Cardiac Myosin Binding Protein C Gene Is Specifically Expressed in Heart During Murine and Human Development

Françoise Fougerousse, Anne-Lise Delezoide, Marc Y. Fiszman, Ketty Schwartz, Jacques S. Beckmann, Lucie Carrier

Abstract—Cardiac myosin binding protein C (MyBP-C) is a substantial component of the sarcomere, with both structural and regulatory roles. The gene encoding cardiac MyBP-C in humans is located on chromosome 11p11.2, and mutations that are most predicted to produce truncated proteins have been identified in this gene in unrelated families with familial hypertrophic cardiomyopathy (FHC). To understand better the pathophysiology of FHC and with a view to the development of animal models for this disease, we have investigated by in situ hybridization the pattern of expression of the cardiac MyBP-C gene during human and mouse development using species-specific oligonucleotide probes. From 4 weeks of human development, a strong labeling of cardiac MyBP-C mRNAs was unambiguously detected in all heart compartments, and no signal could be visualized in somites. In murine embryos, from embryonic day 9.5 until birth, a strong signal was detected exclusively in the heart. Our results showed that during both human and murine development, in contrast to chicken development, the cardiac MyBP-C gene is abundantly and specifically expressed in the heart. **(*Circ Res*. 1998;82:130-133.)**

Key Words: myosin binding protein C ■ cardiac muscle ■ mouse development ■ human development ■ expression

Myosin binding protein C (MyBP-C) is a substantial component of the striated muscles located in the C zone of the A band of the thick filament of the sarcomere. Three isoforms have been identified in adult striated muscle: a fast skeletal, a slow skeletal, and a cardiac isoform.[1,2] For a decade, several lines of evidence have pointed to a potentially important structural and regulatory role of MyBP-C in the sarcomere. MyBP-C participates in the assembly of the thick filaments in striated muscle through several protein-protein interactions: it is intimately associated with myosin subfragment-2 and light meromyosin[3]; it competes with myosin subfragment-1 for binding to F-actin, which results in a modulation of the myosin ATPase activity[4,5]; and last, it binds to titin (or connectin).[6,7] The addition of MyBP-C modulates the actin-activated ATPase activity of skeletal and cardiac myosins[5,8,9]; conversely, partial extraction of MyBP-C from rat skinned cardiac myocytes and rabbit skeletal muscle fibers alters Ca^{2+}-sensitive tension.[10] Cardiac MyBP-C is phosphorylated under adrenergic stimulation,[11–13] and it was recently shown that this phosphorylation alters myosin crossbridges in native thick filaments isolated from rat ventricles, suggesting that MyBP-C can modify force production in activated cardiac muscles.[14] Finally, several mutations in the gene encoding the cardiac isoform in humans (*MYBPC3*) have been identified in unrelated families with FHC.[15–17]

FHC is an autosomal-dominant disease characterized by a ventricular hypertrophy predominantly affecting the interventricular septum and associated with a large extent of myocardial and myofibrillar disarray. It is the most common cause of sudden death in young athletes. Many forms of the disease involve mutations in genes encoding sarcomeric proteins (for reviews, see References 18 to 20), and the finding that *MYBPC3* is one of these disease genes markedly strengthens the hypothesis that cardiac MyBP-C plays an important role in the regulation of cardiac contraction in health and disease. Major insights into the functional consequences of the mutations and the pathogenesis of FHC were obtained through analysis of skeletal muscle biopsies of FHC patients with mutations in the β-MHC gene.[21,22] This approach could be achieved because the β-MHC gene is normally expressed in both cardiac and slow-twitch adult skeletal muscles (see Reference 23 for a review). This is not the case for the cardiac MyBP-C gene: it is not expressed in adult skeletal muscles, and human cardiac biopsies are seldom available. In chickens, it was shown that cardiac MyBP-C is transiently expressed in embryonic skeletal muscles.[24–26] We have investigated by in situ

Received July 1, 1997; accepted November 7, 1997.

From the Laboratoire d'Histo-Embryologie et de Cytogénétique (F.F.), Faculté Cochin Port-Royal, Paris, France; Service d'Histo-Embryologie et de Cytogénétique (A.-L.D.), Hôpital Necker, Paris, France; the Généthon (F.F., J.S.B.), URA1922 C.N.R.S., Evry, France; the INSERM Unit 153 and the IFR de Physiopathologie et Génétique Cardiovasculaire (M.Y.F., K.S., L.C.), Groupe Hospitalier Pitié-Salpétrière, Paris, France.

