Genetic Evaluation of Candidate Genes for the *Mom1* Modifier of Intestinal Neoplasia in Mice

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ABSTRACT

As genetic mapping of quantitative trait loci (QTL) becomes routine, the challenge is to identify the underlying genes. This paper develops rigorous genetic tests for evaluation of candidate genes for a QTL, involving determination of allelic status in inbred strains and fine-structure genetic mapping. For the *Mom1* modifier of intestinal adenomas caused by Apc^{Min}, these tests are used to evaluate two candidate genes: *Pla2g2a*, a secretory phospholipase, and *Rap1GAP*, a GTPase activating protein. *Rap1GAP* passes the first test but is excluded by a single fine-structure recombinant. *Pla2g2a* passes both tests and is a strong candidate for *Mom1*. Significantly, we also find that Apc^{Min}-induced adenomas remain heterozygous for the *Mom1* region, consistent with *Mom1* acting outside the tumor lineage and encoding a secreted product.

MUTATIONS in the adenomatous polyposis coli (*Apc*) gene lead to the development of multiple adenomas in the intestinal tract, in both humans and mice. The best studied mutant allele in the mouse is the *Min* mutation, which was isolated following germline mutagenesis of a C57BL/6J male with the alkylating agent ethylnitrosourea (MOSER *et al.* 1990). The *Min* mutation is a nonsense codon at position 850 in the 2845-amino acid Apc protein (SU *et al.* 1992). Analogous germline mutations in the human *APC* gene lead to familial adenomatous polyposis (FAP) (GRODEN *et al.* 1991; KINZLER *et al.* 1991).

The tumorigenic effect of *Min* is strongly influenced by genetic background (MOSER *et al.* 1992). *Min*/+ mice on the C57BL/6J background (B6-Min) develop an average of 29 ± 10 tumors in the regions of the intestine scored (MOSER *et al.* 1990). By contrast, F_1 hybrids produced by crossing B6 *Min*/+ mice with AKR/J (AKR), MA/MyJ (MA), or *M. m. castaneus* (CAST) mice have an average of four to eight tumors (DIETRICH *et al.* 1993). This difference indicates that these three strains carry dominantly acting modifiers that reduce tumor multiplicity in *Min*/+ mice.

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We previously mapped the major genetic modifier in these strains, Modifier of Min-1 (*Mom1*), to a 15-cM region on distal mouse chromosome *4* (DIETRICH *et al.* 1993). In addition to *Mom1*, the AKR, MA, and CAST strains each carry additional, unmapped dominant modifiers.

The molecular identity of the *Mom1* gene product is unknown. Many genes map to distal mouse chromosome *4* and can be considered as candidates for the *Mom1* locus. Recently, MACPHEE *et al.* have suggested an attractive candidate gene: the secretory phospholipase *Pla2g2a* (MACPHEE *et al.* 1995). It is expressed in Paneth cells of the intestinal crypts and might affect prostaglandin biosynthesis or defense mechanisms against intestinal bacteria (MULHERKAR *et al.* 1991; HARWIG *et al.* 1995). However, the genetic evidence offered in favor of *Pla2g2a* as *Mom1* is limited to two observations: *Pla2g2a* maps to the same broad region of perhaps 30 Mb containing *Mom1* and has a frameshift mutation in B6 but not in AKR, MA, and CAST. From a genetic standpoint, this evidence is tenuous: the reported *Mom1* region is likely to contain several hundred to a thousand genes, and many are likely to have mutations or variants specific to B6. Indeed, another interesting candidate gene in the region, the GTPase-activating protein *Rap1GAP*, also maps to the region and shows amino acid variants specific to B6 (see below).

Formal genetic evaluation of candidate genes for a quantitative modifier locus is a challenging problem that has received little attention. Given a candidate gene that maps to the correct region and shows a specific mutation (or variant) in strains carrying a particular allele at the modifier locus, one can begin to test

the candidate in two ways: (1) study additional strains to determine if genotype for the candidate accurately predicts the allelic state of the modifier locus and (2) perform fine-structure mapping experiments to test if the candidate gene and the modifier locus are recombinationally inseparable.

To this end, we have now determined the *Mom1* status of nine mouse strains, by performing appropriate backcrosses, and compared them with sequence variants in the *Pla2g2a* and *Rap1GAP* genes. We have also performed fine-structure mapping of the AKR allele of the *Mom1* locus ($Mom1^{AKR}$). We have generated a B6.Mom1^{AKR} line, carrying the *Mom1* region from the AKR strain on the B6 genetic background (GOULD *et al.* 1996). Here, we use this line to produce recombinant chromosomes that allow us to refine the positions of *Mom1*, *Rap1GAP*, and *Pla2g2a*.

Finally, we have performed allelic loss analysis of *Min*-induced intestinal adenomas to explore the possibility that *Mom1* may act extrinsically to the tumor lineage.

MATERIALS AND METHODS

Mice: All mice were bred at the McArdle Laboratory for Cancer Research (Madison, WI) from AKR, SWR/J (SWR), DBA/2J (DBA), BALB/cByJ (BALB), and B6 mice purchased from The Jackson Laboratory (Bar Harbor, ME) or from 129/Sv-Pas (129) and BTBR/Pas (BTBR) mice obtained from the Pasteur Institute (Paris, France). In crosses with the inbred strains, F_1 mice were produced by crossing inbred females to B6 *Min*/+ males. The F_1 mice were then backcrossed to B6 to produce the backcross generation. To determine the Mom1 phenotype of the recombinant lines, females from each line were crossed to B6-*Min*/+ males. The B6 *Min*/+ males used in each cross were obtained from our B6 Min colony at different backcross generations: N_5–N_8 for 129 cross, N_7–N_8 for BALB cross, N_{19}–N_{23} for DBA cross, N_{23} for SWR cross, N_{32}–N_{34} for BTBR cross, and N_{29}–N_{32} for recombinant line crosses. The recombinant lines were derived from a B6.Mom1^{AKR} line at the N_6–N_8 generations of backcrossing.

