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The Secretory Phospholipase A2 Gene Is a Candidate for the *Mom1* Locus, a Major Modifier of Apc^{Min}-Induced Intestinal Neoplasia

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Summary

Mutations in the *APC* gene are responsible for various familial and sporadic colorectal cancers. *Min* mice carry a dominant mutation in the homolog of the *Apc* gene and develop multiple adenomas throughout their small and large intestine. Quantitative trait loci studies have identified a locus, *Mom1*, which maps to the distal region of chromosome 4, that dramatically modifies *Min*-induced tumor number. We report here the identification of a candidate gene for *Mom1*. The gene for secretory type II phospholipase A2 (*Pla2s*) maps to the same region that contains *Mom1* and displays 100% concordance between allele type and tumor susceptibility. Expression and sequence analysis revealed that *Mom1* susceptible strains are most likely null for Pla2s activity. Our results indicate that *Pla2s* acts as a novel gene that modifies polyp number by altering the cellular microenvironment within the intestinal crypt.

Introduction

Mutations of the human adenomatous polyposis coli (*APC*) gene are responsible for both sporadic and familial colorectal cancers (Nishisho et al., 1991; Groden et al., 1991). Germline mutations of *APC* are found in inherited familial cancers such as Gardner's syndrome, attenuated APC, and familial adenomatous polyposis (FAP) (Nishisho et al., 1991; Spirio et al., 1992). FAP is an autosomal dominant inherited disease predisposing the patient to colon cancer. Patients inheriting a single mutant allele of *APC* develop hundreds to thousands of adenomatous polyps in the second to third decades of life that, if left untreated, progress to malignant carcinomas (Hamilton, 1989). Genetic linkage analysis localized *APC* to human chromosome 5q21-q22, a region frequently associated with allelic loss of the wild-type 5q allele (Bodmer et al., 1987; Leppert et al., 1987; Kinzler et al., 1991). Mutations in *APC* are also implicated in extracolonic tumors such as gastric and small intestinal polyps, osteomas, sarcomas, and desmoidal tumors (Leppert et al., 1990; Spirio et al., 1992; Klemmer et al., 1987).

A mutant mouse strain is being used as a model to understand better the genetic elements influencing the progression of colorectal tumorigenesis. The multiple intestinal neoplasia (*Min*) strain, established from an ethylnitrosourea-treated C57BL/6J (B6) male mouse, carries a dominant mutation (Moser et al., 1990). Mice heterozygous for the *Min* mutation develop numerous adenomas throughout the small and large intestinal tract. The observed phenotype in *Min* mice closely resembles the clinical features observed in patients with FAP. Genetic linkage analysis localized the *Min* mutation to mouse chromosome 18, in a region known to contain the *Apc* gene, the murine homolog of the human *APC* gene (Luongo et al., 1993). Further studies revealed that the *Min* mutation (hereafter called Apc^{Min}) results from a nonsense mutation in exon 15 of the *Apc* gene; the same type of mutation is frequently found in human FAP kindreds (Su et al., 1992).

The genetic and phenotypic similarities observed between *Min* mice and FAP patients suggest that the *Min* mouse model is a powerful system to study this human disease. Examination of FAP kindreds demonstrate that family members inheriting the same *APC* mutation may differ dramatically in tumor burden (Leppert et al., 1990). Although environmental factors may be partially responsible, modifier genes have been proposed to account for some of this variability (Paul et al., 1993; Giardiello et al., 1994). The use of a genetically defined system, such as the *Min* mouse model, facilitates the identification of these modifier loci. *Min* mice differ greatly in the number of intestinal tumors, depending on their genetic background (Moser et al., 1992; Dietrich et al., 1993). B6 mice heterozygous for the Apc^{Min} mutation exhibit an average of 28.5 polyps throughout their intestines, while their F1 progeny produced from crosses to either AKR/J (AKR), MA/MyJ (MA), or Mus castaneus (CAST) mice exhibit an average of only 5.8, 5.7, and 3.0 polyps, respectively (Moser et al., 1992; Dietrich et al., 1993). These studies indicate that at least one dominantly acting allele is modifying the influence of the Apc^{Min} mutation on polyp susceptibility. Quantitative trait loci (QTL) mapping subsequently identified a locus, *Mom1* (for modifier of *Min* 1), in the distal region of chromosome 4 between *D4Mit16* and *D4Mit13*. *Mom1* is estimated to account for ~50% of the genetic variation in adenoma number in both AKR and MA backcrosses as compared with B6 (Dietrich et al., 1993). Interestingly, *Mom1* also resides in a region of synteny with human chromosome 1p35, a region frequently associated with loss of heterozygosity in neuroblastomas and colon cancer (Martinsson et al., 1989; Leister et al., 1990).

It has not yet been determined whether the *Mom1* gene product acts autonomously or noncell autonomously with respect to the tumor cell lineage (Moser et al., 1993). Polymerase chain reaction (PCR) analysis has shown that 100% of spontaneous *Min*-induced intestinal adenomas have lost their wild-type *Apc* allele, suggesting that *Apc* acts as a classical tumor suppressor gene (Levy et al., 1994; Luongo et al., 1994). Immunocytochemical analysis of these adenomas has demonstrated that the intestinal

lesions consist of a mixture of differentiated enterocytes, enteroendocrine, goblet, and Paneth cells; these findings are similar to what is observed in human adenomatous polyps (Moser et al., 1992; Kirkland, 1988). The presence of multiple lineages in these adenomas suggests that a multipotent stem cell population located at the base of intestinal crypts is the site of initiation for Apc^{Min}-induced tumorigenesis. Therefore, any modifier gene expressed in the crypt microenvironment would have a potential role in altering tumor progression.