This manuscript was sent to Laurence H. Kedes, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

Correspondence to Dr Lucie Carrier, INSERM UR153, Institut de Myologie, Rue du Mur des Fermiers Généraux, Groupe Hospitalier Pitié-Salpétrière, 47 boulevard de l'Hôpital, 75651 Paris cédex 13, France.

E-mail lcarrier@myologie.infobiogen.fr

Selected Abbreviations and Acronyms

DTT = dithiothreitol
E (with number) = embryonic day
FHC = familial hypertrophic cardiomyopathy
MHC = myosin heavy chain
MyBP-C = myosin binding protein C

hybridization the pattern of expression of the cardiac MyBP-C gene during human development with the idea that if this gene is transiently expressed in embryonic and/or fetal skeletal muscles, satellite cells from adult skeletal muscles could be used as a model system to analyze in vitro the transcription, the translation, and the function of cardiac MyBP-C. The expression of the titin gene was used as a control of skeletal muscle development. Furthermore, with a view to the development of animal models for FHC, we have also analyzed the pattern of expression of the cardiac MyBP-C gene in mice.

Materials and Methods

Human Tissues

Normal human embryos (from 4 to 7 postovulatory weeks) were obtained from legal abortions induced by mifepristone (RU486) at the Broussais Hospital in Paris. All procedures were approved by the Ethical Committee of the Necker-Enfants Malades Hospital in Paris. Each sample was first observed macroscopically during dissection under a stereomicroscope; the developmental stage of the embryo was determined using established criteria.[27] The heads and spinal cords of the 10- to 12-week-old fetuses were not available for analysis. Fetal human hearts at 15, 17, and 21 weeks of development were also included in this study.

Murine Tissues

Murine embryos of the NMRI strain, from 9.5 days post coitum to birth were examined. Tissues were collected shortly after delivery and frozen within the first 24 hours post mortem in dry ice and stored at −80°C until used. Ten-micron-thick cryostat sections were mounted on slides previously coated with a 2% 3-aminopropyltriethoxysilane solution in acetone. The sections were postfixed for 20 minutes in 2% paraformaldehyde in a 0.1 mol/L phosphate buffer (pH 7.4) at room temperature, rinsed (once for 2 minutes) in a phosphate buffer, rinsed briefly in water, and dehydrated with a series of washes in ethanol solutions (50%, 75%, and 100%). The sections were then air-dried and finally stored at −80°C. The integrity of each embryo was checked using conventional histological and histochemical staining techniques.

Oligonucleotide Probes

Purified 60mer oligonucleotide antisense cardiac MyBP-C cDNA probes were determined according to either the human sequence (EMBL accession number X84075), 5′-AAC ACG GCA GGG CTG CCT GCG GCC ACT TCC ACT GAC CGT GGC TTC TTG CTA AAG GCT GAG-3′, or the murine sequence,[28] 5′-ACA CGG CAG CAC TGC CAG CAG TCA CCT CCG CTG ACC TTG GCT TCT TGT TGA AGG CTG ACA-3′. These probes were chosen in the 5′ cDNA sequence in order to recognize only the cardiac variant. The probe specific to the titin gene was chosen in the A-band sequence in order to recognize both the cardiac and skeletal isoforms as described by Fougerousse et al.[29] Control sense probes were chosen from the opposite cDNA strands.

Northern Blot Analysis

All oligonucleotide probes (100 ng) were 5′ end–labeled with [γ-^{32}P]ATP (New England Nuclear) using T4 polynucleotide kinase (Promeg) for 1.5 hours at 37°C and then separated from the free nucleotides by chromatography on Biospin 6 columns (Bio-Rad). Mouse and human multiple-tissue Northern blots (Clontech) were prehybridized for 30 minutes at 65°C with ExpressHyb hybridization solution (Clontech). Hybridizations were performed at 65°C for 1.5 hours with the corresponding probes (1×10^6 cpm/mL). The blots were washed for 20 minutes in 2× SSC and 0.1% SDS at room temperature and for 20 minutes in 0.1× SSC and 0.1% SDS at 37°C. The blots were then exposed to X-Omat film (Kodak) at −80°C for several hours.

