Heart-Specific Targeting of Firefly Luciferase by the Myosin Light Chain-2 Promoter and Developmental Regulation in Transgenic Mice

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Based on hybridization studies indicating constitutive expression levels of the endogenous myosin light chain-2 (MLC-2) gene in embryonic, fetal, and adult myocardium, a model system for selective targeting of genes to the heart of transgenic mice has been developed. A 2.1-kb DNA fragment of the 5' flanking region of the rat cardiac MLC-2 gene was fused to the firefly luciferase reporter gene and introduced into fertilized mouse oocytes. In four independent transgenic mouse lines, the expression of the MLC-2–luciferase fusion gene was found exclusively in heart muscle. In contrast to the endogenous MLC-2 gene, no luciferase activity was detectable in slow-twitch skeletal muscle or any other tissue of transgenic mice. This result suggests that the 2.1-kb DNA fragment of the 5' flanking region of the cardiac MLC-2 gene contains the regulatory elements required for selective gene expression in cardiac myocytes in vivo. In contrast to the endogenous steady-state MLC-2 expression during development, transgenic luciferase activity was 10-fold higher during embryogenesis, when formation of the ventricular loop and septum takes place. The enhanced luciferase activity in early heart development may suggest a growth-dependent control mechanism, involving either transcriptional or posttranscriptional regulation. In conclusion, this model system with the 2.1-kb ventricle-specific MLC-2 promoter sequence should facilitate the overexpression of gene products in the developing and mature heart muscle and further elucidate molecular mechanisms of myocardial diseases such as cardiomyopathies. (Circ Res. 1993;73:629-638.)

Key Words: heart muscle • embryogenesis • gene expression • cardiomyopathy • mouse model

Cardiomyopathies constitute a group of heart muscle diseases that present both contractile and electrophysiological abnormalities usually leading to severe heart failure or sudden cardiac death. The majority of cases are diagnosed as ‘idiopathic cardiomyopathies’ because of their unknown etiology. The challenge is to determine the various causes that lead to cardiomyopathies, which are currently the focus of both basic and clinical investigations. To study the potential implications of exogenous and endogenous genetic alterations in cardiomyopathies, an in vivo model system that allows the selective expression of foreign gene products in the normal and diseased heart is necessary. Therefore, it is important to develop a well-characterized transgenic animal model that specifically provides high-level transcriptional activity in the heart muscle but little or no activity in any other tissue. Striated muscle types can be functionally divided into cardiac, slow skeletal, and fast skeletal by their expression of distinct patterns of tissue-specific protein isoforms, which include both myofilibrillar proteins and intracellular enzymes. The expression of many of these muscle-specific proteins is developmentally regulated at the level of transcription. Differentiation into cardiac muscle leads to the activation of muscle-specific genes encoding myosin heavy and light chains, α-cardiac and skeletal actins, the troponins I, C, and T, tropomyosin, and creatine kinase. Since cardiac muscle and other excitable tissues share many electrophysiological, metabolic, and contractile properties, it is not surprising that a defined subset of cardiac genes is coexpressed in cardiac, skeletal, or neural tissues. In contrast to the myosin light chain-1 genes (MLC-1A, MLC-1V), which are coexpressed and developmentally regulated in cardiac and fast skeletal muscle tissue, no developmental studies have been conducted for the myosin light chain-2 (MLC-2) gene.

Several isoforms of the phosphorylatable MLC-2 encoded by the cardiac/slow skeletal muscle, fast skeletal muscle, and smooth muscle/nonmuscle genes have been identified. The smooth muscle/nonmuscle MLC-2 isoform is expressed in smooth muscle tissues such as colon and uterus and in some, but not all, nonmuscle cells. In contrast, it is not expressed in skeletal and cardiac muscle tissues. Transgenic studies of the rat fast skeletal MLC-2 gene have found cis-acting sequences that will target expression specifically in fast-twitch skeletal muscle cells, although the precise cis-regulatory elements required for fast-twitch
skeletal muscle expression have not yet been identified. The cardiac MLC-2 gene has recently been shown to be coexpressed at high levels, both in adult heart and slow-twitch skeletal muscle.\textsuperscript{23} Comparison of the rat cardiac with the rat fast skeletal MLC-2 genes reveals a high degree of conservation.\textsuperscript{24} Each gene contains seven exons of identical size with conservation of intron/exon boundaries. However, the 5' flanking regions, which presumably contain the main determinants for cell-specific regulation of gene expression, are widely divergent.\textsuperscript{25} Previous studies using rat and chicken cardiac MLC-2 regulatory sequences have demonstrated successful reporter gene expression in primary cardiac muscle but not in skeletal muscle cells.\textsuperscript{24,25}

