Localization of modifier loci of the *vl* mutant, a mouse model of spina bifida and congenital cataracts.

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ASTRACT.

During development, neural tube closure and lens formation undergo similar morphogenetic movements. *Vacuolated lens* (*vl*) is a recessive mutation that arose on the C3H/HeSnJ background. *Vl* mutants exhibit congenital cataracts and very occasionally a white belly spot in adults or spina bifida in embryos. We have recently identified the *vl* mutation as an 8bp deletion in a orphan G protein coupled receptor, that is expressed in the developing eye and spinal cord (Desai et al). When the *vl* mutation was crossed onto different genetic backgrounds (*C57BL6/J*, *CAST/Ei* and *MOLF/Ei*) to map the locus, a strain dependent modifying effect on the penetrance and expressivity of the *vl* phenotypes was observed. To map these modifier loci, quantitative trait loci (QTL) analysis was performed on the F2 intercross progeny generated from these crosses. Five QTLs were identified that either suppress the spina bifida or cataracts phenotypes or cause an increase the incidence of a belly spot phenotype. In the *C57BL6/J* cross, one significant QTL (*Modvl1*) for spina bifida was identified on chromosome 5 (44cM, LOD 3.7) with the high allele contributed from the *C3H/HeSnJ* background. Interestingly, the same QTL (LOD 3.7) was identified for the increased incidence of the belly spot phenotype but the high allele was contributed from the *C57BL6/J* background. In the *CAST/Ei* cross, one significant QTL (*Modvl2*) for spina bifida was identified on chromosome 1 (26cM, LOD 3.3). In our largest cross with *Molf/Ei*, one major QTL for congenital cataracts was identified on chromosome 4 (*Modvl3*, 51cM, LOD 4.2) and two significant belly spot QTL were mapped to chromosome 15 (*Modvl4*, 15cM, LOD 4.4) and chromosome 18 (*Modvl5*, 41cM, LOD 5.0). We conclude that several different genes can modify the

different phenotypes caused by the *vl* mutation and that these genes along with the *vl* locus are required for normal neural tube and lens development.

INTRODUCTION.

Neurulation is an important and fundamental process that gives rise to the future brain and spinal cord. Neurulation involves a complex set of genetic and cellular processes that begin with the initiation and bending of the neural plate, a flat sheet of neuroepithilum, to form the neural groove. At the neural groove stage, the two folds of the neural groove elevate, delaminate from the epidermal ectoderm and move towards the midline, finally leading to apposition of the neural folds at the dorsal midline to form the neural tube. During this process of neural tube closure, neural crest cells are generated, which delaminate from the lateral neural plate and migrate away from the neural tube to form different cellular populations including melanocytes. Mutations that affect these complex genetic and cellular processes could lead to neural tube defects, such as spina bifida, or defects in melanocyte development, such as a white belly spot.

Similarly, lens development and morphogenesis also involves coordinated growth, movement and fusion of specialized cells. During lens development the lens placode invaginates to form the lens pit and then fusion of the two folds of the lens pit form the lens vesicle. After the lens vesicle has formed, it delaminates from the overlying epidermal ectoderm. The lens vesicle then undergoes primary lens fiber differentiation and later takes a final step in its differentiation via degradation of cell nuclei and mitochondria. Disruptions in any of these morphogenetic movements lead to congenital eye defects, such as cataracts in the developing lens.

To uncover the genetic and molecular pathways that control these complex morphogenetic movements during development, we have been studying the recessive mouse mutant *vacuolated lens* (*vl*). *Vl* is co-isogenic with the *C3H/HeSnJ* background and on this background adult *vl/vl* mice display cataracts and an occasional white belly spot while *vl/vl* embryos exhibit spina bifida or an attenuation of the dorsal midline of the neural tube. Our positional cloning of the *vl* locus has demonstrated a C-terminal tail truncation of a novel orphan G protein coupled receptor (GPCR) is responsible for the *vl* phenotypes (Desai et al). In accordance with mouse nomenclature, we have named this GPCR *vacuolin*.