We report here the genetic mapping, expression, and sequence analysis of a candidate gene for the *Mom1* locus. The present data are consistent with the hypothesis that *Mom1* is encoded by the secreted form of type II nonpancreatic phospholipase A2 (*Pla2s*). Pla2s is a low molecular mass (14 KDa) phospholipase belonging to a diverse family of enzymes that hydrolyze the sn-2 fatty acyl ester bond of phosphoglycerides to produce free fatty acids and lysophospholipids (reviewed by Kudo et al., 1993; Dennis 1994). Pla2s is one of the enzymes involved in the production of arachidonic acid, which is the rate-limiting substrate for the production of leukotrienes and prostaglandins (reviewed by Glaser et al., 1993). Pla2s has also been implicated in the pathophysiology of acute pancreatitis, rheumatoid arthritis, and asthma (Kaiser et al., 1990). High levels of *Pla2s* expression have been detected in rat and murine small intestine; the *Pla2s* enzyme has been localized to Paneth cells at the base of the crypts of Lieberkuhn (Mulherkar et al., 1991, 1993). Expression of this enzyme coincides with the appearance of mature Paneth cells, suggesting the presence of *Pla2s* during the establishment and maintenance of the villus epithelium (Bry et al., 1994). To study further the role of *Pla2s* in development and pathogenesis, we determined its chromosome location in the mouse. We initially mapped *Pla2s* between the *D4Mit16* and *D4Mit13* loci, which is the same region of mouse chromosome 4 that contains the *Mom1* locus. Analysis of the *Pla2s* gene from inbred mouse strains revealed a 100% concordance between allele type and polyp number. Based on our mapping of *Pla2s* to distal chromosome 4, the correlation between specific *Pla2s* alleles and polyp susceptibility, and the expression of *Pla2s* at the tumor initiation site, we propose that *Mom1* encodes the gene for *Pla2s*. Hence, *Pla2s* is a genetically defined locus that can modify or influence the number of intestinal tumors resulting from a mutation in the *Apc* gene.

Results

Chromosomal Location of the Murine *Pla2s* Gene

An interspecific backcross mapping panel was used to localize *Pla2s* in the mouse. Genomic DNAs from AEJ/Gn and Mus spretus parental control mice were digested with 14 restriction endonucleases and analyzed by Southern blot hybridization using the *Pla2s* probe. An informative restriction fragment length polymorphism (RFLP) was detected with BamHI (see Experimental Procedures). The segregation pattern of the *Pla2s* gene was followed in 195 N2 progeny. The allele distribution pattern of *Pla2s* was compared with known gene and microsatellite markers that scan the entire mouse genome (A. M. B. and L. D. S., unpublished data). The segregation analysis revealed that the *Pla2s* gene resides on mouse chromosome 4. The specific location of *Pla2s* was determined by minimizing the number of multiple recombinations along the chromosome (Figure 1A). The results positioned *Pla2s* between *D4Mit148* and *D4Mit13* in the distal region of chromosome 4 (Figure 1B). The order of the loci and the ratio of the number of recombinants to the total number of N2 offspring examined are centromere–*D4Mit16* (19 of 183)–*D4Mit148* (1 of 170)–*Pla2s* (8 of 172)–*D4Mit13*–telomere. The genetic distance between loci in centiMorgans (± SEM) are centromere–*D4Mit16* (10.4 ± 2.2 cM)–*D4Mit148* (0.6 ± 0.6 cM)–*Pla2s* (4.6 ± 1.6 cM)–*D4Mit13*–telomere. The localization of *Pla2s* to the region between *D4Mit148* and *D4Mit13* coupled with the observation that *Pla2s* is expressed at high levels in the murine small and large

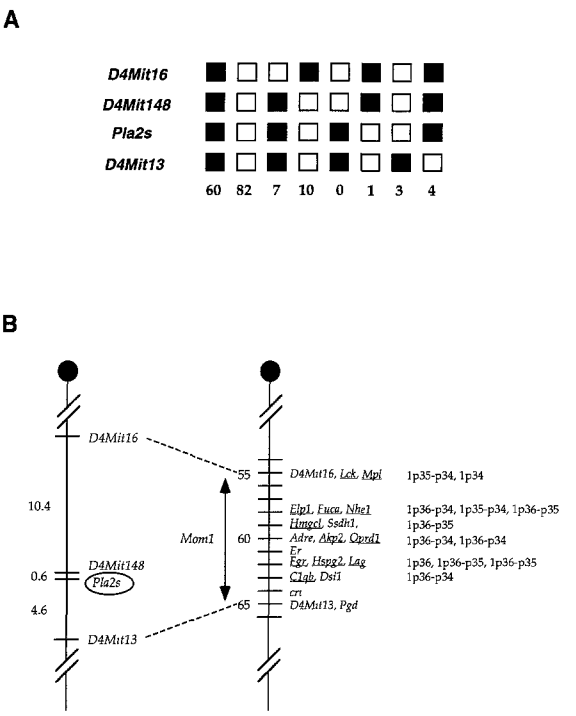

Figure 1. The *Pla2s* Gene Maps to Mouse Chromosome 4

(A) Haplotype analysis of the loci mapped is shown. The loci mapped are listed to the left, with the most proximal locus listed first. A total of 167 N2 progeny were typed for all markers. Each column represents the chromosome inherited from the F1 parent. Closed squares represent the AEJ/Gn allele. Open squares represent the M. spretus allele. The number of N2 progeny carrying each type of chromosome is listed below each column.

(B) Loci mapped in the interspecific backcross (left). Distances between the loci (in centiMorgans) are listed to the left. The interspecific backcross map is aligned (broken line) with the composite map of mouse chromosome 4 (right) (Lyon and Kirby, 1995). The predicted location of *Mom1* is shown by the double-headed arrow (Dietrich et al., 1993). Underlined loci have their human genome localization listed to the right.

intestine (Mulherkar et al., 1991, 1993) suggested *Pla2s* as a candidate for the *Mom1* locus.

Comparison of *Pla2s* Expression Levels between *Mom1*-Resistant and *Mom1*-Susceptible Strains

B6 mice carrying the Apc^{Min} mutation typically have an average of 28.5 ± 7.9 tumors (Moser et al., 1990). However, when B6 Apc^{Min}/+ mice were crossed with AKR, MA, or CAST mice, the F1 progeny inheriting the Apc^{Min} allele demonstrated a decrease in tumor burden (an average of 5.8 ± 4.3, 5.7 ± 4.0, and 3.0 ± 1.8 tumors, respectively) (Moser et al., 1992; Dietrich et al., 1993). QTL mapping suggested that the strains demonstrating resistance to tumor formation have a dominant modifier gene responsible for suppressing the *Min* phenotype (Dietrich et al., 1993). Thus, B6 mice are considered susceptible to multiple intestinal adenomas and carry a $Mom1^s$ allele, while AKR, MA, and CAST mice are resistant to multiple intestinal adenomas and carry a $Mom1^r$ allele. Therefore, we hypothesized that if *Pla2s* was involved, the level or quality of mRNA would differ between $Mom1^s$ and $Mom1^r$ strains. To test this hypothesis, we used Northern blot analysis to compare *Pla2s* expression in the intestines of B6 and AKR mice. *Pla2s* expression was observed in the small and large intestine of AKR mice, where an 800 bp transcript was detected after an overnight exposure to autoradiography film (Figure 2). In contrast, no transcript was detected in the small or large intestine of B6 mice after a similar exposure. However, upon longer exposure, a low level of expression was observed in the small and large intestine of B6 mice (data not shown). These results indicated that *Pla2s* expression was greatly reduced in the intestines of B6 mice compared with AKR mice, consistent with the candidacy of the *Pla2s* gene for the *Mom1* locus.