Since transcription rates of cardiac-specific genes are greatly diminished in primary heart muscle cells and since fundamental differences often exist between permanent in vivo expression in intact tissue and transient in vitro expression in cultured cells,\textsuperscript{26} it is necessary to investigate the activity of the 5' regulatory region of the cardiac MLC-2 gene in vivo. Therefore, we set out to establish transgenic lines containing cardiac-specific promoters linked to the luciferase reporter gene that could be used as a model system to study the selective expression of foreign genes in normal and diseased hearts. Transgene expression was then assessed in various cell types during both embryonic and fetal development. In four independent transgenic mouse lines, the 2.1-kb control region of the cardiac MLC-2 gene restricted expression of a luciferase reporter gene to heart muscle tissue throughout development. In contrast to the endogenous MLC-2 gene, which is constitutively expressed in ventricles during embryogenesis and coexpressed in slow-twitch skeletal muscle in adult mice, luciferase activity was only found in heart muscle, increasing during embryonic ventricular loop and septum formation and then gradually declining to a postnatal steady-state level. These results demonstrate that the 2.1-kb MLC-2 promoter fragment behaves differently from the endogenous MLC-2 expression. Since there is no coexpression in slow-twitch skeletal muscle, it can be used to direct expression of foreign gene products exclusively to cardiac muscle tissue from early embryogenesis.

Materials and Methods

In Situ Hybridization Analysis

Embryos were frozen in liquid nitrogen and embedded in Tissue-Tek OCT (Lab-Tek Products, Chicago, Ill.). Serial cryostat sections (6 μm) were prepared at −20°C and mounted on microscope slides that had been pretreated as previously described.\textsuperscript{27} Sections were fixed for 20 minutes in 4% paraformaldehyde/phosphate-buffered saline (mmol/L: NaCl, 130; Na₂HPO₄, 7; and NaH₂PO₄, 3). Slides were then dehydrated through a series of ethanol. Embryo sections were hybridized according to established procedures\textsuperscript{28} with the following modifications: the hybridization mixture contained 100- to 200-bp fragments of a nick-translated mouse cardiac MLC-2 cDNA (200 ng/mL), derived from a full-length 645-bp fragment.\textsuperscript{29} Probes were labeled with [α-35S]dATP and [α-35S]dCTP (1200 Ci/mmoll) to a specific activity of 1×10⁶ cpm/μg. Control probes were prepared from nonrecombinant vector DNA. Hybridization was performed in 10 mmol/L Tris-HCl, pH 7.4, 50% (vol/vol) deionized formamide, 600 mmol/L NaCl, 1 mmol/L EDTA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.05% bovine serum albumin, 10% dextran sulfate, 10 mmol/L dithiothreitol, denatured sonicated salmon sperm DNA (200 μg/mL), and rabbit liver tRNA (100 μg/mL). Hybridization with DNA probes was performed at 37°C for 36 hours. Slides were then washed as previously described,\textsuperscript{28} followed by incubation in 2× standard saline citrate (SSC) for 1 hour at 55°C. Slides were autoradiographed and stained with hematoxylin/eosin.\textsuperscript{27}

Slot Blot Analysis of Endogenous MLC-2 mRNA Expression

To isolate total RNA, flash-frozen tissues were homogenized with an ultra turrax in a 4 mol/L guanidinium thiocyanate solution followed by phenol-chloroform extraction and propanol precipitation as previously described.\textsuperscript{29} Samples were solubilized in TES buffer containing 0.2% sodium dodecyl sulfate (SDS), 1 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.5). After optical density measurement, equal amounts of RNA (0.5 and 0.25 μg) were deposited onto a sheet of nitrocellulose using a filtration manifold (Minifold II, Schleicher & Schuell, Inc, Keene, NH). Filters were baked in vacuo at 80°C for 2 hours and prehybridized at 42°C for 4 hours in 50% formamide, 2× SSC, 120 mmol/L phosphate buffer (pH 6.8), 20 mmol/L EDTA (pH 7.2), 0.2% SDS, 1% sarcosyl, 4× Denhardt’s solution, and 250 μg/mL denatured herring sperm DNA. Subsequently, the 645-bp EcoRI mouse cardiac MLC-2 cDNA probe,\textsuperscript{23} labeled with [α-32P]dCTP (3000 Ci/mmoll) to a specific activity of 4×10⁶ cpm/μg by random priming,\textsuperscript{30} was added to the prehybridization solution, and hybridization was carried out overnight at 42°C. After posthybridization and washing under stringent conditions (2× SSC and 0.2% SDS at 50°C), filters were exposed overnight to an XAR-5 film (Kodak). To ensure equal binding of total RNA to the filters, the relative quantity of 18S RNA was determined by rehybridization of the slot blots using an oligonucleotide probe (−ACGGTATCTGATCGTCTTCGAACC−) labeled with [γ-32P]dATP (3000 Ci/mmoll) to a specific activity of 2×10⁶ cpm/μg by T4 kinase. The probe was added to the prehybridization solution (5× standard saline phosphate EDTA, 5× Denhardt’s solution, 0.5% SDS, and 250 μg/mL denatured herring sperm DNA), and hybridization was carried out overnight at 42°C. After posthybridization (3× SSC at 42°C), filters were exposed overnight to XAR-5 film (Kodak).

