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Hop Is an Unusual Homeobox Gene that Modulates Cardiac Development

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Summary

Hop is a small, divergent homeodomain protein that lacks certain conserved residues required for DNA binding. *Hop* gene expression initiates early in cardiogenesis and continues in cardiomyocytes throughout embryonic and postnatal development. Genetic and biochemical data indicate that *Hop* functions directly downstream of *Nkx2-5*. Inactivation of *Hop* in mice by homologous recombination results in a partially penetrant embryonic lethal phenotype with severe developmental cardiac defects involving the myocardium. Inhibition of Hop activity in zebrafish embryos likewise disrupts cardiac development and results in severely impaired cardiac function. Hop physically interacts with serum response factor (SRF) and inhibits activation of SRF-dependent transcription by inhibiting SRF binding to DNA. *Hop* encodes an unusual homeodomain protein that modulates SRF-dependent cardiac-specific gene expression and cardiac development.

Introduction

One of the landmark discoveries in developmental biology was the identification of clustered homeobox or *Hox* genes that encode transcription factors required for patterning the embryo along the anterior-posterior axis (reviewed in Duboule, 1994). These clustered *Hox* genes are conserved across millions of years of evolution and are arranged in the genome in the order in which they are expressed along the axis of the embryo. The "Hox code" represents an important paradigm for the definition of positional identity during embryogenesis in which the specific pattern of overlapping *Hox* gene expression defines cellular identity. A plethora of nonclustered *Hox* genes have been identified that are scattered throughout the genome and play additional roles in cell fate specification in all organs and tissues of the body.

Hox genes are defined by the presence of a 60 amino acid domain that mediates DNA binding. This domain has been analyzed by NMR spectroscopy and X-ray crystallography either free or in association with DNA (Kissinger et al., 1990; Qian et al., 1989; Wolberger et al., 1991). The 60 amino acid homeodomain is capable of adopting a fixed structure in solution composed of three α helices in which the second and third helices form a helix-turn-helix motif. The third helix sits in the major groove of DNA and makes critical DNA contacts. Within this third α helix, several amino acids that make direct contact with DNA are conserved amongst all known homeodomains. Other residues vary only slightly and define subclasses of Hox genes. For instance, homeodomains of the goosecoid class contain a lysine at position 9 of the third helix, while those of the paired class contain a serine at this position (Duboule, 1994).

Hox proteins are thought to act by modulating transcription, and all known *Hox* gene products contain domains in addition to the homeodomain that are capable of activating or repressing transcription, mediating protein-protein interactions or binding to DNA. For instance, in the developing heart, *Nkx2-5* encodes a homeodomain protein that includes amino-terminal and carboxy-terminal transcriptional activation domains in addition to the homeodomain (Chen and Schwartz, 1995; Komuro and Izumo, 1993; Lyons et al., 1995; Ranganayakulu et al., 1998). *Nkx2-5* is homologous to the *Drosophila* gene *tinman* that is required for heart formation in the fly (Komuro and Izumo, 1993). In vertebrates, multiple related Nkx2 homeobox genes are expressed in the heart and probably serve redundant functions (Harvey, 1996). In *Xenopus* embryos, injection of dominant-negative forms of Nkx2-5/Nkx2-3 prevent cardiac development (Fu et al., 1998).

Nkx2-5 acts synergistically with other transcription factors expressed in the developing heart to activate expression of cardiac-specific genes. In particular, Nkx2-5, Gata4, and serum response factor (SRF) function together by binding to adjacent sites within the promoter/enhancer region of cardiac genes (Chen et al., 1996; Chen and Schwartz, 1996; Durocher et al., 1997). This particular constellation of binding sites is found in the upstream genomic regions of some, but not all, developmentally regulated cardiac genes. Recently, a cardiac-specific transcription cofactor named myocardin was identified that contains a homeobox-like subdomain and binds to SRF, effecting potent transcriptional activation (Wang et al., 2001). Hence, multiple cardiac-specific gene regulatory networks converge on SRF to

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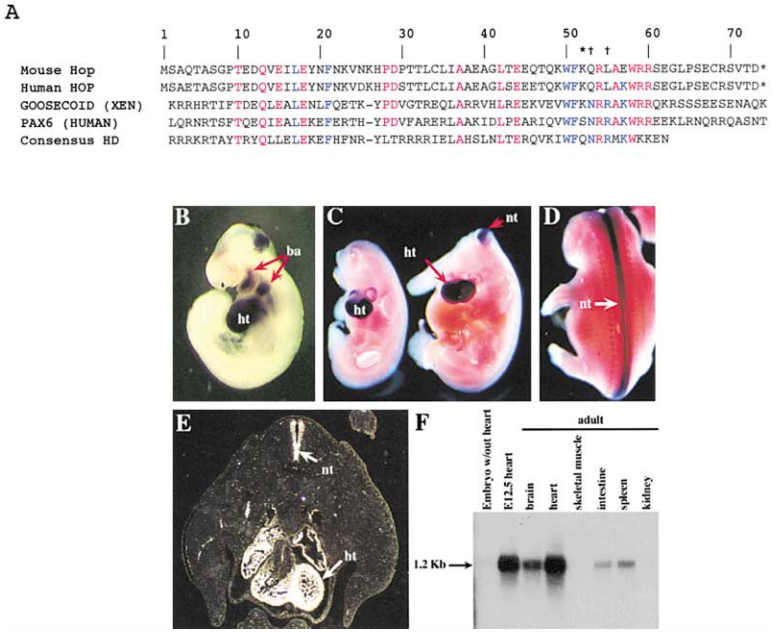

Figure 1. Sequence and Expression of Analysis of Hop

(A) Mouse and human amino acid sequence for Hop. A comparison is made with *Xenopus* Goosecoid, human PAX6, and a consensus homeodomain sequence (Duboule, 1994). Conserved amino acid residues are shown in red. Amino acid residues conserved in all homeodomain are shown in blue. Hop has a lysine at amino acid position 52 (*), which is typical of the Goosecoid protein. Hop also has a glutamine at position 53 (†) and a leucine at 55 (†), which differ from the highly conserved asparagine and arginine, respectively found in all other homeodomains.

(B) Whole-mount in situ at E9.5 revealing expression in the branchial arches (ba) and heart (ht). (Signal in the hindbrain is artifactual due to probe trapping.)

(C) Whole-mount in situ reveals expression of *Hop* in the heart at E10.5 (left) and in the heart and neural tube (nt) at E12.5 (right). The heads have been removed to improve visualization of the heart.

(D) Posterior view at E12.5 indicating expression in the neural tube.

(E) Radioactive in situ hybridization of transverse section at E12.5 reveals strong *Hop* expression in all four cardiac chambers (ht) and in the ventricular zone of the neural tube (nt).

(F) Northern blot of mouse *Hop* demonstrating a 1.2 kb mRNA transcript present in E12.5 heart and not in the embryo with the heart removed. Adult mouse heart, brain, intestine, and spleen express Hop mRNA.

mediate cardiac gene transcription during development and disease.

