Potential Role of Helix-Loop-Helix Proteins in Cardiac Gene Expression

Sylvia M. Evans, Barbara A. Walsh, Cathy B. Newton, Jackie S. Thorburn, Paul D. Gardner, Marc van Bilsen

Because helix-loop-helix (HLH) transcription factors appear to play an important role in mesodermal development, we have investigated the potential role of these factors in cardiac gene expression. HLH proteins interact with DNA at consensus “E-box” sites and may be tissue specific or more widely expressed. We have examined cardiac cells for expression and regulation of widely expressed factors MyoD, MyoD family members, and Pan1/Pan2 and the inhibitor of differentiation (Id) by RNase protection analysis. The effect of MyoD, Id, and Pan1/Pan2 expression on skeletal and cardiac promoters in cardiac cells was examined by transient cotransfection studies. Our results indicate that neonatal ventricular cells are a functional HLH environment, because MyoD can activate a skeletal muscle-specific promoter in these cells. MyoD, however, has no effect on the expression of several genes that are expressed in cardiac cells. In addition, Id may be an early response gene for signal transduction in cardiac cells, because increases in Id mRNA occurred within 30 minutes of stimulation with serum or phenylephrine. Activities of three cardiac promoter elements in primary ventricular myocytes were not downregulated by Id. Surprisingly, expression of Pan1 and Pan2 exhibited a strong negative effect on cardiac expression of the myosin light chain-2 promoter. (Circulation Research 1993;73:569-578)

Key Words: gene transcription • helix-loop-helix proteins • E-boxes • cardiac gene expression

The factors that are involved in determination of cardiac muscle are unknown. In the vertebrate embryo, cardiac muscle derives from the splanchnic mesoderm, part of the lateral mesoderm. Several members of the basic helix-loop-helix (HLH) family of transcription factors appear to play a pivotal role in mesodermal development, including MyoD, MyoD family members,1 and Twist.2 MyoD family is specifically expressed in skeletal muscle lineages, whereas Twist appears to be expressed in the lateral mesoderm. The mesodermal origin of cardiac cells and overlapping expression of many genes in cardiac and skeletal muscle suggest that basic HLH proteins may play a role in cardiac gene expression.

Basic HLH proteins affect gene expression by binding to a consensus DNA binding site, the “E-box.” The DNA binding activity of tissue-restricted HLH proteins such as MyoD may be modulated by their interaction via the HLH domain with more widely expressed HLH family members. Such family members include splice variants of the E2 gene, two of which are E12 and E47.3 E12 and E47 form heterodimers with tissue-specific basic HLH proteins, such as the MyoD family, increasing their DNA binding affinity. Rat homologues of the human E12/E47 genes, Pan2/Pan1, have also been cloned.4 Cotransfection of E47 or Pan1/Pan2 expression vectors with E-box–containing promoter response elements results in an upregulation of promoter activity.4-6

Another family of widely expressed HLH factors negatively modulates tissue-specific expression. One of these is the inhibitor of differentiation (Id).7 Id lacks the basic DNA binding domain of other HLH family members, resembling dominant negative mutants of MyoD. Id mRNA levels are high in skeletal myoblasts grown in high serum and are downregulated in response to serum withdrawal, as myotube formation occurs and skeletal muscle genes are activated. Id cotransfections adversely affect tissue-specific activity of several E-box–dependent promoter elements.7-10 In this way, endogenous levels of E12/E47 and Id may create an environment that is permissive for MyoD or analogous HLH proteins.

We have been interested in investigating the possible role of HLH proteins in cardiac development and in cardiac gene expression (for discussion, see Reference 11). In the present study, we examine the role of Id and Pan1/Pan2 in cardiac gene expression. Our results indicate that Id may be a mediator of cardiac response to physiological stimuli, that MyoD or a MyoD analogue could function in the cardiac environment, and that Pan1 and Pan2 have the potential to negatively regulate cardiac gene expression. In addition, we have found three cardiac-specific promoter elements to be unaffected by cotransfection with Id, indicating that their activation in neonatal cardiac cells is independent of an E-box–mediated pathway that can be countermanded by Id.

Materials and Methods

Cell Culture

Continuous cell lines AR42J, C3H10T1/2, and C2C12 were obtained from American Type Tissue Collection
and cultured according to specifications. Sol8 muscle cells\textsuperscript{12} were obtained from Scott Henderson, University of California, Los Angeles, Rat2 fibroblasts\textsuperscript{13} from Gerry Weinmaster, University of California, Los Angeles, and RMo muscle cells\textsuperscript{14} from Paul Gardner, University of Texas Health Sciences Center at San Antonio. Primary neonatal ventricular myocytes were isolated and cultured as previously described.\textsuperscript{15} Cultures prepared in this manner are more than 95% pure. For continuous cell lines, high-serum conditions included Dulbecco’s modified Eagle’s medium (DMEM, GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO) or 20% FBS for skeletal muscle cells. Low-serum conditions were DMEM with 4% horse serum (Gemini Bioproducts). For primary ventricular cultures, high- and low-serum conditions were DMEM/medium 199 (4-1, GIBCO) supplemented with 10% FBS or 4% horse serum, respectively.