Generation and Breeding of Transgenic Mice

A 2.7-kb EcoRI DNA fragment of the 5' upstream regulatory sequence derived from the rat cardiac MLC-2 gene (Fig 1A) was coupled to the firefly luciferase reporter gene.\textsuperscript{25,31} Transgenic mice were generated using the construct diagrammed in Fig 1B. A 2.1-kb MLC-2–luciferase fusion gene (2.1MLC-2-luc), derived from plasmid pMLCLΔ5′ (−2700 to +12),\textsuperscript{25} was excised at the Kpn I restriction sites as indicated. One picoliter containing 500 to 1000 copies of the fusion gene in 10 mmol/L Tris-HCl (pH 7.5) and 0.2 mmol/L EDTA was microinjected into male pronuclei of fertilized mouse oocytes [B6D2F1]×NMRI) according to established procedures.\textsuperscript{32,33} A total of 341 injected eggs were transplanted into the oviducts of 17 pseudopregnant mice.
The polypeptide and of Preparation DNA crossed and 30 gL female, filter 1. breeding of gastrocnemius, of a-galactosidase 1.8-kb 1.2-kb 1.0-kb 0.8-kb 0.6-kb 0.4-kb 0.2-kb 0.0-kb. 3’ rat cardiac MLC-2 gene 5’

**Fig 1.** A shows the genomic map of the rat cardiac myosin light chain-2 (MLC-2) gene. Seven exons encode a 166-amino-acid polypeptide. The 5’ untranslated region contains the transcriptional start site, TATA box, HF-1b, HF-1a, HF-2, and HF-3 elements, and the cardiac-specific sequence (CSS). B shows the 2.1-kb MLC-2–luciferase fusion gene (2.1MLC-2-luc) used for generation of transgenic mice. Restriction enzymes are indicated as follows: A, AvaII; E, EcoRI; and K, KpnI.

(NMRI). DNA of transgenic founders and their offspring, obtained by backcrosses (F1 generation) or breeding of siblings (F2 and F3 generation), was analyzed by Southern hybridization. For studies of ontogeny, homozygous F2 males of line F0 94 and F0 16 were crossed with nontransgenic female mice. Gestational age of intrauterine mouse embryos was related to the first day of mucous plug formation of the pregnant female, designated day 1 after conception.

**Preparation and Analysis of Genomic DNA**

High molecular weight DNA was isolated by incubating 1 cm of mouse tail in 0.5 ml HMW1 buffer containing (mmol/L) Tris-HCl, 10 (pH 7.5); NaCl, 150; and EDTA, 10; together with 0.4% SDS and 200 µg proteinase K (Merck) for 12 hours at 37°C. DNA was phenol-chloroform–extracted, ethanol-precipitated, and resuspended in 30 µL TE buffer containing (mmol/L) Tris-HCl, 10 (pH 7.5) and EDTA, 1. Five micrograms of EcoRI-digested DNA was electrophoresed and transferred to a nitrocellulose filter in 20x SSC. Prehybridization buffer was used as described above. Filters were hybridized with a [α-32P]dCTP–labeled 1.8-kb HindIII–KpnI luciferase fragment derived from pSV2/LAS5. After posthybridization and washing in 2x SSC, filters were exposed for 48 hours with enhancing screens at −80°C.

**Preparation of Tissue Samples for Luciferase Enzyme Assay and Polymerase Chain Reaction**

For analysis of luciferase expression, muscle tissues such as heart, skeletal muscle (quadriceps femoris, gastrocnemius, and soleus), smooth muscle (uterus), and nonmuscle tissues such as gonads (testis or ovary), lung, liver, spleen, kidney, thymus, salivary gland, brain, skin, stomach, and intestines were removed from ether-anesthetized transgenic mice and nontransgenic control mice. For detailed localization of luciferase expression, the myocardium was separated into atrial and ventricular tissue under a dissecting microscope. For developmental studies, the heart and the remaining thorax, head region, abdomen, and tail region were dissected and immediately flash-frozen in liquid nitrogen. Tissues were then pulverized in liquid nitrogen and homogenized in 200 to 300 µL extraction buffer with 100 mmol/L KH2PO4 (pH 7.8), 1 mmol/L dithiothreitol, and 0.1% Triton X-100. Tissue homogenates were instantly refrozen in a dry-ice bath and lysed by thawing at 37°C for 3 minutes. Cellular debris was pelleted for 15 minutes at 11,000g, and supernatants were stored at −70°C.

