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# A Conserved 28-Base-Pair Element (HF-1) in the Rat Cardiac Myosin Light-Chain-2 Gene Confers Cardiac-Specific and α-Adrenergic-Inducible Expression in Cultured Neonatal Rat Myocardial Cells

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**To study the transcriptional regulatory mechanisms which mediate cardiac-specific and inducible expression during myocardial cell hypertrophy, we have extensively characterized the rat cardiac myosin light-chain-2 (MLC-2) gene as a model system. The MLC-2 gene encodes a relatively abundant contractile protein in slow skeletal and cardiac muscle and is upregulated during in vivo cardiac hypertrophy and α-adrenergic-mediated hypertrophy of neonatal rat myocardial cells. In transient expression assays employing a series of MLC-2–luciferase constructs, recent studies have identified a 250-bp fragment which is sufficient for both cardiac-specific and α-adrenergic-inducible expression. Within this 250-bp fragment lie three regions (HF-1, HF-2, and HF-3), each greater than 10 bp in length, which are conserved between the chicken and rat cardiac MLC-2 genes, suggesting their potential role in the regulated expression of this contractile protein gene. As assessed by substitution mutations within each of the conserved regions, the present study demonstrates that HF-1 and HF-2 are important in both cardiac-specific and inducible expression, while HF-3 has no detectable role in the regulated expression of the MLC-2 gene in transient expression assays. HF-1 sequences confer both cardiac-specific and inducible expression to a neutral promoter-luciferase construct but have no significant effect in the skeletal muscle or nonmuscle cell contexts. Thus, these studies have identified a new cardiac-specific regulatory element (HF-1) which plays a role in both cardiac-specific and inducible expression during myocardial cell hypertrophy.**

Cardiac muscle is a highly differentiated tissue with unique chronotropic, contractile, and metabolic properties essential for the maintenance of normal blood flow. The normal growth and development of the heart may require the tissue- and stage-specific expression of distinct cardiac channels and receptors, specific contractile proteins, and intermediary enzymes. In response to genetic, hormonal, and mechanical stimuli, the myocardium adapts to increased workloads through the hypertrophy of existing myocardial cells, which is manifested as an increase in contractile units, an accumulation of myofibrillar proteins, and a concomitant increase in the expression of contractile protein genes (for a review, see reference 1). The regulated expression of cardiac-specific genes during hypertrophy and normal development is controlled largely at the transcriptional level (28, 33, 35, 49, 61). The question arises whether similar or divergent *cis*- and *trans*-acting elements mediate cardiac-specific expression and inducible expression during myocardial cell hypertrophy. Although the transcriptional regulation of muscle genes in skeletal muscle cells has been extensively studied (1, 2, 4–7, 10, 16, 24, 29, 34, 37, 40, 41, 46, 50, 57, 58, 60), currently little is known about the mechanisms which regulate cardiac gene transcription during myocardial growth and development. It would be of interest to determine whether distinct *cis*- and *trans*-acting elements mediate tissue-specific expression in these two striated muscle types (28, 36).

To address these questions, we have extensively characterized the rat cardiac myosin light-chain-2 (MLC-2) gene as a model system (22, 23, 30). The MLC-2 gene is a relatively abundant contractile protein in slow skeletal and cardiac muscle (11) and is upregulated during in vivo cardiac hypertrophy (31) and α-adrenergic-mediated hypertrophy of neonatal rat myocardial cells (30, 33). In transient expression assays employing a series of MLC-2–luciferase constructs, recent studies have identified a 250-bp fragment which is sufficient for both cardiac-specific and α-adrenergic-inducible expression (30). Within this 250-bp fragment lie three regions (HF-1, HF-2, and HF-3), each greater than 10 bp in length, which are conserved between the chicken and rat cardiac MLC-2 genes, suggesting their potential role in the regulated expression of this contractile protein gene. By assessing substitution mutations within each of the conserved regions, the present study demonstrates that HF-1 and HF-2 are important in both cardiac-specific and inducible expression, while HF-3 has no detectable role in the regulated expression of the MLC-2 gene in transient expression assays. HF-1 sequences confer both cardiac-specific and inducible expression to a neutral promoter-luciferase construct but have no significant effect in the skeletal muscle or nonmuscle cell context. Thus, a new cardiac-specific element (HF-1) which plays a role in both cardiac-specific and inducible expression during myocardial cell hypertrophy has been identified.

## MATERIALS AND METHODS

**Cultured cell techniques.** Cultured neonatal rat myocardial cells were prepared as previously described (38, 52), with minor modifications. Myocytes were dispersed from the ventricles of 1- to 2-day-old Sprague-Dawley rats by digestion with collagenase II (Worthington) and pancreatin

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(GIBCO) at 37°C. Purification of the cell suspensions on a discontinuous Percoll gradient was utilized to obtain myocardial cell cultures with >95% myocytes, as assessed by immunocytofluorescence with MLC antisera. The myocytes were plated in 10-cm-diameter culture dishes (Falcon) at a density of $1.5 \times 10^6$ cells in 5 ml of 4:1 Dulbecco's modified Eagle medium (DMEM)-medium 199 (GIBCO), supplemented with 10% horse serum, 5% fetal calf serum, and antibiotics (ampicillin [34 μg/ml] and gentamicin [3 μg/ml]). Following incubation in serum for 24 h, the cultures were washed and incubated in DMEM-medium 199 with various agents.

