Abstract—Cardiac left ventricular hypertrophy (LVH) is commonly associated with hypertension, but its variance is determined for more than 50% by blood pressure–independent genetic factors. Because it constitutes one of the most important risk factors for cardiovascular mortality, we have performed a genome-wide scan of the F2 progeny of crosses between inbred WKY and WKHA rats to detect quantitative trait loci (QTL) linked to cardiac mass. In addition to left ventricular mass (LVM), we also measured left ventricle (LV) concentration of atrial natriuretic factor (ANF), because we have previously established that there was a genetic link between these two traits in the same animal cross. We found 2 contiguous QTL on chromosome 5 that were linked to LVM (logarithm of odds [LOD] = 3.5) or log (LV ANF) (LOD = 1.2). The 1-LOD support intervals of both QTL shared a region overlapping the locus of natriuretic peptide precursor A (Nppa) (i.e., the ANF-coding gene). We found by sequencing 2 single nucleotide polymorphisms (SNPs) within the first 650 bp of the Nppa minimal promoters of the genes from both strains. One of these SNPs increased the transcriptional activity of the Nppa minimal promoter in transfected neonatal cardiomyocytes in keeping with the higher LV concentration of ANF observed in WKY versus WKHA rats. Taken together with the previous reports showing that ANF may protect cardiomyocytes against hypertrophy, our genetic data single out Nppa as a strong candidate gene for the determination of LVM. (Circ Res. 2001;88:223-228.)

Key Words: left ventricular hypertrophy • genetics • atrial natriuretic factor • natriuretic peptide precursor A gene • quantitative trait locus

Cardiovascular diseases are the principal cause of mortality and morbidity in industrialized countries. In recent years, left ventricular hypertrophy (LVH) has emerged as a powerful independent risk factor for cardiovascular mortality and morbidity.1-3 In humans, cardiac mass may be determined in part by environmental factors (including diet habits and level of physical activity)3 as well as underlying disease state (including diabetes, coronary insufficiency, renal failure, and increased cardiac workload).2,4,5 However, the sum of all these factors can account for only a small fraction of the variance of cardiac mass,6 a fact suggesting the existence of heritable genes that modify cardiac mass independently of the other factors. Accordingly, studies performed with 254 pairs of twins of Caucasian origin have estimated that genetic factors accounted for 63% of the total variance in boys and 71% in girls.7 Likewise, a study using 23 inbred strains of rats and crosses between them has estimated that the portion of the variance of cardiac mass that was genetically determined ranged from 45% to 65%.8

In humans, the distribution of cardiac mass is mostly continuous within populations of unselected normotensive or hypertensive patients,9 which defines this variable as a complex quantitative trait. Such traits typically result from the action of multiple genes that each have modest effects on their own and thus are difficult to detect by linkage analysis in human populations.10 One experimental alternative is to perform crosses of inbred (i.e., genetically pure) animal models that present quantitative differences for the phenotypic trait of interest. Using this approach, several investigators have identified quantitative trait loci (QTL) linked to left ventricular mass (LVM).11 In most of these studies, the parental strain presenting left ventricular hypertrophy (LVH) was hypertensive as well, so that the QTL linked to heart weight were the same as the ones linked to blood pressure.11-15 Nonetheless, some of the QTL identified in such crosses were found to be linked to LVM independently of blood pressure.14,16,17

Recently, others have departed from previous studies by using crosses between 2 strains of rats that showed differences in LVM but not in blood pressure (WKY and F344).18 This strategy led to the identification of one QTL on chromosome 3 that was linked to LVM in the absence of elevated blood pressure. Our previous work has shown that the WKY and WKHA rat strains might also be suitable for the
detection of such QTL, because LVM is 10% to 15% higher in WKHA than in WKY rats despite the fact that mean blood pressure is identical in both strains.\textsuperscript{19} We therefore performed a genome-wide scan of the F2 progeny of male rats originating from a cross between these 2 strains. In addition to measurements of LVM, the phenotypic characterization of each animal in the cross included the measurement of left ventricle (LV) concentration of atrial natriuretic factor (ANF), because we have shown previously by cosegregation analysis that low LV ANF was genetically linked to high LVM in WKHA/WKY crosses.\textsuperscript{19}

