Multiple quantitative trait loci modify the heart failure phenotype in murine cardiomyopathy

Philippe Le Corvoisier¹,², Hyun-Young Park¹,², Kerri M. Carlson², Douglas A. Marchuk² and Howard A. Rockman¹,²,*

¹Department of Medicine and ²Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC, 27710, USA

Received July 29, 2003; Revised September 17, 2003; Accepted September 23, 2003

The variability in outcome of heart failure patients depends on a number of factors including differences in their genetic background. To identify novel genes that modify the human heart failure phenotype, we used a strategy of quantitative trait locus (QTL) mapping in an experimental mouse model of dilated cardiomyopathy induced by cardiac-specific overexpression of calsequestrin and characterized by a strong strain-specific variability in the phenotype. We identified two novel QTLs, Hrtfm3 (heart failure modifier 3) on chromosome (Chr) 4 and Hrtfm4 on Chr 18, significantly linked to survival with likelihood ratio statistics (LRS) of 19.9 and 23.6 respectively (corresponding to LOD scores of 4.3 and 5.1). Two other QTLs, Hrtfm5 on Chr 2 and Hrtfm6 on Chr 13, were significantly linked to cardiac function as measured by echocardiographic fractional shortening (LRS 22.1 and 15.2 respectively, LOD score 4.8 and 3.3) and left ventricular end-diastolic diameter (LRS 23.5 and 18.8, LOD score 5.1 and 4.1). Importantly, Hrtfm5 was not significantly linked to survival. A significant interaction was found between Hrtfm4 and two other QTLs (Hrtfm6 and a QTL near to the marker D19Mit88) for fractional shortening with a LRS of 34.6 and 26.5 respectively (LOD score 7.5 and 5.8). These data show that the effect of genetic background on murine heart failure is complex and result from the action of several loci that differentially modify the cardiac phenotype. The identification of these novel modifier genes will serve as strong candidates for the discovery of modifiers in human heart failure.

INTRODUCTION

Heart failure is a major public health problem affecting nearly 5 million patients in the USA (1). Despite the development of novel treatments, the prognosis of these patients remains poor with a one year mortality estimate as high as 42% (2). Heart failure commonly presents with a wide range of symptoms and outcome between patients that cannot be completely explained by environmental factors. Increasing evidence suggests that differences in genetic background can account for an important part of this variability (3–9). The effect of genetic background on outcome is due to the existence of modifier genes whose multiple alleles differentially affect the heart failure phenotype (10).

The identification of modifier genes is a complex task that requires multiple and complementary approaches. The strategy most commonly used is based on association studies between the severity of measured heart failure traits and genetic variation of selected candidate genes (3–8). While some studies have reported a correlation between the survival of heart failure patients and polymorphisms of several genes (3–8), their statistical power is limited since parameters other than genetic background contribute to the phenotypic variability (etiology, treatment and environmental factors) (11). In addition, this strategy cannot identify epistatic interactions between different modifier genes (12). Therefore, it is unlikely that a purely candidate gene approach will be able to identify novel heart failure modifier genes among the ~25 000 cardiovascular genes (13).

By contrast, linkage studies provide an unbiased approach to localize in the genome the genes responsible for a phenotype without prior hypotheses on their function in the pathophysiology of the disease (14). This strategy has allowed the identification of a number of mutations responsible for monogenic diseases such as familial dilated cardiomyopathy (15). However, its application in humans for the identification of modifier genes is more complex because of its low statistical power due to the modest phenotypic effect exerted by modifier genes and to the heterogeneity of the population (11).
To overcome this difficulty, a genetic linkage strategy can be performed using animal models that are sensitized to develop the disease phenotype. The authors and others (9,11,16) have reported that a large number of experimental murine models show strong strain-specific phenotypic variability due to differences in genetic background. A backcross and/or intercross strategy among the different strains using disease-sensitized animals allows mapping of the loci contributing to these phenotypic differences. In addition, this approach allows standardization of environmental variables that might otherwise confound the analysis.

