Our previous study of homozygous mutants of the ventricular specific isoform of myosin light chain 2 (mlc-2v) demonstrated that mlc-2v plays an essential role in murine heart development (Chen, J., Kubalak, S. W., Minamisawa, S., Price, R. L., Becker, K. D., Hickey, R., Ross, J., Jr., and Chien, K. R. (1998) J. Biol. Chem. 273, 1252–1256). As gene dosage of some myofibrillar proteins can affect muscle function, we have analyzed heterozygous mutants in depth. Ventricles of heterozygous mutants displayed a 50% reduction in mlc-2v mRNA, yet expressed normal levels of protein both under basal conditions and following induction of cardiac hypertrophy by aortic constriction. Heterozygous mutants exhibited cardiac function comparable to that of wild-type littermate controls both prior to and following aortic constriction. There were no significant differences in contractility and responses to calcium between wild-type and heterozygous unloaded cardiomyocytes. We conclude that heterozygous mutants show neither a molecular nor a physiological cardiac phenotype either at base line or following hypertrophic stimuli. These results suggest that post-transcriptional compensatory mechanisms play a major role in maintaining the level of MLC-2v protein in murine hearts. In addition, as our mlc-2v knockout mutants were created by a knock-in of Cre recombinase into the endogenous mlc-2v locus, this study demonstrates that heterozygous mlc-2v cre knock-in mice are appropriate for ventricular specific gene targeting.

Sarcomeres, the functional units of striated muscle, are composed of >20 known proteins, precisely organized in a nearly crystalline fashion. Mechanisms by which these proteins are assembled remain largely unknown. The genetic approach of inactivating genes encoding specific sarcomeric proteins has been used to understand the role of stoichiometry in sarcomeric assembly. One important issue is the gene dosage effect of these proteins on muscle organization and function. *Drosophila* heterozygous mutants with one functional copy of either actin or myosin heavy chain (MHC)1 gene have dysfunctional flight muscle, cannot fly, and have abnormal sarcomeric structure (1). Heterozygous mutants display a 50% reduction in either actin or MHC protein, respectively. Interestingly, the sarcomeric structure of muscle from double heterozygous mutants with single copies of both actin and MHC genes is less abnormal than muscle from either of the single heterozygous mutants. Consequently, double heterozygotes are capable of weak flight (1). These data indicate that variations in sarcomeric protein stoichiometry can affect myofibrillar organization and function. Recently, similar studies have been performed in mouse knockout models. Heterozygous α-MHC knockout mice have ~50 and 25% reductions in α-MHC mRNA and protein, respectively, compared with wild-type littersmates. Heterozygous mice have impaired cardiac function and abnormal sarcomeric organization (2). On the other hand, heterozygous α-tropomyosin knockout mice display a 50% reduction in mRNA but similar levels of α-tropomyosin protein compared with wild-type littersmates and have no detectable cardiac abnormalities (3, 4). However, to date, no studies have been performed with these mutant mice to examine the consequences of the heterozygous state on cardiac function under conditions that induce hypertrophy.

Myosin light chain 2 (MLC-2), the regulatory myosin light chain, plays an essential role in vertebrate smooth muscle contraction (5–7). In vertebrate striated muscle, MLC-2 is thought to have only a modulatory effect on contraction (8–11). However, a recent gene targeting study has revealed a selective requirement for the ventricular specific isoform of mlc-2 (mlc-2v) in embryonic murine heart function (12). Homozygous mlc-2v mutant embryos die at approximately embryonic day 12.5. The null mutants have a significantly reduced left ventricular ejection fraction. Ultrastructural analysis revealed defects in sarcomeric assembly. These data indicate that mlc-2v plays an important role in cardiac function and myofibrilllogenesis during mammalian heart development. Moreover, it has been shown that missense mutations in the human MLC-2v gene result in a rare form of hypertrophic cardiomyopathy (13). However, there are no data to distinguish whether the disease results simply from “loss” of function (gene dosage) or an alternative mechanism. *Drosophila* has a single mlc-2 gene, and homozygous mlc-2 mutants are embryonic lethal. Heterozygous mutants can survive, but cannot fly due to dysfunctional flight muscles that display abnormal sarcomeric structure (14). These heterozygous mutants display a 50% reduction in mlc-2 mRNA with a 25% reduction in MLC-2 protein compared with wild-type light chain 2; MLC-2v, ventricular MLC-2; MLC-2a, atrial MLC-2; TAC, transverse aortic constriction.
terminals. To determine whether mlc-2v exhibits a similar gene dosage effect in mammalian hearts, we have performed in-depth analyses of heterozygous mlc-2v mutant mice.