Counting of tumor number: All mice were sacrificed by CO_2 asphyxiation. The intestinal tract was removed, prepared, and scored for tumors as described previously (MOSER *et al.* 1990). In this method, three 4-cm sections of the small intestine, approximately one-third to one-half of the total length of the small intestine, and the entire large intestine were examined for tumors. Each cross was scored by a single observer, whose initials are indicated in parentheses. All *Min*/+ mice from SWR and DBA crosses were sacrificed when moribund or at 150 days of age (M.K.M.). All *Min*/+ mice from 129 cross were sacrificed when moribund or at 200 days of age (A.R.M.). All *Min*/+ mice from BALB cross were sacrificed when moribund or at 240 days of age, with one exception (A.R.M.). The exceptional animal from the BALB backcross was sacrificed at 320 days of age and had only five tumors. All *Min*/+ mice from BTBR cross were sacrificed when moribund or at 200 days of age (A.S.). All *Min*/+ mice from crosses with the recombinant lines 6, 21, 29, and 25 were sacrificed at 120 days (K.A.G.). All *Min*/+ mice from crosses with the recombinant lines 2, 20, 26, and 32 were sacrificed at 80 days (K.A.G.).

Statistics: All statistical analyses were performed with the Wilcoxon rank sum test. One-sided *P* values are given.

Genomic DNA isolation for genotype analysis: Genomic DNA was isolated by two distinct methods. In the first method, DNA was isolated from 50 μl of blood as described previously (DIETRICH *et al.* 1993). The second method involved isolation of genomic DNA from spleen by a modification of a method described previously for blood (PHILLIPS and NADEAU 1984).

DNA extraction for allelic loss analysis: DNA from tumor tissue and normal tissue samples was prepared as described previously (LUONGO *et al.* 1994).

Genotyping of *Apc* locus: Mice was genotyped to identify carriers of the *Min* mutation by a PCR assay described previously (DIETRICH *et al.* 1992).

Genotyping of chromosome *4* markers: Mice were typed as described previously (DIETRICH *et al.* 1993; GOULD *et al.* 1996). The markers flanking the *Mom1* locus used in crosses with inbred strains were as follows: *D4Mit12* and *D4Mit13* for the SWR and DBA crosses; *D4Mit12* and *D4Mit33* for the 129 cross; *D4Mit12* and *D4Mit189* for the BALB and BTBR crosses. *D4Mit13* was not used for analysis in the crosses with the 129, BALB, and BTBR because it was not polymorphic between B6 and these strains. The nearest polymorphic marker was used in its place. Each recombinant line was genotyped using the two markers that define the end points of the region of heterozygosity in that strain.

The markers *D4Mit12*, *D4Mit13*, *D4Mit134*, and *D4Mit249* were amplified under conditions described previously for *D4Mit12* (GOULD *et al.* 1996). The markers *D4Mit54*, *D4Mit64*, *D4Mit68*, *D4Mit71*, *D4Mit203*, *D4Mit283*, and *D4Mit284* were amplified under analogous conditions but with 0.53 μM of each primer and 1.5 mM $MgCl_2$. The products from these reactions were resolved by electrophoresis through 3.5–4% agarose (SeaKemLE, FMC Corp., Rockland, ME) gels and visualized by staining with ethidium bromide. The markers *D4Mit170* and *D4Mit283* were recombinationally inseparable in our experiments and were used interchangeably.

For the crosses with inbred strains, mice were assigned a genotype at the *Mom1* locus on the basis of genotyping at the flanking chromosome *4* markers. Mice that were heterozygous at both markers were designated as *Mom1* heterozygotes. Likewise, mice homozygous for the B6 allele at both markers were designated as $Mom1^{B6/B6}$ homozygotes. Mice recombinant in the *Mom1* region were excluded from this analysis.

For crosses with strains carrying recombinant chromosomes, mice were initially designated as carrying the recombinant chromosome for a given line if they were heterozygous at the two markers that define the maximal region of heterozygosity in that line. At first, mice recombinant between these two markers were excluded from analysis. After localization of *Mom1* to the region between *D4Mit54* and *D4Mit13*, these omitted recombinants were included, provided that they were not recombinant between these two markers.

SSLP analysis of tumors: For each SSLP marker (*D4Mit12*, *D4Mit170*, and *D4Mit13*), amplification and analysis were carried out in duplicate as described previously (LUONGO *et al.* 1994). Reconstruction experiments demonstrated that the relative abundance of PCR products was linear to the input ratio of genomic DNAs from different strains for ratios in the range of 0.4 to 1.0, although there was a slight bias of amplification for the AKR *vs.* B6 allele. To compensate for any differential amplification between alleles, the AKR:B6 ratio observed in tumor tissue was normalized by dividing it by the AKR:B6 ratio observed in a control sample of normal intestinal epithelium from within 1 cm of the tumor.

Genotyping of *Pla2g2a* locus in mice: The *Pla2g2a* mutation is easily genotyped, since the 1-bp insertion in B6 abolishes a *Bam*HI site present in the wild-type sequence. A fragment of ~500 bp containing this site was amplified with two primers, (5′^{12}GTCCAAGGGAACATTGCG) and (5′^{254}AGAACAGGTGATTTGGCCC), and then incubated with *Bam*HI,

which cleaves the wild-type allele into two fragments of 400 and 100 bp, but fails to cleave the mutant allele.