**RNase protection studies.** Total RNA was isolated from cultured cells and intact mouse myocardium by a previously described method (54). RNAse protection studies were performed essentially as previously described (39). The rat cardiac MLC-2 cDNA (23) was cloned into pGem32 (Promega); digestion with *Bam*HI and transcription with T7 RNA polymerase generated a 686-nucleotide probe which, when hybridized to rat MLC-2 RNA, yielded a 660-bp protected fragment. A mouse cardiac MLC-2 cDNA (48a) was cloned into pBluescript SK (Stratagene) and restricted with *Dde*I, and transcription with T7 RNA polymerase generated a 173-nt probe which, when hybridized to mouse MLC-2 RNA, yielded a 119-bp protected fragment. The RNA probes were labeled with [$^{32}$P]CTP and purified on an 8 M urea–6% polyacrylamide gel. Four micrograms of total RNA was hybridized at 45°C overnight with 5,000 to 15,000 cpm of the purified MLC-2 RNA probes. The unprotected RNA was subsequently digested with RNase A at 25°C for 60 min. The reaction was terminated with proteinase K, and the reaction mixture was phenol extracted and precipitated in ethanol. The RNase-resistant hybrids were electrophoresed on an 8 M urea–6% polyacrylamide gel. The gel was dried and exposed to X-ray film for various periods.

**Construction and transfection of mutant MLC-2–luciferase constructs.** A 250-bp fragment of the MLC-2 5′ flanking region was subcloned into a pBST phagemid vector, and a synthetic oligonucleotide primer containing a mutagenizing sequence corresponding to HF-3, HF-2, or regions of HF-1 was phosphorylated with T4 polynucleotide kinase. The phosphorylated oligonucleotide was annealed to the complementary single-stranded template by heating at 65°C for 10 min followed by gradual cooling to 25°C. The first strand was synthesized by primer extension in 40 μl of a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 7 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mM dNTPs, 10 mM ATP, 25 μg of Gene 32 protein per ml, 1.5 U of Klenow enzyme, and 4 U of T4 DNA ligase at room temperature for 4 h. The reaction mixture was diluted 10-fold with $H_2O$ and was used to transform 200 μl of competent XL-1 Blue *Escherichia coli* cells. The *E. coli* transformants were screened for mutated MLC-2 promoter sequences by a colony lift technique (20). Briefly, the individual *E. coli* transformants were lifted from the selective agar plates onto nitrocellulose filters and lysed with 0.3 N NaOH. After neutralization with 1 M Tris-HCl (pH 8.0) and drying in a vacuum oven, the filters were prehybridized in a solution containing 6× SSC (1× SSC is 150 mM NaCl–15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll–0.02% polyvinylpyrrolidone–0.02% bovine serum albumin), 20 mM $NaH_2PO_4$, and 0.5 mg of salmon sperm DNA per ml at $T_m$ − 5°C for 2 h. Hybridization was performed in 6× SSC–20 mM $NaH_2PO_4$–0.4% sodium dodecyl sulfate (SDS)–0.5 mg of salmon sperm DNA per ml–$10^6$ cpm of mutagenizing oligonucleotide probe per filter at $T_m$ − 5°C for 12 h. The filters were washed twice with 6× SSC–0.1% SDS at room temperature and then once with 6× SSC–0.1% SDS at $T_m$ − 5°C for 10 min and were exposed to XRP film. The mutants were verified by sequencing the double-stranded MLC-2 promoter fragments in the pBST vector. One picomole of each plasmid candidate was denatured with 0.3 N NaOH, neutralized with 2 M ammonium acetate (pH 7.0), and precipitated with 2.5 volumes of ethanol. The denatured plasmid was annealed to 5 pmol of sequencing primer at 37°C for 20 min and then exposed to room temperature for 10 min. Primer extension was carried out in 20 μl of [α-$^{35}$S]dATP–dNTPs–2.8 U of T7 DNA polymerase at room temperature for 5 min. Subsequently, 4.5 μl of the primer extension reaction mixture was divided into aliquots to prepare four termination mixes containing ddATP, ddGTP, ddCTP, or ddTTP. Sequencing reactions were continued at 37°C for 5 min and then terminated by adding 5 μl of sequencing gel loading buffer. The samples were heated at 80°C for 2 min, chilled on ice, and resolved on 8% or 6% sequencing gels. The gels were fixed in 10% hydrogen acetate–10% methanol, dried, and exposed to XRP film. The MLC-2 promoters bearing mutations were recovered as *Hin*dIII-*Eco*RI fragments from pBST and blunt-ended with Klenow enzyme. The expression vector pSVOALΔ5′ (15) was restricted with *Hin*dIII and blunt-ended with Klenow enzyme and ligated to the *Hin*dIII-*Eco*RI MLC-2 promoter. The orientation of the promoter was verified by double-stranded sequencing as described above.

