BOP, a regulator of right ventricular heart development, is a direct transcriptional target of MEF2C in the developing heart

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Summary

The vertebrate heart is assembled during embryogenesis in a modular manner from different populations of precursor cells. The right ventricular chamber and outflow tract are derived primarily from a population of progenitors known as the anterior heart field. These regions of the heart are severely hypoplastic in mutant mice lacking the myocyte enhancer factor 2C (MEF2C) and BOP transcription factors, suggesting that these cardiogenic regulatory factors may act in a common pathway for development of the anterior heart field and its derivatives. We show that *Bop* expression in the developing heart depends on the direct binding of MEF2C to a MEF2-response element in the *Bop* promoter that is necessary and sufficient to recapitulate endogenous *Bop* expression in the anterior heart field and its cardiac derivatives during mouse development. The *Bop* promoter also directs transcription in the skeletal muscle lineage, but only cardiac expression is dependent on MEF2. These findings identify *Bop* as an essential downstream effector gene of MEF2C in the developing heart, and reveal a transcriptional cascade involved in development of the anterior heart field and its derivatives.

Key words: Cardiac gene expression, Skeletal muscle gene expression, MEF2 binding site, E-box, Cardiogenesis, Mouse, Anterior heart field

Introduction

Heart development is a precisely orchestrated process in which even subtle perturbations can have catastrophic consequences for the organism (reviewed by Fishman and Olson, 1997). During vertebrate embryogenesis, heart development begins when mesodermal progenitor cells within a bilaterally symmetric region of the embryo, known as the cardiac crescent or primary heart field, adopt a cardiac fate in response to inductive cues from the surrounding tissues (Fishman and Olson, 1997; Marvin et al., 2001; Schneider and Mercola, 2001; Schultheiss et al., 1997; Tzahor and Lassar, 2001). Soon thereafter, these cells converge along the midline of the embryo to form a linear heart tube that initiates peristaltic contractions. Generation of the mature multi-chambered heart from the linear heart tube involves a complex series of events that include rightward looping and diversification of cardiac cell types, balloon-like growth and septation to form the cardiac chambers, and connection to the inflow and outflow tract vasculature (Christoffels et al., 2000; Moorman et al., 2000).

Recent studies have revealed a population of cardiac precursor cells, referred to as the anterior or secondary heart field, which is derived from a region of the splanchnic mesoderm medial to and distinct from the primary heart field that makes up the cardiac crescent (Abu-Issa et al., 2004; Cai et al., 2003; Kelly et al., 2001; Kelly and Buckingham, 2002; Mjaatvedt et al., 2001; Waldo et al., 2001). Cells from the anterior heart field are added to the anterior region of the heart tube at the onset of looping and give rise to the outflow tract (OFT) and right ventricle (RV). By contrast, the primary heart field, which generates the linear tube, serves as the source of precursors of the left ventricle (LV) and atrial chambers. The existence of two populations of cardiac precursor cells that contribute to different regions of the heart provides a potential explanation for many cardiac abnormalities in mice and humans in which specific cardiac structures are malformed or missing, leaving the remainder of the heart unperturbed (Bruneau et al., 2001; Cai et al., 2003; Fishman and Olson, 1997; Gottlieb et al., 2002; Lin et al., 1997; Srivastava et al., 1997; von Both et al., 2004). Further evidence for heterogeneity of cardiac precursors has come from the analysis of cis-regulatory elements associated with cardiac genes, which often direct expression in highly restricted regions of the developing heart (Biben and Harvey, 1997; Thomas et al., 1998).

Numerous transcription factors have been implicated in heart development based on cardiac phenotypes of mutant mice, zebrafish and fruit flies, as well as congenital heart defects in humans (Fishman and Olson, 1997; Hoffman and

Kaplan, 2002). Deletion of the gene encoding the myocyte enhancer factor 2C (MEF2C) transcription factor in mice results in severe abnormalities in the formation of the right ventricle and outflow tract (Lin et al., 1997), which mimic the cardiac defects observed in mice lacking islet 1 (ISL1), a LIM homeodomain transcription factor expressed in the anterior heart field and its derivatives (Cai et al., 2003). ISL1 was recently shown to bind a cardiac-specific enhancer that controls *Mef2c* transcription in the anterior heart field, establishing a direct transcriptional link between these cardiac control genes (Dodou et al., 2004). The phenotype of *Mef2c* and *Isl1* mutant embryos also resembles that of embryos lacking the basic helix-loop-helix (bHLH) transcription factor Hand2 (Srivastava et al., 1997). Similarly, mice lacking the *Bop* (*Smyd1* – Mouse Genome Informatics) gene, which encodes a muscle-restricted transcriptional repressor and putative histone methyltransferase, die from cardiac abnormalities similar to those of *Isl1*, *Mef2c* and *Hand2* mutant embryos (Gottlieb et al., 2002).

The intriguing similarity between the phenotypes of mice lacking *Mef2c*, *Hand2* and *Bop* raises the possibility that these transcription factors act in a common developmental pathway. Indeed, prior studies have shown that *Hand2* is downregulated in the hearts of embryos lacking *Mef2c* or *Bop* (Gottlieb et al., 2002; Lin et al., 1997). However, it remains unclear whether the downregulation of *Hand2* expression in these mutant embryos reflects a direct influence of MEF2C or BOP on the *Hand2* gene, or a secondary consequence of the loss of *Hand2*-expressing cells in these mutant embryos.

The *Bop* gene is expressed in cardiac precursor cells beginning at ~E8.0. Thereafter, expression is maintained throughout the linear and looping heart tube, as well as in the atrial and ventricular chambers of the heart (Gottlieb et al., 2002). *Bop* is also expressed in the myotomal compartment of the somites and in differentiated skeletal muscle. In an effort to further define the potential regulatory relationship between *Mef2c* and *Bop*, we investigated whether cardiac expression of *Bop* was dependent on *Mef2c*. Here, we show that *Bop* expression in the developing heart is downregulated in *Mef2c* mutant embryos, and we identify a MEF2-response element that controls expression of *Bop* in the anterior heart field during mouse embryogenesis. These findings identify *Bop* as an essential downstream effector gene of MEF2C during formation of the right ventricular chamber and OFT of the heart, and reveal a transcriptional cascade involved in development of the anterior heart field and its derivatives.