Furthermore, we created the original mlc-2v knockout mice (12) by a knock-in of Cre recombinase into the endogenous mlc-2v locus to achieve two goals: 1) to understand the physiological importance of mlc-2v in mammalian hearts and 2) to use the heterozygous mlc-2v cre knock-in mice for ventricular cardiomyocyte-specific gene targeting, as described by Chen et al. (15). In this report, we demonstrate that heterozygous mlc-2v cre knock-in mice are indistinguishable from wild-type mice in all aspects and display normal levels of MLC-2v protein despite a 50% reduction in mRNA. Thus, we have validated the potential utility of the mlc-2v cre knock-in system as a valuable approach for ventricular restricted gene targeting.

MATERIALS AND METHODS

Generation of mlc-2v Knockout/cre Knock-in Mice—The mlc-2v targeting construct has been described elsewhere (12). By homologous recombination, Cre recombinase cDNA was placed into the endogenous mlc-2v locus. This study used the heterozygous mlc-2v mutants, which have one copy of Cre recombinase cDNA in the mlc-2v locus.

Echocardiographic Analysis—Mice were anesthetized with 20 ml of 2.5% Avertin/kg of body weight given intraperitoneally, and transesophageal echocardiography was performed before and 7 days after transverse aortic constriction (TAC) as described in detail elsewhere (16).

Induction of Pressure Overload Cardiac Hypertrophy—Pressure overload was produced in mice at 8 weeks of age by TAC as described previously (17). At 7 days following surgery, the gradient of the arterial blood pressure between the constriction was measured. Six heterozygous mlc-2v mutants and six wild-type mice showing an adequate pressure gradient (>25 mm Hg) were subjected to further studies.

Northern Blot Analysis—Ten micrograms of total RNA from adult left ventricles was used for Northern blot analyses. To characterize the expression of mlc-2v and the atrial isoform of MLC-2 (mlc-2a), the same probes described previously (18) were used. Expression of atrial natriuretic factor and skeletal a-actin mRNAs was examined as a molecular marker for hypertrophy.

Western Blot Analysis—Five micrograms of myofibrillar protein extracts from left ventricles (19) was electrophoresed on 12.5% SDS-polyacrylamide gels and either stained with Coomassie Blue or subjected to Western blotting analysis. Anti-MLC-2v and anti-MLC-2a polyclonal antibodies have been described previously (18).

Myocyte Shortening Measurement (Edge Detection)—Ventricular myocytes were prepared from the left ventricles of heterozygous mlc-2v and wild-type littermates at 10 weeks of age. The mouse heart was excised, and the aorta was cannulated and mounted onto a Langendorff perfusion apparatus. The heart was perfused for 4 min in modified Joklik’s minimal essential medium (Life Technologies, Inc.) consisting of 113 mM NaCl, 4.7 mM KCl, 0.6 mM KH2PO4, 0.6 mM Na2HPO4, 1.2 mM MgSO4, 12 mM NaHCO3, 2 mM d-glucose, 10 mM HEPES, 30 mM taurine, 2 mM creatine, and 2 mM carnitine (pH 7.4). Perfusion was then switched to a modified Joklik’s minimal essential medium plus 0.1% (v/v) collagenase type II ( Worthington). After 15 min of enzymatic digestion, the atria and right ventricle were removed; the left ventricle was cut into several pieces; and the cells were dispersed by gentle agitation. Myocytes were filtered and washed, and 1 mM calcium was reintroduced gradually to the cells.

Isolated cardiomyocytes were transferred to a temperature-controlled perfusion chamber (HCB-101, Crescent Electronics) located on the stage of an inverted microscope (Nikon Diaphot-TMD). Cell shortening was measured using a video edge motion detector (Crescent Electronics) interfaced to a standard CCD camera (Philips Technologies), which was attached to the microscope. A 40× Nikon phase II objective was used. Cells were stimulated at 0.5 Hz with a 5-ms pulse duration by a BMS 414 electrostimulator (Crescent Electronics). Edge detection measurements were performed on cardiomyocytes at 22 °C in continuously circulating Tyrode’s solution on cells. The concentration of calcium in Tyrode’s solution was subsequently changed from 1 to 2 to 4 mM.