In this report, we describe and characterize an unusual type of homeodomain protein that functions during cardiac development called Hop (*h*omeodomain *o*nly *p*rotein, previously referred to as Toto and identified independently by others as Cameo; Shin et al., 2002 [this issue of *Cell*]). Hop is coexpressed with and functions downstream of the mammalian *tinman* homolog *Nkx2-5*. Hop is the smallest known homeodomain protein. It is composed of a divergent homeodomain that is incapable of sequence-specific DNA binding. Instead, Hop functions by interacting with SRF to inhibit DNA binding. This interaction results in decreased transcription of SRF-dependent cardiac-restricted genes. Genetic inactivation of *Hop* in mice results in a partially penetrant phenotype of embryonic heart failure and lethality, while morpholino antisense oligonucleotide inactivation of *Hop* in zebrafish results in impaired cardiac contractility and function. Our results identify an atypical homeodomain protein and describe an unusual function for this conserved structural motif in heart development.

Results

We identified mouse *Hop* in an expressed sequence tag (EST) database search for transcripts encoding proteins related to paired-type homeodomains expressed in the heart. Further analysis of EST databases revealed a human homolog of *Hop* (designated *HOP*), encoding a protein with 92% identity to the mouse sequence (Figure 1A). Homologs were also present in rat, cow, pig, chick, *Xenopus,* and zebrafish, but we failed to identify *C. elegans* or *Drosophila Hop* genes.

The Hop protein contains a 73 amino acid open reading frame with a 60 amino acid motif homologous to *Hox* gene products. At the amino acid sequence level, Hop is most closely related to homeodomain proteins Pax6 and goosecoid, with ~40% identity and ~57% similarity evident within the homeodomain-like domain (Figure 1A). Optimal alignment strategies suggest that Hop has a single amino acid insertion near the end of the predicted first α helix when compared to other homeodomain proteins. To model the Hop protein against other known protein structures, we subjected the 73 amino acid Hop sequence to protein threading calculations using the ProCeryon algorithm (Sippl and Weitckus, 1992; Jones et al., 1992). Results indicated that some 6 homeodomain structures from species as diverse as fruitfly and man scored higher than any other protein in the PDB, making it highly probable that Hop belongs to the homeodomain superfamily. The highest scoring homeodomain folds were those of *Drosophila* paired and engrailed, and human PBX1 and HOX-B1, showing 19%–28% residues both aligned in sequence and structurally superimposed. Least squares (best fit) superimpositions showed these 4 homeodomains to be highly similar in their detailed structures (average root-mean-square error of 1.4 Å). This group of homeodomains therefore provides an excellent structural template for the Hop fold.

Like goosecoid, Hop has a Lys at position 52 (see numbering in Figure 1A; this position in Hop corresponds to position 50 of the goosecoid homeodomain). This amino acid has been identified as an important contributor to DNA binding specificity of most homeodomains (Treisman et al., 1989). Furthermore, Hop contains a Trp at position 50 and Phe at position 51, residues that are conserved amongst all known homeodomains. While these conservations and results from the thread-

ing calculations suggest that Hop is highly likely to adopt a homeodomain-like fold, comparison of the protein sequence with sequences from other homeodomains suggests that Hop is unlikely to have retained DNA binding function. First, the amino acids at positions 53 and 55 (Gln and Leu, respectively) diverge from the highly conserved Asn and Arg that are found in all other homeodomains and which make important binding contact in the major groove of DNA (Duboule, 1994). Second, a number of residues that commonly make ion-pair interactions with DNA in homeodomains are absent in Hop (Billeter et al., 1993 and Wolberger et al., 1991). Third, Tyr27 and Ile49 take part in extensive hydrophobic interactions with nucleotide bases, and neither of these amino acids is conserved in Hop. While not all homeodomains exhibit all of these interactions, the fact that Hop is missing many of them provides strong support for the hypothesis that Hop has lost its DNA binding function. Thus, we consider Hop to be an atypical member of the homeodomain superfamily.

Hop Expression in the Embryo and Adult

Whole-mount in situ hybridization indicates that *Hop* is strongly expressed throughout the myocardium at E9.5 and in the branchial arches (Figure 1B). At E12.5, *Hop* expression is evident in the heart and in the ventricular zone of the neural tube (Figures 1C–1E). At E13.5, low level expression is also seen in the intestinal epithelium, and by E16.5, expression is seen in the airway epithelium of the lung (not shown). Northern analysis of adult and embryonic tissues indicates that Hop is encoded by a 1.2 kb mRNA transcript present in the embryonic and adult heart and in the adult brain, intestine, and spleen (Figure 1F). Throughout embryonic and postnatal development, *Hop* is expressed in the myocardium.

Hop Functions Downstream of Nkx2-5

In situ hybridization analysis reveals that *Hop* is not expressed at E7.5 in the lateral plate mesoderm (arrows, Figure 2A) when cardiogenic precursors are specified, as evidenced by expression of *Nkx2-5* (arrows, Figure 2B). Shortly thereafter (E8.0), *Hop* is expressed in the bilateral clusters of precardiac mesoderm (Figure 2C) and expression persists in the myocardium. Of note, the expression of *Nkx2-5* in lateral plate mesoderm (Figure 2D) is slightly broader than that of *Hop* and includes the maturing ventral foregut endoderm (compare Figures 2E and 2F).

Since *Hop* expression overlaps that of *Nkx2-5* and initiates just after that of *Nkx2-5* during cardiac development, we examined the expression of *Hop* in *Nkx2-5* null embryos. *Hop* mRNA transcripts were downregulated, although not eliminated, in *Nkx2-5* null hearts at E9.5 (Figure 3A). These results indicate that Nkx2-5 acts to induce or maintain expression of *Hop* during cardiac development.

We examined the possibility that transcription of the *Hop* gene might be activated by Nkx2-5. We identified a genomic region upstream of *Hop* that was capable of mediating cardiac-specific expression in vivo using transgenic mice. Five kb of upstream sequence was sufficient to direct expression of a *lacZ* reporter gene in the right ventricle of three independent E10.5 transient transgenic embryos (Figure 3B). Interestingly, left ventricular expression was not seen, suggesting that *Hop* transcription is regulated by distinct modular enhancers directing chamber-specific expression, as has been demonstrated extensively for other cardiac-specific genes including *Nkx2-5* (Schwartz and Olson, 1999). We constructed a human growth hormone reporter construct using this 5 kb *Hop* genomic sequence and tested the ability of Nkx2-5 to activate reporter gene expression in transient transfection assays using NIH3T3 cells. Cotransfection of Nkx2-5 resulted in a roughly dose dependent increase in reporter activity (Figure 3C), consistent with the hypothesis that Nkx2-5 directly regulates *Hop* expression. The 5 kb *Hop* genomic sequence contains 12 potential high affinity Nkx2-5 binding sites conforming to the consensus TNNAGTG (Chen and Schwartz, 1995). We examined the ability of Nkx2-5 to bind to the most proximal putative binding site using EMSA. Nkx2-5 was able to bind to this site in a sequence-specific fashion and mutation of the core sequence eliminated binding (Figure 3D). We next constructed a reporter plasmid that contained only the proximal 261 bp of *Hop* genomic sequence from the 5 kb *Hop* genomic promoter used above that includes a single Nkx2-5 binding site. Nkx2-5 was able to induce reporter gene activation when cotransfected in NIH3T3 cells, and introduction of the identical missense mutation that abolished Nkx2-5 binding in the EMSA greatly reduced the ability of Nkx2-5 to induce transactivation (Figure 3E). These results strongly support the hypothesis that Nkx2-5 is a direct regulator of *Hop* gene expression.

