

FULL LENGTH RESEARCH PAPER

Fine mapping of murine asthma quantitative trait loci and analyses of *Ptgs1* and *Mrc1* as positional candidate genes

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Abstract

Asthma is a common respiratory disease that is driven by both genetic and environmental factors. Identification of the genes underlying asthma will provide insight into the mechanisms and treatment of this important disease. Previous studies in this laboratory identified two distinct quantitative trait loci for the asthma-related parameter, allergen-induced airway hyperresponsiveness, in a murine model by means of a genome-wide linkage analysis. The present study focuses and refines the map location of these two loci. Additionally, we explore prostaglandin-endoperoxide synthase-1 (*Ptgs1*) and mannose receptor C-type 1 (*Mrc1*) genes as two new positional candidate genes for allergen-induced airway hyperresponsiveness through comparative sequence analysis and mRNA expression studies of mouse strains with genetically mediated airway responsiveness.

Keywords: Asthma, genetics, airway hyperresponsiveness, mouse, prostaglandin-endoperoxide synthase-1, mannose receptor C-type 1

Asthma is a chronic respiratory disease that affects more than 17 million people in the US alone (Hartert and Peebles 2000) and is characterized clinically by recurrent wheezing due to airway narrowing, such as occurs with airway hyperresponsiveness. Airway hyperresponsiveness is an important hallmark of asthma and, because it is quantifiable, it is an asthma-related parameter often studied. Underlying airway hyperresponsiveness and asthma is a persistent airway inflammation characterized by pulmonary infiltration of eosinophils, mast cells and lymphocytes, with overexpression of T helper cell type 2 (Th2) cytokines, such as interleukin (IL)-4, IL-5, and IL-13, and increased serum immunoglobulin E (IgE). Prostaglandins have also been implicated in the inflammatory process underlying asthma.

The expression of asthma is affected by both together environmental and genetic factors, which together lead to the presentation of disease symptoms (Lemanske and Busse 1997). While genetic predisposition to asthma is widely accepted, the precise genes that confer susceptibility remain unclear. Thus, in the current study an inbred murine model was used to examine the genetic basis of asthma. Here we report fine mapping of two quantitative trait loci (QTL) for allergen-induced airway hyperresponsiveness and comparative sequence and expression analyses for two positional candidate genes.

Prior to the initiation of the present study, two QTL for allergen-induced airway hyperresponsiveness were identified on murine chromosome 2: *Abhr1* (lod = 4.2) and *Abhr2* (lod = 3.7) (Ewart et al. 2000).

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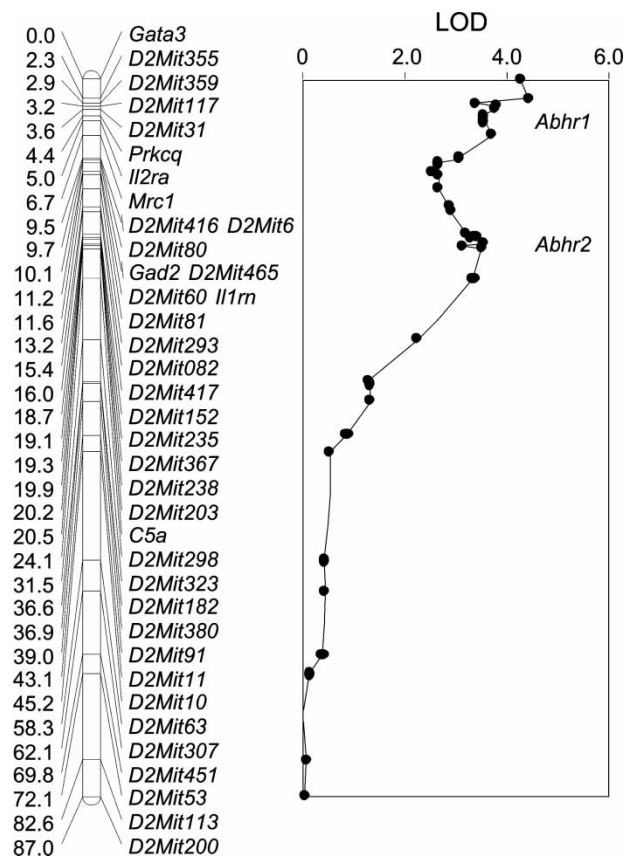


Figure 1. Murine chromosome 2 genetic linkage map (left) and lod score curve for allergen-induced airway hyperresponsiveness (right) in backcross mice ($n = 450$) derived from C3H/HeJ and A/J strains. The map was saturated with informative markers surrounding *Abhr1* and *Abhr2* to refine their map locations.

