

Quantitative trait loci for steady-state platelet count in mice

Carol C. Cheung,^{1,2} Ian C.A. Martin,³ Kyall R. Zenger,⁴ Jenny A. Donald,⁴
 Peter C. Thomson,³ Christopher Moran,³ Michael F. Buckley^{1,2}

¹Centre for Vascular Research, University of New South Wales, Kensington 2052, Sydney, Australia

²Molecular & Cytogenetics Unit, Department of Haematology, South Eastern Area Laboratory Services, Prince of Wales Hospital, High Street, Randwick 2031, Sydney, Australia

³Centre for Advanced Technologies in Animal Genetics and Reproduction, University of Sydney, 2006, Sydney, Australia

⁴Department of Biological Sciences, Macquarie University, North Ryde 2109, Sydney, Australia

Received: 18 May 2004 / Accepted: 8 June 2004

Abstract

Platelet count in humans is a strongly genetically regulated trait, with approximately 85% of the interindividual variance in platelet numbers attributable to genetic factors. Inbred mouse strains also have strain-specific platelet count ranges. As part of a project to identify novel factors that regulate platelet count, we identified two inbred mouse strains, CBA/CaH and QSi5, with substantial differences in platelet count (mean values of 581 vs. $1062 \times 10^9/L$). An F₂ intercross resource of 1126 animals was bred from these two parental strains for a genomewide scan for quantitative trait loci (QTL) for platelet count. QTL were identified on MMU1 (LOD 6.8, $p < 0.0005$) and MMU11 (LOD 11.2, $p < 0.0005$) by selectively genotyping animals from the extremes of the F₂ platelet count distribution. Three other QTL of suggestive statistical significance were also detected on MMU7, 13, and 17. It is noteworthy that no QTL were detected in the vicinity of the genes encoding thrombopoietin (*Thpo*), and its receptor (*c-Mpl*), both known to influence platelet production. Comparison of gene expression levels between the parental mouse strains by microarrays also showed little difference in the mRNA levels of these known candidate genes. These results represent the first published use of a genetic linkage-based approach in a mouse model toward the identification of genetic factors that regulate platelet count.

Platelets are anucleate cellular particles that play a crucial role in the initiation of blood clotting. A severe lack of platelets (thrombocytopenia) is potentially life-threatening as the rate of spontaneous internal hemorrhages is inversely proportional to the platelet count. The established medical management for severe thrombocytopenia is the transfusion of platelet concentrates. Each transfusion episode involves the administration of platelets from between 4 and 10 blood donors, with attendant risks of febrile reactions, transmission of viral agents, alloimmunization, as well as the difficulties and costs associated with product manufacture, stability, distribution, and maintenance of supply. Given the potential clinical risks of thrombocytopenia and those associated with platelet transfusions, there is a clear requirement for the discovery of one or more therapeutic agents that can increase platelet numbers without the hazards and infrastructure problems associated with multiple platelet transfusions.

Thrombopoietin (THPO) is a hematopoietic growth factor whose main role is to promote platelet production. Clinical benefit of administering THPO remains unproven in those patients most likely to require platelet transfusion support, namely those receiving dose-intensive chemotherapy for acute leukemia or stem cell transplantation (Kuter and Begley 2002). Other cytokines, such as GM-CSF, IL-1, IL-3, IL-6, IL-11, leukemia inhibitory factor, and oncostatin M, are known to have appreciable but non-specific effects on promoting platelet production. However, since these factors have a broad spectrum of physiologic activities, among which thrombopoiesis is but one, their side effect profiles have precluded their widespread application as pharmacological enhancers of platelet production (Burstein 1997). While the thrombopoietin (THPO)/thrombopoietin receptor (c-MPL) system is one of the best

Correspondence to: Michael F. Buckley, E-mail: buckleymf@sesahs.nsw.gov.au

characterized physiological regulators of platelet production, there is now considerable evidence from gene-targeting studies that it is neither the sole physiological regulator of platelet production (Burstein 1997; Gainsford et al. 1998; Gainsford et al. 2000), nor of steady-state platelet count, which is a reflection of the homeostatic balance between platelet production, sequestration, and destruction.

One approach to the identification of factors that regulate platelet count is gene mapping, now widely applied to identify genetic factors responsible for variation in continuously distributed traits (quantitative traits) (Lander and Botstein 1989; Moore and Nagle 2000). There are several lines of evidence suggesting that genetic factors play a large part in platelet count variation. Monozygotic twins have a higher concordance rate for platelet count than do dizygotic twins (Whitfield and Martin 1985; Dal Colleto et al. 1993; Yokoyama and Akiyama 1995; Evans et al. 1999). Longitudinal studies on volunteer blood donors also indicate that transient environmental effects on steady-state platelet count accounts for less than 15% of the variance (Buckley et al. 2000). Differences in platelet counts have been described in populations of different ethnic origin who share the same environment (Bain and Seed 1986; Siebers et al. 1989; Gader et al. 1995; Bain 1996). Taken together these studies indicate the magnitude of the genetic control of variation in platelet count (the heritability) to be up to 87%. The genetic basis of variation in a quantitative trait is generally considered to be multifactorial due to the effects of multiple genes together with environmental factors. In the case of steady-state platelet count, its near-normal distribution in both human and mouse populations supports the notion that variation in steady-state platelet count is likely to reflect the effects of many genes (Falconer and Mackay 1996; Glazier et al. 2002).

The molecular basis of complex hematological traits such as platelet count is amenable to analysis by genomewide linkage studies if an appropriately powerful model system is used (Glazier et al. 2002). This approach has had several recent successes, including the identification of genomic regions that regulate lymphocyte numbers and hematopoietic cell proliferation (Chen and Harrison 2002; de Haan et al. 2002). The observation that inbred strains of laboratory mice have characteristic platelet counts has been well-established [The Jackson Laboratory Mouse Phenome Database (<http://aretha.jax.org/pub/cgi/phenome/mpdcgi?rtm=vdocs/home>); Ault et al. 1997]. So far there has been no reported QTL for platelet count (or indeed any of the standard hematological cellular parameters) in the literature. We

undertook a strain survey of seven inbred lines that were readily available in Sydney. In this survey, we identified a locally produced inbred mouse strain, QSi5 (derived from an outbred Quackenbush–Swiss line), which had a platelet count that was substantially greater than the other strains tested. This strain had the additional advantage of very high fecundity, enabling rapid breeding of mapping resource pedigrees (Holt et al. 2004). We have undertaken genetic mapping studies on the F₂ progeny of an QSi5 × CBA cross and have identified loci on mouse Chromosomes 1 and 11 that we propose contain genes which contribute to the regulation of platelet count. Comparison of mRNA levels between the parental mouse strains was performed with selected organ tissues (spleen, bone marrow, liver, kidney) using microarray techniques.

Materials and methods

This study was performed under Animal Care and Research Ethics approval N02/2-2001/2/3337 from the University of Sydney.

Mouse maintenance and breeding. Parental inbred strains were obtained from the Centre for Advanced Technologies in Animal Genetics and Reproduction, University of Sydney (QSi5), and the Animal Resource Centre, Perth, Western Australia (all other inbred strains). Mice were kept in a rodent facility at the University of Sydney in a purpose-built air-conditioned room with a 12-h light/dark cycle and *ad libitum* access to food and water until sampling at 8 weeks of age.