For the transient expression assays, a modified calcium phosphate transfection technique was utilized (8). This method resulted in a marked increase in the efficiency of transfection in the myocardial cells, as previously described (22). Approximately 24 μg of DNA was utilized in each transfection, representing a combination of a luciferase reporter vector and a cytomegalovirus β-galactosidase plasmid (9) as a control for the efficiency of transfection. After various periods, the cells were harvested for luciferase or β-galactosidase assays or both. The luciferase and β-galactosidase assays were performed by a previously described method (15, 48).

## RESULTS

**Expression of the endogenous MLC-2 gene in cardiac and skeletal muscle cells.** As assessed by two-dimensional gel electrophoresis of muscle extracts, previous studies have suggested that the rat cardiac MLC-2 gene is expressed in both slow-twitch skeletal muscle and cardiac muscle tissue (11). Using primer extension with an oligonucleotide complementary to unique sequences within the 5′ untranslated region of the rat cardiac MLC-2 gene, we have recently documented that the cardiac MLC-2 gene is expressed in soleus and cardiac muscle, while fast white muscle (vastus lateralis) does not express the gene (21a). To identify appropriate recipient skeletal muscle cell lines to test the muscle cell specificity of normal and mutant rat cardiac MLC-2 promoter constructs, the expression of the endogenous cardiac MLC-2 gene in the C2 and *Sol*8 muscle cell lines was examined. Since the coding region of the MLC-2 gene is highly conserved, RNase protection analyses were performed with probes containing untranslated sequences. As shown in Fig. 1, hybridization with a 686-nt rat cardiac MLC-2 probe results in a 600-bp protected hybrid following RNase treatment, indicating that cultured neonatal rat myocardial cells express the rat cardiac MLC-2 gene; this result is consistent with previous studies (33). Similarly, hybridiza-

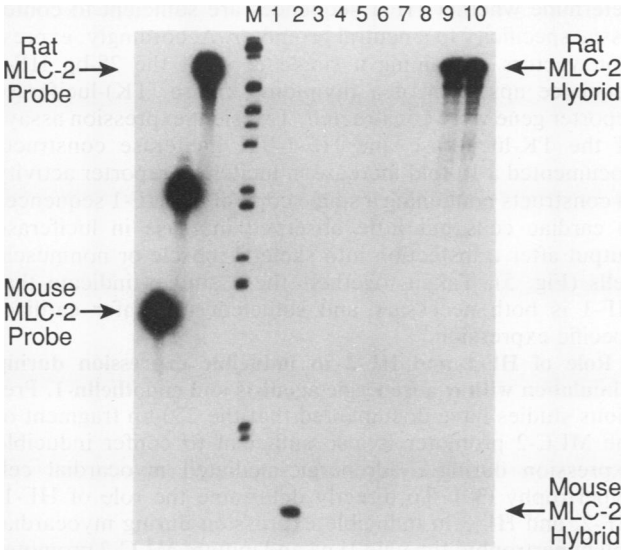


FIG. 1. Assessment of cardiac MLC-2 gene expression in rat and mouse muscle cells with RNase protection analyses. Total RNA was derived from neonatal rat myocardial cells, mouse myocardium, *Sol*8 muscle cells, and C2/C12 mouse muscle cells as described in Materials and Methods. RNase protection analyses were performed with single-stranded RNA probes generated in T3 and T7 vectors and labeled with $^{32}P$. Following hybridization overnight, the samples were treated with RNase, and the hybrids were separated by acrylamide gel electrophoresis. Lanes 1 through 7 represent the mouse MLC-2 probe hybridized to RNAs from the following sources, respectively: tRNA, mouse ventricular cells, log-phase *Sol*8 cells, fused *Sol*8 cells, log-phase C2 cells, fused C2 cells, and rat ventricular myocytes. Lanes 8 through 10 represent the rat MLC-2 probe hybridized to tRNA, rat ventricular myocyte RNA in high serum, and rat ventricular myocyte RNA in low serum, respectively. Lane M contains molecular weight markers.

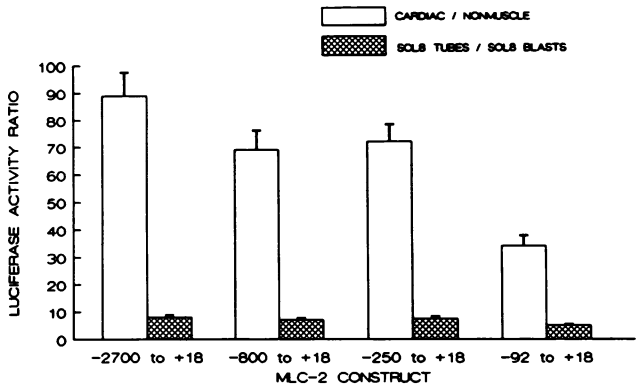


FIG. 2. Relative activity of MLC-2–luciferase fusion genes in transient expression assays in neonatal rat myocardial cells and *Sol*8 skeletal muscle cells. The muscle cells were transfected with chimeric luciferase reporter plasmids containing nested deletions in the 5′ flanking region of the rat cardiac myosin light-chain-2 gene. To control for the variation in transfection efficiency, a β-galactosidase expression vector under the direction of the human cytomegalovirus promoter was used as previously described (22). The cells were harvested for luciferase and β-galactosidase assays as described in Materials and Methods. The results are representative of three separate experiments with three separate plasmid preparations. Results obtained in the cardiac context have been previously reported (22) and are included in the figure for comparison purposes. The relative activity of the MLC-2–luciferase fusion genes was normalized to the level of luciferase expression directed by a Rous sarcoma virus (RSV) promoter in each cell type to control for differences in the efficiency of transfection between the various cell types. For example, for pMLC (−250 to +18) Ld 5′ was 5.1% ± 0.4% of RSV luciferase expression in cardiac cells, 0.07% ± 0.01% in CV-1 cells, 0.92% ± 0.02% in *Sol*8 tubes, and 0.12% ± 0.01% in *Sol*8 blasts (mean ± standard error of the mean).