**RNase Protection Analysis**

The Weintraub laboratory provided a riboprobe vector for Id, pMH18AR.\textsuperscript{7} Restriction of this plasmid with *Xba*I and transcriptions with T7 RNA polymerase generates a 1080-nt riboprobe that hybridizes to 927 nt of mouse Id mRNA. Hybridization of the Id probe to rat mRNAs resulted in three protected fragments of 330, 260, and 220 nt. The human U1 snRNA plasmid pSP65U\textsuperscript{16} was cut with *Hind*III and transcribed with SP6 RNA polymerase to generate an antisense probe of 315 nt, 164 nt of which encodes U1 snRNA.

RNA isolation and RNase protection assays were performed as previously described.\textsuperscript{15} For quantitation of Id mRNA and U1 snRNA signals, autoradiograms were scanned overnight by a radioanalytic scanner (AMBIS Systems, Inc, San Diego, Calif).

**Transient Transfections**

Expression vectors for MyoD and Id were generously provided by Hal Weintraub and members of his laboratory and include the parent vector pEMSV, the MyoD expression vector pEMc11s, the antisense Id expression vector pE:Id(A), and the sense Id vector pE:Id(S).\textsuperscript{7,17} Pan1 and Pan2 expression vectors pCMVPN1 and pCMVPN2 and parental vector plPASA were kindly sent by Chris Nelson, University of California, Riverside.\textsuperscript{4} Eric Olson, M.D. Anderson Cancer Center, Houston, Tex, generously provided his pCKCATE4 construct,\textsuperscript{18} from which the muscle creatine kinase (MCK) 302-bp enhancer was excised with *Bam*HI and subcloned into the luciferase expression vector pT109.\textsuperscript{19} The α-ACHRLuc plasmid is a 720-bp fragment of the α-achtyline receptor subunit (α-AChR) promoter subcloned into the luciferase vector pXP1 as previously described.\textsuperscript{20} The parent plasmid for atrial natriuretic factor (ANF) – and myosin light chain-2 (MLC2)–luciferase promoter expression vectors is pSV-OAL5.\textsuperscript{15,21} The MLC2 deletion construct pMLC50Luc contains nucleotides –30 to +20 of the MLC2 promoter region cloned into the multiple cloning site of luciferase vector pXP1. The cytomegalovirus (CMV) –β-galactosidase expression vector used for normalization of transcription efficiency was pON249,22 and the control Rous sarcoma virus (RSV) –luciferase plasmid has been described.\textsuperscript{23}

Transient transfection analyses were performed by a modification of the calcium phosphate precipitation method\textsuperscript{24} as previously described.\textsuperscript{15} Neonatal ventricular myocytes were plated at a density of 2.7×10\textsuperscript{5} cells per 60-mm dish and incubated in low-serum medium for 48 hours after washing the calcium phosphate precipitate. C3H10T1/2 cells were transfected in high serum at 80% confluence in 60-mm dishes and were also incubated for 48 hours in low-serum medium after washing the calcium phosphate precipitate. Skeletal muscle cell lines Sol8 and C2C12 were transfected in high serum at 80% confluence, were placed in low-serum medium after transfection, and were allowed to fuse before harvesting (usually after 48 to 72 hours in fusion medium). Cells were harvested by adding a 0.5% Triton lysis buffer as previously described.\textsuperscript{25} Luciferase and β-galactosidase activities were assayed as previously described,\textsuperscript{15} and protein concentrations were determined by protein assay (Bio-Rad Laboratories, Richmond, Calif).

**Results**

**Pan1 and Pan2 Isoform mRNAs Are Expressed in Cardiac Muscle**

Previously, Northern blot analysis of cardiac RNA has shown a 3.0-kb mRNA species that hybridizes to probes for Pan1 or Pan2.\textsuperscript{4} However, the shared sequences of these isoforms do not allow a conclusion to be reached from these data as to which isoform is expressed. Pan1 and Pan2 are two of several isoforms of the E2A gene. Because there appear to be functional differences between E2A isoforms that may play a role in tissue-specific expression,\textsuperscript{3,26} we examined which of these isoforms is expressed in cardiac muscle cells by RNase protection analysis. Probes from the HLH regions, specific for Pan1 or Pan2 mRNAs, were used to analyze RNAs from AR42J cells (the parent rat pancreatic cell line), RMo cells (a rat muscle cell line), rat primary neonatal ventricular myocytes, and adult rat ventricle and atrium. Our results with Pan1- and Pan2-specific probes were identical and indicated that both isoforms are expressed in all cell types examined. Levels of Pan1 and Pan2 mRNAs in primary neonatal ventricular cells were comparable to those seen in parental AR42J cells and in RMo myoblasts and myotubes. Levels of Pan1 and Pan2 mRNAs in RNA from whole ventricle or atria were comparatively lower (data not shown).