In Situ Hybridization

All probes were 3′ end–labeled with [α-^{35}S]dATP (Dupont-Nemours) using terminal deoxyribonucleotidyl transferase (BRL) and purified on Biospin columns (Bio-Rad). The in situ hybridization medium contained 50% formamide, 4× SSC, 1× Denhardt's solution, 0.25 mg/mL sheared salmon sperm DNA, 0.25 mg/mL polyA, 0.25 mg/mL tRNA, 10% dextran sulfate, 100 mmol/L DTT, and [α-^{35}S]dATP–labeled probe (6×10^6 cpm/mL). One hundred microliters of the hybridization solution was deposited on each section, which was then covered with a parafilm coverslip and incubated in a humidified chamber at 43°C for 20 hours. After hybridization, the sections were washed, twice for 15 minutes each at 55°C in 1× SSC containing 10 mmol/L DTT, twice in 0.5× SSC containing 10 mmol/L DTT, and finally in 0.5× SSC containing 10 mmol/L DTT at room temperature. The sections were then dehydrated with a series of graded concentrations of ethanol, exposed to Amersham Betamax x-ray films for 12 days, and then dipped in Kodak NTB-2 nuclear track emulsion and exposed for 2 months before development. The sections were examined using a Zeiss Axiophot microscope. Negative controls of in situ hybridization were tested with the sense probes on adjacent slides.

Results

Northern blot analysis of polyA$^+$ RNA isolated from several adult murine and human adult tissues with the cardiac-specific MyBP-C oligonucleotide probes showed that an mRNA of ≈4.5 kb was detected abundantly in cardiac muscle, although it was absent in skeletal muscle, brain, spleen, lung, liver, kidney, and testis in both humans and mice (data not shown). The size and tissue specificity of this mRNA are in full agreement with previous cloning data,[11,28] and this clearly shows that the chosen oligonucleotide probes are specific to the cardiac MyBP-C isoform in both animal species.

At 4 weeks of human development (Fig 1A through 1D), a strong labeling of cardiac MyBP-C mRNAs was unambiguously detected in the whole embryonic heart (Fig 1B). No signal could be detected in the myotome compartment (Fig 1B). No signal was detected with the human control sense probe (Fig 1D). In contrast, titin mRNAs were clearly present both in the heart and in the myotome compartment (Fig 1C). In 10- to 12-week-old fetuses (Fig 1E through 1G), cardiac MyBP-C mRNAs were detected throughout the four-chambered heart (Fig 1F). At this stage, cardiac MyBP-C mRNAs were not expressed in fetal skeletal muscles, whereas titin mRNAs were detected at significant levels (Fig 1G). Transcription of the cardiac MyBP-C gene persisted uniformly in the later hearts studied but was not present in skeletal muscles (15, 17, and 21 weeks old) (data not shown).

Sagittal sections of murine embryos were investigated systematically with the cardiac MyBP-C oligonucleotide probe from E 9.5 to E 18.5 (Fig 2). At E 9.5, the primitive heart tube was strongly and specifically labeled (Fig 2D). No signal was detected with the murine control sense probe (Fig 2E). This cardiac-specific pattern of hybridization was also seen at E 10.5