In order to map novel modifier genes affecting the heart failure phenotype, we used an experimental murine model of heart failure induced by cardiac specific overexpression of calsequestrin (CSQ), a sarcoplasmic reticulum calcium binding protein (9,17). We conducted a genome-wide scan in N2 CSQ progeny resulting from a backcross strategy between two genetically distant inbred strains, DBA/2J (DBA) and AKR/J (AKR), and identified a number of quantitative trait loci (QTL) that are linked to the severity of heart failure. Our results should lead to the identification of novel candidates that can be tested as heart failure modifier genes in human studies.

**RESULTS**

**Effect of the genetic background on heart failure phenotype**

Cardiac specific overexpression of calsequestrin resulted in dilated cardiomyopathy and premature death in all transgenic mice. However, the severity of the phenotype was strongly dependent on the strain indicating an effect of genetic background (Fig. 1A). While the average survival of DBA CSQ mice was limited to 156.9 ± 49.2 days, the survival of the hybrid F1 CSQ progeny showed a significant increase to 230.5 ± 62.9 days ($P < 0.001$). The improvement in survival between homozygous (DBA/DBA) and heterozygous (AKR/DBA) animals indicates that the AKR genetic background has a protective effect on the phenotype. Compared with the F1 CSQ progeny, N2 CSQ showed an additional increase in average survival from 230.5 ± 62.9 to 290.0 ± 82.9 days ($P < 0.001$), however with a wide distribution of the phenotypic values. The effect of genetic background is estimated to be responsible for 53.7% of the total variability of survival in the N2 CSQ progeny. These results suggest a segregation of the phenotype depending on the genotype for putative modifier genes (AKR/DBA or AKR/AKR), allowing us to map the QTLs responsible for this variability. As for many complex phenotypic traits in a genetically heterogeneous population, the survival of N2 CSQ did not segregate into two distinct groups, but rather showed a continuous distribution indicating the presence of multiple QTLs.

Measurement of cardiac function by echocardiography showed the same strong strain-specific variability as survival (Fig. 1B–D). The average left ventricular end diastolic diameter (LVEDD) and fractional shortening (FS) of F1 CSQ progeny were significantly better compared with mice on a pure DBA genetic background (4.6 ± 0.2 versus 5.5 ± 0.4 mm for LVEDD, $P < 0.001$ and 22.1 ± 4.6 versus 15.3 ± 4.6% for FS, $P < 0.05$). Moreover, additional improvement was found between the F1 CSQ and the N2 CSQ progeny (4.6 ± 0.2 versus 4.2 ± 0.4 for LVEDD, $P < 0.001$ and 22.1 ± 4.6 versus 27.1 ± 7.6 for FS, $P < 0.05$). The genetic variation is estimated to be responsible for 49.7 and 63.0% of the LVEDD and FS variability among the N2 CSQ progeny.

The relationship between survival, LVEDD and FS for the N2 CSQ mice is shown in Figure 2. Survival was significantly correlated with both LVEDD ($P < 0.0001$) and FS ($P < 0.0001$) (r-values = −0.42 and 0.49, respectively). However, some mice showed discordance between echocardiographic parameters and survival, indicating that in addition to systolic function, the occurrence of sudden cardiac death likely influenced survival.

Importantly, we found no differences between the level of overexpression of CSQ in hearts with a different genetic background, indicating that the variation in the phenotype cannot simply be accounted for by variation in the level of protein expression (Fig. 3A). Finally, the echocardiographic parameters of wild-type DBA or AKR mice were not different, indicating no preexisting differences in cardiac function in the absence of the CSQ transgene (Fig. 3B and C).