Sample collection for QTL mapping. Blood samples were collected from equal numbers of male and nonpregnant female mice. Sampling of the F₂ resource occurred on a total of 34 sample days over a period of 10 months. Mice were killed by exposure to 100% CO₂. Between 0.5 and 1 ml of blood was collected by ventricular stab using 1-ml syringes pre-anticoagulated with 0.1 ml ETP (2.8 μM prostaglandin E₁, 12 mM theophylline, 108 mM Na₂EDTA, pH 6.2) and stored on ice in 1.5-ml microtubes until further processing. Spleens were dissected and snap-frozen in liquid nitrogen for future DNA extraction. Blood samples were diluted threefold with serum-free Ham's F10 medium (Invitrogen) to bring the platelet count into the linear range of the Sysmex XE9000 automated hematology analyzer (Roche Diagnostics), and a standard set of cellular hematological data recorded.

Sample collection for microarray studies. Six adult male animals of each parental strain (CBA and QSi5) were killed by CO₂ asphyxiation. Only male animals were used in an attempt to limit the confounding effect of estrus cycles on the interpretation of results. Liver, spleen, and kidneys were dissected and snap-frozen in liquid nitrogen. Bilateral femurs were removed and flushed with 0.5 ml phosphate-buffered saline repeatedly to dislodge and suspend marrow cells, and the eluted cells were kept on ice until the RNA extraction process.

Phenotype data normalization. Analysis of the data using a general linear model (GLM) identified significant effects of dates of collection ($p < 0.001$), sex ($p < 0.001$), and coat color ($p < 0.001$) on the distribution of platelet count data in the F₂ resource. The residuals, after eliminating the effects of sex and dates of collection, were used in all subsequent analyses. Since coat color is genetically determined, its effect was not adjusted for. A strategy of selective genotyping was employed. Since animals in the phenotypic extremes contain most of the genetic information, those samples within the top and bottom 8% of the residual platelet count distribution were chosen for genotyping. Statistical analyses were conducted using the software MinitabTM.

Microarray study design. A direct comparison of CBA spleen with QSi5 spleen mRNA, CBA marrow with QSi5 marrow mRNA, and so on was used. For each organ tissue, six animal samples were available from each parental mouse strain. The total RNA of these samples was grouped into two pools: for instance, a pool of spleen RNA from 3 CBA animals would, after reverse transcription, be labeled with Cy3 and cohybridized with a pool of spleen RNA from 3 QSi5 animals, labeled with Cy5. The two pools then serve as biological replicates for each organ tissue. Pooling of RNA samples not only masks environmentally induced variability in gene expression among inbred animals, but it also ensures that adequate amounts of RNA would be available for technical replication and dye swap experiments.

Molecular genetic methods. Candidate microsatellites were selected from the Whitehead Institute database and from local resources, and their informativeness confirmed by screening with DNA from the parental mouse strains. One hundred one informative loci were selected to span the mouse genome, yielding an average intermarker distance of ~15 cM. Those genes whose protein products

have known effects on platelet production (e.g., *Thpo*, *c-Mpl*, *IL11*, *IL6*, *IL3*) were also specifically targeted by the selection of microsatellite markers within close proximity to these loci. Fluorescently labeled PCR primers were purchased from Applied Biosystems.

Genomic DNA was extracted from the spleens of the selected animals using a modified salt precipitation protocol (Miller et al. 1988; Zenger 2001). PCR amplifications were performed using FTS-960 and PC-960C Thermal Cyclers (Corbett Research) and AmpliTaq Gold *Taq* polymerase (Applied Biosystems). Cycling conditions were 94°C for 30 sec, 63°C for 45 sec, and 72°C for 60 sec, dropping the annealing temperature by 1°C for each of the next 5 cycles, followed by 30 cycles at the annealing temperature of 58°C. A 5-min elongation at 72°C was added at the end of the PCR. Each sample was amplified in 10- μ l volume. PCR product detection was performed using an ABI 3700 Genetic Analyzer at the Molecular Genetics Laboratory of the Institute of Medical and Veterinary Sciences, Adelaide, South Australia. Results were visualized with GeneScan[®] software, and genotypes were manually interpreted based on product size.

RNA extractions from liver, spleen, kidney, and bone marrow were all performed using TRIZOLTM (Invitrogen) in accordance with the manufacturer's instructions. An indirect-labeling method for oligonucleotide microarrays was adopted from the Ontario Cancer Institute website (<http://www.oci.utoronto.ca/services/microarray/protocols/>). Superscript II and Superscript IIITM (Invitrogen) were used for reverse transcription at the respective recommended temperatures. Starting with 30 μ g of total RNA per sample pool per slide, the resultant mRNA was coupled to either Cy3 or Cy5 dye labels according to study design. Oligonucleotide spotted array slides manufactured using the Compugen Mouse 22K library (containing representative oligonucleotide sequences from over 21,600 mouse genes) were purchased from The Clive and Vera Ramaciotti Centre for Gene Function Analysis, University of New South Wales. Hybridized slides were scanned with arrayWoRx^{eTM} Biochip Reader (Applied Precision, LLC) at 10- μ m resolution at wavelengths of 595 and 685 nm.

Analytical software. Linkage analyses were performed using the Mapmaker/QTL software package (Lander and Botstein 1989; Lincoln et al. 2003). For a pointwise significance threshold, the conventional LOD > 3.0 is widely accepted as evidence for linkage. In view of this being a genome-wide linkage analysis with 20 chromosome pairs or

independent linkage groups, we applied a conservative Bonferroni correction raising the threshold by a factor of 20. A logarithm of odds (LOD) score of >4.3 was therefore set as the threshold for significance. All linkage analyses were performed in parallel with MapManager QTXb13 software (Manly et al. 2001; Meer et al. 2004), and the results were concordant.

Microarray images were displayed and interpreted with GenePix Pro 3.0 (Axon). Annotations and statistical analyses were performed using GeneSpring 6 (Silicon Genetics). The resultant gene lists were then checked against the raw data for aberrant values.

Results and discussion

Strains survey. Seven inbred mouse strains were phenotyped for their platelet counts: A/J, C3H, C57Bl/6, CBA/CaH, DBA2, QSi5, and SJL. All mice used for the survey were nonpregnant females between 8 and 9 weeks of age. Each inbred strain was demonstrated to have a characteristic mean platelet count (Fig. 1). QSi5 and CBA showed the largest difference in their platelet counts. QSi5 was found to have a mean platelet count of $1062 \times 10^9/L$ (SD = $8.2 \times 10^9/L$, $n = 16$) versus that of $581 \times 10^9/L$ (SD = $79 \times 10^9/L$, $n = 23$) in CBA mice. The pooled standard deviation for platelet counts was $80 \times 10^9/L$, giving a separation of 6 phenotypic standard deviations between the means of QSi5 and CBA, a substantial and highly significant difference. Platelet volume and mean red cell volume also showed highly significant interstrain differences, but the magnitudes were less than those observed for platelet count (data not shown).