Materials and methods

Generation of *Bop* reporter constructs

A mouse genomic DNA fragment covering the region from –3824 to +196 relative to the *Bop* transcription initiation site was isolated from a 129S6/SvEvTac mouse BAC library (CHORI, Oakland, CA) using the *Bop* full-length cDNA as a probe (Gottlieb et al., 2002). This 4-kb *Bop* promoter fragment was subcloned into the *Pst*I site of the pBluescript SKII(+) plasmid (pBSKII-4kbBOP). Using *Pst*I and *Sal*I restriction enzymes, a DNA fragment extending from –3304 to +196 bp relative to the transcription initiation site of the *Bop* gene was excised from the pBSKII-4kbBOP plasmid, blunt ended using the Klenow fragment of DNA polymerase and inserted into the *Sma*I site of *hsp68-lacZ* vector (Kothary et al., 1989) to generate construct 1 (see Fig. 2B). Construct 2 was generated by cloning an 833-bp fragment encompassing the region from –637 to +196 of the *Bop* gene using blunt-end ligation into the *Sma*I site of the *hsp68-lacZ* vector (Kothary et al., 1989). Construct 3 was generated by cloning a *Sal*I/*Eco*RI fragment (–3304 to –637) into the *Sma*I site of the *hsp68-lacZ* vector. Construct 4 was generated by cloning the *Bop* fragment (–986 to +75) using blunt-end ligation into a *Sma*I site of the promoterless AUG-β-gal reporter. Constructs 5 and 6 containing mutations of the MEF2 site were created by mutating the MEF2 site to the sequence shown in Fig. 5B by PCR-based site-directed mutagenesis. The PCR product was subcloned into pCR2.1Topo vector (Invitrogen, Carlsbad, CA) and sequenced to confirm the MEF2 site mutation. This plasmid was digested with *Eco*RI and *Pst*I and the ends were filled in with Klenow fragment and blunt-end ligated into the *Sma*I site of the hsp68-lacZ vector. The same strategy was used to mutate the E boxes in construct 7. In this case, the CA and TG in the CANNTG consensus sequences were mutated to TG and CA, respectively. The correct orientation of all constructs was confirmed by DNA sequence analysis.

Mef2c mutant mice

Mef2c null mice have been described previously (Lin et al., 1997). The *Mef2c* mutant allele was maintained in a C57Bl6×129SvEv hybrid background.

Generation of transgenic mice

The reporter constructs containing *hsp68-lacZ* were digested with *Sal*I, whereas construct 4 was digested with *Xho*I and *Not*I to remove the vector backbone. DNA fragments were purified using a QiaQuick spin column (QIAGEN, MD), injected into fertilized eggs from B6C3F1 female mice, and implanted into pseudopregnant ICR mice as previously described (Lien et al., 1999). Embryos were collected and stained for β-galactosidase activity (Cheng et al., 1993). Sectioning histology and Nuclear Fast Red staining were performed on the embryos as previously described (McFadden et al., 2000).

Southern blot analysis of PCR-amplified cDNA

PCR-amplified cDNA was prepared from embryonic hearts as previously described (Liu et al., 2001). The membranes containing amplified cDNAs were hybridized in Rapid-hyb buffer at 65°C with a *Bop* cDNA that was [^{32}P]-labeled using the Radprime DNA labeling system (Invitrogen). After overnight hybridization, the membranes were washed in 0.1×SSC, 0.1% SDS at 65°C for 10 minutes. Signals were visualized by autoradiography. α-enolase cDNA probe was used as a loading control.

In situ hybridization of embryonic mouse tissue sections

In situ hybridization was performed on mouse sections at embryonic day (E) 9.0 using ^{35}S-UTP-labeled *Bop* riboprobes (Maxiscript, Ambion), as previously described (Shelton et al., 2000).

Electrophoretic mobility shift assays

Oligonucleotides corresponding to the conserved MEF2-binding site in the *Bop* muscle regulatory region, the mutated MEF2-binding site, and a muscle creatine kinase (MCK) MEF2-binding site (Gossett et al., 1989) were synthesized (Integrated DNA Technology) as follows (+ strand sequences are shown with the MEF2 site in bold and the mutation underlined):

Bop MEF2 oligo, 5′-AGGCACCTGGAGG**CTAAAAATAG**CCTACTGACCAAGTG-3′;

Bop MEF2mut oligo, 5′-AGGCACCTGGAGG**CTAGGGGGTAG**CCTACTGACCAAGTG-3′; and

MCK MEF2 oligo, 5′-GATCGCT**CTAAAAATAA**CCCTGTCG-3′.

Annealed oligonucleotides were radiolabeled with [^{32}P]dCTP using the Klenow fragment of DNA polymerase and purified using G50 spin columns (Roche). Nuclear cell extracts were isolated from Cos-1 cells that were transfected with pcDNAMYC-MEF2C. Reaction conditions

of the gel mobility-shift assays were previously described (McFadden et al., 2000). Unlabeled oligonucleotides used as competitors were annealed as described above and added to the reactions at the indicated concentrations. DNA-protein complexes were resolved on 5% polyacrylamide native gels and the gels were exposed to BioMax X-ray film (Kodak).

Results

Downregulation of *Bop* expression in *Mef2c* mutant embryos

Mouse embryos homozygous for a *Mef2c* null mutation die at E9.5 from severe cardiac abnormalities that include a failure of growth of the RV and OFT (Lin et al., 1997). These abnormalities have been proposed to reflect an essential role of *Mef2c* in development of the anterior heart field and its derivatives (Dodou et al., 2004). Because *Bop* mutant mice display a similar, albeit less severe phenotype (Gottlieb et al., 2002), we wondered whether *Mef2c* might act 'upstream' of *Bop* in a cascade of cardiac control genes responsible for development of the anterior heart field. Consistent with this notion, *Bop* expression, as detected by Southern blot analysis of cDNA derived from mRNA from hearts of E9.0 mouse embryos, was downregulated approximately 5-fold in hearts from *Mef2c* mutant mice (Fig. 1A). Similarly, in situ hybridization showed a significant reduction in cardiac expression of *Bop* transcripts throughout the developing heart of *Mef2c* mutant embryos, although residual expression was detectable (Fig. 1B). These findings suggested that MEF2C was required for normal expression of *Bop* in the developing heart. The expression of other MEF2 factors in the early heart (Edmondson et al., 1994) may be sufficient to support a reduced level of *Bop* expression in the *Mef2c* mutant.

Identification of cardiac and skeletal muscle regulatory regions of the *Bop* gene

To determine whether the reduction in *Bop* expression in *Mef2c* mutant embryos reflected a role for MEF2C in the control of *Bop* expression, we sought to identify the cis-regulatory elements responsible for cardiac expression of *Bop*. The *Bop* gene is located on mouse chromosome 6 immediately 5′ of the *CD8b* (*Cd8b1* – Mouse Genome Informatics) gene and was originally identified as a gene of unknown function transcribed in the opposite direction to *CD8b*, hence the name CD8b opposite (*Bop*) (Hwang and Gottlieb, 1995). The *Bop* gene encodes protein products with distinct amino-terminal sequences that are expressed specifically in T lymphocytes (referred to as tBOP), and in cardiac and skeletal muscle (referred to as mBOP) (Hwang and Gottlieb, 1997). The structure of the 5′ region of the gene is shown in Fig. 2A. The first exon for the muscle-specific *mBop* transcript is located ~70 kb 3′ of the first exon for the tBOP isoform. The DNA sequence immediately upstream of the muscle-specific *mBop* first exon can be accessed at NCBI using accession number AC115777 (with the *Bop* transcription start site located at 54866 bp and the 5′ end-point of our construct 1, –3.3 kb at 51570 bp). A TATA box resides between –21 and –24 bp relative to the transcription initiation site of the *mBop* first exon, and the ATG codon for translation initiation is located at +91 bp. In this report, we will refer to muscle-specific *mBop* as *Bop*.