This was accomplished by means of a genome-wide linkage analysis for airway hyperresponsiveness in 176 A/J backcross mice $[(A/J \times (C3H/HeJ \times A/J)F1]$ and $[(C3H/HeJ \times A/J)F1 \times A/J]$ using microsatellite markers located at regular intervals throughout the genome. The 1-*lod* support interval for these linked QTL was

quite broad because *Abhr1* and *Abhr2* are juxtaposed. Therefore, further progress in identifying the genes underlying these QTL required additional refined mapping.

Our strategy in the current study to refine the map positions of *Abhr1* and *Abhr2* was to genotype an expanded population of backcross mice for densely clustered markers on murine chromosome 2. Thus, to pinpoint and more closely identify target *Abhr*-related genes, 37 markers on chromosome 2 were genotyped. Furthermore, these markers were examined in more than twice the number of segregating $[(C3H/HeJ \times A/J)F1 \times A/J]$ backcross mice ($n = 450$) as were examined in the original study.

Fourteen chromosome 2 markers genotyped in the previous study in a limited number of mice were genotyped in the current study in up to 450 mice (Figure 1): *D2Mit359*, *D2Mit416*, *D2Mit6*, *D2Mit465*, *D2Mit60*, *D2Mit81*, *D2Mit82*, *D2Mit417*, *D2Mit152*, *D2Mit238*, *D2Mit298*, *D2Mit323*, *D2Mit380*, and *D2Mit91*. Nine previously untyped polymorphisms on chromosome 2 were genotyped in 450 mice: *D2Mit355*, *D2Mit117*, *D2Mit31*, *D2Mit80*, *D2Mit293*, *D2Mit235*, *D2Mit367*, *D2Mit203*, *D2Mit182* and polymorphisms in *Gata3*, *Prkcq*, *Il2ra*, *Mrc1*, *Gad2* and *Hc* genes. Eight chromosome 2 markers were genotyped in an average of 84 mice in the previous study for which no additional mice were used in this study as these markers were remote from the site of linkage: *D2Mit11*, *D2Mit10*, *D2Mit63*, *D2Mit307*, *D2Mit451*, *D2Mit53*, *D2Mit113* and *D2Mit200*.

Of the 37 polymorphisms examined in this study, 31 were commercially available microsatellite markers, one was a restriction fragment length variation based on a previously described mutation in the complement factor 5 gene (*Hc* or *C5*) (Wetsel et al. 1990), and the remaining were microsatellites that were original to this study. These previously unreported markers are located within genes in the *Abhr*-linked region and contain sequence length polymorphisms between A/J and

Table I. New intragenic markers in the *Abhr* linkage interval.

Gene	Primers (5'–3')	T_A (°C)	Size (bp)	
			A/J	C3H/HeJ
<i>Gata3</i>	(Forward) AGTG TAGACAGCGCAAAGT (Reverse) CCTAGAATTCTATGGCTCCGT	56	192	196
<i>Prkcq</i>	(Forward) GGCCAAGGAACCTAAAC (Reverse) TCCCACCCACAGAAATCATAC	51	310	322
<i>Il2ra</i>	(Forward) GATGCGGCATGGTTCAG (Reverse) CCATCCTAGCATAGGCGTTGT	53	315	311
<i>Mrc1</i>	(Forward) AAAAATCCAATAGAAGTTTGT (Reverse) ATATGTTCTCCACATCTATC	51	318	313
<i>Gad2</i>	(Forward) CACATTGTAAAAGTATCCC (Reverse) TCACATTAAATAAGCAAATCT	44	119	107

T_A , annealing temperature.

C3H/HeJ mouse strains (Table I). The sequence polymorphisms were identified by designing primer pairs flanking simple nucleotide tandem repeats in numerous gene sequences obtained from the Celera database (Celera 2005) and comparing product sizes on sequencing gels. In this manner, polymorphisms were detected in *Gata3*, *Prkcg*, *Il2ra*, *Mrc1* and *Gad2* genes.