Hop Encodes a Nuclear Protein that Does Not Bind DNA

Most homeodomain-containing proteins are nuclear transcription factors that bind to short specific DNA sequences containing ATTA or related motifs (Treisman et al., 1989). We confirmed that *Hop* encodes a nuclear protein by transfecting NIH-3T3 cells with an epitope-tagged version of Hop and performing immunohistochemistry (data not shown). We performed electrophoretic mobility shift assays (EMSA) to determine if Hop was able to bind to previously identified consensus binding sites for members of the bicoid/goosecoid, NK, even skipped, or paired families of homeodomain proteins. While the isolated Pax3 homeodomain bound to all of these sequences (with varying affinities), Hop did not bind to any of them, even under nonstringent conditions (data not shown). Since Hop contains divergent amino acid residues at positions 53 and 55 not found in other related proteins, we replaced these residues with an Asn at position 53 and an Arg at position 55, thus conforming to the homeodomain consensus sequence. The modified Hop proteins were expressed efficiently in bacteria, but these alterations were insufficient to confer upon Hop the ability to bind consensus homeodomain sequences, presumably because of other divergent residues (see above and Figure 1A).

To confirm that Hop does not bind directly to DNA, we performed an unbiased binding site selection assay (Epstein et al., 1994) with Hop, using the Pax6 homeodomain in parallel as a control. After five rounds of selection with pooled randomers containing a central core of 12

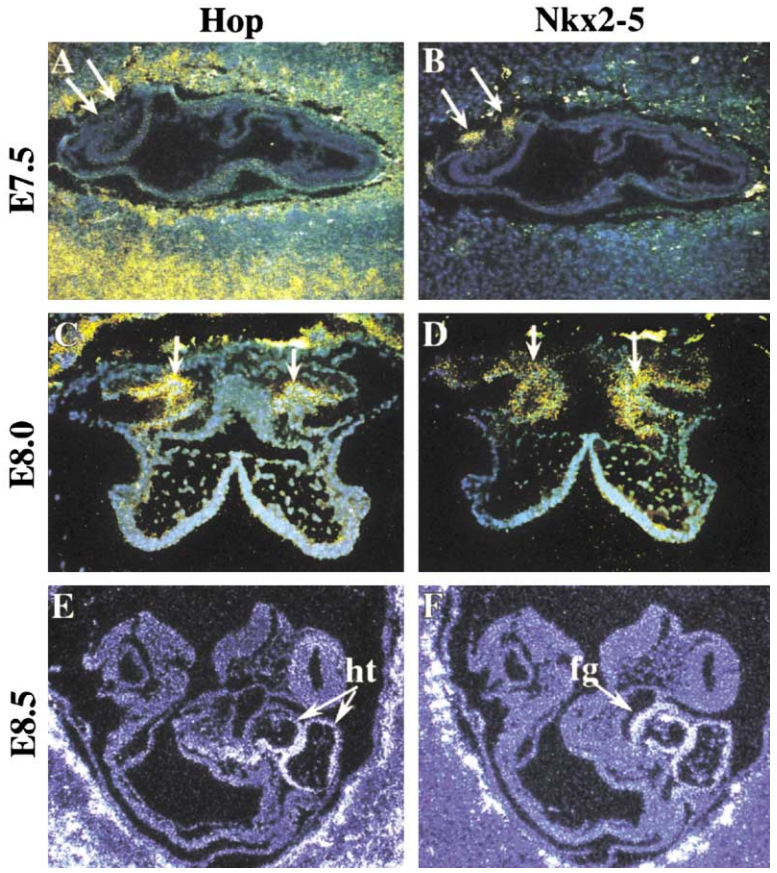

Figure 2. *Hop* Expression Overlaps with that of *Nkx2-5*

Radioactive in situ hybridization of mouse embryos in their decidua from E7.5 (A and B), E8.0 (C and D) and E8.5 (E and F). (A and B), (C and D), and (E and F) are from adjacent sections.

(A) *Hop* expression is not detected in precardiac mesoderm at E7.5 (arrows).

(B) *Nkx2-5* is expressed at E7.5 in this tissue (arrows).

(C) At E8.0, *Hop* is expressed in the forming bilateral heart tubes and is restricted to the cardiogenic region (arrows).

(D) *Nkx2-5* is expressed in a similar pattern, though expression extends ventrally beyond that of *Hop*.

(E) At E8.5, *Hop* expression is restricted to the heart (ht).

(F) *Nkx-2.5* is coexpressed with *Hop* in the heart. *Nkx2-5* is also expressed in foregut endoderm (fg).

random nucleotides, the Pax6 homeodomain selected sequences containing a palindromic ATTA motif, as previously described (Wilson et al., 1993). No specific sequences were selected with Hop. The results were similar to that seen with GST alone. Thus, we conclude that Hop does not possess DNA binding ability under

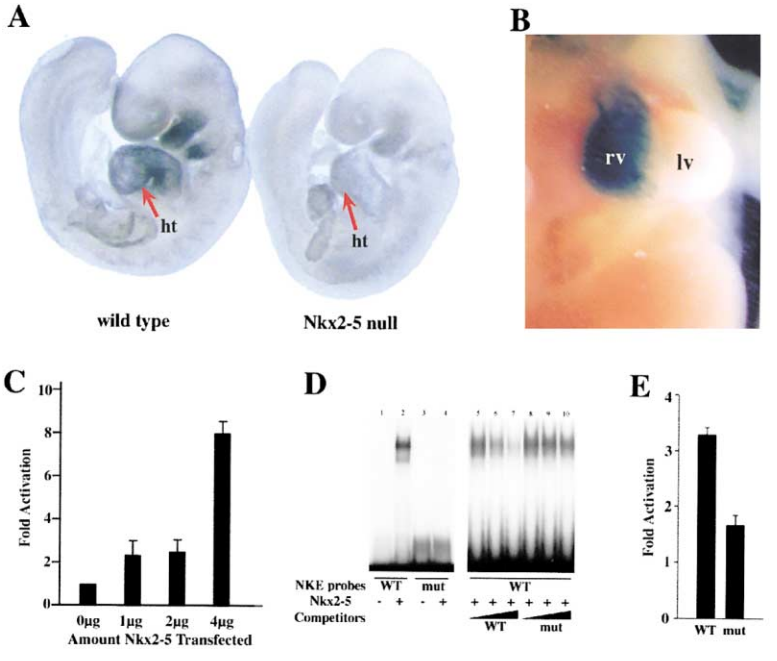

Figure 3. *Hop* Functions Downstream of *Nkx2-5*

(A) Whole-mount in situ hybridization indicates *Hop* expression at E9.5 in wild-type (left) and *Nkx2-5* null (right) littermates. Hop expression is significantly downregulated in the heart (ht) and branchial arches in *Nkx2-5* deficient embryos.