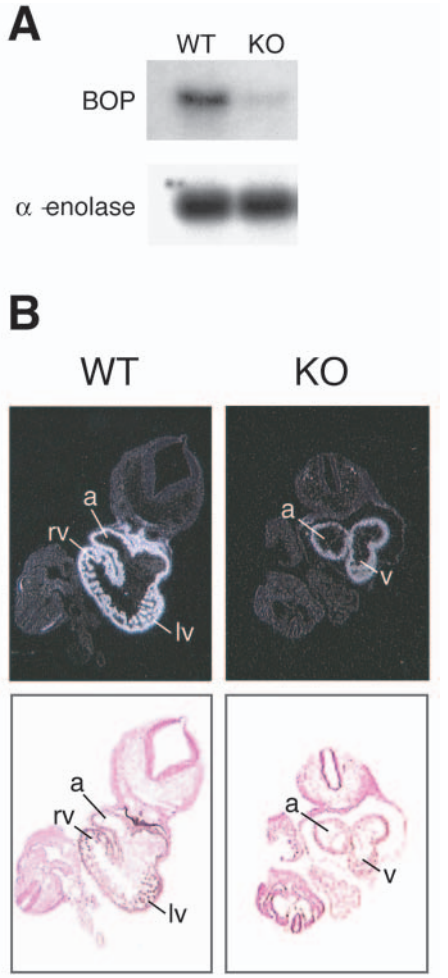

Fig. 1. Downregulation of cardiac *Bop* expression in *Mef2c* mutant embryos. (A) Southern blot of PCR-amplified cDNA derived from hearts of wild-type and *Mef2c* mutant embryos at E9.0 demonstrated a decrease of *Bop* expression in hearts from *Mef2c* mutant embryos. The α-enolase transcript, which is expressed ubiquitously and is independent of *Mef2c* expression, was used as an internal control. (B) Differential *Bop* expression is detected by in situ hybridization in sections of wild-type and *Mef2c* mutant embryos at E9.0. *Bop* transcripts are significantly downregulated in the *Mef2c* mutant. a, atrium; lv, left ventricle; rv, right ventricle; v, ventricle.

We began by fusing a genomic fragment extending from –3304 to +196 bp relative to the transcription start site of the muscle-specific *Bop* exon to the *hsp68* basal promoter upstream of a *lacZ* reporter (Fig. 2B, construct 1). This was tested for expression in F0 transgenic embryos at E11.5. Embryos harboring this transgene showed strong expression of β-galactosidase throughout the developing cardiac chambers and OFT, as well as in developing skeletal muscle cells within the somite myotomes (Fig. 3A-D). The expression pattern of β-galactosidase at this stage recapitulated that of the endogenous *Bop* transcript (Gottlieb et al., 2002).

A DNA fragment extending from –637 to +196 bp fused to *hsp68-lacZ* also directed expression specifically in skeletal muscle and the heart (Fig. 2B, construct 2; Fig. 3E-H). However, the pattern of β-galactosidase expression produced in the heart by this regulatory region was different from that of