To explore further the candidacy of *Pla2s* for *Mom1*, we examined *Pla2s* expression in MA and CAST, two strains determined to contain the $Mom1^r$ allele (Dietrich et al., 1993). High levels of the 800 bp transcript were detected in the small and large intestines of these strains (Figure 2A). We next examined six additional strains to determine whether the absence of *Pla2s* expression was unique to B6. Northern blot analysis identified three strains, C3H/HeJ (C3H), CBA/J (Figure 2A), and DBA/2J (data not shown), with high levels of *Pla2s* expression in the large and small intestine. In contrast, four strains, P/J, A/J, C58/J (Figure 2B), and 129/SvJ (data not shown), were identified that had greatly reduced levels of *Pla2s*, similar to the expression level observed in B6 mice. Though none of these strains have been tested for their *Mom1* phenotype, we predict that C3H and CBA/J mice carry a $Mom1^r$ allele, while P/J, A/J, C58/J, and 129/SvJ mice carry a $Mom1^s$ allele. Consistent with this prediction, it was recently reported that 129/SvJ mice carrying a targeted mutation in the *Apc* gene develop high numbers of intestinal adenomas, indicating that 129/SvJ mice carry the $Mom1^s$ allele (Fodde et al., 1994; Laird et al., 1995). The reduced level of *Pla2s* expression observed in $Mom1^s$ strains compared with the high level of *Pla2s* expression observed in $Mom1^r$ strains is consistent with the hypothesis that the *Pla2s* gene represents the *Mom1* locus.

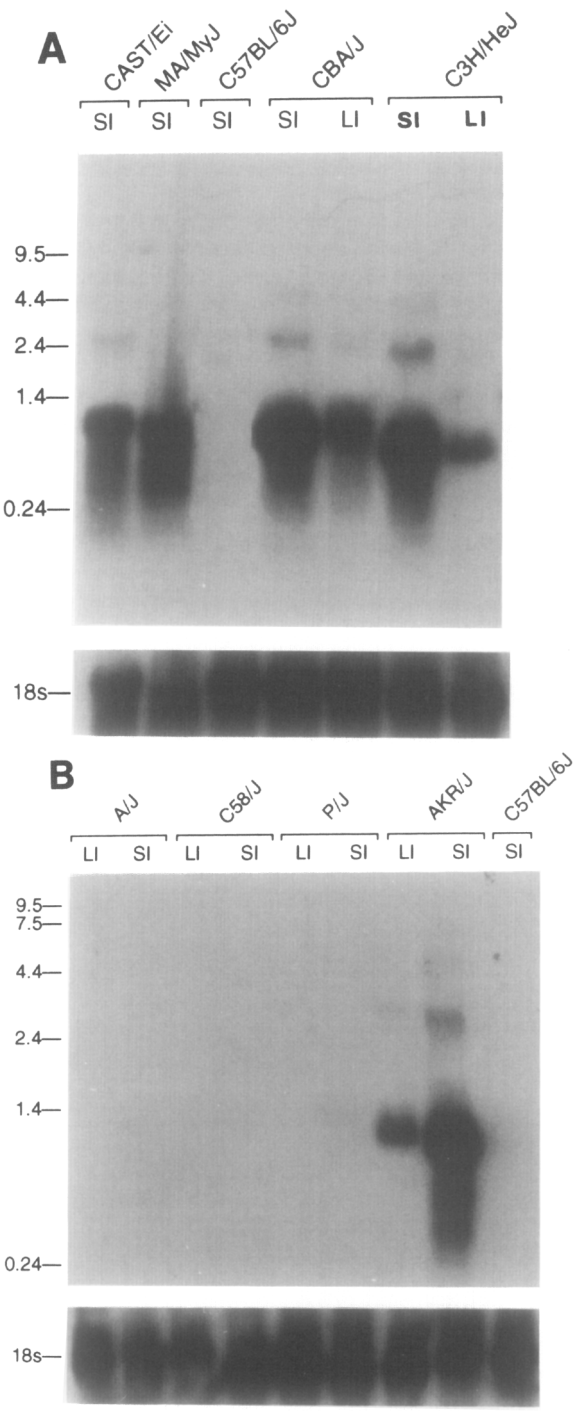

Figure 2. Expression of Intestinal *Pla2s* in Nine Inbred Mouse Strains

Expression of *Pla2s* mRNA in the small intestine (SI) and large intestine (LI) of nine inbred strains. Total RNA (20 μg) was subjected to Northern blot analysis. The membrane was hybridized to a *Pla2s* cDNA probe. After exposure, the membranes were rehybridized to 18S ribosomal RNA to demonstrate the presence of RNA in each lane. Size markers are shown to the left.

(A) Strains that exhibit high levels of *Pla2s* expression, with B6 mice included as a control.

(B) Strains that exhibit low levels of *Pla2s* expression, with AKR mice included as a control. Strains that expressed high levels of *Pla2s* consistently had higher expression levels in the small intestine compared with the large intestine. The 2.6 kb transcript is of unknown origin and was consistently detected in strains expressing *Pla2s*.