(B) 5 kb of the *Hop* promoter driving lacZ expression results in right ventricular (rv) expression of β-galactosidase activity at E10.5 in transgenic embryos. No expression is seen in the left ventricle (lv).

(C) Transient transfection assays demonstrate that a human growth hormone reporter gene driven by the 5 kb mouse *Hop* promoter is activated by cotransfection of Nkx2-5 in a roughly dose-dependent manner. Results are normalized for transfection efficiency in NIH-3T3 cells.

(D) (left) EMSA revealing that Nkx2-5 can bind to a Nkx2.5 binding site (NKE-WT) present in the proximal Hop promoter, while it can not bind a mutant Nkx2.5 binding site (mut). (right) Binding of Nkx2.5 protein to the NKE is competed by unlabeled wild-type but not the mutant competitor oligonucleotide.

(E) Transient transfection assays demonstrate that a human growth hormone reporter gene driven by the 261 bp proximal mouse *Hop* promoter is activated by cotransfection of Nkx2-5, while activation is significantly reduced by mutation of the NKE.

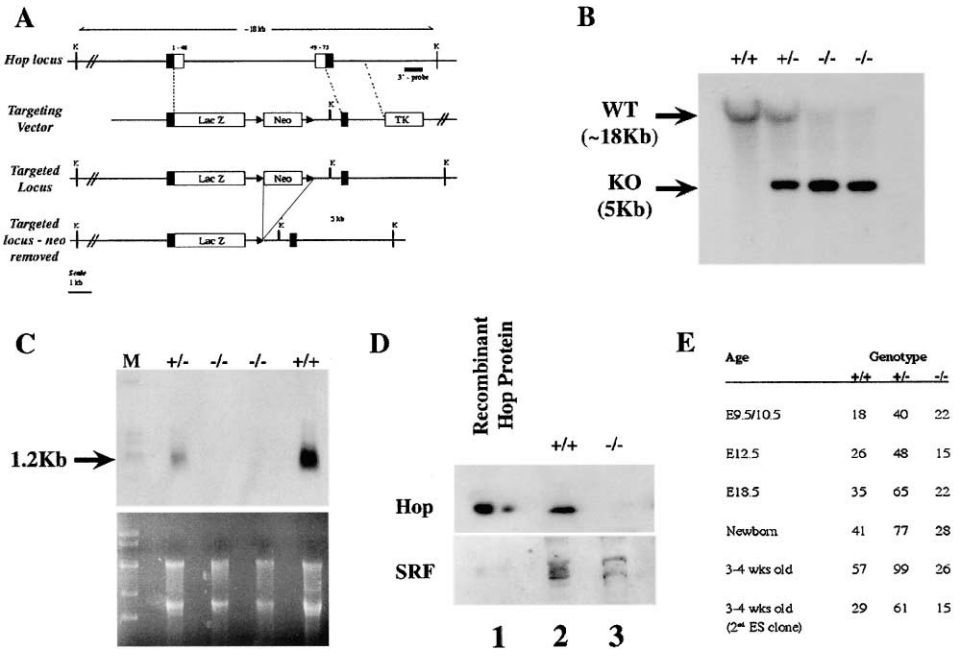

Figure 4. Inactivation of the *Hop* Gene

(A) Structure of the wild-type murine *Hop* locus, targeting vector, targeted locus, and targeted locus with the neomycin-resistance gene removed. K, *KpnI*.

(B) Southern blot of *KpnI* digested genomic DNA hybridized with the 3′ external probe.

(C) Top: Northern blot of total RNA from hearts isolated from newborn mice hybridized with a *Hop* cDNA probe. Bottom: Ethidium bromide stained agarose gel.

(D) Western blot with rabbit polyclonal antiserum raised against bacterial Hop recognizes an 8 kDa band from bacterial extracts expressing Hop (lane 1) and from E11.5 mouse hearts (lane 2). No Hop protein is detected in extracts from hearts of $Hop^{-/-}$ embryos (lane 3). An α-SRF antibody was used to demonstrate approximate equal loading of protein in lanes 2 and 3 (bottom).

(E) Partial embryonic lethality of homozygous *Hop* embryos occurs between E10.5 and E12.5. Offspring resulting from heterozygous *Hop* intercrosses were genotyped as indicated. Mendelian ratios were found prior to E10.5, but only approximately half the expected numbers of homozygotes were identified at E12.5 and thereafter.

conditions in which other homeodomain proteins exhibit sequence specific DNA binding properties.

Inactivation of Hop in Embryonic Stem Cells

We constructed a targeting vector designed to replace the entire Hop coding sequence with *lacZ* (Figure 4A). We identified 4 correctly targeted R1 ES cell clones out of 150 G418/gancyclovir-resistant colonies. Germline transmission was achieved from 2 independent clones and confirmed by Southern blot analysis (Figure 4B).

Heterozygous Hop-deficient mice are viable and fertile. We intercrossed heterozygous mice to obtain homozygous offspring. Northern blot analysis of total RNA extracted from newborn wild-type, heterozygous, and homozygous hearts using the entire *Hop* cDNA as probe confirmed the absence of *Hop* mRNA transcripts in homozygous-deficient mice (Figure 4C). A polyclonal rabbit antibody raised against bacterially expressed Hop detected endogenous Hop protein in heart extracts of wild-type embryos (Figure 4D). In contrast, no immunoreactivity was detected in extracts from $Hop^{-/-}$ embryos (Figure 4D). Thus, our targeting strategy that removed the entire coding region of *Hop* results in a null allele.

Our targeting approach results in the insertion of the bacterial *lacZ* gene and replacement of the *Hop* gene. In heterozygous *Hop* embryos, β-galactosidase activity was detected in the same pattern as that of endogenous *Hop* mRNA transcripts (Figures 5A and 5B). At E9.5, β-galactosidase activity was evident in the branchial arches and in the myocardium (Figure 5A). Activity persisted strongly in all four chambers of the heart (Figure 5B) and was detected in the neural tube and lung at later stages (not shown).

Partial Embryonic Lethality and Heart Failure in Hop Null Embryos

We genotyped 182 three-week-old pups resulting from crosses between heterozygous *Hop* mice on a mixed 129/Sv x C57BL/6 genetic background (Figure 4E). We identified approximately half the number of expected homozygous *Hop*-deficient pups. Similar results were obtained using mice derived from a second independently targeted ES cell line. We examined litters at various stages of gestation and just after birth to determine the timing of partial demise of homozygous *Hop*-deficient mice. At E9.5/10.5 we found the expected number of homozygotes, while at E12.5 significant loss had occurred indicating that Hop deficiency results in partial embryonic demise between E10.5 and E12.5.

At E9.5 and earlier, we could not detect phenotypic abnormalities in Hop deficient embryos. However, by E10.5, significant developmental defects were apparent.