tion of a mouse cardiac MLC-2 probe with RNA derived from mouse heart tissue results in a 119-bp protected fragment. However, myotubes from two separate continuous mouse skeletal muscle cell lines (C2/C12 and *Sol*8) do not express the mouse cardiac MLC-2 gene.

**The myosin light-chain-2 gene is not upregulated during the differentiation of *Sol*8 myoblasts.** Previous studies employed a series of nested MLC-2 deletions fused to a luciferase reporter gene to identify a 250-bp fragment of the MLC-2 promoter which is sufficient to confer cardiac-specific expression to the reporter gene in transient expression assays in neonatal rat myocardial cells (70-fold-higher level of expression in cardiac myocytes than in a nonmuscle cell line, CV-1 [22]). To determine whether these sequences would confer skeletal muscle-specific expression, the MLC-2 constructs were utilized in transient expression assays in *Sol*8 myoblasts and myotubes. As shown in Fig. 2, transient expression assays with a series of MLC-2 luciferase constructs in *Sol*8 cells revealed no significant increase in luciferase reporter activity during the conversion of myoblasts to myotubes in this skeletal muscle cell line. In parallel experiments, a luciferase reporter gene containing the creatine kinase enhancer region was appropriately upregulated (10-fold upregulation in myotubes compared with myoblasts). Accordingly, the 250-bp MLC-2 fragment does not appear to be activated during the differentiation of *Sol*8 muscle cells.

**Role of the conserved HF-1, HF-2, and HF-3 *cis*-regulatory sequences in cardiac-specific expression.** The 250-bp MLC-2 fragment which confers cardiac-specific expression contains three regions (HF-1, HF-2, and HF-3), each >10 bp in length, which are conserved between the chicken and rat cardiac MLC-2 genes. Region I (HF-1) consists of a 28-bp element which is conserved in sequence (27 of 28 bp) and position between the rat and chicken cardiac MLC-2 genes and contains sequences with homology to CArG, AP-2, and MEF-2 motifs. This element lies 14 bp upstream of the TATA element in the MLC-2 promoter (Fig. 3). To directly assess the role of the HF-1, HF-2, and HF-3 sequences in the regulated expression of the cardiac MLC-2 gene, we constructed multiple substitution mutations in each of the three conserved regions within the 250-bp fragment, including separate mutations in the sequences corresponding to the putative CArG (−72 to −62), AP-2 (−60 to −53), and MEF-2 (−52 to −43) motifs within HF-1. The mutant MLC-2 promoters were subsequently fused to the luciferase reporter gene (Fig. 4) and assayed in transient expression studies with primary neonatal rat myocardial cells, *Sol*8 myoblasts, *Sol*8 myotubes, and the nonmyocardial cell line CV-1. Relative to the luciferase activity observed with the wild-type construct (defined as 100%), mutations within HF-3 resulted in no significant decrease in luciferase reporter activity in transient expression assays in neonatal rat myocardial cells. However, mutations within HF-1 and HF-2 reduced expression of the reporter to 5 to 30% of the control levels in cardiac cells (Fig. 4). The mutations in HF-1, HF-2 and HF-3 had little effect on the expression of the luciferase reporter gene in either *Sol*8 cells (myoblasts and myotubes) or

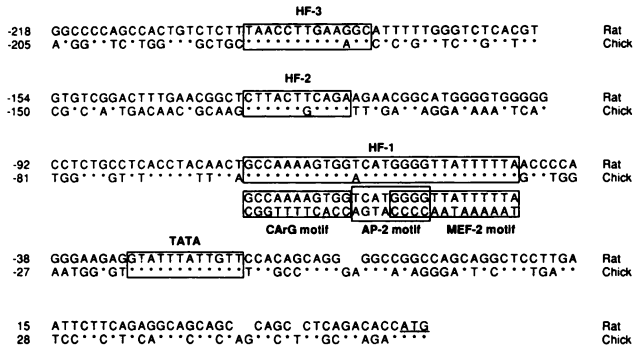


FIG. 3. Conserved HF-1, HF-2, and HF-3 sequences in the 5′ flanking region of the rat and chicken cardiac MLC-2 genes. Comparisons of the nucleotide sequences in the 5′ flanking region of the rat cardiac MLC-2 and the chicken cardiac MLC-2 genes reveal three regions which contain conserved *cis* elements. Sequence identities between the two genes are indicated by asterisks in the chicken sequence, while nucleotide differences are denoted by the insertion of an appropriate base. Brackets indicate conserved HF-1, HF-2, and HF-3 sequences which are 10 bp or greater in length. The HF-1 region contains sequences which are homologous to the CArG, AP-2, and MEF-2 motifs reported to be important in the transcriptional regulation of other genes (see text for details).

nonmuscle cells (CV-1), documenting the specificity of the HF-1 and HF-2 sequences for cardiac muscle cells (Fig. 4).