Specifically, 20–50 ng of genomic DNA was amplified in a 10-μl reaction containing 10 mM Tris-HCl (pH 9.0 at 25°), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.4 mM each of dCTP, dGTP, dTTP, and dATP, 1 mM of each primer, and 0.7 units of Taq polymerase (Promega Corp, Madison, WI). Samples were amplified in a thermocycler (MJ Research, Watertown, MA) under the following conditions: one cycle at 94° for 3 min followed by 30 cycles at 94° for 15 sec, 55° for 2 min, and 72° for 1.5 min, followed by one cycle at 72° for 10 min. Five microliters of each PCR reaction was then incubated at 37° for 1 hr in a 10-μl reaction containing 20 U *Bam*HI restriction enzyme (New England BioLabs, Beverly, MA), 150 mM NaCl, 10 mM Tris-HCl (pH 7.9 at 25°), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin. The products from the PCR/digestion reactions were resolved by electrophoresis through 2% agarose gels and visualized by staining with ethidium bromide.

Genotyping of *Pla2g2a* locus in tumors: The primers used for allelic loss analysis were *Pla2s*8 Forward (F) (5′[8]ACAGGTCCAAGGGATCCTTGCGCAG) and *Pla2s*144 Reverse (R) (5′[144]GGTCTGTGGCATCCTTGGG). The bases that differ from the *Pla2g2a* coding sequence are underlined. These base changes were necessary for the generation of a *Bam*HI restriction site.

Each 2 μl DNA sample (20–50 ng) was amplified in a 10-μl reaction containing 3 mM of each primer, 1.2 mM each of dCTP, dGTP, dTTP, and dATP, 3.3 mM [α-^{32}P] dCTP (3000 Ci/mmol) (Dupont, Boston, MA), 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0 at 25°), 50 mM KCl, 0.1% Triton X-100, and 1.0 U Taq polymerase. Samples were amplified in duplicate as described above for *Pla2g2a* genotyping of mice. Reconstruction experiments demonstrated that the relative abundance of PCR products was linear to the input ratio of AKR:B6 genomic DNAs over the range of 0.25 to 7.5.

Ten microliters of each PCR was incubated at 37° for a minimum of 3.5 hr in a 13-μl reaction containing 26 U *Bam*HI restriction enzyme, 150 mM NaCl, 10 mM Tris-HCl (pH 7.9 at 25°), 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 μg/ml bovine serum albumin. This digestion results in the production of a 125-bp fragment from the B6 allele and a 101-bp fragment from the AKR allele.

Five microliters of each *Bam*HI digestion was electrophoresed through 0.4-mm-thick 7.5% denaturing polyacrylamide gels that were prepared and analyzed as described previously (Luongo *et al.* 1994). The mean ratio of undigested/total was 0.04 ± 0.02 and the maximum ratio was 0.12.

Genotyping of *Rap1GAP* locus in mice: The allelic variation in the *Rap1GAP* was typed by a PCR- and allele-specific oligonucleotide hybridization. Specifically, 100 ng of genomic DNA was amplified in a 50-μl reaction containing 10 mM Tris-HCl (pH 9.0 at 25°), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each of dCTP, dGTP, dTTP, and dATP, 1 mM of each primer, 25 U of Amplitaq DNA ploymerase, and 5% final concentration of DMSO. Samples were amplified in a thermocycler under the following conditions: one cycle at 94° for 5 min followed by 30 cycles at 94° for 10 sec, 60° for 30 sec, and 72° for 30 sec, followed by one cycle at 72° for 5 min. Ten microliters of each PCR was electrophoresed on a 2% agarose gel to verify the presence of the correct amplification product. The remaining 40 μl was blotted onto duplicate filters. Each filter was hybridized with a B6-specific probe (5′GCCTCGGCGGCGACGAC) or an AKR-specific probe (5′GCCTCGGCAGCGACGAC), which differ at the underlined nucleotide. Blotting and hybridizations were carried out as described in Hussussian *et al.* (1994). In addition, genotypes were confirmed by direct sequencing of PCR products by use of an ABI 373 DNA Sequencer.

RESULTS

***Mom1* alleles in nine inbred strains:** Characterizing the *Mom1* allele carried by an inbred strain involves two steps: (1) testing whether the strain carries any dominant modifiers of *Min,* by comparing tumor number in F_1 progeny with the B6-Min mice and (2) testing whether any dominant modifier maps to the same region as *Mom1* by backcross analysis. Mapping a modifier to the *Mom1* region in a backcross does not formally prove that it is identical with *Mom1,* but it is strong evidence, and we tacitly make this assumption.

We previously used backcrosses to establish that B6 carries a recessively acting susceptibility allele at *Mom1,* while AKR, MA, and CAST carry dominantly acting resistance alleles. To characterize five additional inbred strains (SWR, DBA, BALB, 129, and BTBR), we crossed females of the given strain to B6 *Min*/+ males and counted tumors in the resulting *Min*/+ F_1 progeny. To determine whether any reduction in tumor multiplicity mapped to the *Mom1* region, we backcrossed the *Min*/+ F_1 mice to B6 in all five cases and compared the distribution of tumor number in backcross progeny heterozygous across the *Mom1* region with that observed in progeny homozygous for the B6 alleles across this region.

The F_1 *Min*/+ mice showed a significant reduction in average tumor multiplicity with four strains: SWR, DBA, BALB, and 129 (Table 1), indicating the presence of a dominant modifier allele. No reduction of tumor number was seen in the F_1 progeny with BTBR.