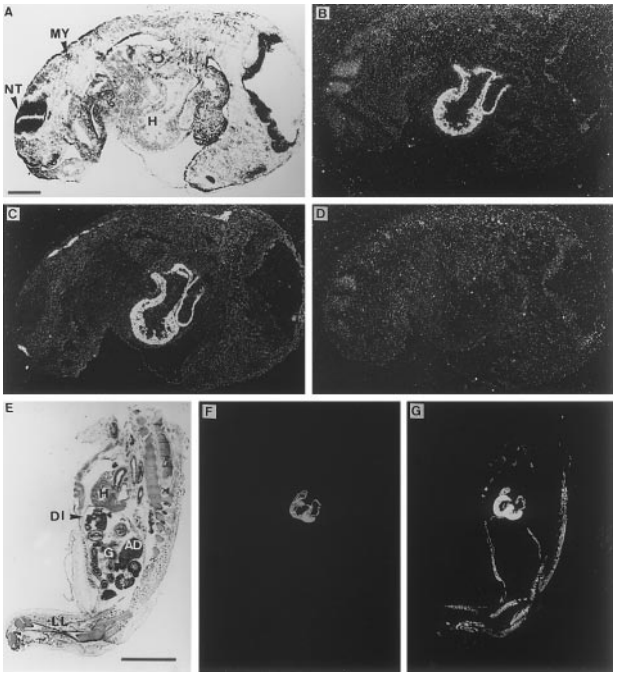

Figure 1. Neighboring transverse sections of a stage 13 human embryo (4 weeks old) (A through D) and sagittal sections of a 10- to 12-week-old fetus (E through G). A and E, Phase-contrast microscopy. C and G, Dark-field microscopy, in situ hybridization with a titin antisense probe.[29] B and F, Dark-field microscopy, in situ hybridization with the cardiac MyBP-C antisense probe. D, Dark-field microscopy, in situ hybridization with the cardiac MyBP-C sense probe. H indicates heart; MY, myotome; NT, neural tube; LL, lower limb; DI, diaphragm; AD, adrenal gland; and G, gut. Bars=600 μm (A) and 5 mm (B). Panels B through D are photomicrographs, and panels F and G are autoradiographic reproductions.

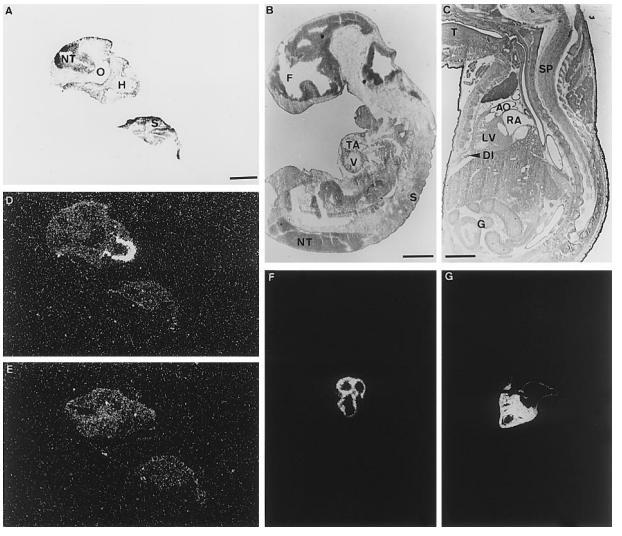

Figure 2. Sagittal sections of murine embryos at E 9.5 (A, D, and E), E 10.5 (B and F), and E 18.5 (C and G). A, B, and C, Phase-contrast microscopy at E 9.5, E 10.5, and E 18.5, respectively. D, F, and G, Dark-field microscopy, in situ hybridization with the cardiac MyBP-C antisense probe. E, Dark-field microscopy, in situ hybridization with the cardiac MyBP-C sense probe. H indicates outflow tract of heart; V, ventricular chamber of heart; TA, truncus arteriosus; RA, right atrium of heart; LV, left ventricle of heart; AO, aorta; S, somite; F, forebrain; NT, neural tube; SP, spinal cord; T, tongue; DI, diaphragm; O, oropharynx; and G, gut. Bars=600 μm (A) and 5 mm (B and C). Panels D and E are photomicrographs, and panels F and G are autoradiographic reproductions.

and E 18.5 (Fig 2F and 2G), without any expression of the cardiac MyBP-C gene in skeletal muscle territories.

Discussion

The present study is the first analysis of the expression pattern of the cardiac MyBP-C gene during human and murine development. We show that the message for cardiac MyBP-C is detected in the ventricles and atria at very early stages in both human and mouse development, but we did not find any expression of the cardiac MyBP-C gene during skeletal muscle development. Furthermore, using reverse-transcriptase polymerase chain reaction, we were unable to detect cardiac MyBP-C transcripts during the differentiation of C2-C12 skeletal muscle cells in culture, hence confirming that there is no expression of cardiac MyBP-C gene in skeletal muscle (data not shown). This result is at variance with what has been described in the chicken: it was found that cardiac MyBP-C is expressed in both cardiac and skeletal muscle during embryonic development.[24–26] This probably reflects a difference in the organization of the promoter region of the avian and the mammalian genes. After the embryonic stages in chicken skeletal muscles, cardiac MyBP-C is replaced subsequently by both slow skeletal and fast skeletal isoforms in late embryonic development, after which the slow skeletal isoform disappears and only the fast skeletal isoform persists during postnatal development. Two splice variants of the cardiac MyBP-C gene were found in chicken development, and further investigations will be required to determine whether splice variants also exist in mammals. Nevertheless, it is reasonable to assume that even if several variants are transcribed from the human and murine cardiac genes, they would follow the same pattern of expression.