**Id mRNA Is Expressed in Cardiac Muscle and Is Responsive to Serum Levels**

To determine whether Id mRNA is expressed in cardiac muscle, we performed RNase protection analysis using a full-length (0.9-kb) probe derived from the mouse Id 1 cDNA.\textsuperscript{7} Because we were interested in determining whether Id was expressed in rat primary neonatal ventricular cells for future experiments, we examined Id mRNA levels in a variety of mouse and rat tissues to ensure that results for the mouse Id probe with rat RNAs were consistent with those seen for mouse RNAs. We also asked whether Id mRNA levels would be regulated in primary cardiac cultures in response to serum levels, as they are in skeletal muscle cell lines. To quantitate our results, we analyzed each RNA sample with the Id probe and a probe for the ubiquitously expressed, highly conserved U1 snRNA\textsuperscript{16} to normalize for the amount of RNA. The data are
mRNA levels were fourfold higher in cardiac myocytes incubated for 48 hours in high serum than for those incubated 48 hours in low serum. This was comparable to the 10-fold difference observed between Id mRNA levels in rat skeletal muscle cells grown in high serum and those differentiated in low serum, consistent with previous observations.7

Time Course of Id mRNA Induction in Response to Serum and Phenylephrine

Having observed that Id mRNA levels were responsive to serum levels, we investigated the time course of this response to determine whether Id could be implicated as an early mediator of signal transduction. In addition, we investigated the response of Id mRNA levels to phenylephrine (PE) stimulation of cardiac myocytes. PE stimulation of primary ventricular myocytes is a well-defined model system for cardiac hypertrophy.27 In response to hypertrophic stimuli, isoform switching and upregulation of a number of cardiac specific genes occurs.15,21,28,29 Primary ventricular myocytes from neonatal rats were incubated without serum for 24 hours and then stimulated with serum (10% fetal calf serum) or PE (10^{-6} M) for ½, 1, 2, 4, or 48 hours. Control cultures were incubated in serum-free medium. The RNA from these cells was analyzed by RNase protection assays using probes for Id mRNA and U1 snRNA (Fig 2). Our results indicated that Id mRNA was induced by PE, as with serum, but to a lesser extent. Serum stimulation resulted in a threefold increase of Id mRNA after 1 hour, which peaked to sixfold at 2 hours and remained threefold higher than control Id mRNA levels after 48 hours. Id mRNA increase with PE was threefold at 1 hour and gradually returned to control levels at 48 hours. These results suggest that Id could be involved in the gene switching that occurs in the PE stimulation of cardiac cells.

Effect of MyoD on Skeletal/Cardiac Muscle–Specific Reporter Gene Expression

Although MyoD transcripts have not been observed in embryonic or neonatal cardiac cells (Reference 30 and our unpublished observations), we wished to examine whether neonatal ventricular myocytes are permissive for MyoD activation of a skeletal muscle–specific
Table 1. Effect of MyoD Expression on Luciferase Reporters in C3H10T½ Cells

<table>
<thead>
<tr>
<th>Luciferase reporter</th>
<th>HLH</th>
<th>Luc/β-gal (mean±SEM)</th>
<th>Fold increase</th>
</tr>
</thead>
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<td>pEMells</td>
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<td>pEMells</td>
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<td>pEMells</td>
<td>&lt;0.100</td>
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<td>148 000±9460</td>
<td>×7</td>
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HLH, helix-loop-helix expression vector; Luc, luciferase activity; β-gal, β-galactosidase activity; NE, no effect.

Transient transfection assays were performed as described in "Materials and Methods." Luciferase reporter (5 µg) was cotransfected with 15 µg HLH and 2 µg pON249, a β-galactosidase expression vector. pEMSV is the parental vector for pEMells, the MyoD expression plasmid. Data shown are the average values obtained from one experiment performed in triplicate and are representative of at least three separate experiments. Absolute values of Luc varied from experiment to experiment, but fold decreases or increases were consistent. Separate experiments were performed with different plasmid DNA preparations. Luc is shown normalized to β-gal.

In addition, we wanted to investigate the effect of MyoD on cardiac-specific promoters and on a cardiac promoter that may be expressed in slow skeletal muscle. In these studies, we have performed transient cotransfection experiments with a MyoD expression vector and cardiac/skeletal muscle promoter–luciferase reporter constructs. We have used the following muscle promoter regions: one exclusive to skeletal muscle, a 720-bp fragment of the γ-AchR promoter;³⁰ one expressed in both skeletal and cardiac muscle, the 302-bp MCK enhancer;³⁸ and two expressed in cardiac muscle, ANF and MLC2 promoters (3003 and 2700 bp, respectively).¹⁵,³¹ The MCK enhancer has been shown to be expressed in skeletal and cardiac cells in transgenic mice, although, as with the endogenous MCK gene, its level of expression is much lower in cardiac muscle than in skeletal muscle.³¹ The MLC2 gene is expressed in slow skeletal muscle, and its mRNA levels are comparable in the two tissue types.³² However, it is unknown whether the 2700-bp promoter region in our luciferase reporter construct can confer slow skeletal muscle expression. Both the γ-AchR promoter and the MCK enhancer have been shown to bind MyoD at specific E-box sites and are activated in response to cotransfection with a MyoD expression vector.²⁻²⁰