In three of the four strains showing resistance in the F_1 progeny, the backcross revealed a clear effect attributable to genotype in the *Mom1* region (Table 1). The mean tumor numbers in heterozygotes *vs.* homozygotes in the *Mom1* region were 8.5 *vs.* 19.1 for SWR ($P < 1 \times 10^{-6}$), 14.0 *vs.* 29.0 for DBA ($P = 1.5 \times 10^{-6}$), and 18.5 *vs.* 27.9 for BALB ($P = 0.025$). These results indicate that SWR, DBA, and BALB each carry a dominant modifier that maps to the *Mom1* region.

By contrast, the *Mom1* region had no detectable effect on tumor multiplicity in the backcross with 129. The most likely explanation for this observation is that the dominant resistance seen in the F_1 progeny is due to unlinked modifier loci (Table 1). There was no difference in the average age at sacrifice between the *Mom1* heterozygotes (130 days) and the *Mom1* homozygotes (124 days). Thus, the failure to observe an effect of *Mom1* on tumor multiplicity in the 129 cross is not due to skewing of the tumor multiplicity data as a result of differences in age at sacrifice. The results of the 129 cross illustrate the necessity of the backcross test in determining the allelic status at *Mom1.*

Finally, the *Mom1* region had no detectable effect on tumor multiplicity in the backcross with BTBR. Al-

TABLE 1

Effect of *Mom1* on tumor multiplicity in backcrosses

Cross	F_1 mice	Backcross	
		Mom1 heterozygotes	*Mom1* homozygotes
B6 × (SWR × B6 *Min*/+)	3.1 ± 2.0 (48)	8.5 ± 4.1 (26)	19.1 ± 8.9 (21)
B6 × (DBA × B6 *Min*/+)	14.0 ± 5.5 (25)	14.0 ± 7.5 (28)	29.0 ± 12.6 (30)
B6 × (BALB × B6 *Min*/+)	11.7 ± 5.5 (6)	18.5 ± 10.7 (23)	27.9 ± 16.6 (14)
B6 × (BTBR × B6 *Min*/+)	21.0 ± 9.7 (21)	40.0 ± 12.0 (38)	38.4 ± 14.8 (32)
B6 × (129 × B6 *Min*/+)	15.2 ± 6.7 (9)	27.9 ± 13.4 (11)	32.9 ± 19.9 (14)

The average tumor multiplicity (±SD) of each class is given. The number of *Min*/+ mice scored for each class is indicated in parentheses. B6 *Min*/+ mice developed, on average, 29 ± 10 tumors in the regions counted (MOSER *et al.* 1990).

though BTBR showed no reduction in the F_1, it is still worth performing a backcross analysis because the F_1 results could represent offsetting effects of a resistance allele at *Mom1* together with unlinked enhancers of tumor number.

Through backcross analysis, we can determine which inbred strains carry resistance to *Min*-induced intestinal tumorigenesis that clearly maps to the *Mom1* region of distal chromosome *4*. From these analyses, we conclude that AKR, MA, CAST, SWR, DBA, and BALB carry dominantly acting resistance alleles ($Mom1^R$), while B6, BTBR, and 129 carry recessively acting susceptibility alleles ($Mom1^S$).

Modifier loci unlinked to *Mom1*: The backcrosses also provide information about the presence of dominant modifiers unlinked to *Mom1*. If there are no unlinked modifiers, the tumor multiplicity should be the same in the F_1 and backcross progeny heterozygous at *Mom1* (since both groups have the same genotype at the *Mom1* locus). By contrast, substantially lower tumor multiplicity in the F_1 would indicate the presence of additional unlinked modifiers.

Mom1 appears to be the only significant modifier in DBA (relative to B6), since the F_1 progeny and the *Mom1* heterozygous backcross progeny both showed an average tumor number of 14. In BALB, the tumor number showed some difference between the two groups, but the effect fell short of statistical significance (11.7 *vs.* 18.2, $P = 0.09$); there is thus no compelling evidence for unlinked modifiers in BALB. By contrast, SWR and 129 clearly show evidence for unlinked dominant modifiers (SWR: 3.1 *vs.* 8.5, $P < 1 \times 10^{-6}$; 129: 15.2 *vs.* 27.5, $P = 0.017$) (Table 1). However, analysis of mice from the 129 cross with 98 SSLP markers has failed to detect linkage of a 129 modifier locus with major effect (data not shown). This suggests that the 129 strain carries a number of modifier alleles, each of which has a small effect on tumor multiplicity. Finally, BTBR failed to show significant evidence for any dominant modifiers (linked or unlinked), since neither the F_1 or backcross animals showed a decrease in average tumor multiplicity relative to the B6 *Min*/+ mice.

Fine-structure mapping of the *Mom1* region: To localize more finely the position of *Mom1* on distal chromosome *4*, we generated a fine-structure map of $Mom1^{AKR}$. To characterize *Mom1* in the absence of any unlinked modifiers, we had constructed a B6.Mom1^{AKR} line carrying a 35-cM interval around *Mom1* from AKR on an otherwise B6 background. Studies with this strain have shown that heterozygosity for $Mom1^{AKR}$ corresponds to a twofold reduction in tumor number (GOULD *et al.* 1996). Crosses with this line suggest that the only modifier(s) of Min in this line maps within the region of heterozygosity on chromosome *4* (GOULD *et al.* 1996). To carry out fine-structure mapping of *Mom1*, we performed a B6.$Mom1^{AKR/B6}$ × B6 cross and identified progeny in which recombination had narrowed the AKR region present.