**Identification of QTLs**

Low resolution linkage maps were constructed by genotyping N2 CSQ mice with the most extreme phenotype for survival ($n = 70$), FS ($n = 69$) and LVEDD ($n = 60$). Three loci on chromosomes (Chrs) 2, 13 and 18 were significantly linked with the variability of the different phenotypic traits (Fig. 4). In addition, one locus with a likelihood ratio statistic (LRS) value suggestive for a linkage with LVEDD was localized on Chr 4. On the basis of the preliminary genetic linkage maps, we performed high-resolution mapping within each of the four potential QTLs using 35 additional microsatellite markers and the entire N2 CSQ progeny ($n = 155$ for survival and $n = 130$ for echocardiographic parameters). The results were analyzed by composite interval mapping and are represented in Figures 5 and 6.

**QTLs linked to mice survival.** We identified two QTLs on Chrs 4 and 18 significantly linked to survival and supported by a LRS of 19.9 and 23.6 respectively (Fig. 5). These QTLs are named Hrtfm3 and Hrtfm4 (Hrtfm for heart failure modifier) and are centered on the microsatellite markers D4Mit236 (support interval, SI, 8–18 cM) and D18Mit28 (SI, 17–38 cM). Hrtfm3 and Hrtfm4 account for ~10 and 12% of the total variance in N2 CSQ survival (18.6 and 22.3% of the genetic variability). Hrtfm3 is linked only to survival and has no statistically significant linkage with cardiac function. By contrast, Hrtfm4 shows significant linkage with FS (LRS, 17.7) and a LRS value suggestive for a linkage with LVEDD (LRS, 9.2). We screened the genome for an interaction between each pair of markers using survival as the phenotypic trait, but no significant interaction was found.

The allele effects of Hrtfm3 and Hrtfm4 on the phenotype are shown in Figure 7. For these QTLs, a homozygous genotype (AKR/AKR) was associated with an improvement of the prognosis compared with a heterozygous genotype (DBA/AKR), explaining the important differences in survival between the three different generations of this study.
QTLs linked to the echocardiographic parameters. We identified two other QTLs on Chrs 2 and 13 significantly linked only to cardiac function (LVEDD, LRS 23.5 and 18.8 respectively; and FS, LRS 22.1 and 15.2 respectively; Fig. 6). They are named Hrtfm5 and Hrtfm6 and are centered on the microsatellite markers D2Mit138 (SI, 67–98 cM) and D13Mit213 (SI, 59–75 cM). Hrtfm5 and Hrtfm6 are estimated to be responsible for 14 and 11% of LVEDD variability (28.2 and 22.1% of the genetic variability, respectively) and 12 and 8% respectively of FS variability (19.0 and 12.7% of the genetic variability, respectively). While Hrtfm5 shows no linkage with survival, the LRS value for Hrtfm6 was suggestive for a linkage...
(LRS 9.5), indicating that the effect of this QTL on survival is probably too weak to reach the significance level.

Interestingly, we found a statistically significant interaction \( P < 0.006 \) between two of the identified QTLs (Hrtfm4 and Hrtfm6) for their effect on FS with a LRS value of 34.6 for the total effect of the two QTLs. In addition, we observed another interaction \( P < 0.002 \), LRS for interaction 26.5) between Hrtfm4 and a locus localized near to the microsatellite marker D19Mit88 that had no significant effect on echocardiographic parameters by itself.

The cosegregation of the phenotype with the microsatellite marker located at the center of each QTL and the consequences on the phenotype of the interaction between loci are reported in Figure 7. As with Hrtfm3 and Hrtfm4, the AKR alleles of Hrtfm5 and Hrtfm6 showed a favorable effect on the echocardiographic parameters of heart failure mice.
DISCUSSION

Using a genome mapping strategy, we identified several novel QTLs that modify the cardiac phenotype in a mouse model of heart failure. Although these loci were identified using the extremes for each phenotype, they are robust since genotyping of all backcrossed animals leads to a further increase in the LRS for the various QTLs. Moreover, this study shows that the

![Figure 4](image.png)