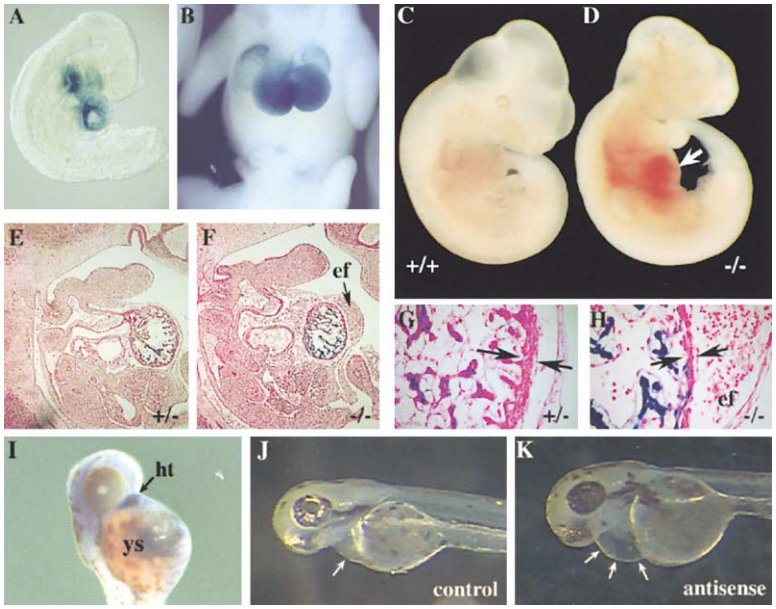

Figure 5. Targeted Replacement of *Hop* with *lacZ* Results in Embryonic Heart Failure

(A) Heterozygous E9.5 *Hop* embryo reveals β-galactosidase activity in the branchial arches and heart.

(B) β-galactosidase activity is detected in all four cardiac chambers at E11.5.

(C) Wild-type E11.5 embryo resulting from a heterozygous *Hop* intercross.

(D) Homozygous *Hop* deficient (Neo-removed) littermate of the embryo shown in (C) with a hemorrhagic pericardial effusion (arrow).

(E) Sagittal section of a heterozygous E11.5 embryo revealing β-galactosidase activity in the ventricle and normal anatomy.

(F) Sagittal section of homozygous littermate reveals a pericardial effusion (ef).

(G) Higher power of myocardium shown in (E).

(H) Higher power of myocardium shown in (F). Note the thinned compact zone (arrows, H) when compared to the heterozygote (arrows, G).

(I) Whole-mount in situ analysis reveals expression of zebrafish *Hop* RNA in the heart (ht, arrow) of this cleared embryo 48 hr post fertilization (hpf; ys, yolk sac). Morpholino-modified antisense oligonucleotides against *Hop* result in impaired cardiac development and function in zebrafish.

(J) Control zebrafish embryo (48 hpf) injected with a control α-*Hop* morpholino antisense oligonucleotide containing a 4 basepair alteration has a normal phenotype. White arrow indicates pericardium.

(K) Zebrafish embryo at 48 hpf after injection with α-*Hop* morpholino with large pericardial effusion (arrows). Video images documenting slow heart rate and significantly impaired circulation are available upon request.

About half of the Hop-deficient embryos examined at E10.5 had gross pericardial effusions, often containing sero-sanguinous exudates. This phenotype was observed both before (not shown) and after germline removal of the floxed pGKneo cassette (Figures 5C and 5D). Histologic analysis confirmed the presence of pericardial effusions, often with associated red blood cells suggesting microperforation of the cardiac wall (Figures 5E and 5F). Indeed, we occasionally noted small perforations of the myocardium. The myocardial wall was thinned with a poorly developed compact zone (Figures 5G and 5H). Hop-deficient embryos without pericardial effusions had histogically normal appearing hearts. These results suggest an incompletely penetrant embryonic lethal phenotype on a mixed 129/Sv x C57BL/6 background resulting from embryonic heart failure.

Inhibition of Hop Expression Results in Defective Cardiac Function in Zebrafish

In order to examine the role of Hop in an animal model in which we could monitor embryonic cardiac function, we perturbed Hop expression in zebrafish embryos using antisense morpholino-modified oligonucleotides (Nasevicius and Ekker, 2000). Zebrafish *Hop* is expressed in the myocardium, as demonstrated by whole-mount in situ hybridization (Figure 5I). Injection of 6 to 9 ng of either of two distinct anti-*Hop* antisense morpholino oligonucleotides into fertilized oocytes resulted in one and two day embryos with dramatic impairment of cardiac function when compared to control, uninjected embryos or to embryos injected with mutated morpholinos containing four nucleotide alterations. At 48 hr post fertilization (hpf), injected embryos were smaller than controls and >75% had large pericardial fluid collections (Figures 5J and 5K). The heart rate was less than half of that of controls and effective circulation was dramatically impaired (Video images available upon request). This phenotype is remarkably similar to that of affected Hop-deficient mouse embryos and indicates that Hop deficiency is associated with impaired cardiac contractile function and ineffective circulation.

Hop Modulates the Transcriptional Activation of Cardiac Promoters

Since Hop was unable to bind DNA independently, we tested the ability of Hop to modulate the transcriptional activity and DNA binding characteristics of other cardiac-specific factors. Nkx2-5, Gata4, and SRF are able to synergistically activate cardiac-specific gene expression by binding to conserved elements in cardiac promoter/enhancer sequences.

We assayed the effects of cotransfecting *Hop* in NIH/3T3 cells with cardiac-specific promoter-driven reporter plasmids and expression vectors for Nkx2-5, Gata4, and SRF. When the α-cardiac actin (α-CA) promoter was used to direct luciferase expression, cotransfection of Nkx2-5, Gata4, and SRF induced 15-fold activation. Cotransfection of Hop results in a dose-dependent reduction in luciferase activity compared with control vector (Figure 6A). A similar inhibitory effect is observed when the atrial natriuretic factor (ANF) promoter was used to direct expression of a human growth hormone reporter gene (Figure 6B). Both the α-CA promoter and the ANF promoter contain SRF binding sites and mediate synergistic activation by SRF, Nkx2-5, and Gata4. However, Hop did not inhibit all cardiac-specific promoter reporter constructs. The −124cTnC promoter does not contain SRF binding sites but is known to be activated by Gata4.