While these results suggest that HF-1 is necessary for cardiac-specific expression, further studies were designed to determine whether HF-1 sequences are sufficient to confer tissue specificity to a neutral promoter. Accordingly, expression vectors containing a single copy of the 28-bp HF-1 sequence upstream of a thymidine kinase (TK)-luciferase reporter gene were constructed. Transient expression assays of the TK-luciferase and HF-1–TK luciferase constructs documented a 10-fold increase in luciferase reporter activity in constructs containing a single copy of the HF-1 sequences in cardiac cells but little observed increase in luciferase output after transfection into skeletal muscle or nonmuscle cells (Fig. 5). Taken together, these studies indicate that HF-1 is both necessary and sufficient to confer cardiac-specific expression.

**Role of HF-1 and HF-2 in inducible expression during stimulation with α-adrenergic agonists and endothelin-1.** Previous studies have documented that the 250-bp fragment of the MLC-2 promoter is also sufficient to confer inducible expression during α-adrenergic-mediated myocardial cell hypertrophy (30). To directly determine the role of HF-1, HF-2, and HF-3 in inducible expression during myocardial cell hypertrophy, the wild-type and mutant MLC-2 promoter constructs were transfected into neonatal rat myocardial cells, and the cells were subsequently stimulated with two distinct agonists which activate the hypertrophic response and the transcription of the MLC-2 gene (the α-adrenergic agonist phenylephrine [30] and endothelin-1 [53]). Relative to the wild-type MLC-2 promoter construct, mutations within HF-3 had no detectable effect on inducibility by endothelin-1 but marginally decreased inducibility by phenylephrine (25 to 30% inhibition). However, mutations

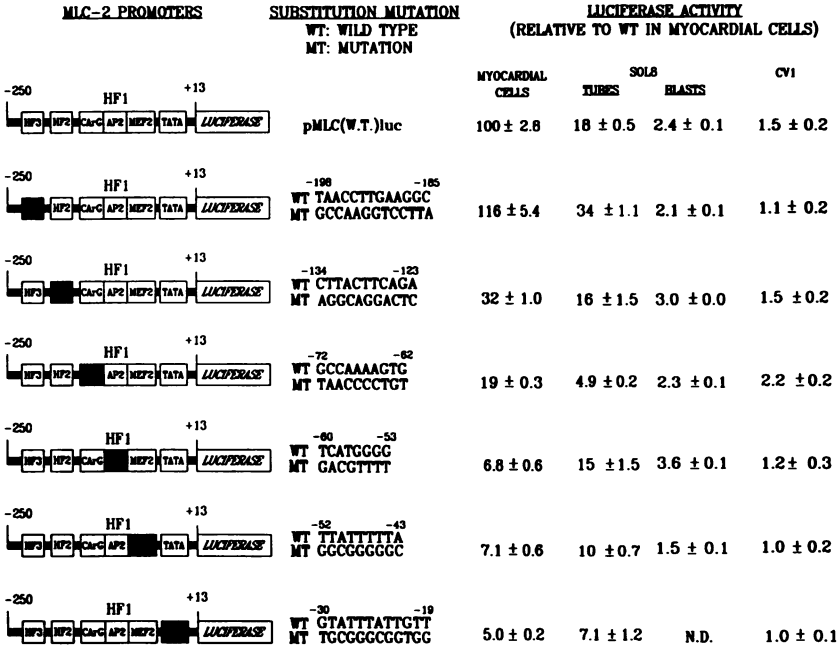


FIG. 4. Effect of mutations within the HF-1, HF-2, and HF-3 conserved sequences on cardiac-specific expression of a MLC-2–luciferase fusion gene. Substitution mutations were created within the HF-1, HF-2, and HF-3 regions of the MLC-2 250-bp promoter fragment (pMLC positions −52 to −43, CCGCCCCCG; pMLC positions −60 to −53, CTGCAAAA; pMLC positions −72 to −62, ATTGGGGACA; pMLC positions −134 to −123, TCCGTCCTGAG; and pMLC positions −198 to −185, CGGTTCCAGAAT). The mutant MLC-2 promoters were fused to a luciferase reporter gene. The activities of the mutant promoters were compared with that of the wild-type promoter in transient expression assays using neonatal rat myocardial cells, *Sol*8 myoblasts, *Sol*8 myotubes, and CV-1 cells. In each case, the efficiency of transfection was controlled by cotransfection with a cytomegalovirus β-galactosidase vector as previously described (22). The relative activity of MLC-2–luciferase fusion genes was normalized to the activity of the RSV luciferase gene in various cell types, and the results were normalized to the wild-type promoter activity in cardiac cells, which was defined as 100%. Results represent the mean ± standard error of the mean of four separate experiments with four separate plasmid preparations. N.D., Not determined.