Materials and Methods

Animals

All procedures on animals were approved by the Institut de Recherches Cliniques de Montréal (IRCM) Institutional Animal Care Committee and conducted according to guidelines issued by the Canadian Council on Animal Care. The nomenclature of the strains is in compliance with the recommendations of the International Rat Genetic Nomenclature Committee. WKHA/Cfd rats originated from a colony that is maintained at the IRCM, as registered with the Institute of Laboratory Animal Resources. WKY/Cfd rats also originated from a colony maintained at the IRCM and were derived from WKY/Cr parents obtained from Charles River (St Constant, Québec, Canada). Adult animals were housed 2 to 3 per cage and given unlimited access to standard chow and water. All animals were used at 12 weeks of age for the purpose of these studies.

Animal Procedures

On the day of tissue collection, each animal was weighed for determination of whole body weight (BW). The rats were then killed by decapitation, and spleens and hearts were collected. Heart ventricles were additionally dissected into right ventricle (without the septal wall) and LV (including the septal wall). Each part was blotted dry and weighed individually. LVM was defined as the ratio of LV weight/BW. All tissues were frozen in liquid nitrogen and kept at −70°C for additional analysis.

ANF Radioimmunoassay

Fragments of LV apex (±200 mg) were weighed, powdered under liquid nitrogen, and boiled for 5 minutes in a volume of 2 mL of 0.2 mol/L acetic acid. The extracts were then centrifuged at 30 000g for 30 minutes. Aliquots of 5 µL of supernatant were assayed using the same procedures and reagents described previously.\textsuperscript{20} The content of ANF immunoreactivity in LV tissue was expressed as fmol/mg wet weight. For phenotype-genotype correlations, a log transformation was performed on the values of LV ANF, because we have shown previously by cosegregation analysis that low LV ANF was genetically linked to high LVM in WKHA/WKY crosses.\textsuperscript{19}

Microsatellite Analysis

For each animal, genomic DNA was extracted from 10 mg of frozen spleen using the QIAamp tissue kit (Qiagen). Analysis of single-sequence-length polymorphisms was carried out by polymerase chain reaction (PCR) amplification of genomic DNA with [32P]-labeled primers using conditions similar to those described previously.\textsuperscript{21} Primers identified with a code containing either Mit, Mgh, or Rat were purchased from Research Genetics (Huntsville, Ala). Primers identified with a code containing Wox were purchased from Genosys (The Woodlands, Tex).

Genetic Crosses and Analyses

A total progeny of 345 male rats was obtained by intercrosses between WKHA and WKY rats. For practical reasons, these animals were generated by 2 separate waves of breeding. For the first wave, male WKHA rats were mated to female WKY rats; the resulting F1 animals were then mated randomly to generate 152 male F2 rats. For the second wave (performed a few months later), male WKY rats were mated to female WKHA rats to produce, ultimately, 193 male F2 animals.

While the second wave of breeding was still in progress, we performed preliminary genetic analyses with DNA samples from some of the 152 male rats that had already been generated. Within this group, we observed a normal distribution of the values of LVM, whereas a log transformation was necessary to normalize the distribution of the values of ventricular ANF concentration. For the purpose of a preliminary screening, it has been shown that selection of the highest and lowest phenotypic values of a progeny provides genetic contrast and makes it possible to detect potential QTL with a specificity and sensitivity rivaling that obtained with the genotyping of the whole population.\textsuperscript{14,22} We therefore selected the individuals that had the 23 highest and lowest phenotypic values for either LVM (defined as the ratio of LV weight to whole-body weight) or ln (LV ANF concentration). Taking into account the partial overlap between the 2 subgroups of 46 individuals, the combined group amounted to 74 individuals. A panel of 80 markers that were polymorphic between WKY and WKHA rats was then used for linkage mapping. These markers covered all autosomes and defined the intervals that averaged 14.7±12.7 centimorgan (cM) (mean±SD), with the exception of chromosome 6 (which contained only one polymorphic marker) and chromosome 10 (which contained only 2 closely spaced polymorphic markers). On the basis of preliminary results, additional analyses were performed with 10 polymorphic markers linked to chromosome 5, using DNA samples from all 345 male F2 rats from the combined total progeny.