The appearance of the sarcomeric protein gene transcripts that interact directly or indirectly with MyBP-C in the newly formed cardiac tube is very similar to that reported in the present study for cardiac MyBP-C. In humans, the β-MHC, cardiac α-actin, and titin genes are expressed at a high level in the embryonic heart from ≈4 weeks of development (the present study, Reference 30, and, for a review, Reference 23). In the mouse embryo, mRNAs encoding β-MHC, titin, and cardiac α-actin are all detected at high levels at 8 days post coitum (for review, see Reference 31). One might thus hypothesize that the potential impairment of sarcomerization associated with FHC occurs very early during development for the two major causative genes, β-MHC and cardiac MyBP-C. In this context, mice expressing the β-MHC mutant are good models for FHC.[32,33] Since the cardiac MyBP-C gene is expressed uniquely in the heart, it is clear that analyses of the consequences of the mutations at molecular and cellular levels will not be possible in human skeletal muscle biopsies and that animal models will be needed. Our present results suggest that the expression of cardiac MyBP-C mutants in mice should also resemble the expression in human disease and will help to resolve the pathogenesis of FHC.

Acknowledgments

This study was supported by the Association Française contre les Myopathies. We are grateful to Drs E. Aubini, J.-H. di Donato, and R. Taurelle for providing human fetuses and embryos and to Muriel Durand and Laurence Suel for their technical assistance. We also thank Gisèle Bonne and Sophie Venin for fruitful discussion.