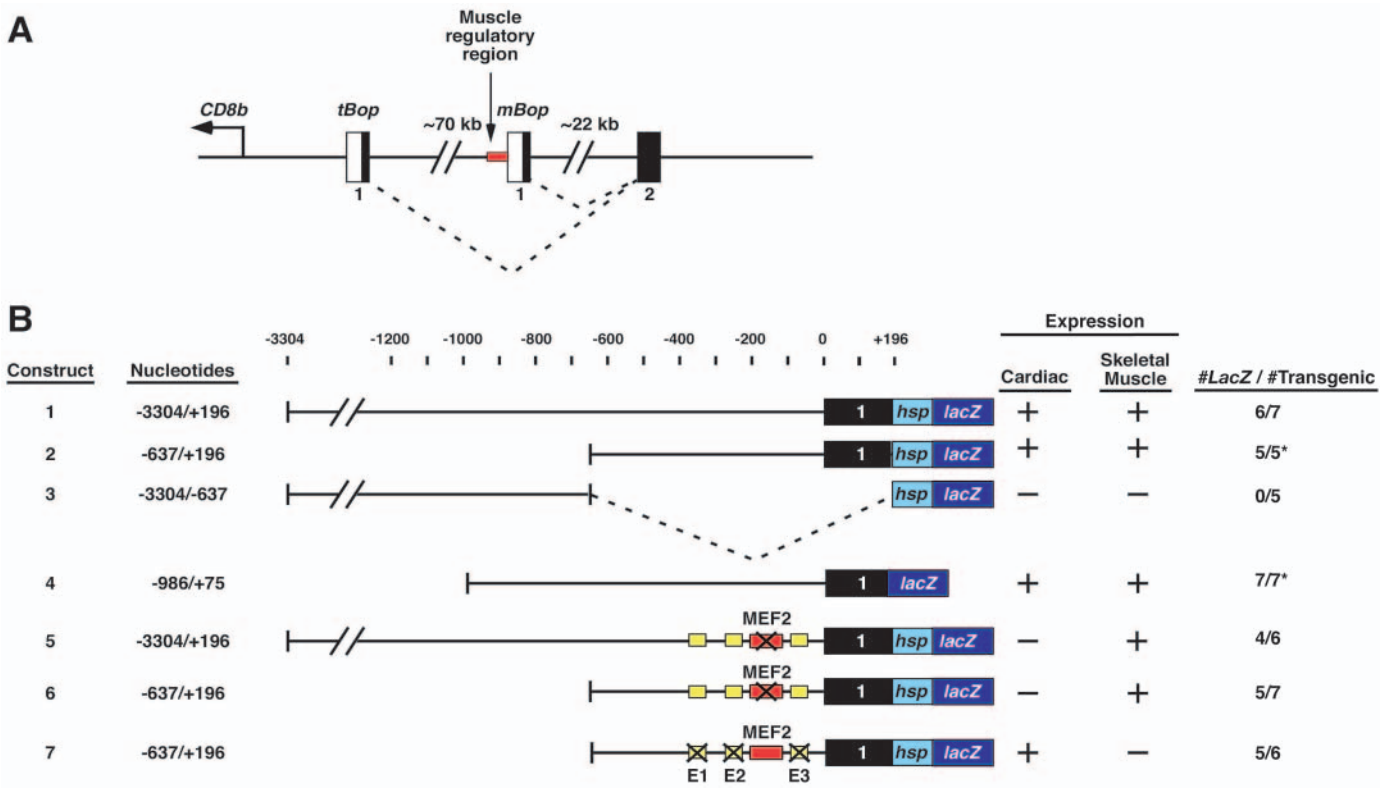

Fig. 2. Transgene constructs used to localize cardiac and skeletal muscle regulatory elements associated with the mouse *Bop* gene. (A) Structure of the 5′ region of the mouse *Bop* gene. The *Bop* gene contains two first exons that code for the amino termini of *Bop* gene products expressed in T lymphocytes (tBOP) and striated muscle (mBOP). These exons, which are separated by an intron of ~70 kb, are spliced to a common second exon located ~22 kb downstream of *mBop* exon 1. Other more 3′ protein coding exons are not shown. White boxes denote 5′ untranslated exon sequence and black boxes denote protein-coding exon sequence. The position of the muscle regulatory region upstream of *mBop* exon 1 is shown in red. (B) Regions of 5′ flanking DNA immediately upstream of *mBop* exon 1 used to create *lacZ* transgenes are shown. Nucleotides are numbered relative to the transcriptional start site, which is designated '0'. Construct number is indicated on the left, and the corresponding expression pattern is summarized on the right. All constructs, except construct 4, contain the indicated upstream genomic regions fused to the *hsp68* basal promoter and the *lacZ* gene. Construct 4 contains the region from –986 to +75 bp fused directly to promoterless *lacZ*. The MEF2 site was mutated in constructs 5 and 6, and the three E-boxes were mutated in construct 7. Asterisk denotes constructs that were also used to generate stable transgenic lines. The numbers of F0 transgenic embryos compared with the total number of transgene-positive embryos are shown.

the larger genomic fragment. Whereas the –3304/+196 region directed expression throughout both the ventricular and atrial chambers, the –637/+196 region was active only in the RV and OFT. The region from the –3304 to –637 bp upstream region showed no transcriptional activity when fused to *hsp68-lacZ* (Fig. 2B, construct 3; data not shown).

The above results indicated that expression of *Bop* in the anterior heart field and skeletal muscle is dependent on the region extending upstream from the muscle-specific exon 1 of the gene to –637 bp. This region appears to be necessary, but not sufficient, for expression in the LV and atrial chambers, as evidenced by the complete loss of cardiac expression when this region was deleted in construct 3. We chose to focus on the cis-regulatory elements involved in expression in the anterior heart field and skeletal muscle, and did not further pursue the LV or atrial regulatory elements.

The temporospatial pattern of expression of construct 2 was further defined by generating stable transgenic lines and analyzing expression on successive days of embryogenesis. Robust *lacZ* expression was seen in the heart tube at E8.0 and thereafter with construct 2 (Fig. 4A-H). Between E8.0 and 9.0, *lacZ* expression was especially strong in the anterior region of the heart tube, including the OFT and conotruncus, with weaker expression extending to the posterior region of the heart tube and into the sinus venosus. After E9.0, *lacZ* expression became localized to the right ventricular region and showed a sharp demarcation at the interventricular groove (Fig. 4G,H). Construct 2 was also expressed in the anterior somite myotomes beginning around E8.75 and was clearly seen at E9.0 (Fig. 4E). Somitic expression progressed caudally in parallel with somite maturation.

To ensure that the *hsp68* basal promoter had no influence on the timing or tissue-specificity of *Bop* regulatory sequences, we created a transgene in which the region from –986 to +75 bp relative to the transcriptional start site was fused to a promoterless *lacZ* reporter (Fig. 2B, construct 4; Fig. 4I-P). Stable transgenic lines harboring this transgene showed β-galactosidase staining throughout the linear heart tube at E9.0 (data not shown), and in the RV and OFT, as well as in the somite myotomes (Fig. 4I-K). Construct 4 was also expressed in the RV and OFT at E11.5, E13.5 (Fig. 4L-N,P) and E15.5 (data not shown). Expression was most prominent along the

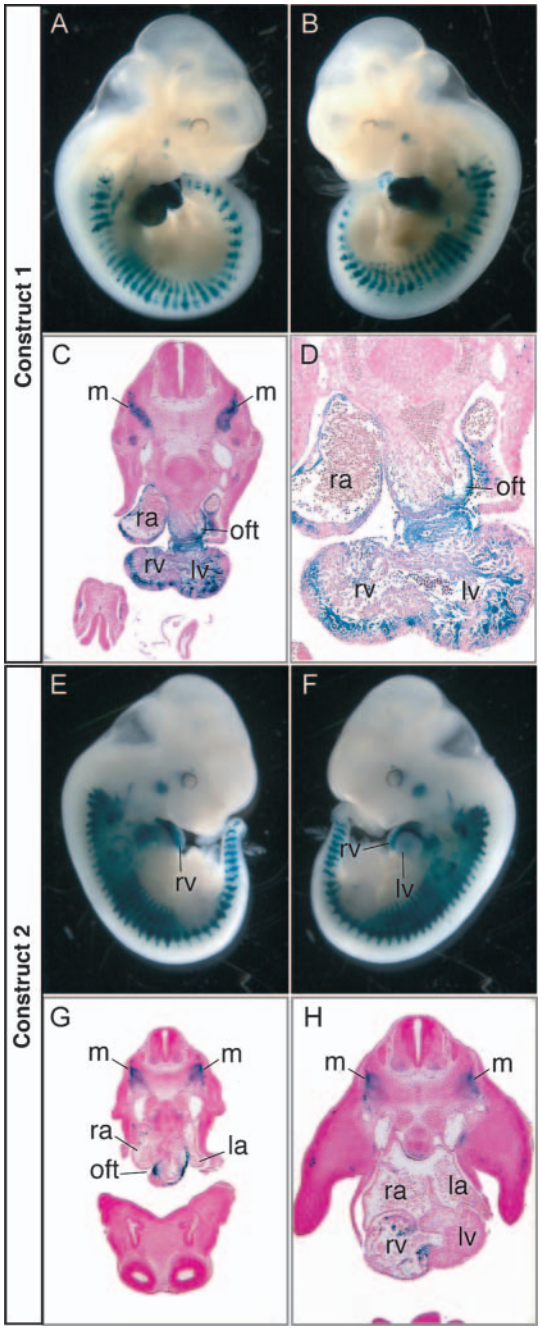

Fig. 3. Expression patterns of transgenes directed by distal (–3303/+196) and proximal (–637/+196) regulatory regions of *Bop* at E11.5. Whole-mount views of F0 transgenic embryos at E11.5 with construct 1 (–3304/+196; A-D) and construct 2 (–637/+196; E-H) are shown (see constructs in Fig. 2). (A,B,E,F) Left and right sides of embryos are shown. (C,D,G,H) Histological sections. (C,D) Robust expression of construct 1 is seen in the ventricular and atrial chambers of the heart and somite myotomes. Identical expression patterns were seen in 6 out of 7 embryos with this construct (data not shown). (G,H) Construct 2 is expressed specifically in the right ventricular chamber and somite myotomes. Identical expression patterns were seen in 5 out of 5 embryos with this construct (data not shown). m, myotomes; oft, outflow tract; ra, right atrium; rv, right ventricle; lv, left ventricle; la, left atrium.

outer curvature of the OFT and at the outlet region of the RV. Construct 4 also showed intense expression throughout developing skeletal muscle (Fig. 4I-P).