Linkage maps were constructed by computing the linkage data with the MAPMAKER/EXP 3.0 software package.\textsuperscript{23} After verifying potential errors by repeating the genotyping for all double recombinants and constructing final maps, QTL affecting phenotypes were mapped relative to genotypes using the MAPMAKER/QTL 1.1 software package.\textsuperscript{24} The percentage of total variation accounted for by a given QTL was also calculated by MAPMAKER/QTL and corresponded in fact to the $r^2$ value calculated by linear regression.

Gene Sequencing and Mutagenesis

The gene that codes for the precursor of ANF is called the natriuretic peptide precursor A (Nppa) gene according to the official gene nomenclature. Oligonucleotides were designed to amplify by PCR a portion of the Nppa promoter that extends 650 bp from the transcription initiation site, using genomic DNA from either WKY of WKHA rats and the high-fidelity enzyme Pwo DNA polymerase (Roche Diagnostics). The amplification products were subcloned into the KpnI and BgIII sites of the pGL3 luciferase reporter plasmid (Promega). Three different clones were selected from three independent amplifications to avoid potential PCR artifacts, and integrity of each sequence was verified by analysis on a Beckman CEQ automatic sequencer. Site-directed mutations of the promoter were generated by oligonucleotide-directed mutagenesis and PCR amplification of the primed DNA. Four different promoters were generated: (1) a promoter corresponding to the sequence found in WKHA; (2) a promoter where nucleotide (−931)T was mutated to C; (3) a promoter where (−467)G was mutated to T; and (4) a promoter corresponding to the sequence found in WKY rats, where both polymorphisms were present. All promoters were subcloned into the KpnI and BgIII sites of the pGL3 luciferase reporter plasmid (Promega).

Primary Cardiomyocyte Cultures and Transfections

The method for culturing ventricular cardiomyocytes from 3-day-old rats was adapted from previously published protocols.\textsuperscript{25,26} After isolation, the cardiomyocytes were plated on 6-well Primaria dishes (Falcon) at a density of 200 000 cells/well. After overnight maintenance in FBS-supplemented medium, each well was transfected with 3.5 μg of test plasmid by using 5 μg of Lipofectin ( Gibco BRL). In addition to the test plasmid, 0.5 μg pCMV/β (containing sequences...
coding for β-galactosidase placed under the transcriptional control of the cytomegalovirus promoter) was used as an internal control for transfection efficiency.27 The cells were then maintained in serum-free medium supplemented with 5 μg/mg insulin, 5 μg/mL transferrin, and 5 ng/mL selenium (Sigma), 20 mmol/L HEPES and 2.5 μg/mL bovine apotransferrin (Gibco BRL), and fatty acid–free BSA (Roche Diagnostics). Forty-eight hours after initiation of the transfection, the cells were lysed with 300 μL of 100 mmol/L Tris (pH 8.0) containing 0.5% Nonidet P-40. Lysate (100 μL) was used to assay luciferase activity (by luminometer reading), and another 100 μL of lysate was used to assay β-galactosidase activity, using chlorophenol red β-d-galactophranoside (Roche Diagnostics) as a substrate.28

### Results

The preliminary low-resolution genome-wide scan of 74 individuals with 80 markers (see Materials and Methods) allowed us to define 26 linkage groups covering all 20 autosomal chromosomes. Within this map, we detected at the distal end of chromosome 5 one QTL that was linked to LVM (LOD=2.8) and to LV ANF concentration (LOD=8.9). Because both LOD scores where higher than 1.9 (ie, the threshold proposed for suggestive linkage of codominant traits in a rat intercross), we performed a higher-resolution analysis by increasing the number of markers for chromosome 5 and by using all 345 individuals of the combined F2 progeny. This procedure yielded a new linkage map of chromosome 5 where the relative order of all markers and distances between them (Figure 1) were found comparable to those reported in the latest genetic maps.30 There was significant evidence (LOD 12) for a locus (QTL/ANFv) located near D5Rat180 that was responsible for 44.3% of the total variance of loge(LV ANF). There was also significant evidence (LOD 3.5) for a locus (QTL/LVM) located around D5Rat99 that was responsible for 18.6% of the total variance of LVM. Both LOD scores were higher than 3.3, thus indicating statistical linkage for a rat intercross.29 Distribution of quantitative values of both phenotypes confirmed that the traits were influenced in a codominant fashion by the genotypes (Table). The 1-LOD support intervals of QTL/ANFv and QTL/LVM were 12 and 28 cM, respectively, and overlapped a region that contains the marker D5Wox10, which coincides with the locus of the Nppa gene.31 Moreover, individuals who were homozygous for both WKHA alleles at loci corresponding to the peaks of the LOD score curves had both the lowest ventricular concentrations of ANF and highest LV masses (Table).