Figure 4. Genetic linkage maps performed with the N2 CSQ mice with the most extreme phenotype using survival (A) \(n = 70\), fractional shortening (B) \(n = 71\) and left ventricular end-diastolic diameter (C) \(n = 60\) as phenotypic traits. Four loci on Chrs 2, 4, 13 and 18 showing a suggestive or significant linkage with the different phenotypic trait values were identified. Each vertical bar represents the level of significance for one of the 106 microsatellite markers and is expressed as a likelihood ratio statistic. A LRS can be converted into a LOD score by dividing it by 4.6. The horizontal dotted lines represent the significance thresholds and were determined by permutation testing.
The relationship between genotype and heart failure phenotype is complex. Indeed, our data demonstrates an interaction between several QTLs, and shows that some of the QTLs have a differential effect on the multiple phenotypic traits of heart failure.

Recent progress in the understanding of the molecular basis of heart failure has demonstrated that a large number of transduction pathways are involved in the development and progression of this disease (18). Given the complexity of heart failure pathophysiology, it is not surprising that we identified several QTLs modifying the expression of the heart failure phenotype in the same genetic cross. These QTLs contain only one of the heart failure modifier genes previously reported in the literature, the \( \beta_2 \) adrenergic receptor (AR) (4). This is probably explained by the absence of polymorphisms within these genes between the two strains in our study.

We have previously reported the effect of genetic background on the heart failure phenotype of CSQ mice in a DBA\( \times \)C57BL/6 reciprocal backcross (9). In that study, we localized two QTLs affecting survival of CSQ mice on Chr 2 and 3. Importantly, the interval we previously reported on Chr 2 (Hrtfm1, 38.3–43.5 cM) (9) does not overlap with the SI of the QTL identified in the current study on the same chromosome (Hrtfm5). Our current data extends our previous findings by showing how novel QTLs can be identified when sensitized disease models are backcrossed into disparate genetic backgrounds.

Given the complexity of the pathophysiology in multifactorial diseases, it has been argued that epistatic interactions (interaction between genes) are a universal component of their genetic architecture (12). In this study, we identified an interaction between two pairs of loci (Hrtfm4/Hrtfm6 and Hrtfm4/D19Mit88). Interestingly, the genotype at microsatellite marker D19Mit88 has no effect on fractional shortening by itself, but enhances the effect of Hrtfm4. Such modifier loci with a pure epistatic effect have previously been identified in several diseases (19,20) but have not been reported for heart failure. However, it has been shown that polymorphisms of the \( \beta_1 \)AR (Arg389Gly) and \( \alpha_2 \)AR (alpha2CDel322–325) act synergistically to increase the susceptibility to develop heart failure in patients without cardiovascular disease (21). These results are consistent with the concept that some modifier genes may have no direct effect on the phenotype, but can have important consequences by modifying the effects of other polymorphisms.

One of the important findings in our study is the identification of QTLs that are linked to both survival and cardiac function (Hrtfm4 and Hrtfm6) when other QTLs are linked only to survival (Hrtfm3) or cardiac function (Hrtfm5). The immediate cause of death of heart failure patients can be the consequence of two different mechanisms. In some cases, the death results directly

**Figure 5.** Composite interval mapping across the entire Chr 4 and 18. Two QTLs, Hrtfm3 (centered on D4Mit226) (A) and Hrtfm4 (centered on D18Mit28) (B), showed significant linkage with the survival of heart failure mice (maximal LRS of 19.9 and 23.6 respectively; \( n = 155 \)). While Hrtfm3 was linked only to survival, Hrtfm4 showed an additional significant linkage with fractional shortening (LRS: 17.7; \( n = 130 \); C). The position of the microsatellite markers is indicated using the function of Kosambi. The limits of each QTL support interval are indicated by the solid box.
from pump failure. However, recent large scale clinical trials have shown that between 40 and 60% of heart failure patients die suddenly due mainly to ventricular arrhythmias (22,23). Comparable results have been reported in the CSQ heart failure model, where half of the CSQ mice died spontaneously without evidence of pump failure on necropsy (24). While several mutations responsible for rare cases of inherited ventricular arrhythmias have been reported (KVLQT1, minK and SCN5A) (25), none of the modifier genes previously identified in heart failure have been correlated to an increased risk of sudden death.