Selective genotyping of F_2 progeny. The mean platelet count in 1126 F_2 progeny was $985 \times 10^9/L$, with a standard deviation of $135 \times 10^9/L$. Categorizing by sex yielded a mean platelet count of $1033 \times 10^9/L$ ($n = 561$, SD = 130) for male mice and $938 \times 10^9/L$ ($n = 565$, SD = 124) for females. After adjusting for dates of collection and sex, the residual values were found to be distributed normally (Anderson Darling statistic = 0.33, $p = 0.52$). The 186 animals displaying the highest and lowest platelet residual values were genotyped (Fig. 2). A list of all the microsatellite markers used in this study is presented in Table 1. The Whitehead Institute genetic map (<http://www.broad.mit.edu/cgi-bin/mouse/index#genetic>) provides the genetic distances for these markers, and corresponding physical locations of these markers have been obtained through the mouse genome resource provided by the NCBI

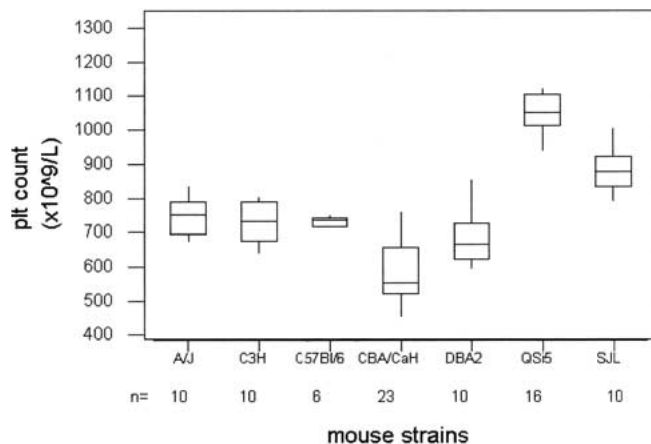


Fig. 1. Distribution of platelet counts in strain survey. Box and whisker plots of platelet counts in seven inbred mouse strains. For each strain, the median and interquartile-range box is shown, with the whiskers covering the observed range of platelet count values. n is the number of observations for each strain. There is a clear difference in the platelet counts between CBA/CaH and QSi5.

(http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=10090).

Linkage results. Two chromosomal regions showed highly significant effects on platelet count (Table 2, Fig. 3). The first is located on MMU1, at the estimated position of 94 cM with a peak LOD score of 6.8. It accounts for 5.6% of the total genetic variance of platelet count in the F_2 progeny of this mouse cross. Compared to the CBA allele, the presence of each QSi5 allele at this QTL is expected

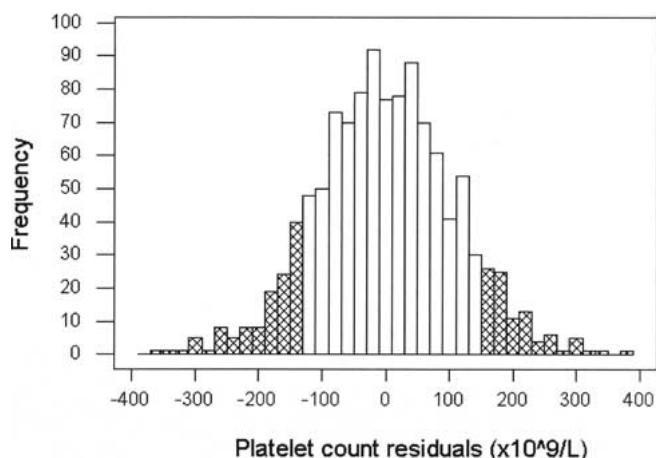


Fig. 2. Distribution of platelet count residuals in F_2 progeny. Frequency distribution of platelet count residual values in the F_2 animals. These are residuals following correction for the effects of dates of collection and sex on platelet count. A normal probability plot (not shown) for these residuals show they are normally distributed. Shaded areas indicate the platelet residual values of animals selected for genotyping.

Table 1. List of informative microsatellite markers

<i>Marker</i>	<i>Locus</i>	<i>Physical (Mbp)</i>	<i>Genetic (cM)</i>	<i>Marker</i>	<i>Locus</i>	<i>Physical (Mbp)</i>	<i>Genetic (cM)</i>
1	D1Mit231	21.1	8.7	53	D10Mit14	118.2	69.9
2	D1Mit322	42.3	23	54	D10Mit103	125.4	76.5
3	D1Mit436	77.5	43.7		total length	130.7	76.5
4	D1Mit396	153.6	80.9	55	D11Mit71	6.8	0.0
5	D1Mit540	169.4	94	56	D11Mit231	35.9	14.2
	total length	195.8	115.8	57	D11Nds9	54.3	25.1
6	D2Mit297	43.3	28.4	58	D11Mit325	87.8	44.8
7	D2Mit380	70.5	40.4	59	D11Mit198	100.3	62.3
8	D2Mit106	134.5	61.2	60	D11Mit167	114.9	75.4
9	D2Mit410	161.9	78.7		total length	122.8	83.7
10	D2Mit517	176.9	97.2	61	D12Mit37	5.2	1.1
	total length	181.4	98.4	62	D12Mit156	74.1	28.4
11	D3Mit130	10.2	4.4	63	D12Mit121	90.7	41.5
12	D3Mit208	55.6	20.8	64	D12Mit8	107.4	56.8
13	D3Mit233	97.4	35		total length	114.4	60.1
14	D3Mit146	137.4	48.1	65	D13MIT55	9.4	0.0
15	D3Mit163	157.3	66.7	66	D13Mit88	38.2	13.1
	total length	160.6	66.7	67	D13MIT24	68.7	25.1
16	D4Mit264	8.8	3.3	68	D13Mit320	92.1	49.3
17	D4Mit345	21.4	10.9	69	D13Mit78	115.5	59.0
18	D4Mit178	64.5	30.6		total length	116.2	59.0
19	D4Mit219	100.9	48.1	70	D14Mit251	16	5.5
20	D4Mit203	127.6	60.1	71	D14Mit56	35.6	20.8
21	D4Mit357	149.7	77.6	72	D14Mit214	44	27.3
	total length	152.9	82	73	D14Mit160	62.4	47.0
22	D5Mit386	26	9.8	74	D14Mit265	92.3	59.0
23	D5Mit278	110.3	45.9	75	D14Mit136	112.1	67.8
24	D5Mit60	133.3	66.7		total length	115.8	69.9
25	D5Mit122	148	80.9	76	D15Mit174	3.2	0.0
	total length	149.7	82	77	D15Mit82	29.2	10.9
26	D6Mit138	12.5	2.2	78	D15Mit184	55.7	24.0
27	D6Mit93	51	12	79	D15Mit198	83.7	40.4
28	D6Mit323	87.4	27.3	80	D15Mit193	98.3	57.9
29	D6Mit105	108.4	41.5	81	D15Mit35	103.2	63.4
30	D6Mit59	139.2	56.8		total length	104.1	65.6
31	D6Mit15	146.7	66.7	82	D16Mit88	12.7	9.8
	total length	149.9	66.7	83	D16Mit146	23.4	17.5
32	D7Mit169	14.2	4.4	84	D16Mit139	66	33.9
33	D7Mit69	37.6	20.8	85	D16Mit219	96.8	51.4
34	D7Mit31	75.9	31.7		total length	98.9	57.4
35	D7Mit330	107.9	43.7	86	D17Mit113	11.1	2.2
36	D7Mit105	117.2	49.2	87	D17Mit176	40.9	12
37	D7Mit259	133.8	67.8	88	D17Mit20	57.4	29.5
	total length	134.4	67.8	89	D17Mit93	72.3	39.3
38	D8Mit64	31.6	15.3		total length	93.5	50.3
39	D8Mit205	49.9	31.7	90	D18Mit67	12.1	2.2
40	D8Mit45	86.6	40.4	91	D18Mit60	32.7	9.8
41	D8Mit120	117.9	63.4	92	D18Mit238	57.3	20.8
42	D8Mit56	128.6	75.4	93	D18Mit144	85.9	38.3
	total length	128.9	75.4		total length	91	39.3
43	D9Mit285	40.9	16.4	94	D19Mit73	23.3	17.5
44	D9Mit6	57.5	29.5	95	D19Mit53	44.8	32.8
45	D9Mit196	86.2	42.6	96	D19Mit105	56.9	47
46	D9Mit212	110.9	60.1	97	D19Mit6	61	57.9
47	D9Mit151	121.7	69.9		total length	67	57.9
	total length	124.4	69.9	98	DXMit55	3.5	1.1
48	D10Mit213	20.1	6.6	99	DXMIT84	91.9	31.7
49	D10Mit87	25.5	14.2	100	DXMit36	129.7	50.3
50	D10Mit213	67.2	27.3	101	DXMit239	131.5	62
51	D10Mit274	67.6	38.7		total length	149.9	70.7
52	D10Mit162	106.5	57.9				