Detection of an RFLP Concordant with *Pla2s* Expression

The low levels of *Pla2s* expression observed in B6 mice could be the result of a mutation in the promoter region resulting in a decreased transcription rate or a mutation in the transcribed region resulting in a decreased half-life of the *Pla2s* mRNA. To distinguish between these possibilities, genomic DNA from B6 and AKR mice was digested with several restriction endonucleases to identify RFLPs for the *Pla2s* gene. Southern blot analysis identified three enzymes, BamHI, MspI, and TaqI, that revealed RFLPs between the B6 and AKR strains (data not shown). Digestion of genomic DNA with BamHI produced a 9.0 kb fragment in the B6 strain and fragments of 2.5 kb and 6.5 kb in the AKR strain (Figure 3). To assess whether this RFLP was linked to the expression of *Pla2s*, nine additional inbred strains were analyzed by RFLP analysis. Interestingly, the six inbred strains expressing high levels of *Pla2s* (AKR, MA, C3H, CBA/J, DBA/2J, and CAST) exhibited the 2.5 kb and 6.5 kb BamHI fragments, while the five inbred strains expressing low levels of *Pla2s* (B6, A/J, P/J, 129/SvJ, and C58/J) exhibited the 9.0 kb BamHI fragment (Figure 3). Thus, there is a 100% concordance between specific BamHI RFLPs and *Pla2s* expression levels. Neither the MspI nor the TaqI polymorphism was concordant with *Pla2s* expression (data not shown). These data indicated that the level of expression observed in the different strains was tightly linked to the BamHI RFLP. The lack of concordance with other identified RFLPs also suggested that the observed expression differences is not the result of a gross chromosomal rearrangement.

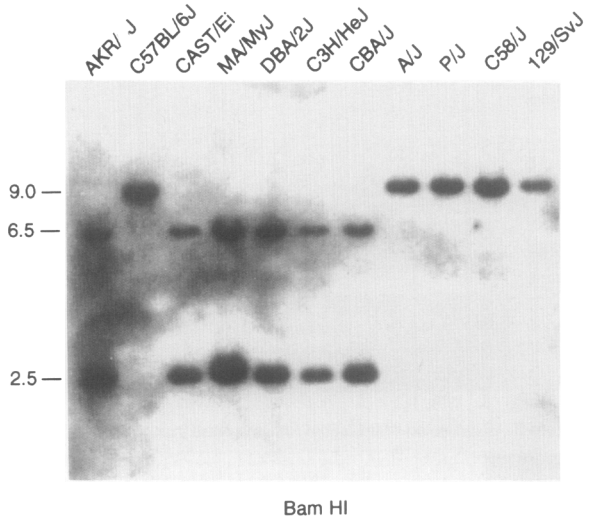

Figure 3. RFLP Analysis of the *Pla2s* Gene Reveals a BamHI Polymorphism Concordant with Expression Levels

Genomic DNA (5.0 μg/lane) from 11 inbred strains was digested with BamHI. The DNA was electrophoresed in a 0.8% agarose gel and transferred to a Hybond N$^+$ filter. Hybridization was carried out under high stringency with [^{32}P]dCTP-labeled mouse *Pla2s* cDNA carrying the entire coding sequence. The sizes of the identified restriction fragments are shown.

Sequence Analysis of the Murine *Pla2s* cDNA

To identify the molecular basis of the *Pla2s* RFLP detected between the AKR and B6 strains, mouse *Pla2s* cDNA was isolated from an ileal cDNA (B6 × C3H)F1 library (Crossman et al., 1994). Screening with a rat *Pla2s* cDNA probe (Ishizaki et al., 1989) yielded two positive clones, MPla2s-2 and MPla2s-6. Both clones were most likely derived from the C3H allele, since *Pla2s* expression is much higher in C3H mice than in B6 mice. Both clones contained the entire *Pla2s* open reading frame including the amino-terminal signal peptide (Figure 4). Clone MPla2s-6 also included a potential polyadenylation site closely followed by a stretch of adenines indicative of a poly(A)$^+$ tail. Sequence comparison indicated that the clones were 99% identical with the BALB/*c Pla2s* nucleotide sequence (Mulherkar et al., 1993; GenBank accession number X74266) and 86% identical with the rat *Pla2s* nucleotide sequence (Ishizaki et al., 1989; Komada et al., 1990).

Identification of an Insertion Mutation in *Mom1^s* Mice

Sequence comparisons among the mouse, rat, and human *PLA2S* cDNAs revealed the presence of a conserved BamHI site at position 207 (Figure 4) (Ishizaki et al., 1989; Kramer et al., 1989). We ascertained whether the BamHI site in the open reading frame (at position 207) was polymorphic between *Mom1^r* and *Mom1^s* strains. Reverse transcription PCR (RT–PCR) of total RNA from the small and large intestines of nine mouse strains (including B6 and AKR) was performed to determine the conservation of the BamHI site located at position 207 of the *Pla2s* gene. All the strains assayed, regardless of the *Pla2s* allele, produced the expected PCR product of ~720 bp (Figure 5). In addition to the 720 bp product, the inbred strains exhibiting the 9.0 kb BamHI allele produced a 610 bp PCR product (Figure 5).

Sequence analysis of the 720 bp RT–PCR product from AKR intestinal RNA and the 720 bp and 610 bp RT–PCR products from B6 intestinal RNA revealed that the BamHI site was present in the AKR product but was absent in the B6 products. A single base pair insertion was detected in the B6 strain within the BamHI site (Figure 6A). The insertion of a thymidine residue at this site results in the destruction of the BamHI site in the B6 strain and the production of the 9.0 kb restriction fragment (see Figure 3). The mutated sequence 5′-GGATTCC-3′ is detected in all strains containing the 9.0 kb BamHI allele and exhibiting reduced expression of *Pla2s*. The insertion results in a frameshift mutation that creates a stop codon 13 amino acids downstream in exon 4 (see Figure 4). In contrast, the RT–PCR product from the AKR strain contained the BamHI site that was observed in both C3H and BALB/c mice (see Figure 4; Mulherkar et al., 1993). Similarly, the BamHI site was detected in all inbred strains possessing the 2.5 kb and 6.5 kb BamHI fragments, consistent with this site causing the polymorphism.

Sequence analysis of the 610 bp B6 fragment revealed the presence of the thymidine insertion at the BamHI site located in exon 3. In addition, the 610 bp fragment was found to result from exon 3 splicing directly into exon 5,