Lines carrying each recombinant chromosome (in a heterozygous state over B6) were established and tested to determine whether the recombinant chromosome carried the resistance allele from AKR or susceptibility allele from B6. Specifically, females from each recombinant line were crossed to B6 *Min*/+ males, and tumors were counted in *Min*/+ progeny. If progeny inheriting the recombinant chromosome showed a distribution of tumor multiplicities that was not significantly different from sibs carrying the B6 chromosome (but was significantly higher than seen in age-matched B6.$Mom1^{AKR/B6}$ *Min*/+ mice produced from the B6.Mom1^{AKR} line), the recombinant chromosome was inferred to carry the susceptibility allele $Mom1^{B6}$. Conversely, if these progeny showed a distribution of tumor multiplicity that was significantly lower than for sibs inheriting the B6 chromosome (but was not significantly different from age-matched B6 $Mom1^{AKR/B6}$ *Min*/+ controls), then the recombinant chromosome was inferred to carry the resistance allele $Mom1^{AKR}$. In this fashion, *Mom1* could be genetically mapped relative to the breakpoints of the recombinant chromosomes.

Eight lines carrying recombinant chromosomes were characterized. The recombinant chromosomes were selected because they had crossovers between *D4Mit12* and *D4Mit13* and the recombinational breakpoint was

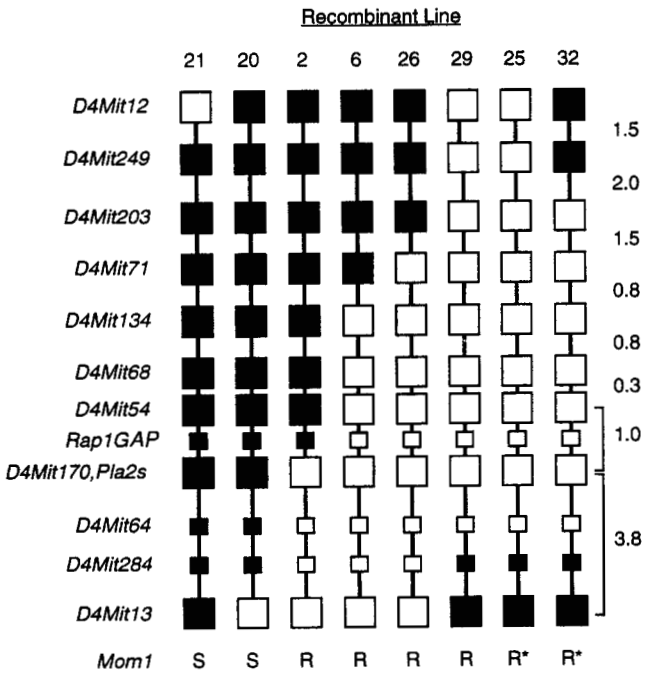

FIGURE 1.—Genotypes of recombinant lines in the *Mom1* region. For each recombinant line, □ indicates positions where each line is heterozygous AKR/B6 and ■ indicates positions at which each line is homozygous B6. Small squares indicate a gene or marker that is known to map within the interval shown, but the precise position within the interval is not known. For each line, the deduced Mom1 phenotype is classified as sensitive (S), resistant (R), or partially resistant (R*). The genetic distance (in cM) for each interval is given to the right of the figure. The genetic distances are based on 400 meioses in an AKR × B6 cross.

mapped relative to genetic markers in the interval (Figure 1). We then determined the *Mom1* allele carried on each recombinant chromosome, as described below (Table 2). For six of the lines, the phenotype was consistent with carrying either $Mom1^{AKR}$ or $Mom1^{B6}$, that is, it was consistent with either a twofold reduction or no reduction in tumor number. Lines 2, 6, 26, and 29 were determined to carry $Mom1^{AKR}$ (Table 2). Lines 20 and 21 were determined to carry $Mom1^{B6}$ (Table 2). From the *Mom1* genotype of the recombinant chromosomes, the *Mom1* locus must lie between the recombination breakpoints of lines 2 and 20. In particular, *Mom1* maps to the interval between *D4Mit54* and *D4Mit284* (Figure 1).

Interestingly, line 25 failed to give a result that was fully consistent with carrying either $Mom1^{AKR}$ or $Mom1^{B6}$: the average tumor multiplicity in the recombinant heterozygotes was intermediate to, but significantly different from, both age-matched, contemporaneous $Mom1^{B6}$ and $Mom1^{AKR/B6}$ controls (Table 2). Moreover, line 32, a derivative of line 25 arising from a crossover between *D4Mit249* and *D4Mit203*, showed a similar intermediate effect (Figure 1, Table 2). We assume that both lines 25 and 32 carry the same recombination breakpoint in the interval between *D4Mit64* and *D4Mit284*. Line 29 also has a recombinational breakpoint in this region, but this line was independently derived and the breakpoint should be different (Figure 1).

The intermediate phenotype in line 25 and its derivative line 32 raises the possibility that *Mom1* may represent a complex locus consisting of two or more genes. The recombination event between *D4Mit64* and *D4Mit284* that occurred in line 25 (and was transmitted to line 32) may have separated these genes, such that the recombinant chromosome would carry AKR alleles at only one (or some) of these genes and thus exhibit a partial effect of $Mom1^{AKR}$. This possibility is particularly intriguing in light of a recent report in which two additional genes encoding secretory phospholipases are mapped to the *Mom1* region of distal mouse chromosome *4* and shown to be tightly linked to *Pla2g2a* (TISCHFIELD *et al.* 1996). It is unclear whether either of these genes affects tumor multiplicity in Min mice. Alternatively, it is formally possible that line 25 acquired a new mutation tightly linked to *Mom1* and affecting tumor susceptibility. It seems unlikely that the partial resistance phenotype in these two lines is the result of undetected AKR alleles unlinked to distal chromosome *4*, as there is no evidence for the existence of AKR alleles that increase tumor multiplicity (DIETRICH *et al.* 1993).