Transient cotransfections were performed in C3H10T1/2 embryonic fibroblast cells, which have been shown to be permissive for the activation of skeletal muscle genes by MyoD. Control transfections were performed with expression vector pEMSV and various luciferase reporters, with and without cloned promoter regions, as indicated in Table 1. All transfections included a β-galactosidase reporter, pON249, as an internal control for transfection efficiency. The results of these cotransfections studies are indicated in Table 1. As expected, the MyoD expression vector pEMc11s activated py-AchRLuc and pMCKLuc (fourfold and sevenfold, respectively), whereas it had no effect on the parental reporter plasmids, pXP.1 and pT109. However, MyoD also activates the ANF and MLC2 promoters. In addition, MyoD activated the RSV-luciferase promoter-reporter plasmid,³³ MyoD also activated pON249 approximately twofold (data not shown). The activation by MyoD of non–muscle-specific promoters has been reported previously.¹³³

In contrast to these results in C3H10T1/2 cells, transient cotransfection studies in primary neonatal ventricular cardiocytes indicated that MyoD activated py-AchRLuc to an extent similar to that seen in 10T1/2 cells but did not activate any of the other luciferase reporters (Table 2). Again, an approximately twofold activation of pON249 by MyoD was observed (data not shown).

Effect of Id on Skeletal/Cardiac Muscle–Specific Reporter Gene Expression

Previous cotransfection studies of Id expression vectors and skeletal or immunoglobulin promoter-reporter constructs have demonstrated a negative effect of Id on E-box–dependent tissue-specific expression.⁷⁻¹⁰ We wanted to examine the effect of Id on the cardiac-specific activity of the MCK enhancer and the ANF and MLC2 promoter regions. The activity of the E-box–containing MCK enhancer in skeletal muscle is downregulated by Id.⁷ This enhancer is active in cardiac muscle, although to a lower level than its activity in skeletal muscle.³¹ If Id can downregulate the MCK enhancer in cardiac muscle, it could indicate that an E-box–dependent pathway is being used for tissue-specific expression in cardiac muscle. The ANF and MLC2 promoter regions contained in the luciferase...
reporter constructs contain several E-box sequences. However, mutation of the E-box within a cardiac-specific 250-bp promoter of the MLC2 gene does not appear to appreciably affect its activity in cardiac cells.34

Transient cotransfections of Id expression vector pE:Id(S)7 and luciferase reporters were performed in primary cardiac cells in low serum, where endogenous Id mRNA levels were lower than in high serum. Control transfections included the expression vector, pEMSV, an Id antisense construct pE:Id(A), and the luciferase reporter vectors. The Id expression vector DNAs were tested with skeletal promoter-reporters in a 6:1 ratio (micrograms Id DNA:micrograms reporter DNA) by transient transfection into skeletal muscle cell lines C2C12 and S018 and behaved as expected, downregulating expression of pγ-AchRLuc and pMCKLuc an average of 20-fold and 4-fold, respectively (data not shown). These results with the MCK enhancer are consistent with those of other investigators using chloramphenicol acetyltransferase reporter constructs.7

Results of Id cotransfections in cardiac cells are shown in Table 3. No effect of Id expression was observed on pMCKLuc, pANF3003Luc, or pMLC2700Luc promoter-reporter constructs. Similarly, cotransfections of PE:Id(S) with promoter deletion constructs pANF638Luc, PANF323Luc, and pMLC250Luc21,35 had no effect on luciferase expression (data not shown). However, cotransfection of the Id expression vector did counteract MyoD activation of pγ-AchRLuc in cardiac cells, indicating that cardiac cells are permissive for Id (data not shown).

Effect of Pan1 and Pan2 on Skeletal/Cardiac Muscle–Specific Reporter Gene Expression

E12 and E47, human homologues of Pan2 and Pan1, respectively, have been shown to interact synergistically with MyoD to activate the MCK enhancer in C3H10T1/2 cells.5 Transfection of E47 expression vectors alone can also activate the MCK enhancer.26 In addition, Pan1 and Pan2 expression vectors can activate an oligomerized E-box–containing enhancer from the chymotrypsin gene, which is contained within the reporter plasmid pCHY7Luc.4 Therefore, activation of a control region by Pan1 or Pan2 in a transient transfection assay could implicate a potential E-box–dependent pathway. We have examined the response of skeletal and cardiac promoters to coexpression of either Pan1 or Pan2 in both 10T1/2 cells and primary cardiac cells. Data from the 10T1/2 transfections are shown in Table 4. As expected from results of previous investigators, both pCMVPan1 and pCMVPan2 activated pCHY7Luc and pMCKLuc approximately fourfold. In addition, pγ-AchRLuc was also activated from 6-fold to 15-fold. Data shown are normalized to total cellular protein rather than β-galactosidase activity, as expression of the CMV–β-galactosidase gene in pON249 was consistently downregulated fourfold to fivefold by Pan1 and Pan2 relative to the Pan expression vector pLPAΔS. Although the same effect of Pan expression vectors on the CMV–β-galactosidase activity was observed in cardiac cell transfections, Pan1 and Pan2 did not activate the other reporter genes in cardiac cells, as shown in Table 5. Cotransfection with Pan expression plasmids did not appreciably affect the expression of pMCKLuc, pγ-AchRLuc, or pANF3003Luc in neonatal ventricular myocytes. However, the expression of pMLC2700Luc was strongly downregulated.

