GENOMICS 28, 280–285 (1995)

Mapping of the 75-kDa Inositol Polyphosphate-5-Phosphatase (*Inpp5b*) to Distal Mouse Chromosome 4 and Its Exclusion as a Candidate Gene for *dysgenetic lens*

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Received March 8, 1995; accepted May 15, 1995

We have determined the chromosomal localization of the murine gene encoding a 75-kDa inositol polyphosphate-5-phosphatase (*Inpp5b*). Using two independent approaches, *fluorescence in situ* hybridization and interspecific backcross analysis, we show that *Inpp5b* maps to distal mouse Chromosome 4. This map position is within the conserved linkage group corresponding to the short arm of human Chromosome 1, where the human homologue, *INPP5B*, has been shown to map previously. The position of *Inpp5b* on mouse Chromosome 4 is in the vicinity of the mouse developmental mutation *dysgenetic lens* (*dyl*). However, using a genetic approach, we show that *Inpp5b* maps distal to *dyl* on mouse Chromosome 4.

INTRODUCTION

Cellular stimulation by various agonists leads to the production of inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by the hydrolysis of membrane-bound 4,5-phosphatidyl inositol phosphate. IP_3 causes the subsequent release of calcium from intracellular stores, while DAG activates protein kinase C (Berridge, 1993). IP_3 can be further phosphorylated to IP_4, which is thought to be involved in regulating calcium entry at the cytoplasmic membrane (Berridge, 1993). These inositol polyphosphate signals can be inactivated by dephosphorylation, thus converting IP_4 and IP_3 into IP_3 and IP_2, respectively (Bansal and Majerus, 1990). These dephosphorylated products are not capable of Ca^{2+} release. This reaction is mediated by inositol polyphosphate-5-phosphatases, and thus the 5-phosphatases serve to terminate the IP_3/IP_4 signals. Cytoplasmic and membrane-bound 5-phosphatases have been described; they appear to predominate in the particulate fraction although the ratio of membrane-bound to cytoplasmic seems to vary from tissue to tissue (Shears, 1989; Laxminarayan *et al.*, 1994). How this partitioning relates to their cellular function is unknown. At least three different types of inositol polyphosphate 5-phosphatases have been described. This classification is based on differences in V_{max} and K_m for their substrates IP_3 and IP_4, as well as their elution properties in an anion exchange column. The type I enzyme has a molecular mass of ~45 kDa, hydrolyzes both IP_3 and IP_4, and has a higher affinity but a lower V_{max} for IP_4. Membrane and cytosolic forms have been described (Takimoto *et al.*, 1989; Laxminarayan *et al.*, 1993). These share immunological and physical properties, but it is unclear whether they are products of the same locus. The type II enzyme (nomenclature according to Irvine, 1992) has an affinity only for IP_3. It was first isolated from bovine brain (Hansen *et al.*, 1987) and has an approximate molecular mass of 115 kDa. The type III enzyme was first isolated from human platelets (Connolly *et al.*, 1985; Mitchell *et al.*, 1989); it has a molecular weight of 75 kDa, and its affinities and activities for IP_3 and IP_4 are similar to those of the type I enzyme (Mitchell *et al.*, 1989). The type III enzyme can also hydrolyze phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 4-phosphate (Matzaris *et al.*, 1994), an activity not reported for types I and II.

The genes for at least two of these enzymes have been cloned. The gene for the 45-kDa enzyme (*INPP5A*) has been cloned from a human placental cDNA library (Laxminarayan *et al.*, 1994) as well as from a canine thyroid cDNA library (Verjans *et al.*, 1994). The gene (*INPP5B*) for the 75-kDa enzyme was cloned from human placental and megakaryocytic cDNA libraries (Ross *et al.*, 1991). Lowe syndrome (*OCRL*) is a rare X-linked developmental disorder, and the affected gene,

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0888-7543/95 $12.00

OCRL1, is highly homologous to *INPP5B* (53% identity) and thus might encode a protein with a similar enzymatic activity. Due to the high sequence homology between these two genes, it is of interest to determine the chromosomal location of *INPP5B. OCRL* maps to Xq25–q26 (Reilly *et al.,* 1990), while *INPP5B* has been shown to map to 1p34 (Jänne *et al.,* 1994), and thus these two genes are products of two different loci. The murine homologue of *OCRL, Ocrl,* also maps to the X chromosome (Jänne and Nussbaum, unpublished observation), whereas the murine homologue of *INPP5B, Inpp5b,* has not been mapped previously. Furthermore, it is possible that mutations in *Inpp5b* or the genes for the other inositol polyphosphate-5-phosphatases are responsible for other genetic disorders in either human or mouse.