References

1. Reinach FC, Masaki T, Shafiq S, Obinata T, Fishman DA. Isoforms of C-protein in adult chicken skeletal muscle: detection with monoclonal antibodies. *J Cell Biol*. 1982;95:78–84.
2. Yamamoto K, Moos C. The C-proteins of rabbit red, white and cardiac muscles. *J Biol Chem*. 1983;258:8395–8401.
3. Starr R, Offer G. The interaction of C-protein with heavy meromyosin and subfragment-2. *Biochem J*. 1978;171:813–816.
4. Moos C, Mason CM, Besterman JM, Feng INM, Dubin JH. The binding of skeletal muscle C-protein to F-actin, and its relation to the interaction of actin with myosin subfragment-1. *J Mol Biol*. 1978;124:571–586.
5. Moos C, Feng INM. Effect of C-protein on actomyosin ATPase. *Biochem Biophys Acta*. 1980;632:141–149.
6. Fürst DO, Vinkemeyer U, Weber K. Mammalian skeletal muscle C-protein: purification from bovine muscle, binding to titin and the characterization of a full-length cDNA. *J Cell Sci*. 1992;102:769–778.
7. Labeit S, Gautel M, Lakey A, Trinick J. Towards a molecular understanding of titin. *EMBO J*. 1992;11:1711–1716.
8. Offer G, Moos C, Starr R. A new protein of the thick filaments of vertebrate skeletal myofibrils: extraction, purification and characterization. *J Mol Biol*. 1973;74:653–676.
9. Hartzell HC. Effects of phosphorylation and unphosphorylated C-protein on cardiac actomyosin ATPase. *J Mol Biol*. 1985;186:185–195.
10. Hofmann PA, Hartzell HC, Moss RL. Alterations in Ca^{2+} sensitive tension due to partial extraction of C-protein from rat skinned cardiac myocytes and rabbit skeletal muscle fibers. *J Gen Physiol*. 1991;97:1141–1163.
11. Gautel M, Zuffardi O, Freiburg A, Labeit S. Phosphorylation switches specific for the cardiac isoform of myosin binding protein C: a modulator of cardiac contraction? *EMBO J*. 1995;14:1952–1960.
12. Hartzell HC, Titus L. Effects of cholinergic and adrenergic agonists on phosphorylation of a 165,000-dalton myofibrillar protein in intact cardiac muscle. *J Biol Chem*. 1982;257:2111–2120.
13. Schlender KK, Bean LJ. Phosphorylation of chicken cardiac C-protein by calcium/calmodulin-dependent protein kinase II. *J Biol Chem*. 1991;266:2811–2817.
14. Weisberg A, Winegrad S. Alteration of myosin cross bridges by phosphorylation of myosin-binding protein C in cardiac muscle. *Proc Natl Acad Sci U S A*. 1996;93:8999–9003.
15. Bonne G, Carrier L, Bercovici J, Cruaud C, Richard P, Hainque B, Gautel M, Labeit S, James M, Beckmann JS, Weissenbach J, Vosberg HP, Fiszman M, Komajda M, Schwartz K. Cardiac myosin binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy. *Nat Genet*. 1995;11:438–440.
16. Watkins H, Conner D, Thierfelder L, Jarcho JA, MacRae C, McKenna WJ, Maron BJ, Seidman JG, Seidman CE. Mutations in the cardiac myosin binding protein-C gene on chromosome 11 cause familial hypertrophic cardiomyopathy. *Nat Genet*. 1995;11:434–437.
17. Carrier L, Bonne G, Bährend E, Yu B, Richard P, Niel F, Hainque B, Cruaud C, Gary F, Labeit S, Bouhour J-B, Dubourg O, Desnos M, Hagège AA, Trent RJ, Komajda M, Schwartz K. Organization and sequence of human cardiac myosin binding protein C gene (*MYBPC3*) and identification of mutations predicted to produce truncated proteins in familial hypertrophic cardiomyopathy. *Circ Res*. 1997;80:427–434.
18. Schwartz K, Carrier L, Guicheney P, Komajda M. The molecular basis of cardiomyopathies. *Circulation*. 1995;91:532–540.
19. Watkins H, Seidman JG, Seidman CE. Familial hypertrophic cardiomyopathy: a genetic model of cardiac hypertrophy. *Hum Mol Genet*. 1995;4:1721–1727.
20. Marian AJ, Roberts R. Recent advances in the molecular genetics of hypertrophic cardiomyopathy. *Circulation*. 1995;92:1336–1347.
21. Lankford EB, Epstein ND, Fananapazir L, Sweeney HL. Abnormal contractile properties of muscle fibers expressing beta-myosin heavy chain mutations in patients with hypertrophic cardiomyopathy. *J Clin Invest*. 1995;95:1409–1414.
22. Malinchik S, Cuda G, Podolsky RJ, Horowits R. Isometric tension and mutant myosin heavy chain content in single skeletal myofibers from hypertrophic cardiomyopathy patients. *J Mol Cell Cardiol*. 1997;29:667–676.
23. Schiaffino S, Reggiani C. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev*. 1996;76:371–423.
24. Bähler M, Moser H, Eppenberger HM, Walliman T. Heart C-protein is transiently expressed during skeletal muscle development in the embryo, but persists in cultured myogenic cells. *Dev Biol*. 1985;112:345–352.
25. Yasuda M, Koshida S, Sato N, Obinata T. Complete primary structure of chicken cardiac C-protein (MyBP-C) and its expression in developing striated muscle. *J Mol Cell Cardiol*. 1995;27:2275–2286.
26. Kawashima M, Kitani S, Tanaka T, Obinata T. The earliest form of C-protein expressed during striated muscle development is immunologically the same as cardiac C-protein. *J Biochem*. 1996;99:1037–1047.
27. O'Rahilly R, Muller F. *Developmental Stages in Human Embryos*. Carnegie Institution of Washington; 1987. Publication 637.
28. Kasahara H, Itoh M, Sugiyama T, Kido N, Hayashi H, Saito H, Tsukita S, Kato N. Autoimmune myocarditis induced in mice by cardiac C-protein: cloning of complementary DNA encoding murine cardiac C-protein and partial characterization of the antigene peptides. *J Clin Invest*. 1994;94:1026–1036.
29. Fougerousse F, Durand M, Suel L, Pourquié O, Delezoide A-L, Roméro NB, Abithol M, Beckmann JS. Expression of genes involved in progressive muscular dystrophies during early human development. *Genomics*. In press.
30. Wessels A, Mijnders TA, de Gier-de Vries C, Vermeulen JLM, Viragh S, Lamers WH, Moorman AFM. Expression of myosin heavy chain in neonatal human hearts. *Cardiol Young*. 1992;2:318–334.
31. Lyons GE. In situ analysis of the cardiac muscle gene program during embryogenesis. *Trends Cardiovasc Med*. 1994;4:70–77.
32. Geisterfer-Lowrance AAT, Christe M, Conner DA, Ingwall JS, Schoen FJ, Seidman CE, Seidman JG. A mouse model of familial hypertrophic cardiomyopathy. *Science*. 1996;272:731–734.
33. Vikstrom KL, Factor SM, Leinwand LA. Mice expressing mutant myosin heavy chains are a model for familial hypertrophic cardiomyopathy. *Mol Med*. 1996;2:556–567.