Total RNA was isolated from frozen tissues as described above. RNA samples from transgenic hearts and muscle tissues were digested by RQ1-DNase (Promega Corp, Madison, Wis) in 50 mmol/L Tris-HCl (pH 7.5) and 5 mmol/L MgCl2. After phenol-chloroform extraction, RNA was reprecipitated and stored at −80°C.

**Quantitative Luciferase Enzyme Assay**

The luciferase assay was performed according to established procedures with the following modifications: A 10-µL sample of protein extract was added to 100 µL of assay buffer containing (mmol/L) glycollic acid, 25 (pH 7.8); MgSO4, 10; EDTA, 2; and ATP, 2.5 (pH 7.5). The tube was placed in a monolight 2001 transilluminometer (Analytical Luminescence Laboratory, San Diego, Calif), and the reaction was initiated by the automated injection of 100 µL of 1 mmol/L D-luciferin. Peak light emission of tissue luciferase activity was recorded over a period of 10 seconds. Three independent measurements were performed for each tissue tested. Luciferase enzyme activity was expressed in relative light units. Enzyme activities were related to the amount of total cellular protein extract in milligrams, determined by the Bradford assay. For each assay, a standard curve with gradually increasing amounts of pure luciferase protein (Analytical Luminescence Laboratory) in 10 µg nontransgenic protein was generated. By double logarithmic display, a regression line linear over a range of 4 logs was generated (see Fig 2). Peak light emission was recorded over four orders of magnitude in the linear range from 0.03 to 300 pg. The limit of detection of luciferase protein (molecular mass, 62 kD) was determined to be 30 fg or 2.9 x 10⁵ molecules. Extrapolation of the light units per milligram logarithm of positive transgenic hearts allowed quantification of the amount of luciferase expression per milligram of total soluble protein. To relate the amount of cardiac protein to 1 g of total heart, the following transformation was performed. Approximately 5 mg of protein derived from one transgenic mouse heart (total
weight, 100 mg) was solubilized in the extraction buffer, which equals 50 mg of protein per 1 g of total heart.

Detection of Luciferase mRNA Expression by Polymerase Chain Reaction

Two unique 23-bp oligonucleotide primers Luc-P2 (from +9 bp to +31 bp) and Luc-P3 (from +363 bp to +387 bp) located within the luciferase coding region were synthesized. These primers amplified a 379-bp fragment of firefly luciferase cDNA. For standard polymerase chain reaction (PCR) analysis, 2 μg of total RNA was reverse-transcribed using the 3′ antisense primer, Luc-P3. One fourth of the reverse-transcribed product was amplified by PCR using 1 μmol/L of each primer in a reaction buffer containing 100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 15 mmol/L MgCl₂, 0.01% (wt/vol) gelatin, and 200 μmol/L of each of the four deoxynucleotide triphosphates. PCR conditions included denaturation at 94°C for 45 seconds, annealing at 64°C for 45 seconds, and polymerization at 72°C for 1 minute; after 40 cycles, an extension step of 7 minutes at 72°C was added. As a positive control, luciferase cDNA, pSV2/LtA5', was transfected into COS cells. Non-reverse-transcribed RNA preparations were used to exclude genomic DNA contamination.

Results

Expression Pattern of the Endogenous Cardiac MLC-2 Gene During Embryogenesis

Previous transgenic experiments have shown that antigen presentation during early development confers self-tolerance by the murine immune system, whereas delayed appearance of the antigen results in an autoimmune response. Therefore, the early onset of gene expression is an important prerequisite for a transgenic model, which allows the introduction of foreign antigens to the heart muscle. Since no data on prenatal expression of the endogenous cardiac MLC-2 gene have been generated, we analyzed tissue specificity of MLC-2 gene expression during embryogenesis. In situ hybridizations using a 35S-labeled mouse cardiac MLC-2 cDNA probe were performed to investigate the temporal and spatial expression pattern of the endogenous cardiac MLC-2 gene during embryogenesis. Sagittal sections of total mouse embryos showed MLC-2 mRNA expression throughout the entire section of the mouse ventricular myocardium as displayed in Fig 3 for day 13 after...
conception. Importantly, no expression was seen within any other muscular or nonmuscular tissue (Fig 3A). As illustrated in Fig 3B, autoradiographic labeling is clearly restricted to ventricular myocardium, demonstrating tissue specificity of MLC-2 expression during embryogenesis. No labeling of myocaridal cells was observed when mouse embryos were hybridized with the nonrecombinant vector control DNA probe (Fig 3C), demonstrating the specificity of in situ hybridization for detection of MLC-2 mRNA. These results indicate that the endogenous MLC-2 gene expression is restricted to the ventricular myocardium during embryogenesis. MLC-2 expression coincides with the education period of B and T cells, which should result in a potential “self-acceptance” of transgenic proteins by the murine immune system.