Table 2. Significant linkage results

Significant QTL			
MMU chromosome	1	11	
Position (cM)	94	45	
Estimated physical location (Mb)	169	88	
Confidence interval (Mb)	160–195.8	74–120	
% variance ^a	5.6	9.2	
Δ platelet count ($\times 10^9/L$) ^b	-36	+48	
LOD score	6.8	11.2	
Suggestive loci			
MMU chromosome	7	13	17
Position (cM)	24	53	20
Estimated physical location (Mb)	51	101	48
Confidence interval (Mb)	15–80	68–116	19–61
% variance ^a	4.1	4.3	3.3
Δ platelet count ($\times 10^9/L$) ^b	+30	+29	+28
LOD score	3.6	3.4	3.5
Noteworthy negative findings			
	<i>Thpo</i>	<i>c-mpl</i>	<i>IL-11</i>
MMU chromosome	16	4	7
Position (cM)	13	56	2
LOD score	0.2	1	1.8

^aPercentage of the F₂ phenotypic variance explained by segregation of the QTL assuming additivity.

^bEstimated impact on the platelet phenotype of an F₂ animal when a QSi5 allele is present instead of the CBA allele at this locus. A positive sign indicates an increase in platelet count compared with the mean platelet count of CBA homozygotes and vice versa. The QTL on MMU1 shows transgressive segregation.

to decrease the phenotypic platelet count by $36 \times 10^9/L$. In single-QTL analyses, another location on MMU1 showed a statistically significant association with platelet count variation, at an estimated position of 63 cM with a LOD score of 4.9. However, analyses with multilocus models within both Map-Maker/QTL and Map Manager did not support the existence of a second QTL on this chromosome.

The second QTL identified is located on MMU11, with a peak LOD score of 11.2 at 45 cM. It accounts for 9.2% of the genetic variance in platelet count, and substitution of a CBA allele with that of QSi5 at this site is expected to increase the phenotypic platelet count by $48 \times 10^9/L$. The microsatellite markers spanned over 90% of MMU11 in this study, and, for almost the entire spanned distance, the LOD test statistic for a single-QTL model analysis exceeded the significance threshold of 4.3. This suggests the presence of multiple QTL on MMU11 that are currently unresolved. Analysis using multiple QTL models yielded a possible second locus at about 8 cM but did not reach statistical significance (LOD = 2.0).

Three other suggestive loci were identified, using LOD > 3.3 as the definition of "suggestive" significance (again applying the same Bonferroni correction to the conventional threshold for suggestive loci at LOD > 2.0). These were located on MMU7 (estimated position 24 cM, LOD = 3.6, 4.1% of variance), MMU13 (estimated position 53 cM,

LOD = 3.4, 4.3% of variance), and MMU17 (estimated position 20 cM, LOD = 3.5, 3.3% of variance). In contrast, at the location of the *Thpo* gene (MMU16, 13 cM), and that of its receptor *c-Mpl* (MMU4, 56 cM), the LOD statistics were unequivocally below the significance threshold. Linkage results for all remaining chromosomes are presented in graphical form in Fig. 4, 5, 6.

Differentially expressed genes. Genes that show significantly different tissue mRNA levels between the parental strains and colocalize to the QTL are listed in Table 3. There are a total of 47 genes on the list, five of which were differentially expressed in two organs. Nineteen of the 47 were classified as "biological process unknown" according to the information supplied with the clone library. Five of these are RIKEN cDNA sequences. Of those with assigned functions, many of them can conceivably play a role in regulating steady-state platelet count:

- Apoptosis (baculoviral IAP repeat and Bcl2-associated X protein);
- Cell cycle control (fibroblast growth factor 10 and cyclin B1);
- Cell growth and maintenance (astrotactin 1);
- Cytoskeleton organization (keratin complex 1 and tubulin-specific chaperone c);
- Protein complex assembly (RNA and export factor binding protein 1);

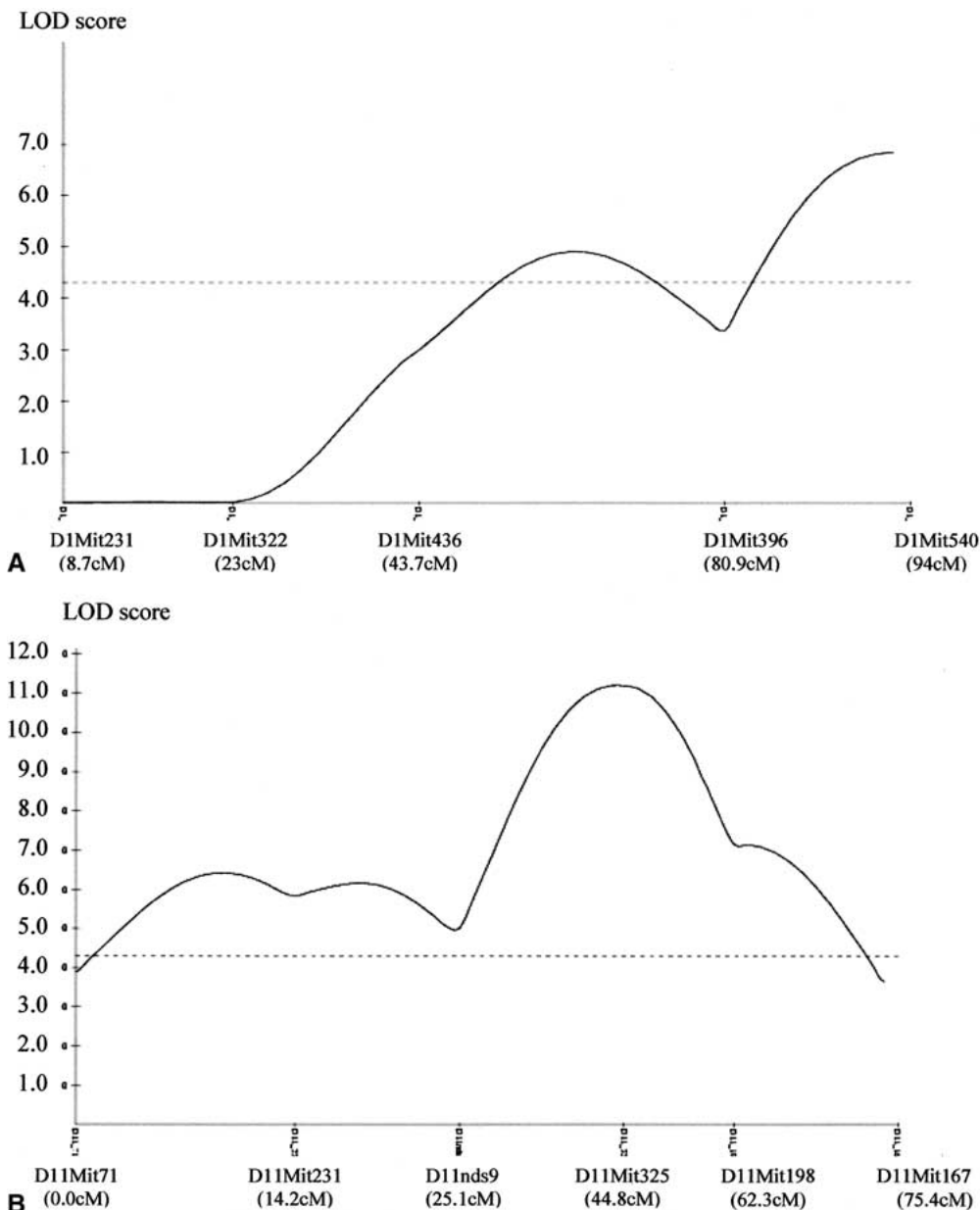


Fig. 3. QTL for platelet count in CBA/CaH \times QSi5 F_2 intercross mice, (a) LOD score distribution on MMU1. (b) LOD score distribution on MMU11. The x axis shows the microsatellite markers used, drawn to a cM scale. The y axis shows the LOD score, and the solid curve indicates variation of LOD at each position of the chromosome for evidence of platelet QTL. The horizontal dotted line indicates a LOD of 4.3, the significance threshold in our study.