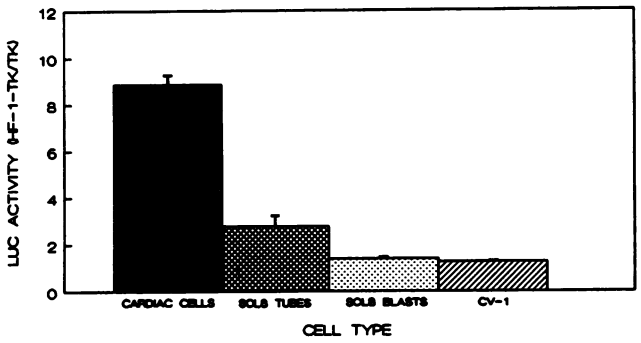


FIG. 5. Effect of HF-1 sequences on cardiac-specific expression of a TK-luciferase (LUC) reporter gene. A duplex oligonucleotide containing HF-1 sequences (5'-GCCAAAAGTGGTCATGGGGTT ATTTTTA-3') was inserted into the TK-luciferase vector PT109 and subsequently analyzed for its ability to confer cardiac-specific expression in transient expression assays of neonatal rat myocardial cells, *Sol*8 myoblasts, *Sol*8 myotubes, and CV-1 cells. In each case, a CMV β-galactosidase vector was cotransfected to control for the efficiency of transfection. Results represent the mean ± standard error of the mean of four experiments with four separate plasmid preparations. The relative activity of the constructs described above was normalized to the RSV luciferase activity in various cell types. As a reference, the relative activity of PT109 was 1.8% ± 0.1% of the RSV luciferase activity in cardiac cells, 8.9% ± 0.5% in *Sol*8 tubes, 5.8% ± 0.1% in *Sol*8 blasts, and 8.7% ± 0.5% in CV-1 cells.

within HF-1 and HF-2 significantly blunted inducible expression (50 to 75% inhibition) by both agonists (Fig. 6). Transient expression assays of the TK-luciferase construct displayed no significant inducibility after treatment with either phenylephrine or endothelin-1. However, TK-luciferase constructs containing a single copy of the HF-1 sequence also displayed α-adrenergic- and endothelin-inducible expression (approximately twofold higher than TK-luciferase [Fig. 7]), demonstrating that HF-1 sequences can confer both inducible and cardiac-specific expression.

## DISCUSSION

**Distinct *cis*-regulatory sequences within the MLC-2 gene mediate cardiac- and skeletal muscle-specific expression.** Re-

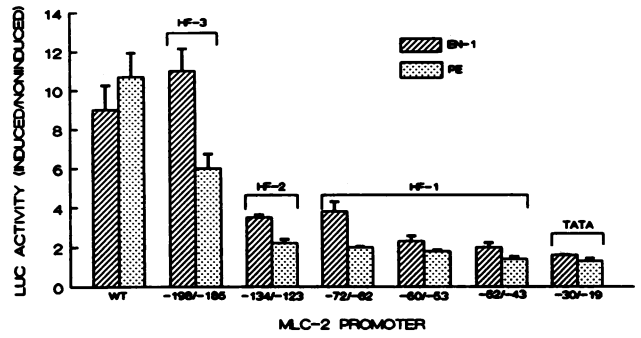


FIG. 6. Effect of mutations in the HF-1, HF-2, and HF-3 regions on inducible expression during stimulation of neonatal rat myocardial cells by phenylephrine (PE) or endothelin-1 (EN-1). The wild-type and mutant MLC-2–luciferase (LUC) constructs shown in Fig. 4 were utilized to assess their effects on adrenergic and endothelin-1 inducibility of the MLC-2 fusion genes. Results represent three separate experiments with three separate plasmid preparations and are expressed as the mean ± standard error of the mean. Inducibility assays were performed as previously described (30).

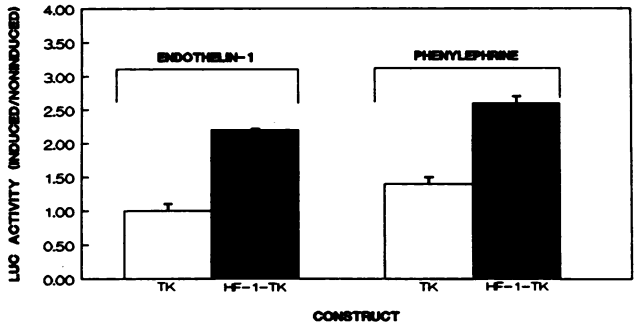


FIG. 7. Effect of HF-1 sequences on inducible expression of a TK-luciferase (LUC) reporter gene following treatment with phenylephrine or endothelin-1. The plasmid constructs are described in the legend to Fig. 5. Results represent three separate experiments with three separate plasmid preparations and are expressed as the mean ± standard error of the mean. Inducibility assays were performed as previously described (30).

cent studies have characterized a number of *cis*-regulatory elements and their corresponding *trans*-acting factors which mediate muscle-specific expression in skeletal muscle cells, including the CArG element in the cardiac α-actin promoter (41), MEF-1 (32), and MEF-2 (19) elements within the creatine kinase 5' flanking region, the M-CAT motif in the troponin T gene (37), and a C-BAR element in the myoglobin (14) and troponin C (45) genes. A combination of genetic and molecular approaches has led to the identification of a host of myogenic determination genes which can serve as dominantly acting master transcriptional regulators, leading to the development of myotubes in several permissive nonmuscle cell lines. myoD, one of the first members of this family to be extensively characterized, plays a critical role in the activation of the muscle gene program in skeletal muscle cells, binding to MEF-1 target sequences and activating several muscle promoters (12, 13, 59). Recently, a number of myogenic determination genes have been isolated, including Myf-5 (4), myogenin (18, 60), MRF-4 (also called herculin or *Myf*-6) (3, 40, 47), and related genes from species other than rats (34).