Quantitative Analysis of the Endogenous Cardiac MLC-2 Expression During Development

Quantitative changes in gene expression during cardiac development are a central feature of myocyte differentiation. So far, cardiac ventricular MLC-2 proteins have been shown to be constitutively expressed after birth. To determine how the endogenous cardiac MLC-2 gene is regulated during prenatal heart development, total RNA was extracted from embryonic, fetal, newborn, and adult mouse hearts. Equal amounts of total RNA were fixed to nitrocellulose and hybridized with a 32P-labeled mouse cardiac MLC-2 cDNA probe. As can be seen by autoradiography, a relatively homogenous signal was detected in prenatal, neonatal, and adult heart samples (Fig 4). Rehybridization of the filters using a 32P-labeled 18S oligonucleotide probe confirmed equal amounts of RNA binding per slot. Comparison of the cardiac MLC-2 transcripts with the signals generated by the 18S RNA slots gave a constant ratio. This demonstrates that, in relation to total RNA, cardiac MLC-2 transcripts do not change during development. Thus, the endogenous MLC-2 promoter reveals a constitutive expression level during the entire period of heart development studied. As a control, the cardiac MLC-2 cDNA probe was hybridized with fast- and slow-twitch skeletal muscle RNA. Specific hybridization was found with slow skeletal muscle RNA, whereas fast-twitch skeletal muscle RNA gave no detectable signals. This result confirmed the coexpression of the endogenous MLC-2 gene in adult mouse tissue of cardiac and slow-twitch skeletal muscle as recently published by Lee et al.

Generation and Characterization of 2.1MLC-2-luc Transgenic Mouse Lines

Since endogenous MLC-2 expression was ventricle specific and occurred during the self-acceptance phase of the murine immune system, the putative regulatory promoter segment of the cardiac MLC-2 gene was assessed for tissue-specific targeting of a reporter gene in transgenic mice. A 2.1-kb 5' upstream MLC-2 DNA fragment (Fig 1A) was coupled to the firefly luciferase reporter gene (Fig 1B). To generate transgenic mice, the 2.1MLC-2-luc fusion gene was microinjected into the male pronuclei of fertilized mouse oocytes. Inoculated zygotes were then implanted into pseudopregnant females, and offspring were screened for transgene integration. A total of 64 pups were born. Seven newborn mice, designated F0 08, 11, 16, 18, 26, 47, and 94, had the luciferase gene integrated into their genome, as determined by Southern blot hybridization analysis (data not shown). The seven positive founders (F0) were mated with nontransgenic mice, and DNA analysis of the 2.1MLC-2-luc offspring showed transmission of the luciferase gene to the F1 generation. According to an autosomal dominant trait, 46% of F1 descendants (120 of 263) were carriers of the transgene. Inbred crossings of hemizygous F1 mice (lines 16, 26, 47, and 94) gave rise to 79% 2.1MLC-2-luc F2 descendants (145 of 178), of which approximately 25% were homozygous and 50% were hemizygous litters. Homozygous inbred crossings of F2 mice (lines 16, 26, 47, and 94) generated 100% 2.1MLC-2-luc–positive progeny (135 of 135). Homozygous F3 mice were used to study MLC-2 promoter activity in response to a double transgenic DNA dose. Outbred crossings of homozygous F2 males produced 100% hemizygous offspring, which were used for subsequent analysis of luciferase expression at different time points during development.

Heart-Specific Expression of the 2.1MLC-2-luc Transgene

Total cellular protein extracts of transgenic hearts from 30- to 60-day-old hemizygous F1 mice, derived from F0 08, 11, 16, 18, 26, 47, and 94, were tested for luciferase enzyme activity. Four independent 2.1MLC-2-luc lines (08, 16, 18, and 94) revealed positive lu-
ciferase activity ranging from 2.7 kLU/mg, where LU indicates light units, (line 94) to 560 kLU/mg (line 18) of soluble protein. Luciferase activities of F1 animals of the two 2.1MLC-2-luc lines 08 and 16 have been contributed to the recent study of Lee et al.25 An average light emission of 161 kLU/mg protein was registered in transgenic hearts, which equals an absolute amount of 14 pg or 1.3x10^8 molecules of luciferase protein per milligram of total heart extract. To relate luciferase activity to the amount of luciferase molecules, light emission was converted to protein concentration by using the standard curve in Fig 2. Extrapolation of the 10 log of 560 kLU/mg of line 18 gave a maximal luciferase protein expression level of 46 pg or 4.5x10^8 molecules per milligram of heart extract; the minimal expression level calculated for line 94 equals 131 fg or 2.9x10^6 molecules per milligram of cardiac protein. To relate luciferase protein expression to 1 g of total heart, a factor of 50x has to be included (see “Materials and Methods”). Thus, the maximal amount of luciferase expression equals 2.3 ng or 2.2x10^10 molecules and the minimal amount equals 6.6 pg or 6.4x10^7 molecules per 1 g of total heart tissue.