Effect of Pan1 and Pan2 on MLC2 Gene Expression

Because Pan1 and Pan2 expression appeared to have such a strong and specific negative effect on expression of pMLC2700Luc, we decided to examine this effect more closely, by varying the amount of pCMVPan1 and pCMVPan2 DNAs. Results are shown in Table 6. Increasing the ratio of Pan1 and Pan2 expression vec-

<table>
<thead>
<tr>
<th>Luciferase reporter</th>
<th>HLH</th>
<th>Luc/β-gal (mean±SEM)</th>
<th>Fold increase</th>
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<td>pT109</td>
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HLH, helix-loop-helix expression vector; Luc, luciferase activity; β-gal, β-galactosidase activity; NE, no effect.

Translational assays were performed as described in "Materials and Methods." Luciferase reporter plasmid (5 μg) was cotransfected with 15 μg HLH and 2 μg pON249, a β-galactosidase expression vector. pEMSV is the parental vector for pEMmlls, the MyoD expression plasmid.25 Data shown are the average values obtained from one experiment performed in triplicate and are representative of at least three separate experiments. Absolute values of Luc varied from experiment to experiment, but fold decreases or increases were consistent. Separate experiments were performed with distinct plasmid DNA preparations. Luc is shown normalized to β-gal.
tors to pMLC2700Luc resulted in a greater decrease in luciferase activity of pMLC2700Luc, indicating that the fold decrease was dependent on the amount of Pan1 and Pan2 protein. To attempt to map the site of the Pan1/Pan2 effect, we performed transfections with various MLC2 promoter deletion-reporter constructs: pMLC2700Luc, pMLC800Luc, pMLC250Luc,35 and pMLC50Luc (see “Materials and Methods”). The negative effect appeared to be targeted to sequences throughout the 2700 bp promoter region, because a progressive decrease in the negative effect was observed with progressive truncations of the promoter. The pMLC250Luc and pMLC50Luc reporters exhibited a twofold decrease with Pan1/Pan2 expression. The latter construct deletes both E-boxes that are contained in the MLC250 promoter region. In addition, we performed

<table>
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<th>Luc/β-gal (mean±SEM)</th>
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HLH, helix-loop-helix expression vector; Luc, luciferase activity;  β-gal, β-galactosidase activity; NE, no effect.

Transient transfection assays were performed as described in “Materials and Methods.” Luciferase reporter (3 μg) was cotransfected with 18 μg HLH. pEMSV is the parental vector for the inhibitor of differentiation (Id) antisense and Id sense expression plasmids, pE:Id(A) and pE:Id(S), respectively. Data shown are the average values obtained from one experiment performed in triplicate and are representative of at least three separate experiments. Absolute values of Luc varied from experiment to experiment, but fold decreases or increases were consistent. Separate experiments were performed with distinct plasmid DNA preparations. Luc is shown normalized to β-gal. Fold decrease is a comparison between results with pE:Id(A) and pE:Id(S).

<table>
<thead>
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<th>Luciferase reporter</th>
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<th>Luc/protein (mean±SEM)</th>
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<td>pPRLLuc</td>
<td>pLPAAS</td>
<td>226±26.1</td>
<td></td>
</tr>
<tr>
<td>pPRLLuc</td>
<td>pCMVPan1</td>
<td>394±25.6</td>
<td>NE</td>
</tr>
<tr>
<td>pPRLLuc</td>
<td>pCMVPan2</td>
<td>235±26.1</td>
<td>NE</td>
</tr>
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<td>pCHY7Luc</td>
<td>pLPAAS</td>
<td>9730±3070</td>
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<td>pCHY7Luc</td>
<td>pCMVPan1</td>
<td>36 460±7170</td>
<td>×4</td>
</tr>
<tr>
<td>pCHY7Luc</td>
<td>pCMVPan2</td>
<td>41 100±5250</td>
<td>×4</td>
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<td>pT109</td>
<td>pLPAAS</td>
<td>15 600±111</td>
<td></td>
</tr>
<tr>
<td>pT109</td>
<td>pCMVPan1</td>
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<tr>
<td>pT109</td>
<td>pCMVPan2</td>
<td>26 700±825</td>
<td>×2</td>
</tr>
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<td>pLPAAS</td>
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<tr>
<td>pMCKLuc</td>
<td>pCMVPan1</td>
<td>189 000±60 150</td>
<td>×15</td>
</tr>
<tr>
<td>pMCKLuc</td>
<td>pCMVPan2</td>
<td>109 000±6030</td>
<td>×8</td>
</tr>
<tr>
<td>pXP.1</td>
<td>pLPAAS</td>
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<td>pXP.1</td>
<td>pCMVPan1</td>
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<td>pCMVPan2</td>
<td>163±18.3</td>
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<td>pLPAAS</td>
<td>654±84.1</td>
<td></td>
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<td>pAchRLuc</td>
<td>pCMVPan1</td>
<td>10 000±3450</td>
<td>×15</td>
</tr>
<tr>
<td>pAchRLuc</td>
<td>pCMVPan2</td>
<td>3645±1050</td>
<td>×6</td>
</tr>
</tbody>
</table>

HLH, helix-loop-helix expression plasmid; Luc, luciferase activity; NE, no effect.