The role of *vacoulin* in the development of the lens and neural tube and the mechanisms by which this truncation leads to spina bifida and cataracts is presently unknown. However, we have noticed that the severity and penetrance of the phenotypes caused by the *vl* mutation depends strongly on the genetic background. For example, while *C3H/HeSn-vl/vl* mice with spina bifida are observed only embryonically and are not viable, when crossed onto the *C57BL/6J* or *CAST/Ei* background, offspring are born with mild to severe spina bifida. However, when the *vl* mutation is crossed onto a *MOLF/Ei* background, the embryonic lethality associated with is not observed. In addition, all *C3H/HeSn-vl/vl* adult mice display cataracts and infrequently accompanied with a small belly spot. On the *MOLF/Ei* background, the penetrance of the cataracts phenotype is also dramatically reduced, while the penetrance of the belly spot phenotype is significantly enhanced. These observations suggest that modifier loci are present in

these strains that interact with the *vl* mutation to affect the penetrance and severity of the *vl* phenotypes.

To map modifer loci, QTL analysis was performed on F2 mice generated from intercross matings between *C3H/HeSnJ-vl/vl* and *C57BL6/J* (Cross A), *CAST/Ei* (Cross B) and *MOLF/Ei* (Cross C). We report the identification of five QTLs that specifically affect the penetrance and/or expressivity of spina bifida, congenital cataracts and belly spot phenotypes. Further characterization of these modifiers will help identify additional genes that act upstream or downstream of *vacuolin* and provide insight into the molecular and cellular mechanisms that control neural tube and lens development.

MATERIALS AND METHODS.

Animals and crosses

Inbred mice were obtained from The Jackson Laboratory, Bar Harbor, ME, for the following crosses: *Cross A*: C57BL/6J females were mated to C3H/HeSn-*vl/vl* males to produce the F_1 progeny; F_1 mice were intercrossed to produce 86 F_2 progeny that were homozygous C3H between *D1Mit143* and *D1Mit15*. *Cross B*: CAST/Ei females were mated to C3H/HeSn-*vl/vl* males to produce the F_1 progeny; F_1 mice were intercrossed to produce 86 F_2 progeny that were homozygous C3H between *D1Mit506* and *D1Mit15*. *Cross C*: MOLF/Ei females were mated to C3H/HeSn-*vl/vl* males to produce the F_1 progeny; F_1 mice were intercrossed to produce 127 F_2 progeny that were homozygous C3H between *D1Mit506* and *D1Mit15*.

Mice were housed in a climate-controlled facility with a 14-hour: 10-hour light-dark cycle with free access to food and water throughout the experiment. After weaning, mice were maintained on a chow diet (Old Guilford 234A, Guilford, CT). All experiments were approved by the Animal Care and Use Committees of the Jackson Laboratory and the UMDNJ-Robert Wood Johnson Medical School.

DNA isolation and genotyping

DNA was isolated from a centimeter of tail digested overnight in 500 μl of 1X digestion buffer (50 mM Tris-Cl pH 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl, 1% SDS, 1mg/ml proteinase K) in a 55°C waterbath. Digests were mixed with 1 volume of 25:24:1 phenol:chloroform:isoamyl alcohol and centrifuged for 5 minutes at 14,000 rpm at room temperature. DNA was precipitated by adding 2 volumes of 100% ethanol to the isolated aqueous phase. The dried DNA pellets were resuspended in 1 ml TE (10mM Tris-HCl, 1mM EDTA, pH 7.5-8.0). Genotyping by PCR using mouse MIT MapPairs primers (Research Genetics, Huntsville, AL) was carried out under standard conditions at an annealing temperature of 55°C. Polymorphisms were detected either by capillary electrophoresis (ABI), standard electrophoresis using 4% agarose (Nusieve) gels or by **JIM?.** Crosses A, B, and C used 75, 60, and 80 markers distributed across the genome, respectively. For cross C, QTL analysis was first performed on 92 F_2 *vl/vl* mice. To confirm and refine the identified QTL intervals, 35 F2 mice were added and flanking SSLP markers to selected QTL regions were genotyped.

Phenotyping

Cross A and B: Mice were scored as having no abnormal phenotype or caudal spina bifida. This was characterized by a protruding spinal cord and paralyzed hind legs (Figure 1). The presence or absence of belly spots was recorded. *Cross C*: No mice with spina bifida were observed in this cross. Cataract (opaque lens) and belly spot were recorded.