- Transcription regulation (RIKEN cDNA 4921501 E09 gene) Erythroid-associated factor was classified as "biological process unknown," but its name clearly implies a role in hemopoiesis. Some of the genes, such as those encoding olfactory receptors or those involved in ergosterol synthesis, may represent genuine difference in gene expression but govern other phenotypic differences in the parental mouse strains. In support of our QTL findings, none of the genes whose products are currently known to govern platelet production was differentially expressed between the parental mouse strains.

Discussion

We have identified two novel QTL affecting steady-state platelet counts, with suggestive evidence for three others. Assuming they are independent, these five regions account for approximately 25% of the variance in platelet count in the F_2 progeny. It is an interesting observation that prime candidate loci for regulatory effects on platelet count such as *Thpo* (MMU16) and *c-Mpl* (MMU4) fall into the class of genetic loci with either very small or undetectable contributions to the difference in platelet count between these two strains. As confirmation there was no significant difference in the mRNA levels of

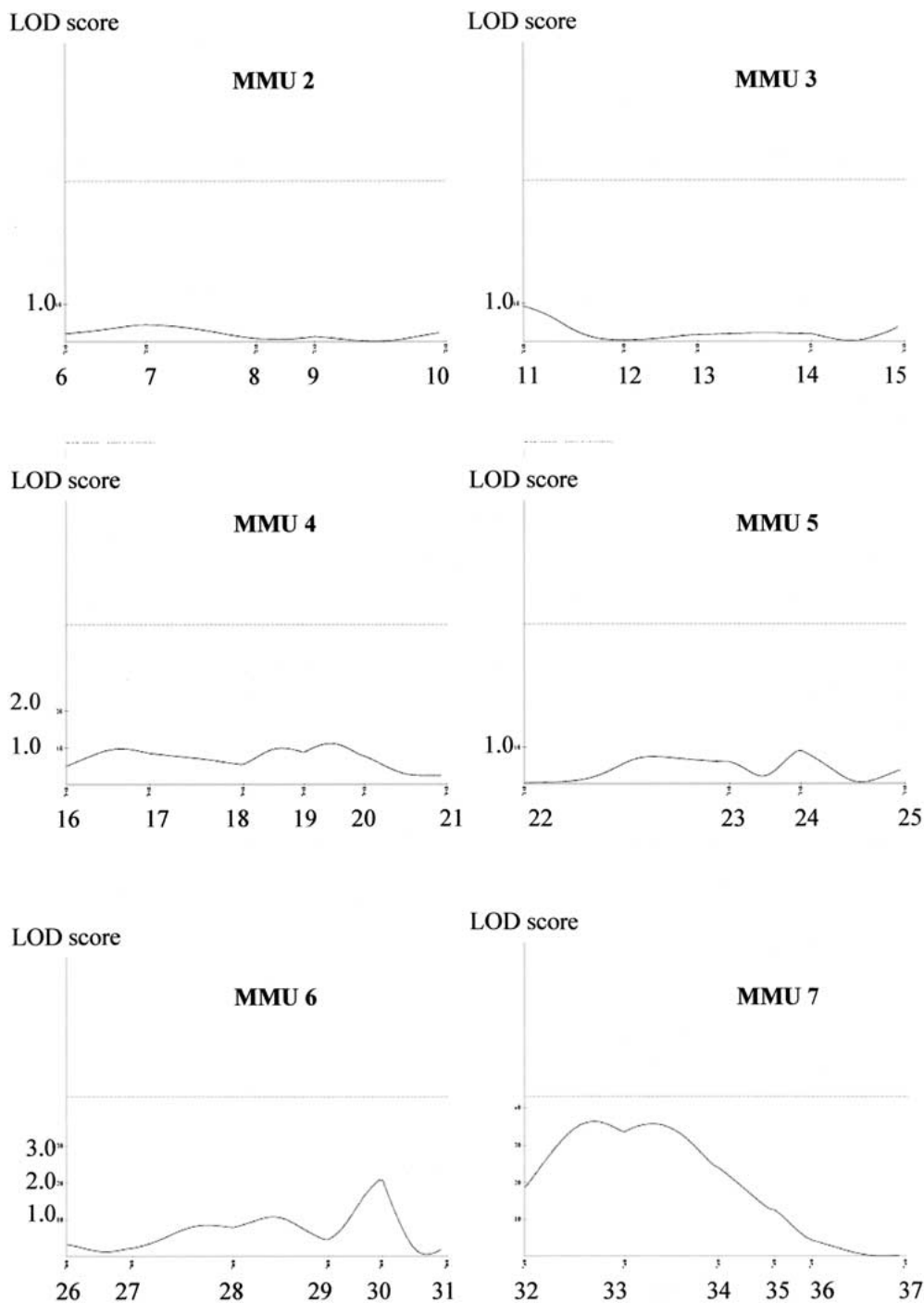


Fig. 4. Genome scan results for MMU2–7.

those genes in several organs. These results are consistent with the negative findings of an association study performed between a 3' untranslated region (UTR) polymorphism in *THPO* and platelet count in humans (Yankowitz et al. 1997). Similarly, genes for other known factors [such as *IL11* (MMU7), *Nf-e2* (MMU15), *Gata-1* (MMUX), or *Fog-1* (MMU8)] that are important in platelet production also map to chromosomal regions where no significant positive

signal was detected. A survey of publicly available mouse genome information has not identified any genes at the positions of our QTL that are currently known to have an effect on platelet count. The result is consistent with our hypothesis that there are numerous as yet undiscovered loci capable of regulating platelet count.

The highest LOD score obtained in this study was on mouse Chromosome 11 at 45 cM. There has

