a transcriptional repressor (Brickman et al., 2000; Tanaka et al., 1999), suggesting that the ectopic activation of gene expression reported here is not a direct effect. Functional dissection of Scl has shown that its DNA binding domain is not required for its activity in vasculogenesis and hematopoiesis (Porcher et al., 1999). One of the models suggested to explain this observation is that Scl sequesters an unidentified repressor of hemangioblast development through the formation of Scl-Repressor heterodimers. Our data indicate that the DNA binding domain of Hhex is required for its activity. One model consistent with these data is that Hhex negatively regulates the aforementioned repressor of hemangioblast development at the transcriptional level. Alternatively, we have previously proposed that Hhex downregulates bmp expression in the gastrulating zebrafish embryo (Ho et al., 1999). Hhex may function similarly in the endothelial lineage and modulate bmp expression. Experiments in chick embryos indicate that Bmp inhibits the appearance of QH1 positive (i.e. endothelial) cells in cardiogenic explants (Yukiko Sugi and Roger Markwald, personal communication), suggesting that regulating the level of Bmp signaling may be critical to endothelial cell differentiation. In this regard, it is interesting to note that tissues directly adjacent to the forming dorsal aorta such as the hypochord and notochord express high levels of collagen, which can bind Bmp through its cysteine-rich domain (Zhu et al., 1999; Larrain et al., 2000). Further analysis should help define the role of Bmp signaling in endothelial cell differentiation and blood vessel formation.

**Hhex function does not appear to be required for early endothelial and blood development**

hhex is deleted in the gamma-ray induced deficiency b16, but the expression of various endothelial and blood genes in b16 mutant embryos appears normal. It is formally possible that a second hhex gene exists in zebrafish although early endothelial differentiation also appears normal in mouse hhex mutants (Martinez Barbera et al., 2000; blood differentiation has not yet been analyzed in these mutants). These data indicate that in zebrafish, as in mouse, Hhex is not required for hemangioblast development and suggest the involvement of another regulator that can compensate for the loss of Hhex function. Scl is potentially this other regulator as it is expressed in a pattern similar to that of Hhex in the endothelial and blood lineages and appears to function similarly based on overexpression experiments in zebrafish. In zebrafish, Scl is required for normal blood development (reviewed by Orkin and Zon, 1997; Barton et al., 1999) and also for the remodeling of the yolk sac vasculature (Visvader et al., 1998). It will be interesting to analyze embryos mutant for both hhex and scl and thereby test whether scl can indeed compensate for the absence of Hhex function during early endothelial and blood differentiation.
**Fig. 8.** Model of hemangioblast development and early endothelial and blood cell differentiation. The cell lineages are shown as colored circles. In this model, hhex and scl function downstream of cloche and cross-regulate each other during hemangioblast development. hhex and scl can induce the expression of the endothelial gene flkl as well as the erythroid gene gata1, suggesting that they positively regulate the differentiation of both endothelial and blood cells. Integrating data obtained in zebrafish and in mouse, the color of the arrows represents results obtained from either gain-of-function (blue), loss-of-function (red) or both types of analyses (green).

A pathway regulating early endothelial and blood differentiation

Similar to all endothelial and blood genes published to date, hhex expression in endothelial and blood precursors is severely reduced in cloche mutants (Fig. 2). In addition, hhex overexpression can induce ectopic expression of endothelial and blood genes in cloche mutants (Table 2). These observations place hhex downstream of cloche. We further show that hhex and scl can cross-regulate each other in a way that does not require cloche function. In addition, the endothelial expression of flil is disrupted in cloche mutants and hhex but apparently not scl can lead to the ectopic activation of flil. Taken together, these results combined with those obtained by Liao et al. (1998) suggest a molecular framework for hemangioblast development as shown in Fig. 8. hhex and scl cross-regulate each other and function downstream of cloche to activate the endothelial and blood genes flil, flkl, and gata1. This framework should facilitate the testing and integration of additional factors thought to regulate hemangioblast development.

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