Statistical analysis

We performed genome-wide scans for QTL by using the method of Sen and Churchill [1], which is similar to the interval mapping procedure of Lander and Botstein [2] but uses a different imputation algorithm. First, we carried out one-dimensional genome scans on a single-QTL basis to detect QTL with main effects. Logarithm of odds ratio (LOD) scores were computed at 2 cM intervals across the genome and significance was determined by permutation testing [3]. Significant and suggestive QTL meet or exceed the 95% and 90% genome-wide thresholds, respectively. Then, simultaneous genome scans for all pairs of markers were implemented to detect epistatic interactions. The search strategy has been described by Sen and Churchill [1], and Sugiyama et al. [4]. Briefly, the genome scan searches through all pairs of loci by fitting a two-way ANOVA model with an interaction item. A LOD score contrasting the full model to a null model (with no genomic effects) is computed and genome-wide significance is established by permutation analysis. A secondary test for the significance of the interaction term is computed only for those pairs that pass the genome-wide screening. A stringent nominal significance level (0.001) is used for interaction test and only those locus pairs passing

both tests are deemed to be interacting. The software package used in this study, R/QTL version 0.97-21, is available at http://www.biostat.jhsph.edu/~kbroman/qtl/ [5].

RESULTS.

Penetrance and expressivity of *vl* phenotypes.

Previous analysis using a limited number of *vl/vl* x *vl/vl* matings suggested that the *vl* mutation was 100% penetrant on the *C3H/HeSnJ* background. To investigate this further and to determine the frequency of each of the *vl* phenotypes, +/*vl* x +/*vl* matings were performed and the progeny were genotyped for the *vl* mutation. 55% of viable adult *C3H/SnJ-vl/vl* mice display cataracts with 4-5% also exhibiting a small white belly spot. Adult *vl/vl* mice are observed **X%** less than +/+ littermates, indicating that this percentage of *vl/vl* embryos die embryonically, which is likely to be due to the lethality associated with neural tube defects. When E9.5-E12.5 litters were sacrificed, 100% of the *vl/vl* progeny display a phenotype. 45% of *vl/vl* embryos display spina bifida (Fig 1B) while the remaining 55% display an abnormal neural tube morphology, including an altered dorsal midline.

When the *vl* mutation is crossed onto a *MOLF/Ei* background, the penetrance and expressivity of the cataracts and belly spot phenotypes are drastically distorted. Most noticeably 48% of the adult *C3H/Molf-vl/vl* mice F2 progeny display no phenotype and are indistinguishable from wild type littermates (Fig 1B). This is in stark contrast to the 100% pentrance of the *vl* mutation in the *C3H/HeSnJ* background.Of the remaining 52% vl/vl mice that exhibit a phenotype, only 14% of *C3H/MOLF-vl/vl* F2 progeny display

cataracts as compared to 55% of *vl/vl* mice in the *C3H/HeSnJ* background (Fig 1B). Furthermore, on all three backgrounds, the *vl* mutation caused a large variation in both the frequency and size of the belly spot. Penetrance and expressivity of the belly spot phenotype is considerably enhanced on the *MOLF/Ei* background, such that 26% of *C3H/MOLF-vl/vl* F2 progeny now displays a belly spot, with a considerable proportion of these mice exhibiting a larger belly spot than ever observed on the *C3H/HeSnJ* background.

Lastly, the embryonic lethality observed in *C3H/HeSnJ-vl/vl* due to spina bifida (45%) is not detected for *C3H/MOLF-vl/vl* mice. In our MOLF/Ei intercross, 22.5% of our F2 mice are genotypically *vl/vl* in the *Molf/Ei* background as compared to the expected 25%. This difference is not statistically significant indicating that the embryonic lethality associated with the spina bifida on the C3H/HeSnJ background is largely rescued by crossing to MOLF/Ei. Although embryonically *C3H/HeSn-vl/vl* embryos exhibit spina bifida, there are no adult *C3H/HeSn-vl/vl* animals observed that display this defect. However, crossing *vl/vl* mice to *C57BL/6J* or *CAST/Ei* genetic background did result in adult *vl/vl* mice with a spina bifida phenotype (Figure 1). These data together suggest the presence of modifiers that affect the penetrance of the *vl* mutation and that these modifiers are able to suppress and enhance the three major phenotypes in the *vl* mouse.

QTL analysis.