Testing *Rap1GAP* and *Pla2g2a* as candidates for *Mom1*: Having characterized the *Mom1* allele in nine inbred strains and performed fine-structure genetic mapping, we sought to evaluate candidate genes for *Mom1*. MACPHEE *et al.* have proposed *Pla2g2a* as a candidate for *Mom1* based on its biological function and the fact that the susceptible B6 strain has a 1-bp insertion in the coding region, creating a mutant allele ($Pla2g2a^{-}$), whereas the resistant AKR, MA, and CAST strains carry the wild-type allele ($Pla2g2a^{+}$) (MACPHEE *et al.* 1995). We developed a PCR assay to distinguish these alleles and determined the genotypes of the strains in which the *Mom1* allele had been characterized. The six strains (AKR, MA, CAST, SWR, DBA, and BALB) with a resistance allele $Mom1^{R}$ all carried the wild-type $Pla2g2a^{+}$ allele, while the three strains (B6, BTBR, and 129) with the susceptible allele $Mom1^{S}$ all carried the mutant $Pla2g2a^{-}$ allele (Table 3).

While a single instance of discordance between *Pla2g2a* genotype and *Mom1* genotype would be sufficient to exclude *Pla2g2a* as a candidate, perfect concordance in the nine strains does not provide definitive support for the hypothesis. The *Pla2g2a* mutation almost surely arose on a single ancestral chromosome that was subsequently transmitted to B6, BTBR, and 129. The actual *Mom1* susceptibility allele could well be a different variation on this ancestral chromosome, at a locus close enough to have remained in linkage disequilibrium with *Pla2g2a* (FRANKEL 1995).

Accordingly, we also sought to study other potential candidates for *Mom1*. One interesting candidate is *Rap1GAP*, which encodes a GTPase-activating protein specific for p21Rap1, a member of the Ras superfamily

TABLE 2

Tumor multiplicity data from crosses with the recombinant lines

Cross	Recombinant heterozygotes	Age-matched controls: Recombinant homozygotes	*P* value	Age-matched controls: Congenic heterozygotes	*P* value	Mom1 phenotype
Rec21 × Min	30.3 ± 12.8 (17)	32.2 ± 7.1 (14)	0.23	15.0 ± 7.8 (77)	$<1 \times 10^{-6}$	S
Rec20 × Min	24.2 ± 8.6 (34)	29.6 ± 10.2 (24)	0.04[a]	10.7 ± 5.8 (11)	2.2×10^{-5}	S
Rec2 × Min	10.7 ± 4.7 (23)	24.6 ± 10.8 (29)	$<1 \times 10^{-6}$	10.7 ± 5.8	0.35	R
Rec6 × Min	13.0 ± 5.9 (24)	31.2 ± 10.6 (13)	6×10^{-6}	15.0 ± 7.8	0.15	R
Rec26 × Min	12.1 ± 6.9 (12)	27.9 ± 9.2 (11)	5×10^{-4}	10.7 ± 5.8	0.43	R
Rec29 × Min	17.8 ± 10.5 (18)	30.2 ± 13.3 (25)	3×10^{-4}	15.0 ± 7.8	0.494	R
Rec25 × Min	21.5 ± 6.9 (20)	30.1 ± 12.8 (14)	0.025[b]	15.0 ± 7.8	3.4×10^{-3}	R*
Rec32 × Min	15.8 ± 5.9 (12)	23.4 ± 8.5 (10)	9×10^{-3}	10.7 ± 5.8	0.02[c]	R*

The average tumor multiplicity (±SD) of each class is given. The number of *Min*/+ mice scored from each class is indicated in parentheses. Mice from crosses with Rec lines 21, 6, 29, and 25 were scored at 120 days of age. Mice from Rec lines 20, 2, 26, and 32 were scored at 80 days of age. The deduced Mom1 phenotype of each line is indicated as S (sensitive), R (resistant), or R* (partially resistant).

[a] To determine whether this result was significant or not, we compared tumor multiplicities between Rec20 heterozygotes and a large group ($N = 73$) of age-matched $Mom1^{B6/B6}$ mice. The difference in tumor multiplicities between these two groups was not significant (24.2 *vs.* 26.5, $P = 0.17$).

[b] To determine whether this result was significant or not, we compared tumor multiplicities between Rec25 heterozygotes and a large group ($N = 55$) of age-matched $Mom1^{B6/B6}$ mice. The difference in tumor multiplicities between these two groups was significant (21.5 *vs.* 31.4, $P = 2.5 \times 10^{-4}$).

[c] To determine whether this result was significant or not, we compared tumor multiplicities between Rec32 heterozygotes and a large group ($N = 73$) of age-matched $Mom1^{B6/B6}$ mice. The difference in tumor multiplicities between these two groups was significant (15.8 *vs.* 26.5 $P = 1.4 \times 10^{-4}$).

of GTPases (RUBINFELD *et al.* 1991). Recently, a protein having similarity to the Rap1GAP catalytic domain and with specific GTPase-stimulatory activity on p21Rap1 was identified as the gene mutated in tuberous sclerosis, a human hereditary cancer syndrome (WEINECKE *et al.* 1995) and in a model of hereditary renal carcinomas in the Eker rat (KOBAYASHI *et al.* 1995). The mouse *Rap1GAP* gene was recently cloned, sequenced, and mapped to mouse chromosome *4* by N. DRACOPOLI (personal communication), who also identified a missense variant involving a Thr/Ala substitution at amino acid 147 in the protein. B6 carries the allele encoding Thr, while AKR carries the allele encoding Ala.

We determined the *Rap1GAP* allele present in the nine inbred strains characterized for *Mom1*. Just as observed for *Pla2g2a,* there was perfect concordance: the six resistant strains carried the AKR allele, while the three susceptible strains carried the B6 allele of this missense variation (Table 3). In summary, the genotypes at *Pla2g2a* and *Rap1GAP* both show perfect concordance with Mom1 phenotype.