Transient transfection assays were performed as described in “Materials and Methods.” Luciferase plasmid (10 μg) was cotransfected with 1 μg HLH. pLPAAS is the parental vector for pCMVPan1 and pCMVPan2 expression plasmids. Data shown are the average values obtained from one experiment performed in triplicate and are representative of at least three separate experiments. Absolute values of Luc varied from experiment to experiment, but fold decreases or increases were consistent. Separate experiments were performed with distinct plasmid DNA preparations. Luc is shown normalized to total cellular protein.
transfections with two mutants of this promoter region, an E-box mutant and an Hf1b/MEF2 site mutant,\textsuperscript{34} to see whether either of these sites was the target for the negative Pan effect. The Hf1b mutant has been previously shown to have a strong negative effect on the MLC250 promotor. Both mutants exhibited the decrease in activity in response to coexpression of Pan1/ Pan2 (Table 6), suggesting that neither site is the target for the repression of activity. These results map a site of action of Pan1/Pan2 for the MLC2 promoter within a 50-bp fragment that does not contain any canonical E-box sites.

**Discussion**

Our results indicate that basic HLH proteins are functional in neonatal cardiac cells. Neonatal ventricular myocytes appeared to express mRNAs for the same isoforms of Pan1, Pan2, and Id as are found in skeletal muscle. As in skeletal muscle, Id mRNA levels varied with serum conditions, increasing a maximal sixfold with serum stimulation. In addition, PE stimulation of neonatal cardiac cells resulted in a threefold increase in Id mRNA levels. PE stimulation of neonatal ventricular myocytes is a well-defined model system for cardiac hypertrophy\textsuperscript{27} and results in upregulation and isoform switching of many genes. Recently, other investigators\textsuperscript{36} have shown that Id mRNA expression in adult ventricular myocytes in culture exhibits an increase after 5 days in serum-containing medium and a twofold increase at 12 hours after PE stimulation. Our results extend these observations to the well-characterized neonatal myocyte model system and in addition demonstrate that upregulation of Id mRNA in response to these stimuli occurred within 30 minutes, suggesting that Id is an early response gene and may play a role in regulating responses of the myocardium to physiological stimuli. Perhaps genes that are downregulated in response to hypertrophic stimuli, such as \textalpha-myosin heavy chain,\textsuperscript{29} are candidates for regulation by HLH proteins. Recent results of other investigators indicate that cardiac activity of an \textalpha-cardiac actin promoter fragment can be downregulated by cotransfection with an Id expression vector.\textsuperscript{37} It would be of interest to examine the activity of this promoter construct in response to PE stimulation, particularly as expression of \textalpha-skeletal actin increases relative to \textalpha-cardiac actin in response to hypertrophic stimuli.\textsuperscript{28} Another member of the Id family, HLH462, is an immediate early response gene for several signal transduction pathways in fibroblasts.\textsuperscript{38} Results of transient cotransfection studies of a MyoD expression vector and skeletal promoter–luciferase constructs into primary cardiac cells indicated that MyoD can activate the skeletal muscle \textgamma-AchR promoter to the same extent in cardiac cells as in C3H10T1/2 cells.