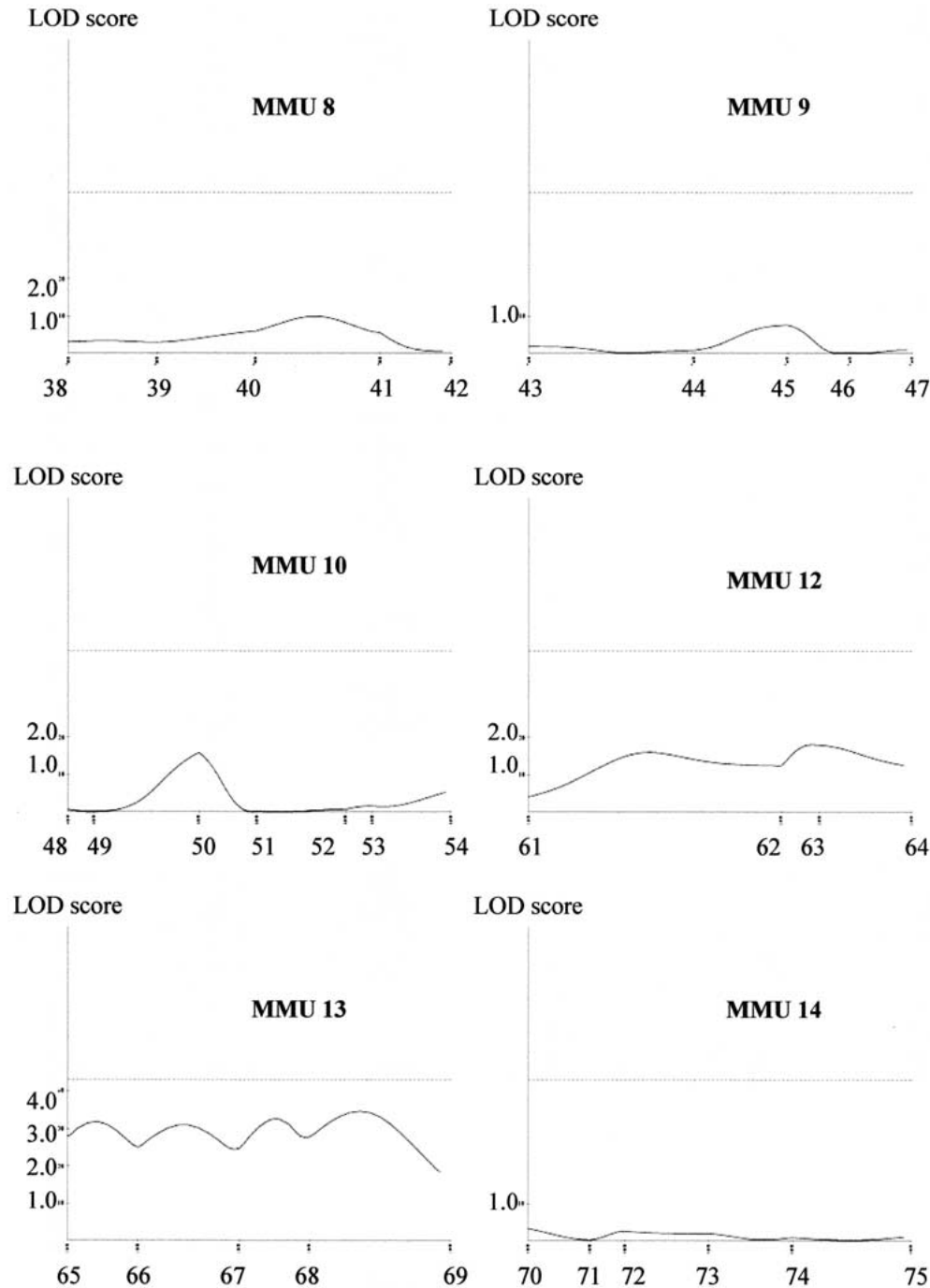


Fig. 5. Genome scan results for MMU8–10, 12–14.

been a report of a QTL for hematopoietic cell turnover at a similar location (47 cM on MMU11) using a cross between C57Bl/6 and DBA2 mice (de Haan et al. 2002). In the vicinity of that QTL were 29 genes that showed differential expression patterns in hematopoietic stem cells. The two strains used by de Haan et al. did not differ significantly in their platelet counts in the strain survey conducted for our study (mean \pm SD: DBA2 = $688 \pm 89 \times 10^9/L$; B6 = $732 \pm 10 \times 10^9/L$; $t = 1.53$, $p = 0.16$). Furthermore, comparisons

of QTL positions obtained using different inbred strains and genetic maps developed by different laboratories are potentially misleading. Nonetheless, one gene did overlap with our findings—eosinophil peroxidase (*epx*) showed a fourfold increased mRNA level in QSi5 bone marrow compared to CBA. This difference is unique to bone marrow; there was no difference in expression in the other organs tested. The relevance of this coincidence will have to be evaluated in the light of future studies.

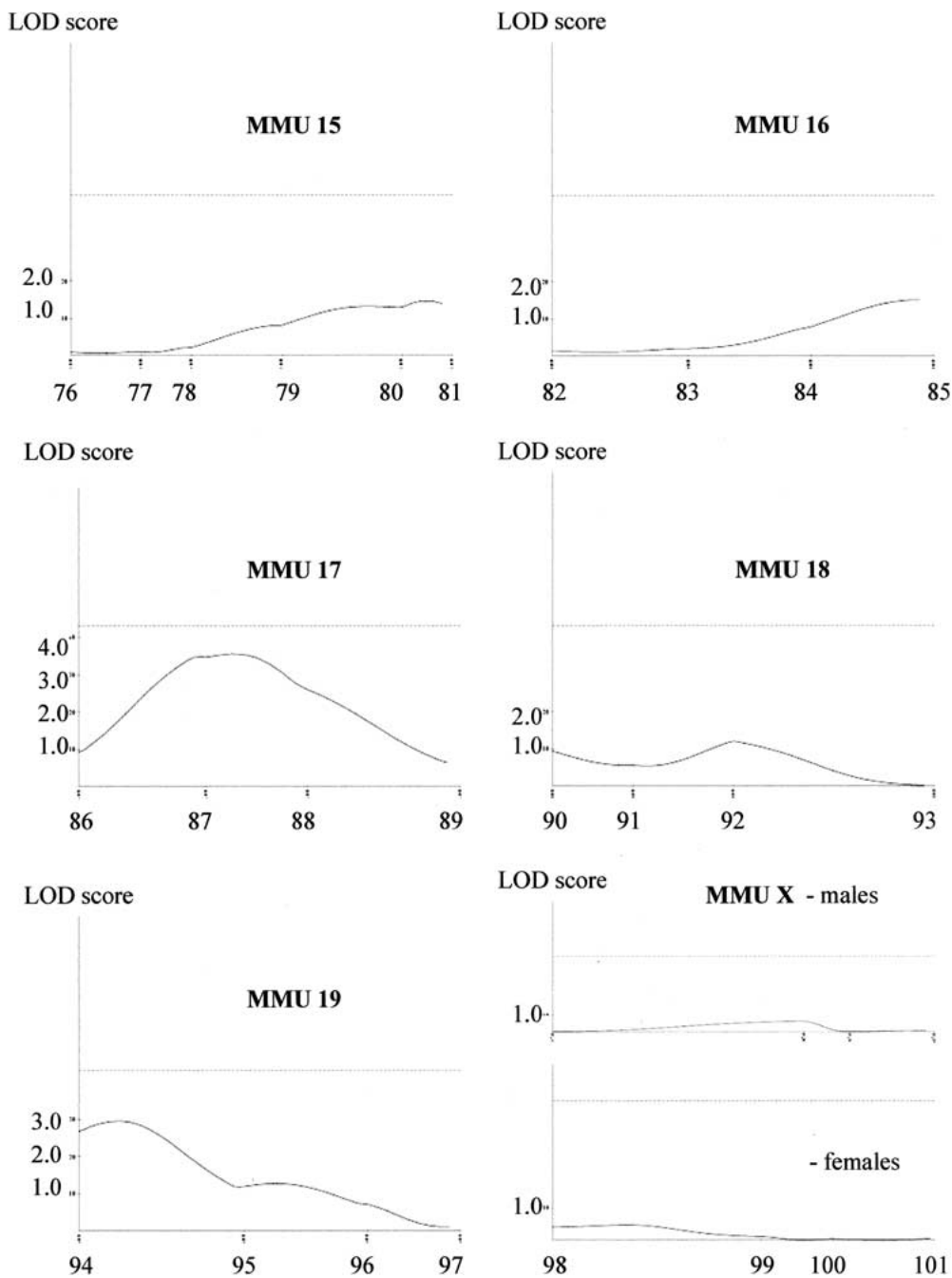


Fig. 6. Genome scan results for MMU15-X. Chromosome X is analyzed separately for male and female progeny.