In comparison with the control of contractile protein gene expression in skeletal muscle cells, surprisingly little is known about mechanisms which dictate the tissue-specific expression of muscle genes in cardiac cells. Since several muscle promoters are expressed in both cardiac and skeletal muscle and both muscle cell types appear early after mesodermal induction (42), the question arises whether their respective muscle gene programs are controlled by similar *cis*- and *trans*-acting elements within these distinct striated muscle subtypes. Recently, several independent lines of evidence have suggested that genes which are expressed in both cardiac and skeletal muscle cells may utilize divergent sets of *cis*-regulatory elements in each muscle subtype. This is evident in the case of the creatine kinase gene: studies of creatine kinase-chloramphenicol acetyltransferase fusion constructs in transgenic mice have identified a segment of the mouse creatine kinase 5' flanking region (positions −3300 to −1256) (28) which appears to mediate a 200-fold increase in reporter activity in cardiac muscle but has little influence on chloramphenicol acetyltransferase expression in skeletal muscle. Transient expression studies with primary chick skeletal and cardiac cells by Mar and coworkers have characterized a 67-bp segment of the cardiac troponin T gene (36) which mediates cardiac-specific expression but has

little effect in the skeletal muscle context. On the other hand, an M-CAT motif within the troponin T gene mediates tissue-restricted expression in skeletal muscle but not in cardiac muscle cells. Finally, while myoD and myogenin appear to play a central role in the activation of creatine kinase gene expression in skeletal muscle (18, 32), to date myoD, myogenin and other myogenic determination factors have not been found in cardiac tissues, implying that the program of muscle gene expression in cardiac tissues may utilize a separate set of cardiac-related myogenic determination genes. Currently, it remains to be determined whether the program of muscle gene expression in cardiac tissue is dictated by transcriptional factors analogous to myoD. Taken together, these studies suggest the possibility that distinct regulatory elements might mediate cardiac-specific expression.

The results of the present study provide further evidence for the divergence of *cis*- and *trans*-acting mechanisms for muscle-specific expression between cardiac and skeletal muscle cells. A 250-bp fragment of the MLC-2 promoter which was found to be sufficient for cardiac-specific expression is not appropriately upregulated during the differentiation of myoblasts to myotubes in a skeletal muscle cell line. In the rat cardiac MLC-2 gene, this result stands in contrast to studies with a 64-bp fragment of the chicken cardiac MLC-2 promoter which was found to confer muscle-specific expression in both primary cardiac and skeletal muscle cells (5). Mutations in HF-1 have little effect in the skeletal muscle cell context but appear to lead to a significant decrease in activity in transient expression assays of neonatal rat myocardial cells. Since the cardiac MLC-2 gene is also expressed in slow skeletal muscle, it became of interest to examine whether the sequences within the 250-bp fragment are sufficient to direct expression of the luciferase reporter in both muscle subtypes in lines of transgenic mice harboring this MLC-luciferase fusion gene. Recently, we have generated transgenic mice which harbor the 250-bp MLC-2–luciferase transgene and which express the luciferase reporter gene in a cardiac-specific fashion with little detectable expression in skeletal muscle or nonmuscle cells (48b), which represents the most critical test of the segregation of cardiac-specific and skeletal muscle-specific regulatory elements in the MLC-2 gene in the in vivo state.

**Conserved *cis*-regulatory elements (HF-1 and HF-2) mediate cardiac-specific expression of the rat cardiac myosin light-chain-2 gene.** Previous studies have led to the identification of a 250-bp fragment in the MLC-2 5′ flanking region which is sufficient to confer cardiac-specific expression (70-fold higher expression in neonatal rat myocardial cells than in CV-1 cells [22]). Within this 250-bp fragment lie three regions, each >10 bp in length, which are conserved between the chicken and rat cardiac MLC-2 genes, thereby implying a potential role for these sequences in the regulated expression of the MLC-2 gene. Using substitution mutations within each of the conserved regions, the present study suggests that the HF-1 and HF-2 regions are important for cardiac-specific expression of the MLC-2 gene, while mutations in the third conserved region, HF-3, appear to have no effect in this transient expression assay system. The results of the present study cannot ascribe any particular function to HF-3 with respect to inducible or tissue-specific expression. Of course, the possibility remains that the conserved HF-3 sequences may be important in regulating the expression of the MLC-2 gene in vivo.