<table>
<thead>
<tr>
<th>Luciferase reporter</th>
<th>HLH</th>
<th>Luc/protein (mean±SEM)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCHY7Luc</td>
<td>pLPAΔS</td>
<td>14 800±1590</td>
<td>NE</td>
</tr>
<tr>
<td>pCHY7Luc</td>
<td>pCMVPan1</td>
<td>16 000±1490</td>
<td>×3</td>
</tr>
<tr>
<td>pCHY7Luc</td>
<td>pCMVPan2</td>
<td>5190±577</td>
<td>×3</td>
</tr>
<tr>
<td>pT109</td>
<td>pLPAΔS</td>
<td>1360±255</td>
<td>×2</td>
</tr>
<tr>
<td>pT109</td>
<td>pCMVPan1</td>
<td>432±44.4</td>
<td>×3</td>
</tr>
<tr>
<td>pT109</td>
<td>pCMVPan2</td>
<td>566±51.1</td>
<td>×2</td>
</tr>
<tr>
<td>pMCKLuc</td>
<td>pLPAΔS</td>
<td>29 100±6320</td>
<td>NE</td>
</tr>
<tr>
<td>pMCKLuc</td>
<td>pCMVPan1</td>
<td>38 700±6210</td>
<td>NE</td>
</tr>
<tr>
<td>pMCKLuc</td>
<td>pCMVPan2</td>
<td>43 242±7570</td>
<td>NE</td>
</tr>
<tr>
<td>pXP.1</td>
<td>pLPAΔS</td>
<td>80.8±5.83</td>
<td>×2</td>
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<td>pXP.1</td>
<td>pCMVPan1</td>
<td>147±22.3</td>
<td>×2</td>
</tr>
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<td>pXP.1</td>
<td>pCMVPan2</td>
<td>90.7±31.5</td>
<td>×2</td>
</tr>
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<td>pLPAΔS</td>
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<td>pCMVPan1</td>
<td>510±89.1</td>
<td>×2</td>
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<tr>
<td>pyAchRLuc</td>
<td>pCMVPan2</td>
<td>347±26.9</td>
<td>×2</td>
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<td>pLPAΔS</td>
<td>103±27.0</td>
<td>×2</td>
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<tr>
<td>pSVOALΔ5'</td>
<td>pCMVPan1</td>
<td>68.0±7.89</td>
<td>×2</td>
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<tr>
<td>pSVOALΔ5'</td>
<td>pCMVPan2</td>
<td>50.0±2.08</td>
<td>×2</td>
</tr>
<tr>
<td>pANF3003Luc</td>
<td>pLPAΔS</td>
<td>250 000±55 200</td>
<td>×13</td>
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<td>pANF3003Luc</td>
<td>pCMVPan1</td>
<td>190 ±86 100</td>
<td>×11</td>
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<td>pANF3003Luc</td>
<td>pCMVPan2</td>
<td>215 000±63 800</td>
<td>×11</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
<td>pLPAΔS</td>
<td>384 000±69 900</td>
<td>×11</td>
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<td>pMLC2700Luc</td>
<td>pCMVPan1</td>
<td>28 000±7850</td>
<td>×11</td>
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<tr>
<td>pMLC2700Luc</td>
<td>pCMVPan2</td>
<td>32 700±9750</td>
<td>×11</td>
</tr>
</tbody>
</table>

HLH, helix-loop-helix expression plasmid; Luc, luciferase activity; NE, no effect.

Transient transfection assays were performed as described in "Materials and Methods." Luciferase reporter plasmid (10 μg) was cotransfected with 1 μg HLH. pLPAΔS is the parental vector for pCMVPan1 and pCMVPan2 expression plasmids.\textsuperscript{4} Data shown are the average values obtained from one experiment performed in triplicate and are representative of at least three separate experiments. Absolute values of Luc varied from experiment to experiment, but fold decreases or increases were consistent. Separate experiments were performed with distinct plasmid DNA preparations. Luc is shown normalized to total cellular protein.
TABLE 6. Effect of Pan1/Pan2 Expression on Myosin Light Chain-2 Promoter-Reporters in Neonatal Ventricular Myocytes

<table>
<thead>
<tr>
<th>Luciferase reporter</th>
<th>HLH</th>
<th>Reporter/HLH (µg:µg)</th>
<th>Luc/protein (mean±SEM)</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMLC2700Luc</td>
<td>pLPAAS</td>
<td>10:1</td>
<td>250 000±25 600</td>
<td>×14</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
<td>pCMVPan1</td>
<td>10:1</td>
<td>17 200±4310</td>
<td>×6</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
<td>pCMVPan2</td>
<td>10:1</td>
<td>9370±283</td>
<td>×27</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
<td>pLPAAS</td>
<td>10:0.3</td>
<td>394 000±74 500</td>
<td>×5</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
<td>pCMVPan2</td>
<td>10:0.3</td>
<td>66 900±38 000</td>
<td>×6</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
<td>pLPAAS</td>
<td>10:0.1</td>
<td>299 000±20 600</td>
<td>×5</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
<td>pCMVPan2</td>
<td>10:0.1</td>
<td>57 700±4580</td>
<td>×5</td>
</tr>
<tr>
<td>pMLC2700Luc</td>
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<td>195 000±27 200</td>
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<td>pCMVPan2</td>
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<td>284 000±123 000</td>
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<td>pMLC800Luc</td>
<td>pCMVPan1</td>
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<td>12 600±310</td>
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<td>pMLC800Luc</td>
<td>pCMVPan2</td>
<td>10:1</td>
<td>25 000±1950</td>
<td>×4</td>
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<tr>
<td>pMLC250Luc</td>
<td>pLPAAS</td>
<td>10:1</td>
<td>93 700±7740</td>
<td>×2</td>
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<tr>
<td>pMLC250Luc</td>
<td>pCMVPan1</td>
<td>10:1</td>
<td>38 800±5620</td>
<td>×2</td>
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<tr>
<td>pMLC250Luc</td>
<td>pCMVPan2</td>
<td>10:1</td>
<td>40 000±9520</td>
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<td>pMLC50Luc</td>
<td>pLPAAS</td>
<td>10:1</td>
<td>162±11</td>
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<td>pMLC50Luc</td>
<td>pCMVPan1</td>
<td>10:1</td>
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<td>pMLC250E-Luc</td>
<td>pLPAAS</td>
<td>10:1</td>
<td>55 000±14 100</td>
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<td>pMLC250E-Luc</td>
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<td>10:1</td>
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<td>×3</td>
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<tr>
<td>pMLC250E-Luc</td>
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<td>24 100±1360</td>
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<tr>
<td>pMLC250H1b</td>
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<tr>
<td>pMLC250H1b</td>
<td>pCMVPan2</td>
<td>10:1</td>
<td>1160±101</td>
<td>×4</td>
</tr>
</tbody>
</table>

HLH, helix-loop-helix expression plasmid; Luc, luciferase activity; NE, no effect.