The choice of organ tissues for microarray examination is based on known physiological processes involved in platelet count regulation: spleen and bone marrow as organs of hematopoiesis, with the spleen having additional functions of sequestration and destruction of senescent platelets. The liver is the major producer of *Thpo*, while the kidneys are subsidiary producers of *Thpo* and major producers of erythropoietin. It is anticipated that in this selection all those organs likely to be implicated have been canvassed. Since platelet count is not the only phe-

notypic difference between the two parental strains of mice, it is expected that there will be some confounding results. Several genes involved in hemoglobin production and red blood cell cytoskeleton scaffolding were identified as differentially expressed and serve to illustrate this issue. Depending on the protocol of elicitation (for instance, whether alternatively spliced products of a genomic DNA sequence are to be counted as one "gene"), the predicted "full size" of the mouse genome may vary from 29,000 to 33,000 genes. Consequently, the oli-

Table 3. Differentially expressed genes that colocalize to QTL

Organ	Map Chr.	Map position	GenBank accession number	Name	GO biological process	Median fold change
Genes expressed at a higher level in QSi5 compared to CBA						
Liver	7	39.6 Mb/23.5 cM	NM_009117	Serum amyloid A 1	Acute-phase response [0006953] phosphate transport [0006817]	2.7
	7	44.4 Mb (B4)	AF324864	Solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), members 6		1.4
	17	38.3 Mb (B1)	AY036118	<i>Mus musculus</i> ETS-related (B1) transcription factor ERF (Erf1) mRNA, complete cds.	Acetyl-CoA metabolism [0006084]	3.8
Spleen	1	162 Mb/H1	NM_007495	Astroactin 1	Cell growth and/or maintenance [0008151]	3.3
	1	171 Mb/H2	NM_025569	Microsomal glutathione S-transferase	Biological_process unknown [0000004]	5.2
	1	178.1 Mb/H3	NM_025470	RIKEN cDNA 1810030J14 gene	Protein complex assembly, multichaperone pathway [0006462]	1.7
	1	185.9 Mb/97.3 cM	BC014835	Lamin B receptor	Ergosterol biosynthesis [0006696]	1.8
	7	24.6 Mb	AK014928	RIKEN cDNA 4921520E09 gene	Biological_process unknown [0000004]	28.1
	7	38.4 Mb/23 cM	NM_007527	Bcl2-associated X protein	Apoptosis [0006915]	4.0
	11	78.0 Mb/45 cM	AK003278	DNA segment, Chr 11, ERATO Doi 18, expressed	Biological_process unknown [0000004]	4.9
	11	99.2 Mb	NM_026807	RIKEN cDNA 1110033F04 gene	Electron transport [0006118]	4.4
	11	99.6 Mb/58.3 cM	NM_010665	Keratin complex 1, acidic, gene 2	Cytoskeleton organization and biogenesis [0007010]	4.0
	11	109.6 Mb/69 cM	AF213393	ATP-binding cassette, subfamily A (ABC1), member 8b	Transport [0006810]	4.6
	11	117.4 Mb	NM_009689	Baculoviral IAP repeat-containing 5	Apoptosis [0006915]	3.5
	11	120.2 Mb/E2	NM_026428	Dicarbonyl L-xylulose reductase	Biological_process unknown [0000004]	2.9
	13	99.2 Mb/56 cM	NM_007629	<i>Mus musculus</i> cyclin B1 (Ccnb1), mRNA	Cell cycle control [0000074]	7.3
	13	117.6 Mb/75 cM	NM_008002	Fibroblast growth factor 10	Cell cycle control [0000074]	3.2

(continued)

Table 3. Continued

Organ	Map Chr.	Map position	GenBank accession number	Name	GO biological process	Median fold change
	13	not placed	AK019652	RIKEN cDNA 4930482J15 gene	Biological_process unknown [0000004]	4.2
	17	24.4 Mb/11 cM	BC004749	Hydroxyacyl glutathione hydrolase	Biological_process unknown [0000004]	9.3
	17	25.4 Mb	AK002535	RIKEN cDNA 0610011F06 gene	Biological_process unknown [0000004]	4.7
	17	31.0 Mb	NM_008804	Phosphodiesterase 9A	Behavior [0007610]	5.2
	17	32.8 Mb/B1	AK014800	RIKEN cDNA 4921501E09 gene	Transcription regulation [0006355]	2.9
	17	46.3 /Mb/C	AK012950	Tubulin-specific chaperone c	Post-chaperonin tubulin folding pathway [0007023]	3.4
	17	47.9 Mb/C	AK011003	Benzodiazepine receptor, peripheral like 1	Mitochondrial translocation [0006628]	3.6
	17	56.8 Mb	AK017377	RIKEN cDNA 5430432H19 gene	Biological_process unknown [0000004]	4.7
	Not placed		AF060220	Erythroid-associated factor	Biological_process unknown [0000004]	11.2
	Not placed		NM_029583	<i>Mus musculus</i> RIKEN cDNA 2810408B13 gene	Biological_process unknown [0000004]	6.1
	Not placed		AJ409470	<i>Mus musculus</i> RNA binding site for Dazl protein, clone kc7	Biological_process unknown [0000004]	7.2
	Not placed		S73844	Gene 154 = olfactory receptor [5' region]	Biological_process unknown [0000004]	22.2
	Not placed		AJ279840	<i>Mus musculus</i> partial mRNA for hypothetical protein, clone mvx11076	Biological_process unknown [0000004]	29.5
	Not placed		X13067	Mouse pEAT11 mRNA with highly repetitive	Biological_process unknown [0000004]	80.0
Marrow	11	87.4 Mb/49 cM	NM_007946	Eosinophil peroxidase	Peroxidase reaction [0006804]	4
Kidney	1	162 Mb/H1	NM_007495	Astroactin 1	Cell growth and/or maintenance [0008151]	2.3
	13	98 Mb	AK005917	RIKEN cDNA 1700012J22 gene	Biological_process unknown [0000004]	2.4
	17	24.4 Mb/11 cM	BC004749	Hydroxyacyl glutathione hydrolase	Biological_process unknown [0000004]	1.7
	17	42.8 Mb/30.4 cM	NM_008585	Meprin 1 alpha	Proteolysis and peptidolysis [0006508]	8.5
	17	47.8 Mb	NM_009694	Apolipoprotein B editing complex 2	mRNA editing [0006381]	6.4
	Not placed		L15321	Mouse T-cell receptor gamma V1 and V2 pr	Biological_process unknown [0000004]	5.5

(continued)

Table 3. Continued

Organ	Map Chr.	Map position	GenBank accession number	Name	GO biological process	Median fold change
Genes expressed at a lower level in QSi5 compared to CBA						
Liver	11	120.1 Mb/E2	NM_011568	RNA and export factor binding protein 1	Protein complex assembly [0006461]	7.72
Spleen	7	16.6 Mb/4 cM	NM_007469	Apolipoprotein C-1	Lipid transport [0006869]	31.64
	17	33.9 Mb/18.66 cM	NM_010382	Histocompatibility 2, class II antigen E beta	Defense response [0006952]	11.51
Marrow	1	171.0 Mb/H2.3	BC011457	RIKEN cDNA 190006A08 gene	Protein metabolism and modification [0006411]	12.88
	7	16.6 Mb/4. CM	NM_009696	Apolipoprotein E	Lipid transport [0006869]	11.70
	11	77.4 Mb/44 cM	S73846	Olfactory receptor 1	Biological_process unknown [0000004]	12.51
Kidney	11	120.1 Mb/E2	NM_011568	RNA and export factor binding protein 1	Protein complex assembly [0006461]	3.81
	7	16.6 Mb/4 cM	NM_007469	Apolipoprotein C-1	Lipid transport [0006869]	16.10
	11	77.4 Mb/44 cM	S73846	Olfactory receptor 1	Biological_process unknown [0000004]	5.15

gonucleotide set used in our experiments, though it may be one of the largest that is currently available, represents only somewhere between 65% and 75% of the mouse genome, and the problem of missing candidate genes due to noninclusion is a relatively small but real issue.