**FIG. 1.** RNA and protein analyses in heterozygous mlc-2v mutants and wild-type littermates. A, Northern blot analysis of adult (2-month-old) ventricular RNA. Ten micrograms of total RNA was used for each sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. B, myofilamentous proteins from adult (2-month-old) ventricles were analyzed by SDS/glycerol-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Five micrograms of protein was loaded in each lane. TM, tropomyosin; TNI, troponin I.

**FIG. 2.** Protein analysis before and after TAC. Myofilamentous proteins from ventricles before and after TAC were analyzed by SDS/glycerol-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Five micrograms of protein was loaded in each lane. Sham, sham operation; TM, tropomyosin; TNI, troponin I.
MLC-2v Exhibits No Gene Dosage Effect in Mice

**RESULTS**

**Characterization of Heterozygous mlc-2v Mutant Mice**—Our previous study demonstrated that homozygous mlc-2v−/− offspring die at ~12.5 days post coitum due to cardiac dysfunction, indicating that mlc-2v is an essential component of the myocardial contractile apparatus (12). Heterozygous mlc-2v mutants can survive a full life span and reproduce normally. We performed an in-depth analysis of heterozygous mlc-2v mutants. There were no significant differences in heart/body weight ratios between wild-type mice (4.24 ± 0.34, n = 13) and mlc-2v mutants (4.20 ± 0.33, n = 13). Histologically, heterozygous mlc-2v mutants displayed normal sarcomeric assembly in the ventricular wall (data not shown), although our previous study demonstrated that homozygous mlc-2v mutants exhibit a disrupted myofibrillar organization during cardiogenesis (12).

**Post-transcriptional Regulation of MLC-2v Expression**—Quantitative Northern blot analysis indicated that the level of mlc-2v transcripts in ventricles of adult heterozygous mlc-2v mutant mice decreased ~50% compared with wild-type littermates (Fig. 1A). However, the amount of MLC-2v protein in ventricular myofibrillar preparations of both wild-type mice and heterozygous mlc-2v mutants was indistinguishable when corrected for the amount of protein loaded by using amounts of total myosin as a standard (Fig. 1B).

To examine whether the regulation of mRNA and protein expression would be modified by cardiac hypertrophic stimuli, we compared the levels of mlc-2v mRNA and protein following induction of hypertrophy by TAC. Both prior to and following TAC, heterozygous mlc-2v mutants exhibited normal levels of MLC-2v protein (Fig. 2) and a 50% reduction in mRNA (Fig. 3) relative to wild-type littermates as revealed by SDS-polyacrylamide gel electrophoresis and Northern blot analyses.

**TABLE I**

|                 | Wild-type (n = 10) | Heterozygous mlc-2v (n = 17) |
|-----------------|------------------|-----------------------------|
| BW (g)          | 23.9 ± 1.3       | 24.0 ± 0.8                  |
| HR (bpm)        | 462 ± 19         | 459 ± 16                    |
| LVEDD (mm)      | 3.36 ± 0.10      | 3.26 ± 0.07                 |
| LVEDD (mm)      | 2.03 ± 0.07      | 2.02 ± 0.07                 |
| PWth (mm)       | 0.67 ± 0.01      | 0.67 ± 0.02                 |
| IVSth (mm)      | 0.68 ± 0.02      | 0.68 ± 0.02                 |
| LV%FS           | 39.4 ± 1.8       | 38.3 ± 1.1                  |
| Mean Vcf (circ/s) | 7.96 ± 0.56    | 8.26 ± 0.47                 |

In addition to MLC-2v, MLC-2a is abundantly expressed in the ventricular chamber at the earliest stages of murine cardiogenesis (18). Our previous studies showed that MLC-2a protein content in the ventricular chamber is dramatically increased in mlc-2v null mutant embryos at 12 days post coitum, although there are no significant differences in the mlc-2a mRNA levels between wild-type and homozygous mlc-2v mutant embryos (12). Therefore, we examined the expression of MLC-2a protein in ventricles from adult heterozygous mlc-2v mutants by Western blot analysis. No detectable MLC-2a protein was found in ventricles of wild-type mice or heterozygous mlc-2v mutants before or after TAC (data not shown).