Transient transfection assays were performed as described in "Materials and Methods." Data shown are the average values obtained from one experiment performed in triplicate and are representative of at least three separate experiments. Absolute values of Luc varied from experiment to experiment, but fold decreases or increases were consistent. Separate experiments were performed with distinct plasmid DNA preparations. Luc is shown normalized to total cellular protein.

demonstrating that the neonatal ventricular cell environment is permissive for MyoD. This observation extends recent results in transgenic mice, where ectopic expression of MyoD resulted in the expression of skeletal muscle markers in embryonic hearts.39 As the transgenic mice died before birth, the effect of expression in the neonatal heart could not be examined. In addition, we have tried to examine more closely the effect of MyoD expression on several genes normally expressed in cardiac cells. MyoD expression could affect cardiac expression by binding to potential recognition sites or could theoretically downregulate expression by competing for a partner required for activation. Recently, low levels of MyoD mRNA have been reported in adult Xenopus heart,40 raising the possibility of low levels of MyoD expression in the mammalian heart.

Although MyoD activated an E-box–containing MCK enhancer in C3H10T1/2 cells, consistent with results of previous investigators,4 MyoD did not activate the same enhancer in primary neonatal ventricular cells. This may be a reflection of the fact that the MCK enhancer is expressed in cardiac cells and that MyoD is unable to increase transcriptional levels further, perhaps because of the lack of E-box accessibility or the inability of MyoD to activate over and above endogenous transcription factors. Similarly, although MyoD was capable of activating cardiac-specific promoters of ANF and MLC2 genes in 10T1/2 cells, it did not affect the activity of these two promoters in cardiac muscle cells. MyoD has previously been shown to activate promoters other than skeletal muscle promoters in transient cotransfection assays.1,3,33 This activation may be direct or indirect. In addition, the MLC2 gene is expressed in slow skeletal muscle,32 although it is not known whether the 2700-bp promoter is sufficient to confer skeletal muscle–specific expression.

Cotransfection of an Id expression vector with MCK, ANF, and MLC2 promoter element–reporter constructs did not appear to affect their expression. However, the Id expression vector can be effective in primary neonatal ventricular cells, as indicated by its ability to counteract MyoD activation of the γ-AchR promoter. Inability of Id to downregulate the three cardiac promoters may indicate that their activity in neonatal ventricular myocytes is largely independent of an E-box–mediated pathway, which can be affected by Id. Consistent with this, activation of the MLC2 promoter has been shown to be mediated by an E-box–independent pathway.34 Recently, other investigators have demonstrated that the Hfla site that mediates cardiac-specific activity of the MLC2 promoter is capable of recognizing an E-box–binding complex, which binds to the α-myosin heavy chain promoter.41 This observation has led to the proposal that the Hfla site is
a modified E-box. Our results indicate that, if Hf1a is an E-box-binding protein, it is one that cannot be inhibited by Id. Both MLC2 and ANF genes are upregulated in response to PE stimulation,15,21 and our results indicate that Id mRNA levels also increase with PE stimulation. These observations are consistent with the inability of Id cotransfection to downregulate activity of these two cardiac promoters. Insensitivity of the MCK enhancer to downregulate activity of these two cardiac promoters differs from the α-cardiac actin promoter context, consistent with results of previous investigations,4,26 no activation by these expression vectors. The ANF-luciferase promoters were cotransfected with Pan1 and Pan2 expression vectors in both C3H10T1/2 embryonic fibroblast cells and in primary neonatal cardiac cells. This is the first report of a negatively acting basic HLH protein also acting as a negative regulator. Members of other transcription factor families have been shown to be capable of both negative and positive gene regulation. With the leucine zipper Fos/Jun family, negative regulation can occur at AP1 sites or at non-AP1 sites, such as, for Jun, the E-box site.43–45 Whether this negative potential of Pan1 and Pan2 plays a role in cardiac gene regulation is not known.

In summary, our results implicate E-box–dependent pathways in regulating changes in gene expression that occur in cardiac cells in response to physiological stimuli but suggest that, at least in neonatal ventricular cells, E-box pathways may play a lesser role in tissue-specific gene expression than in differentiated skeletal muscle. Activity of three cardiac promoter elements appeared to be unaffected by Id, including the MCK enhancer, which is downregulated by Id in skeletal muscle cells. A functional HLH environment in neonatal myocardial cells may indicate that HLH proteins play a role in embryonic and neonatal development of the myocardium. We have recently observed high levels of Id mRNA expression in embryonic and neonatal endocardial cushions, which suggests that HLH proteins may affect endocardial development.46

Acknowledgments

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Potential role of helix-loop-helix proteins in cardiac gene expression.

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