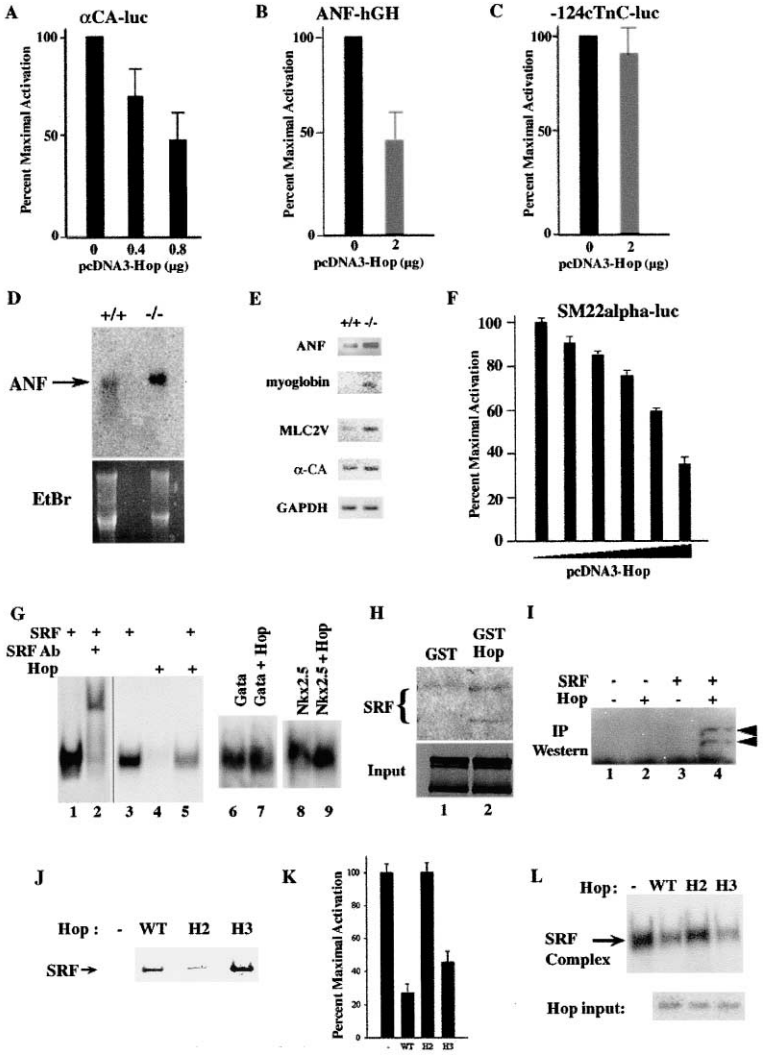

Figure 6. Hop Inhibits SRF-Dependent Cardiac Gene Transcription

(A) COS7 cells were transfected with an α-CA luciferase reporter plasmid (αCA-luc), expression plasmids for Nkx2-5, Gata4, and SRF, and with increasing amounts of Hop expression plasmid (pcDNA3-Hop). Maximal activation (15-fold) with Nkx2-5, Gata4, and SRF was designated 100%. Hop cotransfection resulted in dose-dependent inhibition of transcription. Results shown in panels (A–C), (F), and (K) were normalized for transfection efficiency and represent the average of three independent experiments performed in triplicate, with error bars representing the standard deviation.

(B) Hop also inhibited activation of an atrial natriuretic factor human growth hormone reporter plasmid (ANF-hGH).

(C) Hop did not inhibit GATA4 activation of a cardiac troponin C luciferase reporter plasmid (−124cTnC-luc) that does not contain SRF binding sites.

(D) Northern blot analysis of endogenous *ANF* expression in hearts from wild-type (+/+) and *Hop* knockout (−/−) newborn mice. *ANF* is upregulated in mutant hearts. Ethidium bromide staining of the RNA gel is shown below to indicate equal loading of RNA.

(E) RT-PCR analysis of heart RNA from E10.5 wild-type (+/+) and *Hop* knockout (−/−) embryos. *ANF*, *myoglobin*, *MLC2v*, and α-*CA* expression are increased in the mutant hearts. *GAPDH* expression was used to demonstrate equal amounts of RNA.

(F) Hop transfection of COS7 cells results in dose-dependent inhibition of myocardin induced activation of an SM22α-luciferase reporter construct.

(G) EMSA reveals binding of epitope tagged SRF to radioactively labeled SRE (lanes 1,3). Antibodies specific for epitope tagged SRF (SRF Ab) result in a supershift of the SRF/SRE complex (lane 2). Hop itself is unable to bind to SRE (lane 4). Addition of in vitro transcribed and translated Hop with SRF results in inhibition of DNA binding by SRF (lane 5). Hop does not inhibit binding of GATA4 and Nkx2.5 to their respective binding sites (lanes 6–9). (Lanes 1 and 2, 3–5, 6–9 are from different gels).

(H) GST-Hop interacts with in vitro translated and transcribed SRF. GST or GST-Hop bound to agarose beads was mixed with radioactive SRF and bound SRF was visualized by autoradiography. SRF bound to GST-Hop (lane 2) but not to GST alone (lane 1). The SRF input material is shown below.

(I) SRF and Hop interact in cultured cells. Immunoprecipitation of myc epitope-tagged Hop followed by Western blot for HA epitope-tagged SRF (IP Western) was performed using nuclear extracts from untransfected COS7 cells (lane 1) or cells transfected with Hop (lane 2), SRF (lane 3) or SRF plus Hop (lane 4). SRF was detected by Western blot after immunoprecipitation of Hop only if Hop and SRF were both present (arrowheads, lane 4).

(J) A mutation of 4 amino acids in the second helix of Hop (H2) reduces its ability to bind radiolabeled epitope tagged-SRF containing a carboxy-terminal truncation (amino acids 1–296; lane 3). Wild-type Hop and a mutant containing five altered amino acids in the third helix (H3) are able to bind to this form of SRF (lanes 2 and 4).

(K) Transfection of H2 Hop does not repress myocardin activation of αCA-luc in COS7 cells. However H3 Hop represses similar to wild-type Hop.

(L) (top) EMSA demonstrating that wild-type Hop (WT) and H3 Hop repress binding of SRF to its binding site (SRE), while H2 Hop does not repress. (bottom) Wild-type and mutant Hop (H2 and H3) in vitro translated proteins express at equal levels and were used in 6J and 6L.

Cotransfection of Hop with a −124cTnC-luciferase plasmid had no effect (Figure 6C). Likewise, Hop did not affect the activity of an SV40 promoter-reporter construct (not shown).

If *Hop* functions to modify cardiac-specific gene transcription in vivo, we would expect alterations in the expression of downstream genes in *Hop* null mice. *ANF* message was upregulated in $Hop^{-/-}$ hearts compared to wild-type littermates (Figure 6D). Likewise, transcripts for myoglobin, myosin light chain 2v (MLC2v), and α-CA were all upregulated in $Hop^{-/-}$ embryonic hearts (Figure 6E), consistent with a role for *Hop* in the negative modulation of SRF-dependent cardiac-specific gene expression.

Hop Inhibits the Ability of Myocardin to Activate SRF-Dependent Transcription

Recently, a potent transcriptional cofactor for SRF named myocardin has been described (Wang et al.,

2001). A dominant-negative form of myocardin is able to impair cardiac development in *Xenopus* embryos. We sought to determine if Hop could modulate the ability of myocardin to activate SRF-dependent transcription. As has been previously described, we found that myocardin was able to induce dramatic (500–1600-fold) activation of *ANF* or *SM22*α reporter constructs. Hop was able to significantly inhibit this activation in a dose-dependent fashion (Figure 6F and data not shown).