TABLE 3

Summary of analysis of inbred strains

Inbred strain	Mom1	*Pla2s*	*Rap1GAP*
B6	S	−	B6
AKR	R	+	AKR
CAST	R	+	AKR
MA	R	+	AKR
SWR	R	+	AKR
DBA	R	+	AKR
BALB	R	+	AKR
BTBR	S	−	B6
129	S	−	B6

To further evaluate *Pla2g2a* and *Rap1GAP,* we used genetic mapping. We first used an (AKR × B6) F_2 intercross and an (AKR × B6) × B6 backcross to localize these genes to the 4.8-cM interval defined by *D4Mit54* and *D4Mit13*. We then used the recombinant lines described above to map the candidate genes relative to *Mom1*. *Pla2g2a* was recombinationally inseparable from *Mom1* in these lines, with $Pla2g2a^+$ being present in the lines carrying $Mom1^R$, and $Pla2g2a^-$ being present in those carrying $Mom1^S$. In contrast, *Rap1GAP* had recombined with *Mom1* in one recombinant line: line 2 carries the B6 allele at *Rap1GAP* but carries the $Mom1^R$ allele. This single recombination event eliminates *Rap1GAP* as a candidate for *Mom1*.

Allelic loss analysis of the *Mom1* region and *Pla2g2a:* The *Mom1* region of the mouse is syntenic with the human chromosomal segment 1p35-36, a region that frequently shows somatic deletions in human colon carcinomas (LEISTER *et al.* 1990). The somatic loss indicates the presence of a tumor suppressor gene, but it is unclear whether this locus is the human homologue of *Mom1* or an unrelated gene. We sought to determine whether the *Mom1* region shows somatic loss in intestinal tumors. Frequent somatic loss would suggest that *Mom1* acts as a classical tumor suppressor gene and might also allow fine-structure genetic mapping by deletion analysis.

We performed allelic loss analysis of the *Mom1* region in tumors from (AKR × B6) *Min*/+ F_1 hybrids and (Rec6 × B6-*Min*/+) F_1 mice. Allelic loss was assessed by using a quantitative PCR assay for genetic markers to measure the ratio of AKR:B6 alleles. The allelic ratio measured in tumors was normalized by dividing it by the allelic ratio found in surrounding normal intestinal epithelial tissue. Using analogous methods, we have previously demonstrated that the chromosome *18* homologue carrying the wild-type *Apc* allele is lost in intestinal adenomas from *Min*/+ mice (LUONGO *et al.* 1994).

Seventeen tumors from (AKR × B6-Min) F_1 mice were analyzed for the genetic markers *D4Mit12, D4Mit170,* and *D4Mit13,* which span the *Mom1* region. For the markers *D4Mit170* (average ratio: 1.05 ± 0.20) or *D4Mit13* (average ratio: 1.12 ± 0.10), the adenomas showed no evidence of allelic loss. For *D4Mit12* (average ratio: 0.97 ± 0.23), two of the 17 adenomas showed normalized ratios of 1.6, suggesting that some of the cells in the adenoma had lost the B6 allele of *D4Mit12.* These adenomas were histologically indistinguishable from those retaining the B6 allele of *D4Mit12* (data not shown).

Twenty-six tumors from (Rec6 × B6*Min*/+) F_1 mice were also analyzed. Again, the markers *D4Mit170* (average ratio: 0.96 ± 0.12) and *D4Mit13* (average ratio: 0.97 ± 0.08) showed no evidence of allelic loss. The marker *D4Mit12* could not be used to analyze these tumors, as mice carrying the Rec6 chromosome are homozygous for the B6 allele at this marker (see Figure 1).

Although we did not detect frequent allelic loss with *D4Mit12, D4Mit170,* or *D4Mit13,* it remains a possibility that somatic loss is confined to a region too small to be detected by the markers studied. To test specifically for allelic loss at *Pla2g2a,* we used a quantitative PCR assay for this locus to analyze the same adenomas and control samples studied above. The relative abundance of the AKR allele (*Pla2g2a*$^+$) and B6 allele (*Pla2g2a*$^-$) was determined. The mean adenoma-to-normal-tissue control values were as follows: 1.06 ± 0.16 [(AKR × B6) *Min*/+ F_1 hybrids] and 0.95 ± 0.10 [(Rec6 × B6*Min*/+) F_1 mice]. These values indicate that the *Pla2g2a* locus does not undergo somatic loss in adenomas of *Min*/+ mice.

DISCUSSION

Although the methodology for mapping genes controlling quantitative traits in experimental organisms is becoming well established, there remains a formidable challenge in moving from linkage to locus. Even when a specific candidate has been proposed, considerable care is needed in testing the hypothesis. Transgenic experiments require careful interpretation in the case of quantitative traits. For example, demonstration of a phenotypic effect in transgenic animals does not provide definitive proof of a candidate gene, since the effect could result from copy number or position effects. The gold standard must involve construction of gene disruptions or replacements.

Before undertaking such experiments, it is important to have a battery of genetic tests to employ for evaluating candidates. In this paper, we define and apply such tests to the *Mom1* locus.