In this report we determine the fine map position of the murine homologue of *INPP5B, Inpp5b,* to be distal mouse Chromosome 4 (MGD Accession #MGD-CREX-336). Interestingly, this gene maps in the vicinity of a mouse locus for congenital cataracts, *dysgenetic lens* (*dyl*) (Sanyal *et al.,* 1986). *dyl* is an autosomal recessive developmental mutation that arose spontaneously in a BALB/cHeA colony (Sanyal and Hawkins, 1979) and causes cataracts. Since *INPP5B* is 53% identical to *OCRL1* and since mutations in *OCRL1* cause cataracts in humans, we hypothesized that *Inpp5b* could be the gene responsible for *dyl.* However, in this paper we present evidence that *Inpp5b* maps close to but is genetically distinct from *dyl* (MGD Accession #MGD-CREX-337).

MATERIALS AND METHODS

Isolation and mapping of genomic Inpp5b clones. To map and characterize the *Inpp5b* genomic locus, a 1.4-kb partial mouse cDNA clone of *Inpp5b* (Jänne *et al.,* 1994) was used as a probe to screen a 129Sv cosmid library (Stratagene, La Jolla, CA). Screening was performed using standard techniques, and clones were purified through tertiary screening (Sambrook *et al.,* 1989). Eight positive clones were identified and characterized by restriction mapping and Southern blotting.

Chromosome localization of Inpp5b by interspecific backcross. C3H/HeJ-*gld* and *Mus spretus* (Spain) mice [(C3H/HeJ-*gld* × *M. spretus*)F_1 × C3H/HeJ-*gld*] interspecific backcross mice were bred and maintained as previously described (Seldin *et al.,* 1988). *Mus spretus* was chosen as the second parent in this cross because of the relative ease of detection of informative restriction fragment length variants (RFLV) in comparison with crosses using conventional inbred laboratory strains. DNA isolated from the mouse organs by standard techniques was digested with restriction endonucleases, and 10-μg samples were separated by electrophoresis in 0.9% agarose gels. DNA was transferred to Nytran membranes (Schleicher & Schull, Inc., Keene, NH), hybridized at 65°C, and washed under stringent conditions, all as previously described (Sambrook *et al.,* 1989). For *Inpp5b,* the 1.4-kb cDNA (as described above) was used as a probe. Other clones used as probes in the current study and RFLVs that detect the Lmyc proto-oncogene locus, *Lmyc1,* and the glutamate receptor-7 locus, *Grik5,* were previously described (Gregor *et al.,* 1993). Gene linkage was determined by segregation analysis (Green, 1981). Gene order was determined by analyzing all haplotypes and minimizing crossover frequency between all genes that were determined to be within a linkage group. This method resulted in determination of the most likely gene order (Bishop, 1985).

Mapping of Inpp5b by fluorescence in situ hybridization (FISH). One of the cosmids isolated (as described above), cMIN5P, was purified through a cesium chloride gradient (Sambrook *et al.,* 1989) and used as a probe for the FISH studies. Approximately 150 ng of biotinylated cosmid DNA was precipitated with total mouse genomic DNA (6 μg) and sonicated salmon sperm DNA (12 μg). This mixture was dissolved overnight in the hybridization solution containing 50% formamide and 2× SSC (Hybrisol VII, Oncor, Gaithersburg, MD). Metaphase chromosome spreads were made from mouse spleen lymphocytes (Nagle *et al.,* 1994), which incidentally harbored a Robertsonian (6:16) translocation (purchased from The Jackson Labs, Bar Harbor, ME). The hybridization was carried out as previously described (Nagle *et al.,* 1994), and detection of the signal was carried out using fluorescein-labeled (FITC) avidin and anti-avidin (Oncor). After counterstaining with propidium iodide and diamidinophenylindole (DAPI) in *p*-phenylenediamine (1 mg/ml), the slides were analyzed using a triple bandpass filter (DAPI/FITC/rhodamine) for chromosome identification and a dual wavelength filter (FITC/rhodamine) for signal visualization.