Extending luciferase expression analysis from hemizygous F1 mice to homozygous F3 mice, transgenic lines 94 and 16 were analyzed for their cardiac luciferase activity. Heart extracts of 25 homozygous F3 animals revealed 6.7 or 5.5 kLU/mg protein, which corresponds to an average increase in luciferase enzyme activity of 80% to 90% in homozygous animals containing a double gene dose of the 2.1MLC-2-luc fusion gene. This implies that luciferase gene expression from both alleles takes place in an additive way. To complete luciferase protein expression analysis, various transgenic and control tissues were tested for light activity. Average luciferase activities (LU per milligram protein) detected in the four independent 2.1MLC-2-luc expressor lines (8, 16, 18, and 94) are presented in Fig 5. Total cellular protein extracts of fast skeletal muscle (eg, quadriceps femoris), slow skeletal muscle (eg, soleus), and smooth muscle (eg, uterus), as well as the nonmuscle tissues listed, did not show any light activity above background. Luciferase activity was detected exclusively in cardiac tissue and predominantly in the ventricles (ratio of ventricles to atria, 100:1) with an average activity of 161±65.5 kLU/mg (Fig 5).

To exclude the possibility of low-level expression of luciferase in noncardiac muscle tissues of transgenic mice, PCR analyses were performed. Hemizygous F1 litters derived from high expressor line 18 were used for mRNA expression analysis (Fig 6). After reverse transcription of total cellular RNA using a 3' antisense luciferase primer (Luc-P3), cDNA was amplified by PCR adding the 5' sense primer (Luc-P2). A specific luciferase cDNA fragment of 379 bp was generated from 2.1MLC-2-luc transgenic hearts only (Fig 6, lane 7). In contrast, no specific PCR product was amplified from total cDNA of skeletal muscle, such as soleus, and smooth muscle, such as uterus, even after 40 rounds of amplification (Fig 6, lanes 8 and 9). These results

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FIG 5. Diagram of mean luciferase activities (in light units [LU] per milligram protein) in various organs (quad: fem. indicates quadriceps femoris) of transgenic mice. Columns represent average luciferase activity determined in the four 2.1-kb myosin light chain-2–luciferase lines (8, 16, 18, and 94). From each transgenic line, light activity was determined in at least 10 different positive F1 mice.

FIG 6. Agarose gel of luciferase-specific polymerase chain reaction (PCR) products. The 379-bp band is the amplified PCR product of oligonucleotide primers Luc-P2 and Luc-P3. Lanes are as follows: lane 1, a 123-bp DNA ladder; lanes 2 through 4, PCR products of 100 fg (lane 2), 10 fg (lane 3), and 1 fg (lane 4) pSV2/LAS'-transfected cells; lanes 5 and 6, control study with PCR amplification of reverse-transcribed mRNA (lane 5) and non–reverse-transcribed mRNA (lane 6) from pSV2/LAS'-transfected cos cells; and lanes 7 through 9, reverse-transcribed total cellular RNA from 2.1-kb myosin light chain-2–luciferase transgenic heart (lane 7), soleus muscle (lane 8), and uterus (lane 9) from the F1 generation of transgenic line 18.
demonstrate a selective expression of the luciferase transgene in heart muscle tissue.

Expression of the 2.1MLC-2-luc Transgene During Heart Development

To investigate prenatal expression activity from the 2.1MLC-2-luc fusion gene, hemizygous F3 progenies from transgenic lines F0 94 and F0 16 were analyzed for luciferase enzyme activity at different time points during embryonic, fetal, and postnatal development (Fig 7). Embryonic heart protein extracts were generated as early as day 9 after conception, when atrial trunk and ventricular loop formation takes place.41 Until days 11 to 13 after conception, when the interventricular and interatrial septum develops, luciferase expression levels up to 36 kLU/mg protein were detected in the embryonic heart. From the beginning of fetogenesis at day 14.5 after conception, when the murine circulation system is complete and functioning with an open foramen ovale, luciferase activity gradually declines until postpartum day 15. A steady-state luciferase expression level of 3.3 kLU/mg cardiac protein was observed from postpartum days 15 to 62 (Fig 7). These results demonstrate that during the embryonic period of ventricular loop and septum formation there is a 10-fold higher luciferase activity compared with postpartum steady-state levels. In addition, enzymatic analyses of embryonic protein extracts from the remaining thorax, head, abdomen, and tail and fetal protein extracts from tissues as listed in Fig 5 did not reveal light activity at any time point studied.