Binding of MEF2 to the *Bop* regulatory region

We scanned the –637 bp *Bop* regulatory region for nucleotide sequences conserved in the corresponding regions of the mouse, human, rat and chicken genes, and identified a single MEF2 consensus-binding site ($CTA(A/T)_4TAA/G$) (Gossett et al., 1989) at –329 to –320 bp (Fig. 5A,B). To determine whether this site was a bona fide binding site for MEF2C, we performed gel mobility shift assays using this sequence and extracts from COS-1 cells transfected with a MYC-MEF2C expression plasmid. The MEF2-consensus sequence from the *Bop* control region bound MEF2C avidly (Fig. 5C). This DNA-protein complex was super-shifted by anti-MYC antibody, and was abolished in the presence of an excess of the unlabeled cognate DNA sequence or the MEF2 site from the muscle creatine kinase (MCK) enhancer (Gossett et al., 1989) as a competitor, whereas a mutant sequence failed to compete for MEF2C binding (Fig. 5C). We conclude that MEF2C binds directly to the regulatory region responsible for expression of *Bop* in the anterior heart field.

The MEF2 site is essential for *Bop* expression in the anterior heart field

To determine whether the MEF2-binding site was required for *Bop* expression, the MEF2-binding site was mutated in the context of the –3304/+196 and –637/+196 fragments (Fig. 2B, constructs 5 and 6, respectively) by replacing four consecutive A residues with G residues in the core of the consensus-binding site. Mutation of the MEF2-binding site (Fig. 2B, constructs 5 and 6) abolished *lacZ* expression in the anterior heart field (Fig. 5D,E). Remarkably, however, the MEF2 site mutation expression did not abolish expression in skeletal muscle. We conclude that *Bop* transcription in the anterior heart field is dependent on a single MEF2-binding site, whereas transcription in the skeletal muscle lineage is independent of the MEF2 binding at –329 to –320 bp.

E-boxes are required for *Bop* expression in developing skeletal muscle

Members of the MYOD1 (previously MyoD) family of bHLH transcription factors activate skeletal muscle gene expression by binding E-box consensus sequences (CANNTG) (Olson and Klein, 1994). Within the –637 bp regulatory region of the *Bop* gene, we identified three E-boxes surrounding the essential MEF2 site (Fig. 5A). To determine whether myogenic bHLH proteins bind any of these E-boxes, we performed a gel mobility shift assay using extracts from COS-1 cells transfected with MYOD1/E12 expression plasmids and observed that the region of *Bop* containing the E-boxes bound strongly to the MYOD1/E12 complex (data not shown). Mutation of the E-boxes in the context of the –637/+196 region and the *hsp68-lacZ* transgene (Fig. 2B, construct 7) abolished *lacZ* expression in skeletal muscle, but did not affect cardiac expression (Fig. 6). Collectively, these findings show that the E-boxes within the *Bop* control region are necessary for expression in skeletal muscle but are dispensable for cardiac expression.