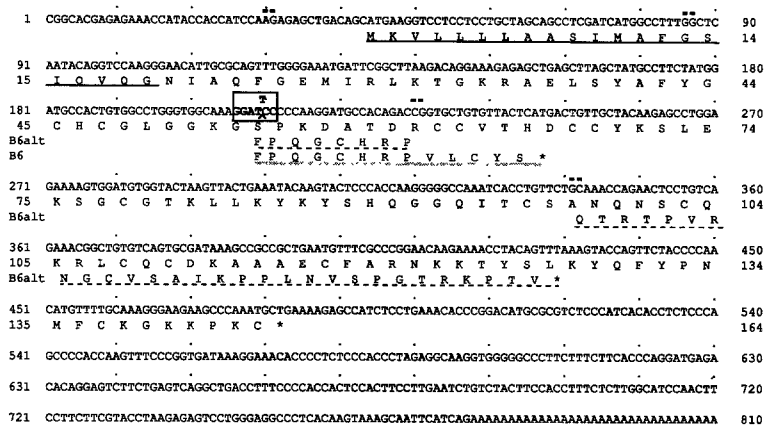

Figure 4. The Sequence of the Mouse *Pla2s* cDNA

The nucleotide and predicted amino acid sequences of mouse *Pla2s* are shown. The paired closed boxes above the nucleotide sequence denote intron/exon junctions as determined by genomic sequencing (data not shown). The single underlined region indicates the signal sequence. The boxed sequence denotes the BamHI site mutated in *Mom1*^{s} strains; the thymidine insertion is shown within the box. The predicted amino acid sequence of wild-type Pla2s is shown immediately below the nucleotide sequence. The two predicted proteins of *Mom1*^{s} strains are shown from the site of the thymidine insertion. The sequence indicated by the broken underline and labeled B6alt is the predicted peptide derived from the splicing of exon 3 into exon 5, thus the gap encompassing exon 4 (see Figure 5). The sequence indicated by the broken underline and labeled B6 is the truncated product generated from the correctly spliced mRNA.

with the exclusion of exon 4 (Figure 6B). To confirm the sequencing results, the 720 bp RT–PCR products from both B6 and AKR intestinal RNA were subjected to BamHI digestion. The RT–PCR product amplified from AKR intestinal RNA was digested into two smaller fragments of ~120 bp and 600 bp in length, while both the 720 bp and 610 bp fragments amplified from B6 mRNA failed to digest with BamHI (data not shown).

The Thymidine Insertion Is Present in Genomic DNA

To rule out posttranscriptional processing as a cause of the thymidine insertion in exon 3, we subjected genomic DNA from AKR and B6 mice to PCR using primers specific for exons 3 and 5 of the *Pla2s* gene. The resultant 2.2 kb products from AKR and B6 were sequenced. The insertion of a thymidine at the BamHI restriction site was observed in the B6 product, while a normal BamHI site was observed in the AKR product (Figure 6C). These results demonstrate that the insertion of a thymidine at the BamHI site is responsible for the RFLP identified by Southern blot analysis. Moreover, these results suggest that this mutation is responsible for the *Mom1*^{s} phenotype.

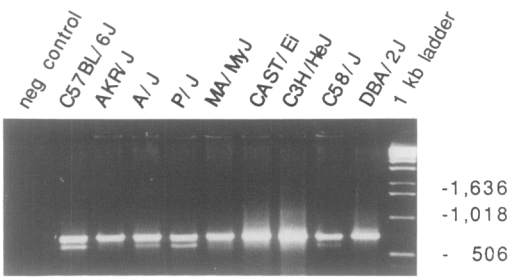

Figure 5. RT–PCR Analysis of Intestinal RNA Reveals the Presence of *Pla2s* in All Strains and an Alternatively Spliced Product in *Mom1*^{s} Strains

RT–PCR products derived from total RNA isolated from the small intestine of nine inbred strains listed at the top. PCR products were electrophoresed on 2.0% agarose gels and visualized with ethidium bromide. The primers used for PCR were 5′-GAAACCATACCACCATCCAA-3′ and 5′-CCAGGACTCTCTTAGGTACG-3′, which amplified nucleotides 11–747 of the murine *Pla2s* cDNA (Figure 4). The size marker is the 1 kb ladder (GIBCO–BRL, Gaithersburg, Maryland). The negative control (neg control) is made of the the same components as all other lanes except that no RTase was added.

Discussion

Mom1 and *Pla2s*

Genetic modifiers of cancer have long been predicted to exist. The generation of mouse models of human cancer coupled with QTL analysis have enabled the identification of these modifier loci. *Mom1* was shown to contribute ~50% of the genetic variance responsible for intestinal neoplasia in *Min* mice (Dietrich et al., 1993). The results described here suggest that allelic variants of the *Pla2s* gene are responsible for *Mom1* phenotypes. Genetic and molecular analysis demonstrated that *Pla2s* maps to the same chromosomal region as *Mom1* and that *Mom1*^{s} strains express a mutated form of *Pla2s*. We also determined the expression pattern of *Pla2s* in seven strains of mice not analyzed for their *Mom1* allele and found that they fell into two distinct classes: the first class was like the wild-type AKR pattern of expression, and the second class was like the mutant B6 pattern.

Probability theory can provide the likelihood that the two alleles of *Pla2s* would segregate with *Mom1* genotypes by chance. The B6 and 129/SvJ strains have the *Mom1*^{s} allele and the *Pla2s* mutation, whereas the AKR, MA, and CAST strains have the *Mom1*^{r} allele and a wild-type *Pla2s* gene. We have also found that the P/J strain carries the *Mom1*^{s} allele, based on the average number of tumors (34.8) present in F1 hybrids from a cross between (P/J × B6-*Apc*^{Min}/+) mice (M. M., L. D. S., and A. M. B., unpublished data). Six additional strains (A/J, CBA/J, C3H, C58/J, DBA/2J, and M. spretus) have been characterized for their *Pla2s* allele, but not their *Mom1* genotype (Figure 3). Thus, if the distribution of mutant and wild-type alleles