Hop Interacts with SRF and Modulates SRF Interactions with DNA

The ability of Hop to inhibit SRF-dependent gene activation by either myocardin or by Nkx2-5, Gata4, and SRF suggested that Hop might affect the ability of SRF to interact with DNA or with other cofactors. We tested the ability of Hop to affect SRF binding to preferred serum response element (SRE) binding sites derived from the *SM22*α or *c-fos* enhancers by performing EMSA using in vitro transcribed and translated SRF and Hop. The predominant complex formed upon addition of SRF to radiolabeled *c-fos* SRE contained SRF protein, since addition of antibodies directed against the epitope tag of SRF resulted in a delayed migration (supershift) of that complex (lane 2, Figure 6G). Hop alone was unable to bind to radiolabeled SRE (lane 4, Figure 6G) and did not result in altered electrophoretic migration of SRF bound to SRE. Rather, Hop inhibited the ability of SRF to interact with the SRE (lane 5, Figure 6G). We obtained similar results using the *SM22*α SRE and nuclear extracts of COS7 cells expressing SRF and Hop (not shown). The addition of reticulocyte lysate alone, or of unrelated proteins expressed in vitro, had no effect on SRF binding to SRE. Hop did not bind to Nkx2-5 and Gata4 binding sequences and did not affect the ability of Nkx2-5 or Gata4 to interact with their cognate binding sites (lanes 6–9, Figure 6G). Hence, Hop specifically impairs the ability of SRF to bind to DNA.

We performed GST pull-down experiments and coimmunoprecipitation assays to determine if Hop interacts directly with SRF. GST alone was unable to interact directly with radiolabeled SRF prepared in vitro (lane 1, Figure 6H), while GST-Hop could bind to SRF (lane 2, Figure 6H). Next, COS7 cells were transfected with epitope-tagged Hop and SRF or with control vectors. Immunoprecipitation with an antibody directed against the Hop epitope tag followed by Western blotting to detect SRF confirmed the ability of Hop to interact with SRF in vivo (Figure 6I). Hence, Hop forms a stable complex with SRF that correlates with the ability of Hop to impair SRF binding to DNA and SRF-dependent gene transcription.

We carried out both targeted and random mutagenesis of the Hop cDNA and examined the ability of several subtly mutated Hop proteins to interact with SRF. Alteration of 4 amino acids in the predicted second α helix of Hop (a mutant called H2 in which amino acids 35–39 LIAAE were changed to AAASM) dramatically reduced interaction with SRF (lane 3, Figure 6J).

Other mutations examined, including a construct encoding a similar mutation in the third predicted α helix (H3, in which amino acids 49–53 KWFKQ were changed to AAASM), retained the ability to interact with SRF (lane 4, Figure 6J). The H2 mutant was unable to inhibit myocardin activation of SRF-dependent transcription (Figure 6K) and did not inhibit SRF binding to DNA (Figure 6L). The H3 mutant (and other mutants not shown) inhibited SRF-dependent transcription and DNA binding (Figures 6K and 6L). Both wild-type and mutant proteins were expressed at equal levels in these experiments. These results suggest that Hop interacts with SRF via the second α helix, and strongly support the conclusion that Hop inhibits SRF-dependent transcription by physically interacting with SRF and affecting SRF interaction with DNA.

Discussion

In this report, we have described the identification and functional analysis of a gene that is closely related to known homeobox genes, yet encodes a protein that fails to bind DNA and contains no other recognizable structural motifs. *Hop* encodes the smallest known homeodomain protein. While all known homeodomain factors are thought to function, at least in part, by binding to DNA, Hop acts to modulate SRF-dependent transcription and cardiac-specific gene expression in the absence of intrinsic DNA binding capability. Our results suggest a model by which Hop modulates SRF-dependent cardiac gene transcription by binding directly to SRF and reducing the ability of SRF to bind to DNA.

Hop expression is first observed at early stages of cardiogenesis just after that of *Nkx2-5*, and it remains strongly expressed in the heart throughout development and adulthood. Our data indicate that Nkx2-5 directly activates *Hop* expression, since *Hop* is downregulated in *Nkx2-5* null embryos, and since Nkx2-5 can activate a functional *Hop* promoter/enhancer construct in cultured cells by binding directly to Nkx2-5 binding sites. Hop acts in a negative feedback loop to inhibit the ability of Nkx2-5 to activate a subset of target genes, namely those that are dependent upon SRF activity (Figure 7). This model suggests a delicate balance of transcriptional regulation that profoundly impacts on cardiac function. In the absence of Hop in mice, cardiac development is disrupted such that about half of the null embryos develop embryonic heart failure and die between E10.5 and E12.5. This is precisely the period of gestation at which numerous cardiac mutant embryos succumb to deficiencies involving a thin-walled myocardium (Auerbach, 1954; Lyons et al., 1995; Srivastava et al., 1997), supporting the conclusion that heart failure accounts for lethality in Hop mutant mouse embryos. Inhibition of Hop protein expression in zebrafish also results in specific cardiovascular functional defects, and the impairment of effective circulation in this model confirms a role for Hop in supporting contractile function.

Interestingly, some null murine embryos survive to birth and appear healthy and fertile up to 6 months of age. Invasive hemodynamic analysis has failed to reveal significant alterations in myocardial function in these adult animals (data not shown). Genetic modifiers may alter the degree of phenotypic penetrance when Hop-deficient mice are bred on distinct genetic backgrounds, as has been amply demonstrated in the case of other mouse mutations (Nadeau, 2001; Wittman and Ham-

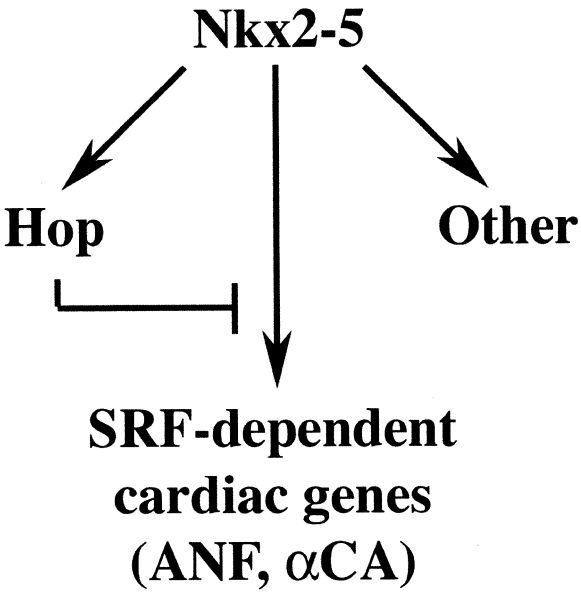

Figure 7. Model for Hop Modulation of Cardiac Transcription

Nkx2-5 activates expression of Hop, which feeds back to inhibit the ability of Nkx2-5 to activate SRF-dependent cardiac-specific genes such as ANF and α CA, while gene activation that is not dependent on SRF ("Other") is unaffected.

burgh, 1968). We have not identified other related Hop-like genes in the mouse or human genomes, although we cannot rule out the possibility of functional redundancy with known or unknown gene products that could account for the partial penetrance of the lethal phenotype. It is possible that Hop deficiency causes a delay in myocardial maturation, which leads to the stochastic occurrence of myocardial rupture as hemodynamic forces increase during midgestation. If rupture and ensuing demise do not occur, subsequent myocardial maturation and adaptive capacity may suffice to allow for normal or near normal development. This type of stochastic event could explain the partially penetrant embryonic lethal phenotype that we observe.