**Changes in Molecular Markers of Hypertrophy**—To elucidate whether there are differences in expression of hypertrophic response genes in wild-type mice and heterozygous mlc-2v mutants, we examined the expression of atrial natriuretic factor and skeletal α-actin mRNAs following TAC. Hypertrophic stimuli resulted in comparable increases in expression of atrial natriuretic factor and skeletal α-actin mRNAs in both groups. Fig. 3 shows a representative Northern blot that examined atrial natriuretic factor expression.

**Examination of Global Cardiac Function by Echocardiography**—Cardiac function was evaluated noninvasively by echocardiography prior to and following TAC. Under basal conditions, all echocardiographic parameters were equivalent in both groups (Table I). Similar pressure gradients between the aortic constriction were produced in both groups (Table II). Left ventricular weight/body weight ratios were also similar in both groups following TAC, confirming that the amount of hypertrophic stimulus was comparable for each group. In both groups, left ventricular wall thickness and percent fractional shortening increased in response to TAC. These data indicate that global cardiac function and response to hypertrophic stimuli are unaffected in heterozygous mlc-2v mutants.

**Mechanical Properties of Ventricular Cardiomyocytes Remain Normal in Heterozygous mlc-2v Mutant Mice**—To characterize contractile properties of cardiac myocytes isolated from wild-type mice or heterozygous mlc-2v mutants, unloaded contractile motion was monitored. Cardiac myocytes isolated from heterozygotes or wild-type littermates were found to be morphologically indistinguishable. Unloaded contractile motion was normalized to resting cell length. Table III shows that percent cell fractional shortening, dL/dt, and +dL/dt were comparable in wild-type and heterozygous mlc-2v mutant cells at various extracellular calcium concentrations. Additionally, Ca2+-ATPase, K+-EDTA-ATPase, and actin-activated ATPase activities in myosin preparations isolated from wild-type mice and heterozygous mlc-2v mutants displayed no statistical dif-

**Fig. 3. RNA analysis before and after TAC.** Ventricular mRNA was analyzed by Northern blotting before and after TAC. Ten micrograms of total RNA was used for each sample. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Sham, sham operation; ANP, atrial natriuretic factor.


**VLCC-2v Exhibits No Gene Dosage Effect in Mice**

**TABLE II**

Comparative echocardiographic measurements before and after induction of hypertrophy by TAC

See the legend to Table I for details and definitions of abbreviations. LVW, left ventricular weight; PG, pressure gradient. Data comparison was carried out before and after TAC in either group. bpm, beats/min; circ, circumference.

|                      | Before TAC | Wild-type (n = 6) | Heterozygous mlc-2v (n = 6) | Wild-type (n = 6) | Heterozygous mlc-2v (n = 6) |
|----------------------|------------|-------------------|-----------------------------|-------------------|-----------------------------|
| BW (g)               | 21 ± 1.4   | 23.7 ± 0.8        | 402 ± 32                    | 450 ± 8           |
| HR (bpm)             | 481 ± 23   | 502 ± 21          | 2.59 ± 0.09°                | 2.85 ± 0.10°      |
| LVEDD (mm)           | 3.37 ± 0.8 | 3.35 ± 0.13       | 1.26 ± 0.09°                | 1.55 ± 0.12°      |
| LVESD (mm)           | 2.05 ± 0.03| 2.05 ± 0.14       | 1.12 ± 0.10°                | 0.87 ± 0.06°      |
| FWH (mm)             | 0.66 ± 0.01| 0.62 ± 0.03       | 0.93 ± 0.07°                | 0.86 ± 0.07°      |
| IVSth (mm)           | 0.64 ± 0.03| 0.62 ± 0.02       | 51.3 ± 2.7°                 | 45.7 ± 3.5        |
| LVth/FS              | 39.0 ± 1.2 | 39.0 ± 2.6        | 8.29 ± 0.35                 | 8.27 ± 1.11       |
| Mean Vcf (circ/s)    | 8.27 ± 0.67| 9.10 ± 1.23       | 4.06 ± 0.21                 | 3.99 ± 0.16       |
| LV/W/BW              |            |                   | 51.6 ± 12.1                 | 54.7 ± 11.1       |
| PG (mm Hg)           |            |                   |                             |                  |