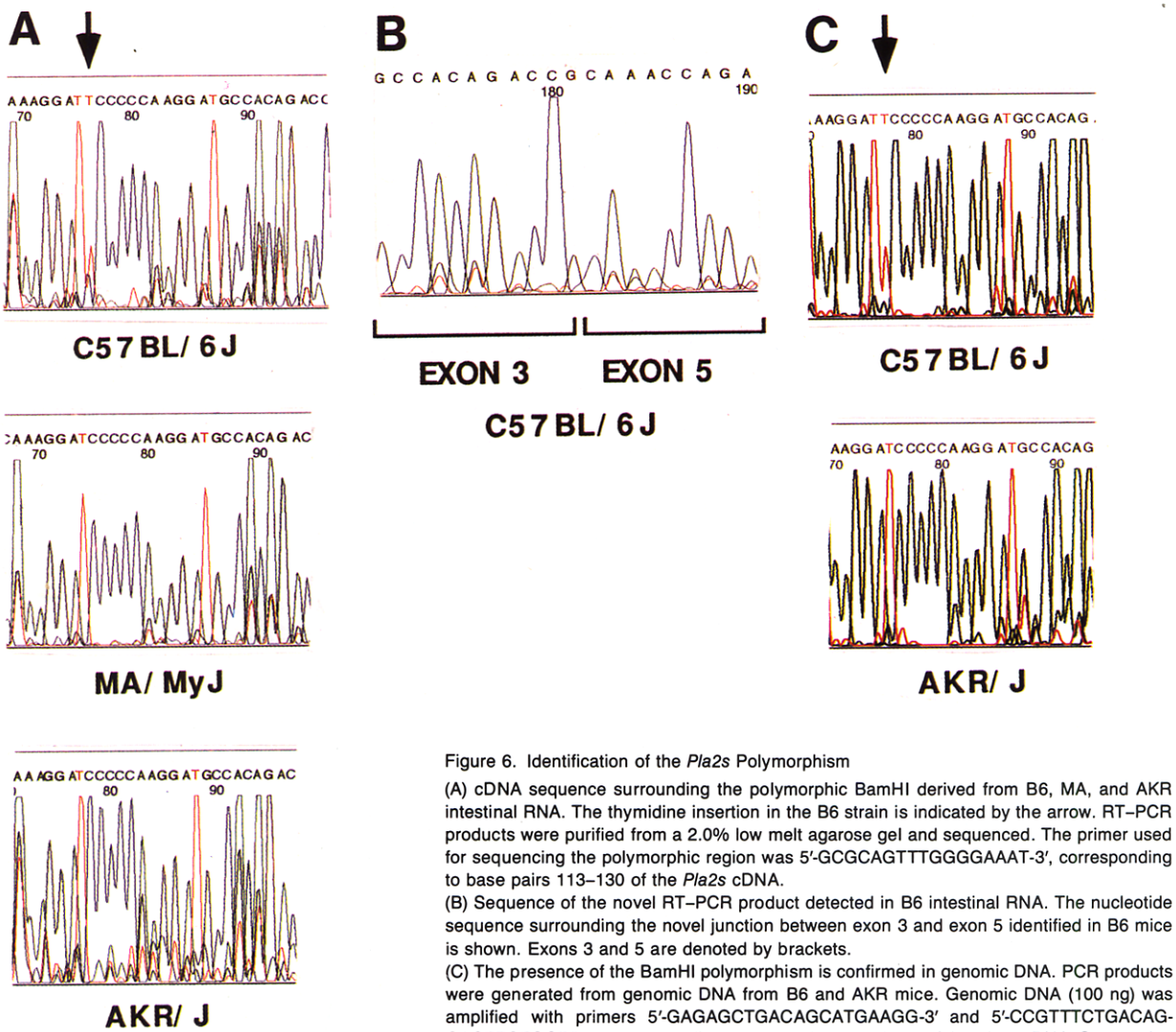

Figure 6. Identification of the *Pla2s* Polymorphism

(A) cDNA sequence surrounding the polymorphic BamHI derived from B6, MA, and AKR intestinal RNA. The thymidine insertion in the B6 strain is indicated by the arrow. RT–PCR products were purified from a 2.0% low melt agarose gel and sequenced. The primer used for sequencing the polymorphic region was 5′-GCGCAGTTTGGGGAAAT-3′, corresponding to base pairs 113–130 of the *Pla2s* cDNA.

(B) Sequence of the novel RT–PCR product detected in B6 intestinal RNA. The nucleotide sequence surrounding the novel junction between exon 3 and exon 5 identified in B6 mice is shown. Exons 3 and 5 are denoted by brackets.

(C) The presence of the BamHI polymorphism is confirmed in genomic DNA. PCR products were generated from genomic DNA from B6 and AKR mice. Genomic DNA (100 ng) was amplified with primers 5′-GAGAGCTGACAGCATGAAGG-3′ and 5′-CCGTTTCTGACAGGAGTTCTGGTT-3′, corresponding to base pairs 31 and 368 of the *Pla2s* cDNA. Sequencing was performed using the primer 5′-GCGCAGTTTGGGGAAAT-3′, corresponding to base pairs 113–130 of the *Pla2s* cDNA. The thymidine insertion in the B6 strain is indicated by the arrow.

reflects the population frequency of the mutation in all mouse strains, the frequency that by chance three *Mom1*^{s} strains would carry the mutant *Pla2s* allele and three *Mom1*^{r} strains would carry the wild-type *Pla2s* allele is (6/12)^6 or 1.56%. Thus, the odds are 63 to 1 that the association between *Mom1* genotype and *Pla2s* allele is not a random event, but rather represents a causal relationship.

Southern blot analysis revealed that the *Pla2s* expression differences detected between the inbred strains was concordant with a BamHI polymorphism. Cloning and sequencing of the *Pla2s* cDNA from both *Mom1*^{r} and *Mom1*^{s} strains revealed that the BamHI polymorphism mapped to the middle of exon 3. The polymorphism results from the insertion of a single thymidine residue in the BamHI site that causes a frameshift mutation resulting in a stop codon in exon 4 (Figure 4). Analysis of frameshift and nonsense mutations has revealed that these mutations often result in decreased steady-state levels of mRNA (Belgrader and Maquat, 1994), consistent with the dramatically lower steady-state levels of *Pla2s* mRNA (Figures 2A and 2B). In addition, analysis of RT–PCR products revealed that a second transcript is present in intestinal RNA isolated from mice carrying the *Mom1*^{s} allele (Figure 5). This second transcript results from the splicing of exon 3 into exon 5, skipping exon 4 (where the nonsense codon occurs) (Figures 4 and 6B). This alternative splicing still results in a +1 shift in the open reading frame, yielding a novel carboxyl terminus. The appearance of an alternatively spliced *Pla2s* transcript in B6 mice is consistent with reports that identified exon skipping due to the presence of nonsense codons within the deleted exon (Dietz et al., 1993; Belgrader and Maquat, 1994).

In *Mom1*^{s} strains, if the two alternative transcripts are translated, the resulting proteins would most likely be nonfunctional. The predicted product of the normally spliced transcript would result in the addition of a novel 13 amino acids followed by a stop codon (Figure 4, B6). This truncated protein would be missing 12 of the 14 conserved

cysteine residues critical for maintaining secondary structure and stability. The predicted product derived from the alternatively spliced form would contain a novel 37 amino acid carboxyl terminus (Figure 4, B6alt). This form would also lack enzymatic activity owing to loss of the structural integrity of the Pla2s protein.