Discussion

On the basis of our finding of early endogenous MLC-2 gene expression at constitutive levels in the embryonic, fetal, and adult myocardium, a 5' regulatory MLC-2 fragment was chosen to establish a transgenic model for heart-specific gene expression. In the present study, we have analyzed the ability of the rat cardiac MLC-2 promoter to selectively target luciferase reporter gene expression to heart tissue of transgenic mice during development. Different from the endogenous cardiac/skeletal muscle MLC-2 gene expression, our data demonstrate two properties of the 2.1-kb rat cardiac MLC-2 sequence: (1) A constant cardiac muscle−restricted luciferase activity was observed postpartum in four transgenic lines. (2) In two lines, luciferase activity was found to be 10-fold higher during the embryonic period of ventricular loop and septum formation. The enhanced luciferase activity in early heart development indicates a growth-dependent control mechanism. Since no coexpression of the luciferase reporter was detected in any other tissue except murine myocardium, the 2.1-kb DNA fragment of the cardiac MLC-2 gene should contain the regulatory elements required for selective gene expression in cardiac myocytes in vivo.

Cardiac-Specific Gene Expression From the 2.1-kb MLC-2 Promoter

Several activating and repressing elements responsible for cardiac-specific gene expression have been mapped within the −2100-bp region of the MLC-2 promoter in tissue cultures.42,44 Five activating DNA elements, HF-1a, HF-1b, HF-2, HF-3, and the TATA box, are located within a region from −198 to −19 bp of the rat cardiac MLC-2 gene.42 A repressing element called the cardiac-specific sequence (CSS) is located further upstream between −1723 and −1686 bp of the rat cardiac MLC-2 gene44 (Fig 1A). The HF-1 region, a conserved 28-bp sequence between −72 and −45 bp,42 contains conserved sequences to known regulatory elements including CC(A+T-rich)GG (CArG) box/serum responsive element (SRE)/element A,45,46 AP-2 box,47,48
and myocyte-specific enhancer-binding factor-2 (MEF-2) box/element B,45,46 the latter being identical with the HF-1b sequence.43 For muscle-specific transcription, only the proximal promoter consisting of HF-1b and TATA box/element C is required.44 Addition of HF-1a and HF-2 domains results in a muscle-specific and inducible gene expression in transient tissue culture assays.42,45 Since the nuclear CARG-, AP-2-, and MEF-2-binding factors are found in differentiated cardiac as well as skeletal muscle cells, they may not be sufficient to account for the heart muscle-specific activity of the MLC-2 promoter observed in transgenic mice. However, specificity might be conferred by a unique combination of factors or through binding of a cardiac-specific cofactor. Alternatively, repression of cardiac MLC-2 promoter activity has been pointed to the 5' flanking CSS element in primary fast-twitch and mouse C2C12 skeletal muscle cell cultures.24 Removal of the CSS sequence from the chicken cardiac MLC-2 promoter allowed transcription in fast-twitch skeletal muscle cells without affecting the transcriptional activity of the promoter in cardiac muscle cells. CSS-binding proteins were identified in nuclear extracts from skeletal muscle but not from cardiac muscle.24 A negative regulatory mechanism may therefore account for lack of expression of the cardiac MLC-2 gene at least in fast skeletal muscle tissue. However, it remains to be determined why endogenous cardiac MLC-2 mRNA is an abundant transcript in both heart and slow-twitch (soleus) muscle of the adult rat and mouse23 and why transgenic mice with a 2.1MLC-2-luc fusion gene display luciferase activity exclusively in cardiac muscle. According to our results, the 2.1-kb MLC-2 promoter contains all cis-regulatory elements required for restricted expression in embryonic and adult cardiac muscle cells but obviously lacks critical sequences necessary for expression in slow skeletal muscle. This may indicate that the cis-acting sequences required for expression in the slow skeletal context either lie further upstream from the 2100-bp fragment or are located in the downstream intronic portion of the gene. Therefore, we suggest that distinct regulatory programs may have developed for the expression of the cardiac MLC-2 gene in heart and slow-twitch skeletal muscle.