It is noteworthy that for the QTL on the distal end of MMU1, the QSi5 allele had a decreasing effect on platelet count in the F₂ progeny (indicated by the negative sign in the Δ platelet section in Table 2). Inspection of allele frequencies at adjacent microsatellite markers confirmed this interpretation. The QSi5 parental strain shows a much higher platelet count compared to the CBA, so intuitively the inheritance of its genetic content might be expected to lead to a higher platelet count in the progeny. This phenomenon is known as transgressive segregation, and the QTL are described as "cryptic." In these situations some F₂ individuals show more extreme phenotypes than are seen in either parental line, or an allele reducing a trait can be traced back to parents from the higher line and vice versa (Lynch and Walsh 1998). In a survey of 58 animal studies that reported phenotypic variation in segregating hybrid populations, 78% described transgressive segregation in 31% of the phenotypic traits examined (Rieseberg et al. 1999). This putative "cryptic" locus may not represent a reduced transcription of a "platelet-raising" gene in the QSi5 (even though this strain displays the higher platelet count phenotype); on the contrary, increased expression of the locus may play a physiological role to reduce platelet count in QSi5.

In summary, we report the identification of two novel QTL for platelet count in inbred strains of laboratory mice. The QTL do not appear to be associated with known genes that regulate platelet production, confirming an evolving view that the regulation of platelet count may involve multiple systems and/or hierarchies of control.

Acknowledgments

The authors acknowledge with gratitude the support of Professor Colin Chesterman, the Haematology Department, and the Molecular & Cytogenetics Unit of South Eastern Area Laboratory Services, Sydney. We would like to thank Dr. Alan Baxter of the Centenary Institute, Sydney, for providing microsatellite markers, Dr. Zbigniew Rudzki and staff at Medvet Science in IMVS, Adelaide, South Australia, for genotyping the samples, and the staff at The Clive and Vera Ramaciotti Centre for Gene Function Analysis at UNSW for technical support with the microarray work. CC holds a Medical

Postgraduate Award of the National Health and Medical Research Council of Australia. The Centre for Vascular Research is supported by a Research Development Infrastructure Grant from the NSW Department of Health and a Program Grant from the National Health and Medical Research Council of Australia. This project received support from the University of New South Wales University Research Support Program, a University of Sydney Sesqui R & D grant, and a Macquarie University research grant.

References

- Ault KA, Knowles C, Mitchell J, Brown CL, Schultz KL, et al. (1997) Genetic control of platelet activation in inbred mouse strains. *Platelets* 8, 235–241
- Bain BJ (1996) Ethnic and sex differences in the total and differential white cell count and platelet count. *J Clin Pathol* 49, 664–666
- Bain BJ, Seed M (1986) Platelet count and platelet size in healthy Africans and West Indians. *Clin Lab Haematol* 8, 43–48
- Buckley MF, James JW, Brown DE, Whyte GS, Dean MG, et al. (2000) A novel approach to the assessment of variations in the human platelet count. *Thromb Haemost* 83, 480–484
- Burstein SA (1997) Cytokines, platelet production and hemostasis. *Platelets* 8, 93–104
- Chen J, Harrison DE (2002) Quantitative trait loci regulating relative lymphocyte proportions in mouse peripheral blood. *Blood* 99, 561–566
- Dal Colleto GMD, Fulker DW, Barretto OCdO, Kolya M (1993) Genetic and environmental effects on blood cells. *Acta Genet Med Gemellol* 42, 245–252
- de Haan G, Bystrykh LV, Weersing E, Dontje B, Geiger H, et al. (2002) A genetic and genomic analysis identifies a cluster of genes associated with hematopoietic cell turnover. *Blood* 100, 2056–2062
- Evans DM, Frazer IH, Martin NG (1999) Genetic and environmental causes of variation in basal levels of blood cells. *Twin Res* 2, 250–258
- Falconer DS, Mackay TFC (1996) Continuous Variation, pp. 100–107 in *Introduction to Quantitative Genetics*. (Essex: Longman)
- Gader A, Bahakim H, Awadalla S, Malaika S (1995) Ethnic variations in the haemostatic system: Comparison between Arabs, Westerners (Europeans and Americans, Asians and Africans). *Blood Coagul Fibrinolysis* 6, 537–542
- Gainsford I, Roberts AW, Kimura S, Metcalf D, Dranoff G, Milligan RC, et al. (1998) Cytokine production and function in c-mpl-deficient mice: No physiologic role for interleukin-3 in residual megakaryocyte and platelet production. *Blood* 91, 2745–2752
- Gainsford I, Nandurkar H, Metcalf D, Robb L, Begley CG, et al. (2000) The residual megakaryocyte and platelet production in c-mpl-deficient mice is not dependent on the actions of interleukin-6, interleukin-11, or leukemia inhibitory factor. *Blood* 95, 528–534
- Glazier AM, Nadeau JH, Aitman TJ (2002) Finding genes that underlie complex traits. *Science* 298, 2345–2349
- Holt M, Nicholas FW, James JW, Moran C, Martin ICA (2004) Development of a highly-fecund inbred strain of mice. *Mamm Genome* 15, in press
- Kuter DJ, Begley CG (2002) Recombinant human thrombopoietin: Basic biology and evaluation of clinical studies. *Blood* 100, 3457–3469
- Lander ES, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using rflp linkage maps. *Genetics* 121, 185–199
- Lincoln SE, Daly MJ, Lander ES (2004) Website for mapmaker at <http://www.genome.wi.mit.edu/ftp/distribution/software/mapmaker3/>, accessed 4th Feb 2004
- Lynch M, Walsh B (1998) *Genetics and analysis of quantitative traits* (Sunderland, MA.; Sinauer Associates)
- Manly K, Cudmore RJ, Meer J (2001) Map Manager QTX, cross-platform software for genetic mapping. *Mamm Genome* 12, 930–932
- Meer JM, Cudmore RHJ, Manly KF (2004) Website for map manager at www.mapmanager.org/mmQTX.html, accessed 4th Feb 2004
- Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16, 1215
- Moore KJ, Nagle DL (2000) Complex trait analysis in the mouse: The strengths, the limitations, and the promise yet to come. *Annu Rev Genet* 34, 653–686
- Rieseberg LH, Archer MA, Wayne RK (1999) Transgressive segregation, adaptation and speciation. *Heredity* 84, 363–372
- Siebers RWL, Carter JM, Wakem PJ, Maling TJB (1989) Racial differences in platelet counts in New Zealand men. *N Z Med J* 102, 588–589
- Whitfield JB, Martin NG (1985) Genetic and environmental influences on the size and number of cells in the blood. *Genet Epidemiol* 2, 133–144
- Yankowitz J, Zeng SM, Murray JC, Widness JA, Strauss RG (1997) A novel sequence-based polymorphism in the 3'-untranslated region of the thrombopoietin gene and its association with human platelet count. *Am J Human Genet* 4S, abstr 2363
- Yokoyama Y, Akiyama T (1995) Intrapair differences of the blood cell components and lymphocyte subsets in monozygotic and dizygotic twins. *Acta Genet Med Gemellol* 44, 203–214
- Zenger KR (2001) Genetic linkage maps and population genetics of macropods [thesis], Macquarie University, Sydney