These results indicate that the increased tumor susceptibility in *Mom1*^{s} strains is either due to the expression of abnormal Pla2s protein(s) or due to the lack of wild-type Pla2s enzyme activity. Since the resistance to tumor formation observed in hybrid F1 mice generated from a cross between *Mom1*^{s} and *Mom1*^{r} mice is dominant, the most likely hypothesis is that the presence of wild-type Pla2s enzyme activity confers resistance to multiple adenoma formation, while the truncated product(s) has no effect on tumor formation.

Pla2s and Inflammation

There is as yet no consensus as to how many *Pla2s*-related genes exist in the mammalian genome. Southern blot hybridization suggests that there is a single *Pla2s* locus in both human and rat genomes (Kramer et al., 1989; Komada et al., 1990). However, Johnson et al. (1990) identified two distinct sequences related to the human *PLA2S* gene. Furthermore, Chen et al. (1993) have sequenced a novel *Pla2s* gene expressed mainly in heart. These results suggest that there exists multiple forms of *Pla2s* in the mammalian genome. *Pla2s* has been widely implicated in being instrumental in the inflammatory process (reviewed by Pruzanski and Vadas, 1991; Mukherjee et al., 1992). However, our results indicate that several inbred strains lack this enzyme activity (B6, A/J, C58/J, P/J, and 129/SvJ). To our knowledge, no data have been presented that indicate that *Mom1*^{s} mice have an altered or impaired inflammatory response. A comparison of the inflammatory process between *Mom1*^{s} and *Mom1*^{r} strains would further elucidate the role of *Pla2s* in inflammation and arthritis.

Nonsteroidal Anti-Inflammatory Drugs and Colon Cancer

Pla2s is one of several enzymes involved in generating arachidonic acid and lysophosphatidic acid. Arachidonic acid is the rate-limiting substrate for the generation of prostaglandins and leukotrienes (reviewed by Glaser et al., 1993). Although previous studies have suggested the potential roles of phospholipase C and prostaglandins in the development of intestinal adenomas, no evidence implicating Pla2s has been found (Rao et al., 1995). In addition, numerous studies in both human and rodent have demonstrated that nonsteroidal anti-inflammatory drugs (NSAIDs) confer a protective effect in the generation of colon adenomas. The mechanism of action of NSAIDs such as Sulindac is presumed to be by inhibiting cyclooxygenase, resulting in a decrease in prostaglandin production (reviewed by Marnett 1992; Thun, 1994). Studies have determined that Sulindac significantly decreased the levels of prostaglandin E2 in colonic mucosa and tumors (Rao et al., 1995). Thus, it has been hypothesized that the decrease of specific prostaglandins contributes to tumor protection and polyp regression. Since our results suggest that increased levels of Pla2s can also yield a protective effect, it suggests that Sulindac and other NSAIDs might ameliorate polyp formation by a more complicated mechanism.

The Role of Pla2s in Modifying Tumor Susceptibility

One potential hypothesis for the mode of action of Pla2s might be in its role in lipid homeostasis. Though it is believed that pancreatic type I Pla2s is responsible for the digestion of fatty acids, the presence of high quantities of type II Pla2s in the intestine is suggestive of a role in the digestion of dietary fats. There is a strong positive correlation between fatty acid intake and an increased susceptibility to colon cancer (reviewed by Reddy, 1993). Thus, dietary lipids might interact with the intestinal villi to stimulate the formation of aberrant crypts. Therefore, the presence of high levels of Pla2s in the intestine could provide a protective effect by inactivating harmful effects of dietary fatty acids. Alternatively, Pla2s may be important for maintaining normal intestinal flora; different types of dietary fat are associated with alteration of the normal intestinal flora. Diets high in saturated fatty acid are associated with increased amounts of the anaerobic bacteria, Bacteroides (Pence, 1985). It has recently been demonstrated that purified type II Pla2s from mouse intestines possesses potent bactericidal activity (Harwig et al., 1995). Since Pla2s is secreted from Paneth cells and these cells secrete many bactericidal proteins, it is not surprising that Pla2s has a role in microbial defense mechanisms. Thus, *Mom1*^{r} strains have high levels of Pla2s in their intestinal lumen that helps protect or control intestinal flora (or both), while *Mom1*^{s} strains lack sufficient levels of Pla2s to affect bactericidal activity. Bacteroides and other anaerobic bacteria have been proposed to produce toxins or convert bile salts to carcinogenic products (van Tassell et al., 1990; Rumney et al., 1993). Thus, the lack of Pla2s in the intestine of B6 mice allows for the proliferation of certain types of bacteria that produce carcinogenic products that facilitate polyp formation and transformation.

Alternatively, murine intestinal Pla2s was independently identified as enhancing factor (Deo et al., 1983; Mulherkar et al., 1993). Enhancing factor was isolated as a low molecular mass heat and acid stable polypeptide that enhanced the binding of epidermal growth factor to cells. Pla2s was also found to induce DNA synthesis and proliferation independent of arachidonate products (Kurizaki et al., 1992). Thus, the presence of high levels of Pla2s in intestinal crypts might provide an environment that would inhibit the formation of transformed crypts owing to the enhanced accessibility of growth factors.

A final potential mechanism of Pla2s action is its role in maintenance of membrane asymmetry. Pla2s activity requires millimolar concentrations of Ca^{2+}, suggesting that Pla2s must be released into the extracellular environment to become fully active. In addition, the specificity of Pla2s in the microenvironment is mediated by membrane asymmetry (Kudo et al., 1993); specifically, Pla2s has a preference for the head group of anionic phospholipids (i.e., phosphatidylserine) and phosphatidylethanolamine located on the external membrane. Normal colonic epithelium has phosphatidylcholine on its external leaflet, a

phospholipid that is a low affinity substrate of Pla2s (Fourcade et al., 1995). Taken together, these data suggest that Pla2s does not catalytically attack the membrane phospholipids of normal intestinal epithelium. In contrast, bacterial cell membranes, comprised mostly of phosphatidylglycerol, are a prime target for Pla2s action, hence its bactericidal properties (see above). However, loss of the membrane asymmetry in intestinal epithelium could lead to increased accessibility of cell membrane phospholipids to Pla2s enzymatic activity. Colon carcinoma cells have elevated levels of phosphatidylserine on their external leaflet, providing a suitable target for Pla2s digestion (Utsugi et al., 1991). If the membrane phospholipid shift occurs at an early point in the transformation of intestinal epithelium, then high levels of Pla2s in the intestine may provide a protective function by eliminating aberrant crypt cells while low levels may allow unbridled growth of transformed cells.