Comparison of Endogenous and Transgenic Promoter Activity During Cardiac Development

Expression of the cardiac MLC-2 mRNA and the luciferase protein has been determined during ontogeny of the mouse heart. The cardiac MLC-2 mRNA was found to be constitutively expressed in relation to 18S RNA during murine heart development. In contrast, a 10-fold increased luciferase protein synthesis driven by the 2.1-kb MLC-2 promoter was detected during embryogenesis, when the ventricular loop and septum formation takes place and the atrioventricular cushions and the atrial septum with the remaining foramen ovale appear. After completion of the fetal circulatory system at day 14.5 after conception, a decrease in luciferase activity was monitored. These findings extend our understanding of the developmental expression of the cardiac MLC-2 gene; simultaneously, they raise the question of a differential regulatory mechanism for luciferase reporter gene expression driven by the 2.1-kb MLC-2 promoter. Little is known about the correlation of MLC-2 mRNA and protein levels in the growing myocardium. A single study performed during the development of chicken fast skeletal muscle reported that MLC-2 protein levels correspond to the accumulation of mRNA.51 In the case of similar protein and mRNA levels, it can be speculated that the 2.1-kb MLC-2 promoter region has lost negative regulatory elements, which are necessary for a constitutive developmental luciferase gene expression. On the other hand, a posttranscriptional mechanism such as a change in the rate of protein synthesis could be possible. For example, the rate of 18S and 28S rRNA transcription has been reported to be upregulated in growing tissues.52,53 Since MLC-2 mRNA levels were related to 18S RNA, a simultaneous increase can be postulated. On the other hand, it is possible that the luciferase mRNA stability, rate of transcription, and posttranscriptional mRNA processing, which are important factors determining the rate of protein synthesis, are changed in the growing myocardium. Therefore, more detailed analyses such as nuclear run-on assays of luciferase and MLC-2 and Western blot assays of MLC-2 protein during myocardial development are necessary to resolve these questions.

Quantitative Analysis of the Luciferase Expression Levels From the 2.1MLC-2-luc Transgene

For further cardiac gene expression studies, it is important to estimate the strength of the 2.1-kb MLC-2 promoter activity. Therefore, the amount of luciferase reporter enzyme in transgenic hearts was compared with a representative tissue-specific household promoter and with the expected amount of MLC-2 mRNA in heart muscle. A household promoter, such as phosphoglycerate kinase 2, revealed testis-specific luciferase activity of 1600 LU/µg in transgenic mice.54 The maximal expression activity of the 2.1-kb MLC-2 promoter falls with 560 LU/µg into the range of household gene expression.54 The concentration of total mRNA in heart muscle (R. Kandolf, unpublished data) was estimated to be 21.3±4.5 µg/g of total heart, which is close to the reported concentration of mRNA in skeletal muscle of 26 µg/g tissue.55 The amount of MLC mRNA constitutes 2.0% of the poly(A) RNA.56 Since the ratio of MLC-1 to MLC-2 is almost 1:1, approximately 1.0% or 213 ng of MLC-2 mRNA per 1 g of total heart can be expected. Based on the assumption that the amount of luciferase protein reflects the level of MLC-2 mRNA, the highest luciferase expression in adult hemizygous F1 mice (2.3 ng per gram of total adult heart) equals 1.1% of the estimated MLC-2 mRNA. When it is taken into account that the amount of MLC-2 mRNA expression was reported in 16-day-old chicken embryos56 and the luciferase protein expression was calculated for adult F1 mice, a factor of 10× regarding the embryonic upregulation and a factor of 2× regarding hemizygosity have to be included. According to this calculation, luciferase protein expression was estimated to be 22% of MLC-2 mRNA levels. This may suggest that most, if not all, cis-acting elements for effective MLC-2 mRNA expression are located in the 2.1-kb regulatory sequence.

Implications for Cardiac-Specific Gene Expression

By the transgenic approach described in the present study, other promoters with potential cardiac specificity,
such as members of the contractile (α-myosin heavy chain, β-myosin heavy chain, MLC-1A, MLC-1V, and troponins C, T, and I), the ion channel (voltage-dependent Na+, Ca2+, and K+ channels), the receptor (α- and β-adrenergic receptors), and the cardiac hormone (atrial natriuretic factor and renin) gene family, can be characterized for their developmental, regional, and quantitative expression activity. So far, cardiac-specific promoter sequences tested (atrial natriuretic factor, α-myosin heavy chain, and creatine kinase) show either coexpression in skeletal muscle or other tissues.1057-59

To address a particular biochemical, physiological, or pharmacological problem using a transgenic model, it is necessary to know the detailed properties of the chosen promoter sequence in order to distinguish tissue-specific influences from a systemic effect. The transgenic model described in this study should provide a powerful in vivo system for selective targeting of foreign gene products to the ventricular myocardium. By this approach, viral gene regions of the cardiotropic coxsackievirus B327,60,61 are being expressed in a heart-specific manner to unravel interference with myocyte function. In addition, overexpression of oncogenes62,63 and altered contractile proteins such as β-myosin heavy chain2-4 can be tested as potential pathogenes in the etiology of cardiomyopathy. The transgenic model using the ventricular myocyte-specific MLC-2 promoter will therefore allow a genetic approach to understanding basic molecular aspects of cardiac development and regulation.

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