To test whether genetic alterations of the Nppa gene could possibly be linked to the higher ventricular concentration of ANF in WKY versus WKHA rats, we sequenced 650 nucleotides of the minimal Nppa promoter32 of both strains. We detected two SNPs: C(-93)T (where the first nucleotide corresponds to that in WKHA rats and the position is defined in reference to the transcription initiation site) and G(-467)T. In WKHA rats, the identity of both nucleotides was identical to that in the published sequences of the Nppa gene from SD rats.33,34 To test whether these SNPs alter the transcriptional activity of the Nppa minimal promoter, we transfected primary cardiomyocyte cultures with DNA constructs containing different variants of the promoter driving the luciferase reporter gene in the pGL3 luciferase reporter plasmid. Preliminary experiments showed that no luciferase activity was detected in extracts of cardiomyocytes transfected with pGL3 alone. When using pGL3 plasmids containing different variants of the Nppa minimal promoter, we observed that the transcriptional activity of promoters containing the C(-93)T single nucleotide polymorphism (SNP) (either alone or in combination with the other SNPs, as found in the WKY promoter) was significantly higher than that of the WKHA promoter, whereas the T(-467)G polymorphism had no effect (Figure 2). Of note, the (-93)T nucleotide found in WKY promoters is contained within a sequence of 18 nucleotides that is fully conserved in the promoters of human, mouse, and bovine Nppa genes, and is immediately adjacent to the reported NK2 core-binding site35 (Figure 3).

### Correspondences Between Genotypes and Phenotypes

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Locus</th>
<th>HH</th>
<th>HW</th>
<th>WW</th>
<th>LOD Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV ANF, fmol/mg w.w.</td>
<td>D5Rat180</td>
<td>37±4.25</td>
<td>77±5</td>
<td>104±11</td>
<td>12</td>
</tr>
<tr>
<td>LVM/BW, mg/g</td>
<td>D5Rat99</td>
<td>2.82±0.04</td>
<td>2.74±0.03</td>
<td>2.54±0.03</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Values are mean±SEM, expressed in units as indicated for each phenotype. w.w. indicates wet weight. Genotypes are either WKHA homozygous (HH), WKY homozygous (WW), or heterozygous for the particular locus specified in each row.
Discussion

Up until now, most genetic investigations on LVM in rats have used crosses where one of the parental strains was hypertensive. By using crosses between parental strains harboring differences in LVM but similar blood pressures, we were able to detect on chromosome 5 a QTL linked to LVM independently of blood pressure. Using other strains with similar characteristics (ie, WKY and F344), other studies have recently detected on chromosome 3 another QTL linked to LVM. Up to 4 genes are contained within the 1-LOD support interval of that QTL, but no data have been provided to indicate whether any of these genes could be a potential candidate. This is not unlike the present status of research for a variety of complex cardiovascular traits, for which multiple QTL have been found but for which no candidate genes have been identified yet.