Primers for the murine *Hc* gene (forward 5'-CAAT-TAAAGCTAACTATAAGAAGGATTTTACAA-3', reverse 5'-CAAGTTAGAACTAAGCACTAGCTACTCAAACAA-3') were based on the report by Wetsel et al. (1990) and flanked a restriction site yielding 180 and 28 bp products in A/J mice and a single uncut 210 bp product in C3H/HeJ mice. The *Hc* gene has physiologic as well as positional significance (Karp et al. 2000) and is, therefore, a positional candidate for the *Abhr2* locus.

Multipoint linkage analysis and calculation of the map order and distance were performed by MAPMAKER, MAPMAKER/QTL 1.1 (Lincoln et al. 1992a,b) and JoinMap 3.0 (Van Ooijen 2001) software packages. Interval mapping and simultaneous search techniques were used to link phenotype with genotype and identify QTL.

These studies considerably refined the *Abhr1* and *Abhr2* linkage regions. Based on our fine mapping results we identified two new positional candidates, *Ptgs1* and *Mrc1*, for the *Abhr* loci and performed comparative sequence analysis of these genes.

Prostaglandin-endoperoxide synthase-1 (*Ptgs1*), also known as cyclooxygenase-1 (*Cox1*) and prostaglandin G/H synthase-1 (*Pghs1*), is a key enzyme in prostaglandin biosynthesis, acting as a limiting enzyme in converting arachidonic acid into prostaglandins and thromboxanes. It is one of two well-described isozymes of *Ptgs* that differ in their regulation of expression and tissue distribution. *Ptgs1* and *Ptgs2*, while related, are distinct as their

genes reside on separate chromosomes. Furthermore, the *Ptgs1* isoform is primarily responsible for constitutive expression of prostaglandins while *Ptgs2* plays a major role in increasing prostaglandin production in inflammatory states (Szczeklik and Sanak 2002). Recent evidence suggests the presence of a third *Ptgs* enzyme, *Ptgs3*, which is a splice variant of *Ptgs1*, retaining intron 1 in its mRNA (Chandrasekharan et al. 2002). By nature of its constitutive expression, *Ptgs1* is responsible for the side effects of nonsteroidal anti-inflammatory medication intolerance, including aspirin-induced asthma. Additionally, *Ptgs1* is likely to have importance in allergic asthma as evidence from murine models indicates that *Ptgs1* products have mainly protective effects in allergic mouse lungs (Gavett et al. 1999; Peebles et al. 2002).

The murine *Ptgs1* gene contains 11 exons with a coding sequence approximately 2.7 kb in length and a protein product of 602 amino acids. It is located on murine chromosome 2, 29 cM from the centromere and on human chromosome 9q32-q33 (MGD 2005). Thus, its location and role in prostaglandin synthesis make it a positional candidate gene for *Abhr2*.

The mannose receptor, C-type 1 gene, *Mrc1*, is located 5 cM from the centromere on murine chromosome 2, while its human counterpart maps to chromosome 10p12.33. Murine *Mrc1* is approximately 10.2 kb in length with 31 coding regions. This gene belongs to a subgroup of the C-type lectin superfamily, which is a large group of transmembrane receptors and soluble proteins (East and Isacke 2002). These lectins play a role in the innate and adaptive immune systems. In addition to having clear importance in host defense against pathogens, some C-type lectin gene family members have also been implicated in allergic airway inflammation (Raes et al. 2005). While little is known about the relationship specific

Table II. *Ptgs1* sequencing primers.