The ability of Hop to repress transcriptional activity induced by Nkx2-5 and myocardin implicates a common mechanism of action involving SRF. Myocardin functions by binding to SRF (Wang et al., 2001), as does Nkx2-5 (Sepulveda et al., 1998). Myocardin contains a SAP domain that bears homology to the first helices of a homeodomain, suggesting an ancestral structural homology between myocardin, Nkx2-5, and Hop.

The small size of Hop and its ability to inhibit the activity of Nkx2-5 is reminiscent of the action of Id and its ability to inhibit the function of other helix-loop-helix (HLH) proteins. *Id* genes encode HLH proteins that lack the adjacent basic domain required for DNA binding in bHLH proteins such as MyoD. Id functions to inhibit DNA binding by other bHLH proteins and hence plays important roles in development and differentiation (Benezra et al., 1990; Kreider et al., 1992).

Mutations in *NKX2-5* have been associated with pediatric and adult structural heart disease and conduction defects (Benson et al., 1999; Schott et al., 1998). Given the functional relationship of Hop with Nkx2-5, perhaps mutations in the human homolog of *Hop* will be identified in association with related cardiac conditions.

Experimental Procedures

Cloning of *Hop*

Hop was identified from the EST database at the National Center for Biotechnology Information using the *Pax3* homeodomain sequence as query. A genomic clone for Hop was obtained by using the *Hop* cDNA to screen a mouse λ genomic library (Stratagene, La Jolla, CA; 129/Sv λFIX II library).

In Situ Hybridization

Whole-mount and radioactive in situ hybridization was performed as described (Schulte-Merker et al., 1992; Wawersik and Epstein, 2000) and our protocols are available at www.med.upenn.edu/mcrc.

RNA Analysis

One μg each of poly (A) RNA from mouse tissues was used for Northern blots. RT-PCR was performed using heart tissue from E10.5 embryos. RNA was prepared using Trizol (Life Technologies, Rockville, MD). Qualitative comparison of gene expression was determined after 25 cycles.

Glutathione *S*-Transferase (GST) Fusion Protein, In Vitro Translated Protein, and Anti-Hop Antibody Production

Proteins for EMSA and coimmunoprecipitation experiments were made using the TNT coupled transcription/translation system (Promega, Madison, WI). GST-Hop fusion protein was prepared by standard techniques and used to immunize rabbits (Cocalico Biologicals, Inc., Reamstown, PA).

Immunoprecipitations/Western Analysis

COS7 cells were transfected with pcDNA3 myc-Hop and/or pCGN-SRF (HA tagged) using Effectene (Qiagen, Valencia, CA). Cell extracts were isolated 48 hr after transfection, and 500 μg was immunoprecipitated using a mouse α-myc monoclonal antibody (α-myc1-9E10) and protein A/G sepharose. Cell extracts were prepared by sonication in GS buffer (40% glycerol, 40 mM HEPES, 100 mM KCl, and 2 mM β-mercaptoethanol). Western blotting with Western Breeze (Invitrogen Co., Carlsbad, CA), mouse monoclonal α-HA antibody (Roche, Indianapolis, IN), and rabbit polyclonal α-SRF (Santa Cruz Biotechnology) were used.

Transfections

NIH-3T3 fibroblast cells were transfected using Effectene (Qiagen) as directed. Reporter constructs included α-CA-Luc (Chen et al., 1996), rat B-type natriuretic peptide promoter cloned into p0GH (Nichols Institute, San Juan Capistrano, CA) (ANF-hGH), SM22α-Luc and −124cTnC-Luc (kindly provided by M. Parmacek), and constructs containing either 5 kb or 261 bp of the *Hop* promoter cloned into p0GH. Mutagenesis utilized QuikChange Site-Directed Mutagenesis and GeneMorph Kits (Stratagene). Myocardin was cloned by PCR from a mouse E15 cDNA library (Clontech, Palo Alto, CA) and is identical to the myocardin A sequence (accession number: AF437877). pSVβ-galactosidase (Promega) was used to normalize for transfection efficiencies and pcDNA3 was used to equalize the amount of DNA in each transfection.

EMSA, DNA Binding Site Selection Assay

Binding site selection conditions have been described previously (Epstein et al., 1994). EMSA experiments utilized γ-^{32}P ATP-labeled oligonucleotides (50,000 cpm), 0–7 μl of in vitro translated proteins, 300 ng of poly(dI-dC) in 0.5 × TBE, incubated for 30 min at room temperature in a final volume of 20 μl. The reaction mixture was loaded on a 5% polyacrylamide gel in 0.5 × TBE. Oligonucleotide sequences are available upon request.

Transgenic Mice Analysis

A 5kb mouse *Hop* promoter fragment fused to a *lacZ* reporter gene was purified using Qiaex II (Qiagen) and an Elutip column (Schleicher and Schuell, Keene, NH). DNA was injected into B6SJL/F1 eggs.

Gene Targeting

The targeting vector was based on the pPNT vector with the neomycin resistance gene cassette that has been modified to contain flanked lox P sites (Tybulewicz et al., 1991). The 5′ arm of the targeting construct consists of a 5 kb *NotI -NarI* fragment that is 5′ of the *Hop* coding region fused to a LacZ reporter gene, and the 3′ arm consists of a 2 kb *KpnI-EcoRI* fragment that flanks the *Hop* 3′ untranslated region. The *NotI*, *KpnI*, and *EcoRI* restriction sites were engineered. The targeting vector was linearized with *NotI* and electroporated into R1 ES cells (kindly provided by Andras Nagy, Toronto, Canada). Genotyping was performed by Southern analysis of *KpnI* digested genomic DNA. Mice were maintained on a mixed 129/Sv X C57Bl/6 genetic background. CMV-Cre recombinase transgenic mice used to remove the floxed neomycin resistance gene cassette kindly provided by P.A. Labosky.

Genotyping *Hop* Mutants

Hop mutant mice were genotyped by Southern blotting and by PCR (primer sequences available upon request).

Morpholino Inhibition in Zebrafish

To deplete Hop in zebrafish, 6 and 9 ng of morpholino antisense oligonucleotide (Gene Tools, LLC, Philomath, OR) directed against the zHop 5′ untranslated region (zHop as1, 5′-TAAACTCCGAACAG CAAACGAGCAG-3′) was injected into the yolk region at the one-cell stage. 7.5 ng of a second morpholino antisense oligonucleotide directed against the zHop amino-terminal coding region (zHop as2, 5′-ACGCCGCGTTTCCATTCGCGCTCAT-3′), and a negative control for zHopas1 containing 4 mutations (zHopas1mut, 5′-TGAACTGC GAACAGCAGACAAGCAG-3′) were also injected.

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Accession Numbers

The myocardin A sequence reported in the paper is identical to GenBank accession number AF437877. The GenBank accession number for the murine and zebrafish Hop sequences reported in this paper are AF536202 and AF536198, respectively.