In the present study, we also measured LV ANF concentration in addition to the LVM phenotype. The primary reason for doing so was because the use of intermediary or associated traits has been proposed as a means to enhance the power of genetic analyses of complex traits. LV ANF concentration seemed to be of particular interest, because we have shown previously that this trait was genetically linked to LVM in WKHA/WKY crosses. Accordingly, we detected QTL/ANFLV that was linked to loge (LV ANF concentration) with a LOD score of 12. QTL/LVM was also detected on chromosome 5, and its 1-LOD-support interval overlapped with that of QTL/ANFLV. Because of the limited resolution of linkage mapping, it is difficult to determine solely on the basis of genetic distances whether the 2 QTL are contiguous or identical. Nonetheless, the statistical chances for finding after random scanning 2 QTL within such close vicinity are small. Moreover, both confidence intervals define a region containing the Nppa locus, which should be considered a strong candidate gene for the following reasons. First, the LOD score by which LV ANF concentration is linked to the locus of Nppa (ie, the very gene that encodes for the ANF precursor) is unusually high, suggesting a direct causal relationship between the Nppa locus and LV ANF concentration. Second, we have detected by direct sequencing of the Nppa promoters of WKHA and WKY the C(−93)T SNP that alters the function of the Nppa promoter in a manner that is consistent with the LV ANF concentration in both strains. This finding makes it likely that the C(−93)T SNP is responsible (at least in part) for the higher LV concentrations of ANF in WKY versus WKHA rats, although it does not rule out any possible contribution of other polymorphisms. We did not find variations in the coding region, but it is possible (and even probable) that other polymorphisms exist in regions of the gene we did not investigate, ie, farther upstream in the promoter or downstream in the 3′-untranslated region. However, single-base mutations causing human genetic disease often consist (as in the present case) of CT transitions within CpG dinucleotides. Thus, even if other polymorphisms did occur elsewhere within the sequence of the gene, it is not certain that they would have the same functional impact as the C(−93)T SNP (by virtue of the nature of the transition and its localization just upstream of the Nkx2.5-responsive element). Finally, a link between low LV concentration of ANF and high LVM makes functional sense in the light of previous reports indicating that ANF or its second messenger cGMP may protect cardiac cells against hypertrophic stimuli. In vitro, the action of hypertrophic agents on cultured neonatal cardiac cells is either inhibited by ANF or cGMP or enhanced by an ANF antagonist. In vivo, knockout inactivation of either Nppa or of the ANF receptor in mice increases ventricular mass disproportionately with the small changes in blood pressure observed in the same animals.

Of note, the fact that an 18-nucleotide sequence in the Nppa promoter of WKY rats was identical to that in human, mouse, and bovine makes it possible that the sequence in WKY rats is the ancestral one (as opposed to the one found in WKHA rats). We have verified by Northern blot analysis that the abundance of ANF mRNA in LV of SD rats (whose minimal Nppa promoter is reportedly identical to that of WKHA) is similar to that of WKHA rats, ie, much lower than that of WKY rats (data not shown). Sequencing of Nppa promoters of several inbred rat strains should provide more information in this regard. Because the SNP is also immediately adjacent to the reported NK2 core-binding site, it is also

Figure 2. Relative activities of various Nppa promoter variants in transfected cardiomyocytes, expressed as percentages of the mean values of the ratios of luciferase/β-galactosidase activities found for the WKHA promoter. The bars represent mean±SE, n=36 individual wells for WKHA and WKY rats, n=18 for single mutations. One-way ANOVA determined that there was a significant difference (P<0.0005) between groups. *P<0.01 compared with WKY rats, as determined by post hoc Fisher's least-significant difference tests.

Figure 3. Comparison and alignment of sequences found around the C(−93)T polymorphism in the Nppa promoters of different species and rat strains. The figure was designed on the basis of data published previously as well as the results of the present study. SD (publ) corresponds to the sequence published previously for SD rats. The shaded area highlights the nucleotides that are different in SD and WKHA than in WKY rats or other mammalian species.
possible that it affects the transcriptional activity of the promoter by altering the interaction of this corresponding DNA segment with a transcription factor that binds within the Nkx2.5 region.\(^\text{15}\)

In summary, the concurrence of genetic, molecular, and functional evidence is such that Nppa emerges as one strong and new candidate for the determination of LVM. Of note, chromosome 5 has never been reported in any of the previous studies to contain a QTL linked to LVM. This may be attributable in part to differences in genetic backgrounds.\(^\text{11}\) Alternatively, it is possible that the parental strains that have been used previously did not harbor the same Nppa polymorphism, making the crosses inappropriate to detect a link between LVM and the Nppa locus. Nonetheless, if the link is confirmed in congenic animals derived from backcrosses between WKY and WKHA rats (or 2 other strains with the Nppa polymorphism), the notion that cGMP (the second messenger of ANF) may play an important role in the protection against ventricular hypertrophy will be reinforced.

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References

Functional Alterations of the *Nppa* Promoter Are Linked to Cardiac Ventricular Hypertrophy in WKY/WKHA Rat Crosses

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