Forward primer (5'-3')		Reverse primer (5'-3')		Product size (bp)
Location	Sequence	Location	Sequence	
5' UTR	TGCCCTCTACAAGGTCTACAT	5' UTR	GTGGGTCAAGTGATTGTCA	843
5' UTR	TTCCTCCCAGATCTCATTATT	5' UTR	GCAGTGAGTGGGATGTAAAGT	810
5' UTR	CAGTAAACAAAATTGCCTTAT	5' UTR	TCCTCCCTCCAGTTGTC	697
5' UTR	CAGAGGAAGTTGGCGGTCAGC	Intron 2	GCGAGCAGAAAGGGGAATCAGGGA	421
Intron 2	GTGGGGTTTCCGCTCTAT	Intron 3	GGAAGTACCCTTAGGAGTGATA	402
Intron 3	TATCACTCCTAAGGGTACTTCC	Intron 5	CTTTCCTTGATCCTAGACA	655
Intron 5	TCCTGCTTGGGCTAGTTTG	Intron 7	TAACCTCCTCCAGCCTTTACTC	566
Intron 7	GCTTAGGGCAGGCACTTAGGGA	Intron 8	GCAAGTCAGCGCGTGTAGGAA	506
Intron 8	TCTGTTTTACATCCCCTTACA	Intron 9	CCTGGCACTGTCACCTAAATTA	567
Intron 9	CATCACGATCACTGGGCTACAC	Intron 10	CACTGTCTTCCCTGACAATGCT	374
Intron 10	TGCAGGTTAAATTTTACGATGT	3' UTR	CAACCACACCCTACCCTATAA	771
3' UTR	TTATAGGGTAGGGTGTGGTTG	3' UTR	GCATCAAAGATAAGGCTTACAT	705
3' UTR	CAGATCTTTGCTTGTGGC	3' UTR	ATATCCGTATGTCTACTCAC	631
3' UTR	CAAATGCTGCTTGTGTACTA	3' UTR	GGGCTGTTACGTAGAGA	630

Annealing temperature (T_A) = 55°C. *Ptgs1* GenBank accession numbers are DQ173699-DQ173707 for A/J mice and DQ173708-DQ173716 for C3H/HeJ mice.

Table III. *Mrc1* sequencing primers.

Forward primer (5'-3')		Reverse primer (5'-3')		Product size (bp)
Location	Sequence	Location	Sequence	
5' UTR	TCCTGTGCCTTAGCTTACGAA	Intron 1	GGGCTTCATCTTGGACAACA	546
Intron 1	CTGCCCTCTCTATGTGAAC	Intron 2	GCTGTTTCTTGTAGGGACCAC	539
Intron 2	GAATGGATGGGTCAACT	Intron 3	AAAGGTAGATAGAGGGTACAT	382
Intron 3	AAGTTGGCTCATGATGAGTCT	Intron 4	GGGACAAACTTTTTTCGTGAC	453
Intron 4	AGCACCTTCTTTGACAACTCG	Intron 5	ATCCAGGTTGCAGATTCCA	358
Intron 5	CCCAAGGATGACGACGTAAG	Intron 6	GCCCTATGTTCCATCCCTATAT	418
Intron 6	GCCATAAAATGAAGAATGTT	Intron 7	ACCGACGTACGATCTACTTA	545
Intron 7	AGCCCAGGACCCTTTT	Intron 8	GGCCATACTGAGATACTCCA	420
Intron 8	AGAGCCAAATATACTGC	Intron 9	TCAAGAAGGGTAGAGTTATC	431
Intron 9	GACAATGTCATGCGAATTT	Intron 10	CCAGGCTGCATAATAAGACC	562
Intron 10	TTTAGCTTCCCTGGATCTATG	Intron 11	ACTTGAAATGTTTCGTTGC	609
Intron 11	CATTTTCATTCTTACATGCAG	Intron 12	TGGGTCTCCATTAAATGAGTC	485
Intron 12	GAGACCTGCATTGGTTAG	Intron 14	GCCAAAATAAATACACTACA	2122
Intron 14	CTAGCACAGCATAGCAACC	Intron 15	ATGTTTTTAAGGACGTGACT	433
Intron 15	GAGGATGGGGCAAGTGTAGA	Intron 16	CTAACAGGGCAACGGGTGT	315
Intron 16	ATCTTTGGCATAGTGCTTAT	Intron 18	TTGGCAAACAAAGGTCTC	1406
Intron 18	CCCCTTCATCTTGATGTAT	Intron 20	CTGTGTCCTCCGAAATAGAG	1171
Intron 20	ATTCTCAACGGAAGT	Intron 21	TCCCTTACTCTATCTTATTA	494*
Intron 21	TTGGGAATACTAACACGAGT	Intron 22	TCACTGCCAACGAAGACT	483
Intron 22	TGATGTTGGGTGAGATAAGC	Intron 23	TCTATGGAGAGGGGTAGAC	345
Intron 23	TGCCAGTTTGGACTTATGA	Intron 24	TGGCACATTATCACTAGGTC	492
Intron 24	AAATACTTTCCAGGTTGTAA	Intron 25	TTCTATCTCCATCATTTTCG	593
Intron 25	GCTATAAAAATAAGATCCC	Intron 26	CACTGGCAGAGCTGTAGAGT	414
Intron 26	CATGTCCCTGGGCTAACA	Intron 28	ACCAATAGAGGGGAACGATA	1054
Intron 28	AGAACTCATCCGATTACAGC	Intron 29	GGATTGTGGCCTAATGATA	314
Intron 29	AAGAATATTCATGCATCAAA	3' UTR	CGCTAGTGCTGTGTATTTCAT	640
3' UTR	CTATCCTGGCAAGATTAGA	3' UTR	CAATATTCAGCCTGTCAAAC	444