Prospectus

Pla2s represents a novel class of genes that influence tumor susceptibility. Several mechanisms to explain how Pla2s activity could contribute to tumor resistance have been proposed. Regardless of the mechanism, it is probable that the mode of protection is noncell autonomous, since Pla2s is most active in the intestinal lumen and not within the cell. Recently, Laird et al. (1995) identified another modifier of murine intestinal cancer that is intrinsic to the transformed cell. This modifier confers tumor resistance by causing DNA hypomethylation (Laird et al., 1995). The identification of this cell-autonomous modifier was accomplished through the use of gene targeting and drug treatment in mice. The proposed models for Pla2s action are testable through the use of mouse mutants and through experimental manipulation of the diet and drug treatment of *Mom1*^{s} and *Mom1*^{r} strains. The ultimate proof of *Pla2s* identity with *Mom1* will be shown by expressing a wild-type copy of the *Pla2s* gene within the microenvironment of the intestinal crypts in *Mom1*^{s} strains and demonstrating a reduction in the number of adenomas or by targeted ablation of the *Pla2s* gene in *Mom1*^{r} strains.

The human homolog of *Pla2s* has been localized to chromosome 1p35 (Johnson et al., 1990), a region that exhibits loss of heterozygosity in a variety of neoplasms (Martinsson et al., 1989; Leister et al., 1990). However, owing to the proposed noncell-autonomous mode of action for Pla2s, it is unclear as to how loss of *PLA2S* in tumor cells would contribute to neoplasia. Thus, one would not expect to find loss of heterozygosity at the *MOM1* locus in human or murine tumors.

The identification of *Pla2s* as a candidate gene for the *Mom1* locus has important implications in human cancer. One possibility of *PLA2S* involvement may be reflected by the variable number of adenomas identified among FAP family members inheriting the same *APC* mutation. This variation in tumor burden could be attributable to the segregation of different *PLA2S* alleles. In addition, population surveys would be useful for both determining the extent of *PLA2S* allelic variation as well as for identifying individuals at risk for developing intestinal cancer. Finally, the identification of *Pla2s* as a candidate for a major modifier of intestinal polyp formation may provide a missing link between high fat diets and increased incidence of colon cancer.

Experimental Procedures

Mice

The chromosomal location of *Pla2s* was determined by interspecific backcross analysis of [(AEJ/Gn-*a bp*^{H}/*a bp*^{H} × Mus spretus)F1 × AEJ/Gn-*a bp*^{H}/*a bp*^{H}] mice (Argeson et al., 1995). Additional inbred strains were purchased from The Jackson Laboratory (Bar Harbor, ME).

Probes

Probes used to map the mouse *Pla2s* gene and screen murine cDNA libraries were derived from a 758 bp rat cDNA fragment cloned into the EcoRI site of a pGEM vector. The pGEM vector containing the rat *Pla2s* gene was a gift from Dr. J. Ishizaki (Ishizaki et al., 1989; Shionogi Research Laboratories, Osaka, Japan). A 456 bp fragment of the rat *Pla2s* coding region was amplified by the PCR using primers 5′-ATGAAGGTCCTCCTGTTG-3′ and 5′-CAGAGAGTGTCTTTTCAGC-3′. The probe was radiolabeled with [α-^{32}P]dCTP using a random prime kit (Boehringer Mannheim, Indianapolis, IN). The *Pla2s* probe hybridized to a 9.0 kb BamHI fragment in the M. spretus parental strain and a 2.5 kb BamHI fragment in the AEJ/Gn parental strain.

cDNA Cloning

Murine *Pla2s* clones (MPla2s1-6) were isolated from a (B6 × C3H)F1 ileal cDNA library made from the terminal 3.0 cM of the small intestine (Crossman et al., 1994); the library was a gift from Dr. J. Gordon (Washington University School of Medicine, St. Louis, MO). Clones were isolated by standard techniques (Sambrook et al., 1989). A 780 bp fragment of the clone was PCR amplified using T3 and T7 primers. PCR products were purified by electrophoresis on a low melt agarose gel and radiolabeled as above.

SSLP Analysis

DNA primers for SSLP analyses were made (Ma et al., 1993). Genomic DNA isolation and agarose gel electrophoresis were as described previously (Siracusa et al., 1987). Primers and fragment sizes for *D4Mit13*, *D4Mit16*, and *D4Mit148* were as described previously (Dietrich et al., 1992). The resulting *D4Mit148* fragments were visualized by EtBr staining of 3% agarose gels, while 10% and 5% polyacrylamide gels were used for *D4Mit13* and *D4Mit16*, respectively.

DNA Isolation and Southern Blot Analysis

Genomic DNAs were isolated from tail biopsies as described previously (Siracusa et al., 1987). DNA (5.0 μg) was digested with the appropriate restriction endonucleases according to the directions of the supplier, fractionated on 0.8% agarose gels, and transferred to Hybond N$^+$ nylon membranes (Argeson et al., 1995). Hybridization to [α-^{32}P]dCTP-labeled probes was as described previously (Church and Gilbert, 1984). Membranes were washed in 1 × SSCP, 0.1% SDS at 65°C and exposed to autoradiography film at −70°C.

RNA Isolation and Northern Blot Analysis

Total mouse RNA was isolated as described previously (Ma et al., 1993). Total RNA (20 μg) was size-fractionated on 1.0% formaldehyde–agarose gels (Sambrook et al., 1989). Northern blots were transferred and hybridized as described above.

RT-PCR and Sequence Analysis

First-strand cDNA was synthesized from 1.0 μg of total RNA using oligo(dT)$_{15}$ and MoMLV RTase (Sambrook et al., 1989). PCR was performed, as described above, using primers specific for murine *Pla2s*. Double-stranded cDNA products were purified using QIAquick PCR purification columns (Qiagen, Chatworth, CA), dried, and resuspended in 10 μl of H_2O.

Long-range PCR of genomic DNA fragments was performed using the KlenTaq I and polymerase II kit (Ab Peptides, St. Louis, MO). PCR amplification of genomic DNA (100 ng) was 25 cycles at 95°C for 5 s, 65°C for 30 s, and 68°C for 7 min. PCR products were purified as

described above. Sequencing reactions of the PCR products were performed using dideoxy terminator reaction chemistry and analyzed on an Applied Biosystems model 373 DNA sequencer.

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