The 28-bp HF-1 element is particularly interesting in that it appears to be not only necessary for cardiac-specific expression but also sufficient to confer cardiac-specific expression when a single copy is placed upstream of a neutral promoter. Sequence analysis of the 28-bp HF-1 element reveals homology to three known transcriptional regulatory elements. At the 5′ boundary of the HF-1 conserved sequences lies a sequence homologous to the CArG box which appears to mediate muscle specific expression of the sarcomeric α-actin genes. Within the middle of the 28-bp sequence lies an AP-2-like motif which has been implicated in inducible expression during phorbol ester and cyclic AMP stimulation (25). At the 3′ boundary of HF-1 lies an AT-rich sequence with homology to the MEF-2 element which has been implicated in skeletal muscle-specific expression of the creatine kinase gene. MEF-2-binding activity is upregulated during the conversion of myoblasts to myotubes and appears to be a marker of the differentiated muscle cell state (57). While MEF-2 appears to be a relatively weak enhancer in skeletal muscle cells, it appears to play an important role in the muscle-restricted expression of the creatine kinase gene (57). The questions arise whether these motifs within HF-1 bind authentic CArG, MEF-2, and AP-2 transcriptional factors and whether a unique combination of these transcriptional factors is responsible for the cardiac-specific expression of the MLC-2 gene. Alternatively, the HF-1-binding activity might reflect the presence of a novel cardiac nuclear binding factor. Studies are in progress to determine the identity of the HF-1-binding activity by employing MEF-2, CArG, and AP-2 competitor plasmids and mobility gel shift assays with appropriate fragments of the MLC-2 promoter.

**HF-1 and inducible expression during myocardial cell hypertrophy.** In response to various hormonal, genetic, and mechanical stimuli, the myocardium adapts to increased work loads through the hypertrophy of individual muscle cells (43). Some of the central features of the myocardial hypertrophic response are the increase in contractile protein content, the induction of contractile protein isoforms, and the expression of embryonic markers (27, 30, 51, 56). These features of the hypertrophic response appear to be largely dependent on the activation of transcription of the corresponding cardiac genes which encode these proteins. Upregulation of contractile protein genes which are constitutively expressed in the myocardium, such as the cardiac myosin light-chain-2 gene, lead to an accumulation of the contractile protein in individual myocardial cells (30). Ventricular hypertrophy is also associated with qualitative changes in contractile protein composition, such as the induction of contractile protein genes which are normally expressed strictly during embryonic development (e.g., the reactivation of skeletal α-actin and β-myosin heavy-chain gene expression in rodent and rabbit models of hypertrophy in adult myocardium [21, 27, 44, 51]). While alterations in the expression of contractile protein and other cardiac genes have been well characterized in both in vivo and cellular models of hypertrophy, relatively little is known about the precise signaling mechanisms which lead to the induction of contractile proteins and embryonic target genes during myocardial cell hypertrophy. Presumably, hypertrophic stimuli must generate signals which ultimately reach the nucleus and activate the transcription of cardiac target genes.

To address this question, the current study has utilized mutant MLC-2–luciferase constructs to identify the role of conserved HF-1, HF-2, and HF-3 regions within the MLC-2 5′ flanking region in the inducible expression of the MLC-2 gene during hypertrophy of neonatal rat myocardial cells following stimulation with two distinct agonists, the α-adrenergic agonist phenylephrine and endothelin-1. Both of these

agonists induce several features of myocardial cell hypertrophy, as determined by several separate morphological, structural, biochemical, and genetic criteria (26, 30, 33, 38, 53, 55), including the activation of a 250-bp MLC-2 promoter–luciferase fusion gene in transfection studies.

As assessed by transient expression assays using neonatal rat myocardial cells, deletion of the HF-3 sequences in the 250-bp MLC-2 promoter has little effect on the induction of the luciferase reporter gene, whereas mutations within HF-1 and HF-2 regions significantly reduce (by 50 to 75%) inducible expression. Studies employing an HF-1–TK luciferase construct which contains a single copy of the HF-1 sequence upstream of the neutral promoter TK-luciferase construct document that HF-1 is not only necessary but also sufficient to confer α-adrenergic- and endothelin-inducible expression. Thus, a conserved *cis*-regulatory element (HF-1) which has been implicated in cardiac-specific expression can also function to confer inducible expression during myocardial cell hypertrophy.

The question of the relationship between these dual roles of HF-1 in the regulated expression of MLC-2 gene arises. The possibility exists that a new inducible binding factor recognizes HF-1 or cooperates with existing transcriptional factors which occupy HF-1. Alternatively, the factors which recognize HF-1 might be covalently modified, leading to an increase in expression during the hypertrophic response of myocardial cells. Since there is an AP-2-like site within the HF-1 sequence which has been implicated in phorbol-inducible expression in other cell types, the question arises whether AP-2 mediates this induction process during the hypertrophic response. This possibility becomes more intriguing given the recent demonstration that phorbol esters can activate the hypertrophic program (17). In addition, recent studies have documented that the cotransfection of a mutant protein kinase C construct which leads to the transcriptional activation of ANF and MLC-2–luciferase reporter genes in neonatal rat myocardial cells (52a). It will become of particular interest to directly address the role of AP-2 and other sequences in the HF-1 element with regard to the induction of the MLC-2 gene. Since HF-1 contains multiple potential regulatory elements, it will become of interest to determine whether one can segregate the cardiac-specific and the inducible functions of HF-1 with fine mutations within the HF-1 sequence.

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