**TABLE III**

Ventricular myocyte mechanical properties with changes of calcium concentration

All data are presented as means ± S.E. Note that measurements were performed on nine ventricular myocytes from three wild-type mice and on ten ventricular myocytes from three heterozygous mice. –dL/dt, the rate of shortening; +dL/dt, the rate of relengthening.

|                      | 1 mCa²⁺ | 2 mCa²⁺ | 4 mCa²⁺ |
|----------------------|---------|---------|---------|
| Shortening (%)       | Wild-type | Heterozygous mlc-2v | Wild-type | Heterozygous mlc-2v | Wild-type | Heterozygous mlc-2v |
| −dL/dt (μm/s)        | 6.6 ± 0.9 | 6.6 ± 0.9 | 10.5 ± 1.0 | 12.8 ± 1.0 | 16.6 ± 1.0 | 17.3 ± 1.2 |
| +dL/dt (μm/s)        | 81.3 ± 7.0 | 94.8 ± 16.5 | 144.7 ± 12.8 | 184.2 ± 21.6 | 209.1 ± 14.1 | 207.7 ± 14.5 |
| Shortening duration (ms) | 55.2 ± 6.7 | 65.0 ± 13.6 | 107.3 ± 15.9 | 131.3 ± 15.3 | 135.4 ± 14.5 | 116.9 ± 9.1 |
| Cell length (μm)     | 123.9 ± 6.4 | 121.6 ± 4.5 | 476 ± 27     | 461 ± 17     | 558 ± 20     | 531 ± 28     |

**TABLE IV**

ATPase activities of heterozygous mlc-2v mutants and wild-type littermates

All data are presented as means ± S.E. Measurements were done on six independent myosin preparations from the left ventricles in each group.

|                      | Wild-type (n = 6) | Heterozygous mlc-2v (n = 6) | Wild-type (n = 6) | Heterozygous mlc-2v (n = 6) |
|----------------------|-------------------|-----------------------------|-------------------|-----------------------------|
| K⁺-EDTA-ATPase (nmol P/mg/min) | 304 ± 20 | 319 ± 13 | 327 ± 38 | 297 ± 26 |
| Ca²⁺-ATPase (nmol P/mg/min) | 227 ± 38 | 297 ± 26 |
| Actin-activated ATPase (nmol P/mg/min) | 18 ± 4 | 20 ± 5 | 197 ± 14 | 183 ± 9 |

**DISCUSSION**

There were two main goals for this study: 1) to determine whether mlc-2v exhibits a gene dosage effect in mammalian heart and 2) to evaluate the validity of utilizing mlc-2v cre knock-in mice for ventricular specific gene targeting. Our previous study revealed that mlc-2v null mutants are embryonic lethal at −12.5 post coitum due to cardiac dysfunction and exhibit abnormal sarcomeric assembly (12), which suggests that mlc-2v plays an essential role in cardiac function and myofibrillogenesis during development. However, this study has shown that, although heterozygous mlc-2v mice display a 50% reduction in mlc-2v mRNA levels, they exhibit no reduction in endogenous MLC-2v protein levels. Heterozygous mice have neither a molecular nor a physiological cardiac phenotype either at base line or following hypertrophic stimuli. These results suggest that, due to post-transcriptional regulation, a single mlc-2v allele is sufficient to produce normal levels of MLC-2v protein and to maintain normal sarcomeric function and development in mouse heart. In addition, our results provide the first evidence that this post-transcriptional regulation can operate following induction of cardiac hypertrophy. In a transgenic approach by other investigators, a 10-fold overexpression of mlc-2v mRNA in murine ventricle failed to increase expression of MLC-2v protein (22). Thus, it appears that mlc-2v is strictly regulated at the post-transcriptional level in murine heart.

A similar post-transcriptional regulatory mechanism has been observed for α-tropomyosin, where overexpression of α-tropomyosin mRNA in murine ventricle failed to increase expression of MLC-2v protein (22). This, thus, appears that mlc-2v is strictly regulated at the post-transcriptional level in murine heart.

There is only one mlc-2 gene in Drosophila. Interestingly, heterozygous mlc-2 mutants can survive but cannot fly due to...
MLC-2v Exhibits No Gene Dosage Effect in Mice

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