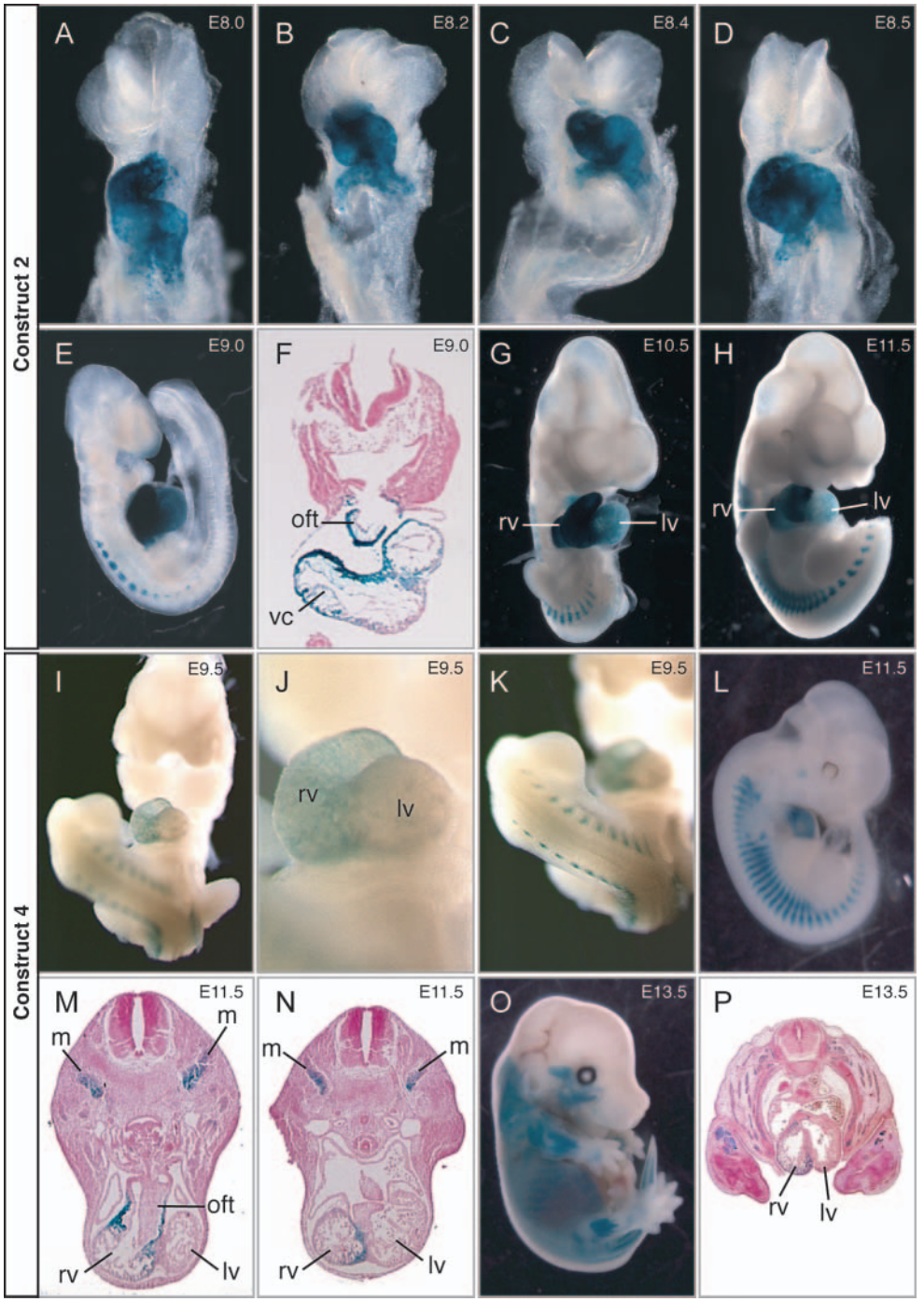

Fig. 4. Expression patterns of transgenes directed by –637/+196 and –986/+75 regulatory regions of *Bop* at different developmental stages. (A-H) Transgenic embryos of the indicated ages from a stable transgenic line harboring construct 2 (–637/+196; see Fig. 2). (A-D,G,H) Frontal views; (E) right-side view; (F) transverse section. Two independent stable transgenic lines displayed identical patterns of *lacZ* expression. (I-P) Transgenic embryos of the indicated ages from a stable transgenic line harboring construct 4 (–986/+75; see Fig. 2). (J,K) Enlarged regions of the embryo shown in I. (M,N) Transverse sections at different levels of the embryo shown in L. (P) Transverse section of the embryo shown in O. rv, right ventricle; lv, left ventricle; oft, outflow tract; vc, common ventricular chamber; m, myotome;

Discussion

Numerous transcription factors have been implicated in cardiac myogenesis and morphogenesis. However, relatively little is known of the regulatory interconnections between cardiac transcription factors, or of the target genes that mediate their actions. The results of this study identify MEF2C as a direct activator of *Bop* transcription in the anterior heart field and its derivatives, and provide a potential explanation for the similarities in cardiac defects seen in mice lacking *Mef2c* and *Bop*, in which the OFT and RV are severely hypomorphic (Gottlieb et al., 2002; Lin et al., 1997).

Control of cardiac *Bop* expression by MEF2C

There are four members of the MEF2 family (MEF2A, MEF2B, MEF2C and MEF2D) in vertebrate organisms and a single MEF2 factor in *Drosophila* (Black and Olson, 1998). MEF2 proteins bind a conserved A/T-rich consensus sequence found in the control regions of the majority of cardiac and skeletal muscle-specific genes, and play numerous roles in growth, differentiation, morphogenesis and remodeling of striated muscles (Black and Olson, 1998). *Drosophila* embryos homozygous for a *Mef2* null allele die during embryogenesis and display a complete loss of differentiation of cardiac, somatic and visceral muscle cells (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995), demonstrating the central role of MEF2 as a regulator of muscle differentiation. Analysis of the functions of the mammalian *Mef2* genes based on loss-of-function phenotypes has been more difficult because the four *Mef2* genes display overlapping expression patterns in developing muscle cell lineages and in other cell types (Edmondson et al., 1994). *Mef2c* is expressed in the cardiac crescent and anterior heart field beginning at E7.75 and subsequently throughout the linear, looping and multichambered heart (Dodou et al., 2004; Edmondson et al., 1994). The other *Mef2* genes are also expressed in the early heart, although their expression is delayed slightly relative to that of *Mef2c* (Edmondson et al., 1994).

Mice lacking *Mef2c* die at E9.5 from severe cardiac defects that include a failure of the RV and OFT to develop (Gottlieb et al., 2002; Lin et al., 1997). These defects resemble those of *Bop* mutant embryos, which die between E9.5 and E10.5 (Gottlieb et al., 2002; Lin et al., 1997). The similarities between the *Mef2c* and *Bop* mutant phenotypes prompted us to investigate whether these genes might act in a cascade of cardiac control genes. Indeed, our results show that cardiac *Bop* expression is dramatically downregulated in *Mef2c* mutant embryos at E9.0, although residual *Bop* expression can be detected in these mutant hearts. The complete loss of cardiac expression of *Bop-lacZ* transgenes lacking the MEF2-binding site suggests that the *Bop* regulatory region we have identified is absolutely dependent on the binding of MEF2. However, it is also possible that other enhancers that are MEF2-independent might support residual expression of the endogenous *Bop* gene in *Mef2c* mutant embryos. Alternatively, or in addition, the residual cardiac expression of the endogenous *Bop* gene in *Mef2c* mutants could reflect functional redundancy between *Mef2c* and other *Mef2* genes, which continue to be expressed in the heart of the *Mef2c* mutant (Lin et al., 1997).