Our results suggest that \textit{Hrtfm3}, which correlates only to survival, may affect the phenotype of heart failure by modifying the risk of sudden death. In contrast, the effect of \textit{Hrtfm4} and \textit{Hrtfm6} on outcome is more likely to be linked to a modification in the progression of left ventricular systolic dysfunction. We hypothesize that the function of the modifier gene(s) localized within the QTLs \textit{Hrtfm4} and \textit{Hrtfm6} may be quite different from that identified in \textit{Hrtfm3}. For example, an ionic channel could modify the risk of sudden death, whereas a structural or calcium handling protein could affect both survival and cardiac function. However, since the relationship between \textit{Hrtfm3} and the risk of sudden death has only been determined indirectly, we cannot definitively exclude a non cardiac effect of \textit{Hrtfm3} on survival.

By contrast, another QTL identified in our study (\textit{Hrtfm5}) is linked only to echocardiographic parameters without a significant effect on survival. Although the correlation between cardiac function and survival in human heart failure is well established (26), some experimental models, such as knock-out mice of the muscle LIM protein, have shown that the relationship between these two parameters is more complex, and that severe systolic dysfunction may not always be associated with

Figure 6. Composite interval mapping across the entire Chrs 2 and 13. Two QTLs on Chrs 2 and 13 (centered on \textit{D2Mit138} and \textit{D13Mit213} respectively), named \textit{Hrtfm5} and \textit{Hrtfm6}, were significantly linked with left ventricular end-diastolic diameter (LRS 23.5 and 18.8 respectively) (A and B) and fractional shortening (LRS 22.1 and 15.2 respectively; \(n = 130\)) (C and D).
reduced survival (27). Our results demonstrate that linkage studies provide a genetic tool that will enable dissection of the molecular basis underlying the different phenotypic traits of complex diseases such as heart failure.

The identification of modifier genes contained within a QTL remains a challenge that requires the use of several complementary approaches (28–31). The different intervals identified in this study contain numerous genes, with some of them considered good candidates. For example, Hrtfm6 includes the gene for MEK kinase 1 (MEKK1) that is essential for JNK (c-Jun N-terminal kinase) activation in vivo (32) and for the development of cardiac hypertrophy in response to Gq overexpression (33). However, the number of genes in our intervals is still quite large and it would be impracticable to evaluate all

Figure 7. Allelic effects of the different QTLs on survival (A), left ventricular end-diastolic diameter (B) and fractional shortening (C). The effect of the interaction between Hrtfm 4 and Hrtfm 6 and between Hrtfm 4 and D19Mit88 is reported in D and E. The allelic effects are shown for homozygous AKR/AKR (AA) and for heterogeneous AKR/DBA (AD) CSQ mice. Values are expressed as mean ± SD. *P < 10^{-4} and **P < 10^{-5} determined by t-test for the effect of a single locus. "P < 0.05 and "P < 0.001 determined by ANOVA followed by Tukey tests for the interaction between two loci.
genes for polymorphic variations. Strategies to further refine each QTL that include in silico sequence comparison and the generation of several lines of congenic mice, should allow us to isolate the phenotypic effects of each QTL and to reduce the number of candidate genes within the interval.

The survival of heart failure patients has become the standard for the evaluation of interventions in clinical trials. The identification of the gene(s) responsible for modifying survival of mice with severe heart failure would therefore be of major interest and of potential clinical application. Nonetheless, survival may not be the ideal phenotypic trait to perform fine mapping because of the possibility that death in some mice could result from non-cardiac causes. This however, should be overcome with the use of multiple phenotypic traits to map modifier gene(s) that are localized within the QTLs. By contrast, the identification of gene(s) linked only to cardiac function (Hrtfm5) will mainly be useful to provide novel mechanistic insights into the physiopathology of heart failure.