*Annealing temperature (T_A) for this PCR was 52°C; T_A for all other PCRs was 55°C. *Mrc1* GenBank accession numbers are DQ358917–DQ358942 for A/J mice and DQ358943–DQ358968 for C3H/HeJ mice.

to *Mrc1* and airway inflammation or asthma, receptor-mediated endocytosis through mannose receptors (among others) is a mechanism used by dendritic cells for antigen uptake (Deslee et al. 2002). Furthermore, dendritic cells from patients with house dust mite allergy expressed more mannose receptor. These allergic patients were also more efficient in mannose receptor-mediated Der p 1 uptake by dendritic cells. As selective depletion of dendritic cells in allergen-sensitized mice leads to an ablation of the Th2 response (Lambrecht et al. 1998), a link between the mannose receptor and allergic asthma seems plausible. Based on function and location *Mrc1* is a viable positional candidate gene for *Abhr1*.

To determine if strain-specific genetic differences existed in *Ptgs1* and *Mrc1* positional candidate genes, DNA from the murine strains used for mapping, A/J and C3H/HeJ, was sequenced and compared. Primers were designed to sequence all exons, splice sites and both the 5' promoter and 3' untranslated regions (UTR) for these genes (Tables II and III). All sequences were determined on both strands. Over 8.5 kb of *Ptgs1* sequence was examined, while total sequence for *Mrc1* was 15.5 kb. No sequence polymorphisms were detected in the regions examined for either gene. These results indicated the absence of coding variants between A/J and C3H/HeJ strains;

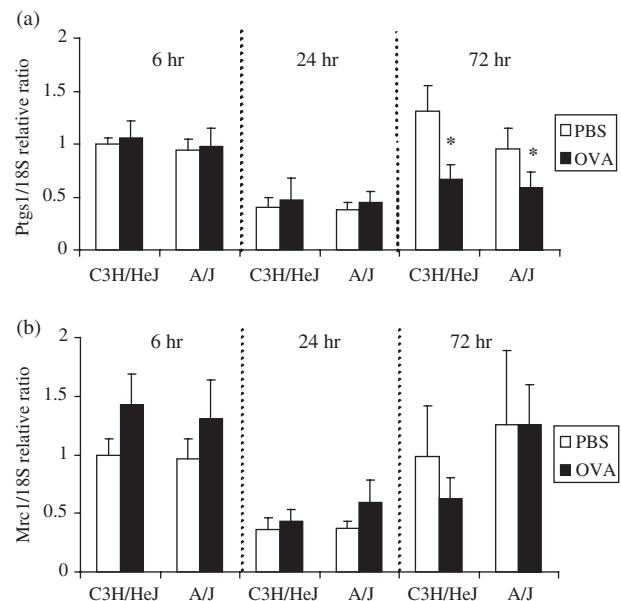


Figure 2. The expression of *Ptgs1* (a) and *Mrc1* (b) was measured by real-time PCR in lung tissue harvested from A/J and C3H/HeJ mice following OVA exposure to induce experimental asthma or PBS as a control. The oligos utilized to measure *Ptgs1* were forward primer 5'-CCAGAACCAGGGTGTCTGTGT-3'; reverse primer 5'-GTAGCCCGTGCGAGTACAATC-3'; and probe 5'-CGCTTTGGCCTCGACAACCTACCAGTG-3'. A gene expression Assay-on-Demand (Applied Biosystems, Branchburg, NJ) was used to quantify *Mrc1*. *Indicates significant difference due to treatment, $p \leq 0.05$.