QTL analyses were employed to map the major modifiers of the *vl* phenotypes. All F2 *vl* homozygotes were individually genotyped for 65 SSLP markers spaced evenly throughout the genome, enabling us to detect both additive and interactive QTL in our three crosses. This genome wide scan identified significant QTLs in all three crosses (Cross A, B, and C). We analyzed our data with a one QTL and interacting QTL models, and did not detect any interacting QTLs and therefore only single QTLs are presented. Permutation testing was used to determine significance thresholds.

Cross A: We were able to detect only one significant QTL in the cross with B6 for spina bifida (Figure 2A). This locus, which we named *Modifier of vl 1* (*Modvl1*), mapped to chromosome 5 (peak at 44 cM) (Figure 2B) was also detected for belly spot. This suggests the underlying gene influences both spina bifida and belly spot. The allele effect showed that the allele for high penetrance of spina bifida is contributed by C3H in a dominant fashion (Figure 2C), while the high allele for the belly spot is contributed by B6 and is recessive (Figure 2D).

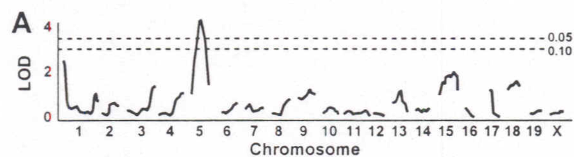
Cross B: In the cross with CAST we found only one significant QTL for spina bifida. This QTL, named *Modvl2*, mapped to chromosome 1 with a peak at 26 cM (Figures 3A and B). The allele effect showed that the high allele came from C3H and was additive (Figure 3C).

Cross C (MOLF intercross): One significant QTL for congenital cataracts and two significant QTL for the belly spot phenotype were identified. A QTL (*Modvl3*) for

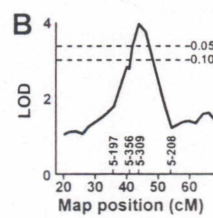
congenital cataracts was mapped to chromosome 4 with a peak at 51 cM linked to marker *D4Mit168* (Figures 4A and B). For this QTL, the high allele contributed from *Molf/Ei* determined the presence of cataracts in an additive fashion and accounted for **X** percent of the F2 phenotypic variation (Figure 4C). When the belly spot phenotype was used as a covariant, two QTLs (*Modvl4* and *Modvl5*) were identified that mapped to chromosomes 15 and 18 (Fig 5A). *Modvl4* mapped to chromosome 15 with a peak at 15 cM linked to *D15Mit252* and *Modvl5* mapped to chromosome 18 with a peak at 41 cM linked to *D18Mit50* (Figures 5A, B, and D). *Modvl4* accounts for **X** percent of the F2 phenotypic variance, whereas *Modvl5* accounts for **Y** percent of the F2 phenotypic variance. For both loci the *C3H* allele was responsible for an increase in the incidence of the belly spot phenotype in an additive fashion (Figures 5C and E).

A

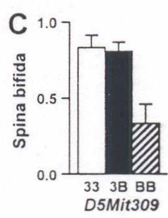
B



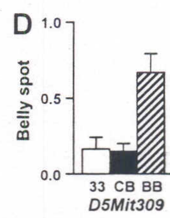
A
LOD
0.05
0.10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X
Chromosome



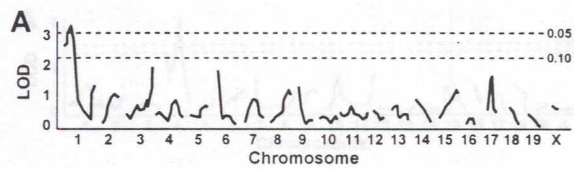
B
LOD
0.05
0.10
5-197
5-356
5-309
5-208
Map position (cM)

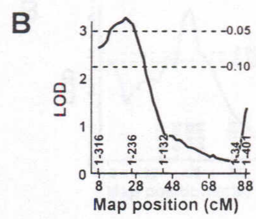


C
Spina bifida
33 3B BB
D5Mit309



D
Belly spot
33 CB BB
D5Mit309


A
LOD
0.05
0.10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 X
Chromosome


B
LOD
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0.10
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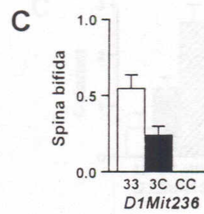
1-236
1-132
1-34
1-401
8 28 48 68 88
Map position (cM)


C
Spina bifida
1.0
0.5
0.0
33 3C CC
D1Mit236