One potential limitation of our study is the experimental model of heart failure we used. It could be hypothesized that the modifier loci identified will be specific to the cardiomyopathy induced by CSQ overexpression. Although expression of CSQ has not been shown to be upregulated in heart failure (34), this model reproduces a number of key features of the disease including modifications of the β-adrenergic receptor signaling pathway, electrophysiological abnormalities, left ventricle hypertrophy, left ventricular systolic dysfunction and sudden death (17,24,35). Furthermore, a mutation in the CSQ gene has been associated with polymorphic ventricular tachycardia in humans (36). Since QTL mapping requires a reproducible phenotype with a low mouse to mouse variability, we believe that it will be difficult to map modifier genes using other classical models of heart failure such as myocardial infarction by coronary artery ligation (37) or pressure overload (38).

In conclusion, we have identified a number of novel QTLs that modify the severity of the heart failure phenotype. These data will allow a better understanding of the complex relationship between the variability of clinical presentation of heart failure patients and their genetic background.

MATERIALS AND METHODS

Animals

Animals were handled according to protocols and animal welfare regulations approved by the Institutional Review Board at Duke University Medical Center. The nomenclature of the crosses is in compliance with the recommendations of the International Committee on Standardized Genetic Nomenclature for Mice. Both DBA and AKR strains were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). These strains were chosen because of the relatively large phenotypic and genotypic differences between them that allowed us to perform linkage analysis. A line of transgenic mice overexpressing CSQ and inbred on a DBA genetic background was generated as previously described (9,17). Briefly, full-length canine cardiac CSQ cDNA was fused to the α-myosin heavy chain promoter to drive cardiac-targeted expression and micro-injected into fertilized mouse eggs.

Animals positive for the transgene were identified at each generation by Polymerase chain reaction (PCR).

Genetic crosses

DBA CSQ males (F₀) were mated with wild-type AKR females, leading to a F₁ progeny of hybrid mice [AKD2F1/J-Tg(CSQ)]. Fifteen F₁ males were selected randomly as breeders and chronically treated with beta-blockers (350 mg/kg of body weight per day) administrated in the drinking water (2 mg/ml) in order to improve their survival and the breeding efficiency. F₁ breeders were backcrossed to wild-type AKR females to produce 172 N2 mice [AK(AKD2F1)/J-Tg(CSQ)]. According to Lander and Botstein (39), this number of animals is sufficient to detect three QTLs of equal phenotypic effect with 90% of power when an interval mapping strategy is used to analyze the data.

Western blot

The level of CSQ overexpression was compared between mice with different genetic background or age by western blot. Briefly, left ventricles were homogenized and 10 μg of cytosolic extracts were resolved by 10% SDS–PAGE, transferred to nitrocellulose membranes and probed with antibodies specific for CSQ (1 : 2000; gift from Dr L.R. Jones).

Determination of heart failure phenotype

Transthoracic two-dimensional guided M-mode echocardiography was performed at 12 weeks of age in conscious mice using an HDI 5000 echocardiograph with a 15 MHz probe (ATL, Bothell, Washington, DC, USA) (35). Echocardiograms of good technical quality were obtained in 130 mice and were analyzed off-line using the Access Point 2000 software (Freedland Systems, Kilburn Court, IN, USA). Because all mice presented a pulsus alternans characteristic of CSQ overexpression (40), left ventricular end diastolic diameter, left ventricular end systolic diameter, and left ventricular fractional shortening were measured by averaging six consecutive beats.

All animals were maintained in the same environment with a 10 h light/14 h dark cycle and received unlimited access to the same standard diet. The cages were inspected daily to determine survival. Dead mice were autopsied and animals with evidence for a cause of death other than heart failure (i.e. leukemia, tumors or infection) were excluded from all statistical analysis using survival as the phenotypic trait. The observers gathering the phenotypic data were blinded to the genotype data.