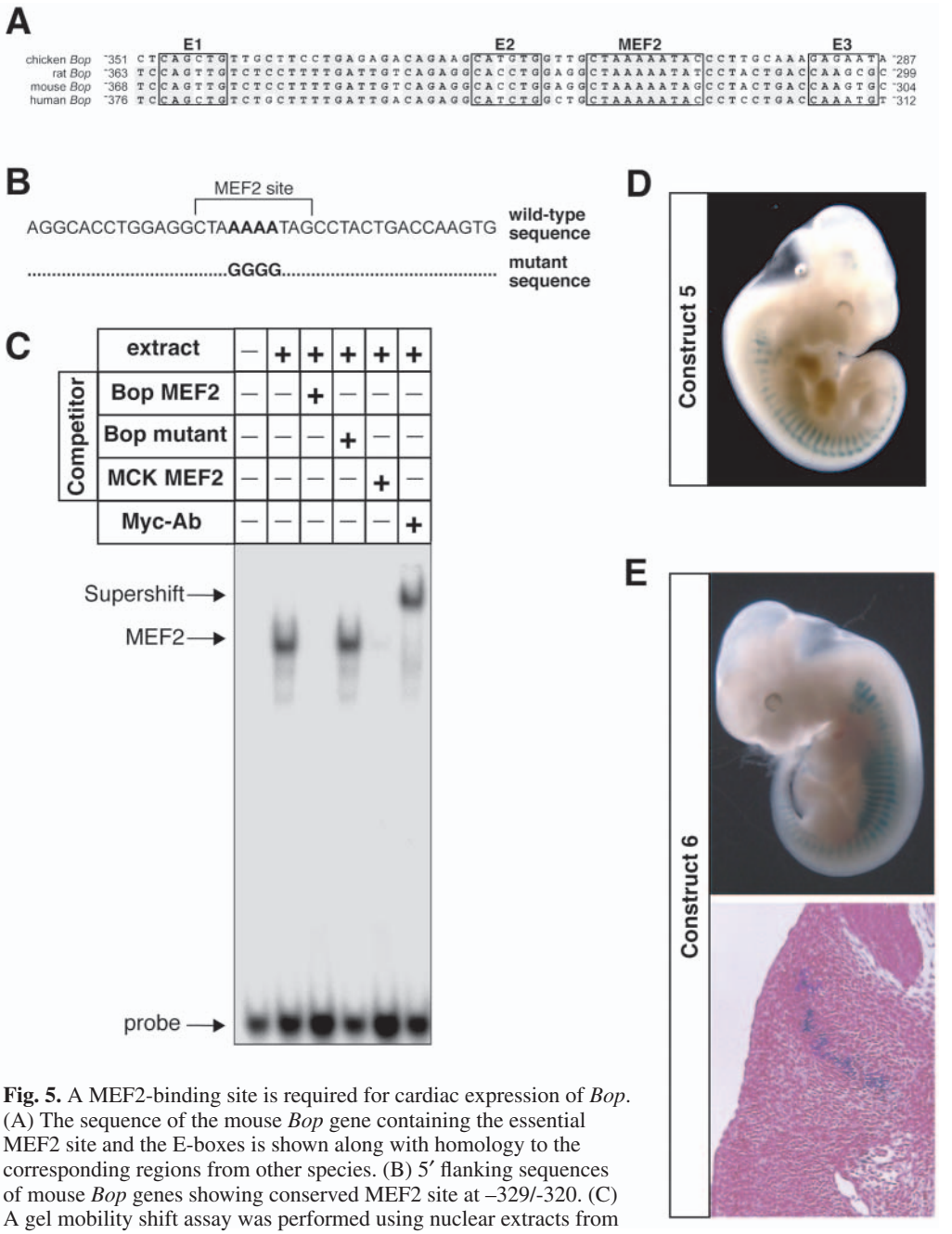

Fig. 5. A MEF2-binding site is required for cardiac expression of *Bop*. (A) The sequence of the mouse *Bop* gene containing the essential MEF2 site and the E-boxes is shown along with homology to the corresponding regions from other species. (B) 5′ flanking sequences of mouse *Bop* genes showing conserved MEF2 site at –329/-320. (C) A gel mobility shift assay was performed using nuclear extracts from COS-1 cells transfected with a MYC-MEF2C expression plasmid and the radiolabeled MEF2 site shown in panel A as probe. Specific and nonspecific competitors were used at 50-fold molar excess. Antibody supershift used 1 μg of polyclonal anti-MYC antibody. (D,E) MEF2 site is essential for *Bop* expression in the anterior heart field in vivo. F0 transgenic mouse embryos were generated using constructs 1, 2, 5 and 6 (see Fig. 2B), and stained for expression of β-galactosidase at E11.5. Mutation of the MEF2 site abolished expression in the anterior heart field without affecting expression in skeletal muscle. Four independent F0 transgenic embryos were analyzed with construct 5 (D) and five independent embryos were analyzed for construct 6 (E). All embryos with each transgene showed comparable expression patterns.

Modular control of *Bop* transcription in the developing heart

The 637 bp of DNA sequence immediately upstream of the muscle-specific *Bop* exon 1 is sufficient and necessary for transcription in the anterior heart field and its derivatives – the RV and OFT. However, in contrast to the larger 3.3-kb upstream region, this smaller region does not direct expression in the LV or atrial chambers. In *Mef2c* mutant embryos, *Bop* expression is reduced throughout the heart, not just in the anterior heart field. Because mutation of the MEF2 site in the context of the 3.3-kb region eliminates all cardiac expression, we conclude that this site is required for expression of *Bop* throughout the heart, but regulatory sequences between –637 bp and –3.3 kb must also be required for left ventricular and atrial expression. This type of modularity of cis-regulatory elements, in which transcription in different regions of the heart depends on separate enhancers, is emerging as a common theme of cardiac

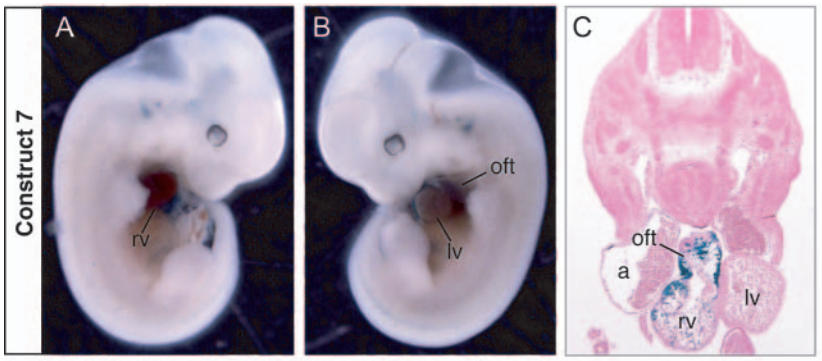

Fig. 6. E-boxes are required for *Bop* expression in developing skeletal muscle. Mutation of the three E-boxes in the *Bop* upstream region (construct 7 in Fig. 2B) caused a loss of skeletal muscle expression without affecting expression in the heart, as seen in a whole-mount lateral view (A,B) and transverse sections (C) of five independent F0 transgenic embryos at E11.5. a, atrium; rv, right ventricle; lv, left ventricle; oft, outflow tract.

gene regulation (Firulli and Olson, 1997). Such modularity is likely to reflect combinatorial interactions among positive and negative regulators expressed in different anatomical regions of the developing heart. In this regard, it should be emphasized that, while MEF2 is clearly an essential activator of *Bop* transcription, there must be additional regulatory factors that cooperate with MEF2 to control cardiac *Bop* expression because MEF2 is highly expressed in other cell types (e.g. neurons) in which *Bop* is not expressed. Whether there are repressors of *Bop* expression in other tissues or additional co-activators that cooperate with MEF2C in cardiac muscle are interesting questions for the future.

Independent regulation of *Bop* transcription in cardiac and skeletal muscle

In addition to its expression in the developing heart, *Bop* is expressed in developing skeletal muscle cells within the somite myotome and in differentiated skeletal muscle fibers (Gottlieb et al., 2002; Hwang and Gottlieb, 1997). Members of the MYOD1 family of bHLH proteins cooperate with MEF2 factors to activate skeletal muscle transcription (Molkentin et al., 1995). Mutational analysis of the *Bop* control region revealed that E-boxes surrounding the MEF2 site are required for expression of the gene in skeletal muscle. The necessity of these E-boxes sites suggests that *Bop* is a direct target of myogenic bHLH proteins. Indeed, MYOD1 binds these sites as a heterodimer with the ubiquitous bHLH protein E12 (data not shown). The MEF2-binding site in the *Bop* control region is not required for expression in skeletal muscle. Conversely, the E-boxes are not required for cardiac expression of *Bop*. The apparent MEF2-independence of *Bop* transcription in skeletal muscle contrasts with the mechanisms that control many other skeletal muscle genes in which E-boxes and adjacent MEF2 sites cooperate to direct skeletal muscle expression (Li and Capetanaki, 1994; Wang et al., 2001). However, MEF2 has also been shown to be capable of activating transcription by associating with myogenic bHLH proteins bound to E-boxes, without a requirement for direct binding of MEF2 to DNA (Molkentin et al., 1995). Thus, the cluster of E-boxes in the *Bop* promoter may obviate the necessity for MEF2 to bind its site in the promoter to activate skeletal muscle transcription.

