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 Isll 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 Isll, 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 PstI site of the pBluescript SKII (+) plasmid (pBSKII-4kbBOP). Using PstI and SalI 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 Smal 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 Smal site of the hsp68-lacZ vector (Kothary et al., 1989). Construct 3 was generated by cloning a SalI/EcoRI fragment (~3304 to ~637) into the Smal site of the hsp68-lacZ vector. Construct 4 was generated by cloning the Bop fragment (~986 to ~75) using blunt-end ligation into a Smal 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 EcoRI and PstI and the ends were filled in with Klenow fragment and blunt-end ligated into the Smal 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 SalI, whereas construct 4 was digested with XhoI and NotI 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 using a Rapid-hyb buffer at 65°C with a Bop cDNA that was [32P]-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-rolease 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 35S-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., 1998) 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′-AGGCCACCTGAGGCTTTAAAATAGCC-TACTGACCAAGTG-3′;

Bop MEF2mt oligo, 5′-AGGCCACCTGAGGCTATGGGCTAG-CCTACTGACAAAGTG-3′; and

MCK MEF2 oligo, 5′-GATCGCTTAAATATACCCGTCGG-3′.

Annealed oligonucleotides were radiolabeled with [32P]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 pcDNA/MYC-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
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 fused to promoterless \( lacZ \) (Fig. 2B, construct 4; Fig. 4I-P). Stable transgenic lines harboring this transgene showed \( \beta \)-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
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.
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-αlacz 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).

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 transcriptional regulation of Bop by MEF2.

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.
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.

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 in other proteins has been shown to possess histone methyl transferase activity (Lachner and Jenuwein, 2002), and which in other proteins has been shown to possess histone deacetylase-dependent transcriptional repressor by methyl transferase activity (Lachner and Jenuwein, 2002). BOP contains a SET domain (Gottlieb et al., 2002), 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 reprogrammed during adulthood to maintain cardiac function.

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