Table IV. Positional candidate genes prioritized for future analysis in the *Abhr* region.

Gene symbol	Gene name	Chromosome 2 map position*	Polymorphisms†	Function
<i>Prkcg</i>	Protein kinase C, theta	11088123–11217236	rs27084582 (mRNA-UTR), rs27084468 (intron)	Intracellular signaling cascade, TCR-mediated T cell activation, activation of NF-κB
<i>Il1f8</i>	IL-1 family member 8	24085324–24092682	rs27219201 (locus-region), rs27219199 (locus-region), rs27219198 (mRNA-UTR), rs27219196 (mRNA-UTR), rs27219193 (intron), rs27219191 (intron), rs27219190 (intron), rs27219189 (intron), rs27219188 (intron), rs27219185 (intron), rs27219181 (intron), rs27219179 (intron), rs27219176 (w/in 2 kb downstream), rs27219174 (w/in 2 kb downstream), rs27219172 (w/in 2 kb downstream)	Immune response, cytokine activity
<i>Il1f9</i>	IL-1 family member 9	24118645–24125730	rs27219168 (locus-region), rs27204767 (intron), rs27204766 (intron), rs27204765 (intron), rs27204764 (intron), rs27204762 (intron), rs27204758 (locus-region)	Immune response, cytokine activity, interleukin-1 receptor binding

* Physical map position in basepairs as reported in the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/>); † Reported sequence differences between A/J and C3H/HeJ strains in the Mouse Genome Informatics database (<http://www.informatics.jax.org>).

however, they did not exclude the possibility of sequence variation in more distant regulatory elements of *Ptgs1* and *Mrc1* that may result in strain-specific expression differences of these genes. Therefore, we also tested for strain-specific quantitative differences in the mRNA expression of these positional candidate genes in A/J and C3H/HeJ mice following experimental allergic asthma induction.

Mice ($n = 6/\text{group}$) were sensitized (day 0) and challenged (day 14) with ovalbumin (OVA) or phosphate buffer saline (PBS) as previously described (Ewart et al. 2000). Tissues were harvested at multiple time points after OVA challenge, RNA was extracted (Trizol) from lungs and reverse transcribed (Applied Biosystems, Branchburg, NJ). *Ptgs1* and *Mrc1* expression levels were measured by real-time PCR (Figure 2) (ABI 7700 Sequence Detection System, Applied Biosystems, Branchburg, NJ) with 18S rRNA as the endogenous reference using the standard curve method. The data were analyzed by two-way ANOVA with Student-Neuman–Keuls tests for *post-hoc* pairwise comparisons. No strain-specific expression differences were identified in either of these two candidate genes; however, we found that the expression of *Ptgs1* was significantly lower in both strains due to OVA treatment at the 72 h time point.

In summary, we have refined the map locations of two important loci, *Abhr1* and *Abhr2*, underlying a cornerstone of the asthma phenotype in a murine model, with the result being the improved ability to reduce the positional candidate genes in the linkage interval. The *Ptgs1* and *Mrc1* genes were subsequently prioritized. The results of sequence analysis indicated a lack of strain-specific coding difference in these genes. Likewise, strain-specific expression differences

were not identified in these genes. Additional candidate genes in the refined *Abhr* intervals can be identified based on both function and known polymorphisms in the relevant strains. There is high conservation between A/J and C3H/HeJ mice in the region spanning the *Abhr* loci on chromosome 2, with much of this region contained in haplotype blocks that are shared by these strains as reported in the Roche Mouse SNP Hapmap (Liao et al. 2004). This significantly reduces the number of additional genes prioritized going forward as candidates for our phenotype (Table IV). We have prioritized the subset of genes in the *Abhr* region with potential for functional relevance to immune mechanisms underlying asthma in which polymorphisms have been reported between A/J and C3H/HeJ strains. Therefore, future studies may reasonably focus on analyses of these genes.

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