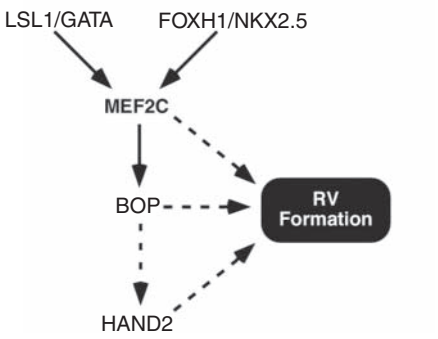

Fig. 7. Putative network of transcription factors involved in ventricular development. Direct interactions of transcription factors with regulatory sequences associated with downstream target genes are shown by solid lines; steps in which transcription factors have been implicated, but direct target genes have not been identified, are shown by broken lines.

The results of this study raise interesting questions about the role of MEF2 in muscle gene regulation and the mechanisms through which it controls cardiac or skeletal muscle transcription. Three tandem copies of a MEF2 site are sufficient to direct expression of a *lacZ* reporter specifically in cardiac and skeletal muscle during mouse development (Naya et al., 1999). The MEF2 site in the *Bop* control region is required for cardiac expression, but apparently does not influence expression in skeletal muscle. By contrast, there are several cases in which a single MEF2 site has been shown to be required specifically for expression in skeletal muscle, but not in heart, such as in the myogenin and *Mrf4* promoters, and in the skeletal muscle-specific promoter of the *Mef2c* gene (Black et al., 1995; Cheng et al., 1993; Naidu et al., 1995; Wang et al., 2001; Yee and Rigby, 1993). Furthermore, there are many genes in which a single MEF2 site is required for expression in both cardiac and skeletal muscle (Anderson et al., 2004; Kelly et al., 2002; Kuisk et al., 1996). Together, these findings underscore the central role of MEF2 in muscle-specific transcription, and indicate that the context of MEF2 sites dictates the specificity of expression of MEF2 target genes.

A cascade of cardiac transcription factors in the anterior heart field

The results of the present and prior studies have begun to reveal a transcriptional pathway involved in development of the anterior heart field and its cardiac derivatives, as schematized in Fig. 7. The LIM-homeodomain transcription factor ISL1 is expressed in cells of the anterior heart field, and is required for RV and OFT formation (Cai et al., 2003). The forkhead transcription factor FOXH1 is also expressed in the anterior heart field, and *Foxh1* mutant embryos, like embryos lacking *Isl1* and *Mef2c*, display defects in the RV and OFT (von Both et al., 2004). ISL1 and FOXH1 directly activate transcription of *Mef2c*

in the anterior heart field by activating two independent cardiac enhancers in collaboration with GATA factors and NKX2.5, respectively (Arceci et al., 1993; Dodou et al., 2004). Thus, these factors appear to act at the top of a cascade of cardiac transcription factors in the anterior heart field. Our data demonstrates that *Bop* is a direct target of MEF2C during anterior heart field development, implying that *Bop* is indirectly regulated by ISL1/GATA factors and FOXH1/NKX2.5. It should be pointed out that the phenotype of *Mef2c* mutant embryos is more severe than that of *Bop* mutants, suggesting that *Bop* is not the sole downstream target of MEF2C in the developing anterior heart field, and that it may act together with other MEF2C target genes.

It is intriguing that, although both *Mef2c* and *Bop* are expressed throughout the developing heart, the phenotypes associated with null mutations in these genes are largely confined to the anterior heart field and its derivatives. This anatomic restriction of cardiac defects could reflect redundant transcriptional mechanisms that operate outside the anterior heart field. Alternatively, an arrest in anterior heart field development may be a general consequence of diverse cardiac anomalies at E9.5.

The *Bop* and *Mef2c* mutant phenotypes show an intriguing similarity to that of *Hand2* null mice, including the absence of the right ventricular chamber and a defect in looping morphogenesis (Lin et al., 1997; Srivastava et al., 1997). However, thus far there is no evidence indicating that MEF2C directly activates *Hand2* transcription (McFadden et al., 2000). In fact, *Hand2* expression is eliminated in the heart of *Bop* mutant mice (Gottlieb et al., 2002), suggesting that *Hand2* expression is governed by BOP. As our results indicate that *Bop* is a direct target of MEF2C, it is likely that MEF2C regulates *Hand2* expression indirectly via *Bop* in a transcriptional cascade during chamber-specific heart development (Fig. 7).

Although the essential role of *Bop* in development of the anterior heart field has been clearly established based on the phenotype of *Bop* mutant embryos (Gottlieb et al., 2002), the precise mechanism of action of BOP and its transcriptional targets remains unclear. BOP does not bind DNA directly and, instead, acts together with other chromatin-remodeling factors and transcriptional regulators. BOP has been shown to associate with the transcriptional co-activator skNAC, which is expressed specifically in cardiac and skeletal muscle (Sims et al., 2002). BOP contains a SET domain (Gottlieb et al., 2002), which in other proteins has been shown to possess histone methyl transferase activity (Lachner and Jenuwein, 2002), and a MYND domain, shown to function as a protein-protein interaction domain in other transcription factors (Lutterbach et al., 1998). Previous in vitro data suggest that BOP functions as a histone deacetylase-dependent transcriptional repressor by interacting with class I HDACs (Gottlieb et al., 2002). Thus, BOP is likely to modulate the expression of key cardiac effector genes via its association with other components of the transcriptional machinery required for development of the anterior heart field.

Potential roles of MEF2 and BOP in the adult heart

In addition to the roles of MEF2 factors in myogenesis and morphogenesis in the developing heart, MEF2 factors have been implicated in hypertrophic growth of the adult heart in response to stress signaling (Zhang et al., 2002), and in the control of genes involved in cardiac energy metabolism (Czubryt et al., 2003; Moore et al., 2003). *Bop* expression is maintained in the adult heart, although its functions at that stage remain unknown. In the future, it will be of interest to determine the extent to which the transcriptional circuits involved in the development of the heart are redeployed during adulthood to maintain cardiac function.

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