Statistical analysis of the phenotypic traits

All quantitative values were expressed as mean± SD. Differences in phenotypic trait values between mice with a different genetic background were analyzed by analysis of variance followed by Tukey tests. A value of $P < 0.05$ was considered significant. The proportion of the total N2 variance due to variations in genetic background was determined according to the following formula: (total N2 variance – environmental variance) × 100/total N2 variance, where the environmental phenotypic variance was
estimated by averaging the variance of the two non-segregating generations (F₀ and F₁) (41).

Genotyping

The genomic DNA was isolated from mouse tails using DNeasy Tissue Kit (Qiagen, Chatsworth, CA, USA). One hundred and six microsatellite markers polymorphic in the DBA × AKR cross and regularly spaced (14.6 ± 3.9 cM) throughout the 19 mouse autosomes were selected using the 2003 mouse genome informatics database from Jackson Laboratory (www.jax.org). Fluorescent labeled primers were purchased from Research Genetics (Carlsbad, CA, USA) and Applied Biosystems (Foster City, CA, USA). PCR was performed following the recommendations of the suppliers, and PCR products were separated by capillary electrophoresis in an ABI PRISM® 3100 Genetic Analyzer. Genotyping was performed using only microsatellite markers that amplified reliably and distinctly the two parental alleles. In order to avoid any misgenotyping, we repeated the PCR for all double recombinants within a short genetic distance.

Linkage analysis

The Map Manager QTX program (version b13) was used to localize the QTLs responsible for the variability of mice heart failure phenotypes (survival, FS, LVEDD). The source codes are available on line at http://mapmgr.roswellpark.org/mmQTX.html. The analysis of the linkage between the phenotype of the N2 CSQ mice and their genotype for the different microsatellite markers was performed in several steps as we previously described (9).

As most of the statistical information required for QTL mapping is located in the extremes of a trait distribution (39,42), we constructed the preliminary genetic linkage maps by genotyping only the N2 progeny with the most extreme phenotype (±0.5 SD from the average). The level of significance of each potential association was estimated using the LRS proposed by Haley and Knott (43). This logarithmic index can be converted to a LOD score by dividing it by 2 ln(10).

The significance thresholds for each phenotypic trait were empirically determined by permutation tests performed with all the informative markers and 10,000 permutations of data (44).

The results obtained by this method were identical to the thresholds determined by the method proposed by Piepho (45). On the basis of the preliminary genetic linkage maps, high-density mapping was performed using the entire N2 population within the area suggesting linkage with the phenotype. The composite interval mapping function of Map Manager QTX was used to more accurately determine the position of the QTLs and to estimate the statistical level of significance at multiple analysis point across each interval between two markers. When several QTLs where identified for the same phenotypic trait, one of them was used as genetic background in order to evaluate the effect of each QTL independently (46).

The support interval of a QTL was defined as the region where the highest LOD score decrease by a value of 1 on either side of the peak (1 – LOD).

In the last statistical analysis, we examined the interaction or epistatic effect between all pairs of marker for each phenotypic trait. Two loci show interaction when the genotype at one locus affects the effect of the other locus (47). The significance of the interaction between the loci was assessed by a permutation tests for the total effect (leading to a threshold comparable to the stringent recommended P-value < 10⁻⁵) and by a P-value under 0.01 for the interaction itself.

The genes localized within the support interval of the different QTLs have been identified using the available databases (database of the University of California, Santa Cruz; www.genome.ucsc.edu and Celera Discovery system; www.celeradiscoverysystem.com/index.cfm).

ACKNOWLEDGEMENTS

We thank Dr Lan Mao for her expert technique of echo-cardiography, Kristine Porter for her technical assistance and Nancy Golson, Tracey Howell and Christopher Clayton for their dedicated work on animal handling. We also thank Drs Tom Coffman and Thu Le for active discussion and interpretation of data. This work was supported by National Institutes of Health grant HL69230 (H.A.R.) and by a grant from the French Federation of Cardiology (P.L.). H.A.R. is a recipient of a Burroughs Wellcome Fund Clinical Scientist Award in Translational Research.

REFERENCES