We determined the allelic status of *Mom1* in inbred strains by examining F_1 progeny and then analyzing backcrosses. The strains AKR, MA, CAST, SWR, DBA, BALB, and 129 all showed reduction of tumor number in the F_1 hybrid with B6-Min. One might be tempted to conclude that all seven strains carry the resistant *Mom1*^{R} allele. However, backcross analysis showed that the reduction maps to the *Mom1* region only in the case of the first six strains, but not in the case of strain 129. Accordingly, AKR, MA, CAST, SWR, DBA, and BALB carry *Mom1*^{R}, while B6, 129, and BTBR carry *Mom1*^{S}. It is important to note that no inference about *Mom1* can be drawn from the observation of a reduction of tumor number in F_1 mice, since modifiers unlinked to *Mom1* are known to exist (DIETRICH *et al.* 1993). Thus the report by MACPHEE *et al.* (1995) that the P/J mouse strain carries a resistance allele at *Mom1* because F_1 progeny between P/J and B6-Min show reduced tumor numbers must be interpreted with caution. These results underscore the crucial importance of using backcrosses to assign allelic status in a polygenic trait, in which phenotypic modification could reflect the effect of unlinked loci. The situation requires considerably more care than for simple Mendelian traits with distinct qualitative phenotypes. Analysis of F_1 and backcross populations also revealed that SWR, but not BALB and DBA, carries modifiers of the Min phenotype in addition to *Mom1.* As in the AKR, CAST, and MA strains, the map position of these additional modifiers is unknown.

It is worth noting the somewhat contradictory reports in the literature concerning strain 129. LAIRD *et al.* (1995) detected no difference in tumor multiplicity in age-matched (129 × B6)F_1 *Min*/+ and B6 *Min*/+ mice. This discrepancy may be due to the small number of animals examined: six B6 mice and two F_1 mice. Alternatively, differences in husbandry conditions, which can have significant effects on tumor multiplicity in *Min*/+ mice, may also be involved (KIM *et al.* 1993). It is also possible that the difference between our observations and those of LAIRD *et al.* (1995) are attributable to differences in the 129 substrain used.

We also note that OSHIMA *et al.* (1995) reported that mice heterozygous for a targeted disruption *Apc*Δ*716* on either a 129 background or a (129 × B6) F_1 background had no colonic tumors, although the total tumor number along the length of the intestine was not significantly different from that seen in B6 *Min*/+ (OSHIMA *et al.* 1995). These authors postulated that 129 has a modifier effect specifically acting in the colon.

Having determined the allelic status of *Mom1* in nine inbred strains, we evaluated two candidate genes,

Pla2g2a and *Rap1GAP*. Specific variants in each showed a perfect correspondence with the *Mom1* allele. This result is probably the consequence of the two variants (insertion in *Pla2g2a* and base-substitution in *Rap1GAP*) having been present on an ancestral chromosome and having remained in linkage disequilibrium in the lineages that led to B6, 129, and BTBR. This result underscores that a perfect correlation with allelic status does not provide reliable proof of the identity of a candidate gene.

We also generated a fine-structure genetic map of the *Mom1* region by constructing a B6.Mom1^{AKR} line, isolating recombinant chromosomes with breakpoints in the *Mom1* region, and then determining the *Mom1* allele present on each chromosome by progeny testing. In contrast to the situation for simple Mendelian traits, testing of multiple progeny is required to ascertain the *Mom1* allele on a recombinant chromosome. For six of the recombinant chromosomes, the *Mom1* allele could be unambiguously determined. By comparing the alleles at *Mom1* with the alleles at various SSLP genetic markers, the *Mom1* locus could be localized to the 4-cM interval between the markers *D4Mit54* and *D4Mit13*. Both *Pla2g2a* and *Rap1GAP* also mapped to this interval. However, *Pla2g2a* showed perfect concordance with the *Mom1* allele in the six recombinant lines, whereas *Rap1GAP* was discordant for one of the lines (line 2). *Rap1GAP* is thus eliminated as a candidate for *Mom1*, demonstrating the power of fine-structure mapping of quantitative traits. *Pla2g2a* satisfied all the tests and thus remains a strong candidate.

The phenotype of one recombinant chromosome (line 25) and its derivative (line 32) did not completely reflect the phenotype of either the *Mom1*^{R} or *Mom1*^{S} allele. This finding raises the possibility that *Mom1* may be a complex locus. Further investigation of these lines is important; any complexity of *Mom1* would affect both the design and interpretation of further tests of any single candidate locus. Based on the recent mapping of two additional genes encoding secretory phospholipases to the *Mom1* region (TISCHFIELD *et al.* 1996), it is of particular interest to investigate the possibility that either or both of these genes also influence tumor multiplicity in Min mice.

We explored the use of somatic genetics as a potential means to refine the map position of *Mom1* and test candidate loci. However, no allelic loss of markers flanking the *Mom1* locus was observed in intestinal adenomas from *Min*/+ mice. This indicated that there is not frequent chromosomal loss or large deletion, but the possibility of small deletions is left open. We tested this possibility directly for *Pla2g2a*, but found no allelic loss. Since there is no somatic loss of heterozygosity, somatic genetics cannot be used for mapping or candidate testing.

The maintenance of heterozygosity at *Mom1* in adenomas would be expected if *Pla2g2a* is *Mom1* (DOVE *et al.* 1994). Since *Pla2g2a* is produced and secreted by Paneth cells, it should act in a cell nonautonomous fashion. Thus, loss of *Pla2g2a* would not be expected in tumor lineages (MACPHEE *et al.* 1995). Recently, RIGGINS *et al.* (1995) reported the absence of somatic mutation of the human homologue of *Pla2g2a* in sporadic colon tumors. However, as no adenomas from FAP patients were analyzed, it is unclear whether mutation of *PLA2G2A* is involved in hereditary colon cancer in humans.

The results presented here are consistent with *Pla2g2a* being a strong candidate for the *Mom1* locus. Although these analyses cannot prove identity, they present a strong series of tests that were able to distinguish between two potential candidate genes. In the end, definitive proof of the identity of *Mom1* will require the construction of transgenic or, more importantly, an allelic substitution for the candidate gene.

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