Southern, Northern, and RT-PCR analysis of normal and dysgenetic lens (dyl) mice. Genomic DNA was isolated from livers of BALB/cJ, C57BL/6J, and *dyl* (in a BALB/cHeA background) mice (all purchased from The Jackson Laboratories) as described above. The DNAs were digested with 18 different restriction endonucleases, and the fragments separated by electrophoresis in 0.85% agarose and transferred onto nylon membrane (Hybond-N, Amersham) as previously described (Sambrook *et al.,* 1989). The membranes were hybridized with the 1.4-kb *Inpp5b* cDNA, washed at high stringency, and exposed to X-ray film for 24–36 h at −70°C. Total RNA was isolated from the brains of C57BL/6J and *dyl* mice using the guanidinium isothiocyanate method (RNA STAT-60, Tel-Test Inc.) followed by DNase I treatment (Boehringer Mannheim). Fifteen micrograms of RNA was separated by electrophoresis in an agarose formaldehyde gel, transferred to nylon membrane (GeneScreen, NEN, Boston, MA), and hybridized to the *Inpp5b* cDNA as previously described (Sambrook *et al.,* 1989). For RT-PCR analysis, 1 μg of total RNA was reverse transcribed using MMLV-RT (Gibco–BRL) with primer INPRT (5′ ATG TCT TCA CAA GTC AGC ATG 3′) at 37°C for 1 h under the manufacturer's suggested conditions. Following reverse transcription, 10% of the mixture was taken for PCR analysis. This PCR was nested using primers INPF (5′ GAT AGA AGA GCT GGA TGT GGG 3′) and INPR (5′ AGG TCT AGG CTC AGG TAG AAG AAA C 3′). The PCR was performed in a Perkin Elmer thermal cycler for 30 cycles (94°C 1 min, 56°C 1 min, 72°C 2 min). The PCR products were separated by electrophoresis in a 1.3% agarose gel and visualized with ethidium bromide.

Identification, isolation and characterization of $(CA)_n$ repeat regions from the Inpp5b genomic locus. To identify the presence of $(CA)_n$ repeat regions, a poly(TG) probe (Pharmacia) was used to screen a Southern blot of cMI5P that had been digested with various restriction endonucleases. Hybridization was carried out at 65°C using Church buffer (0.5 *M* sodium phosphate, pH 7.2, 7% SDS, 1% BSA, 1 m*M* EDTA) for 16 h. The blot was washed to high stringency and exposed to X-ray film at −70°C for 5 h. Positive fragments were subcloned and sequenced using the ABI automated sequencer with fluorescent dideoxy terminators. Two $(CA)_n$ were found, and their location with respect to the body of the gene was determined. PCR primers (forward, 5′ TGC AGT GCA CAT ACA CAT ATG C 3′; reverse, 5′ ACT GCC TTA GGT GTT GTT CCA 3′) flanking one of these repeats, INPCA1, were made to determine whether it is polymorphic between various inbred mouse strains. DNA samples for strains DBA/2J, SJL/J, and *Mus musculus castaneus*/Ei (CAST/EI) were provided by M. Bartolemei. The forward primer was end labeled using [γ-^{32}P]ATP. PCR was performed using ~20 ng of genomic DNA from various mouse strains for 30 cycles (95°C 15 s, 56°C 30 s, 72°C 30 s). The products were electrophoresed in an 8% acrylamide/urea gel, which was then exposed to X-ray film at −70°C.

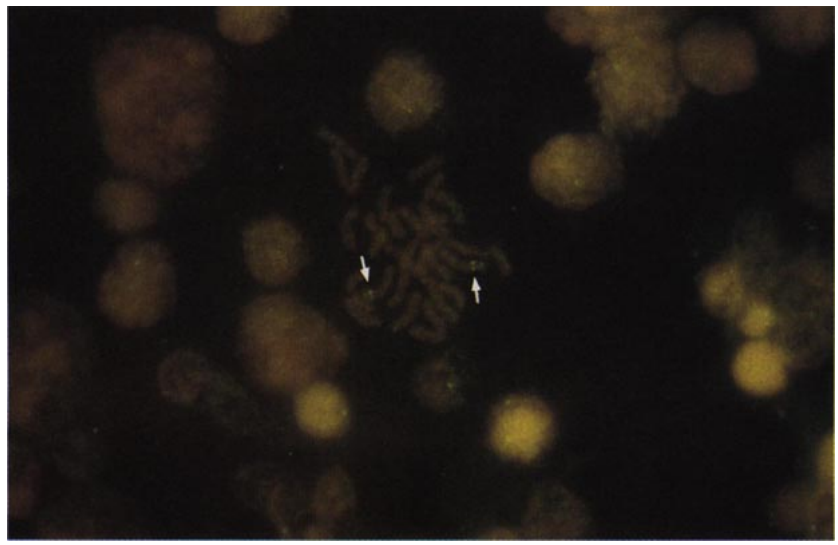

FIG. 1. Metaphase from Rb (6:16) mouse following fluorescence *in situ* hybridization with the biotinylated cosmid cMI5P. The arrows mark the positions of the hybridization signals, which can be seen on both chromatids on each of the chromosomes. Fluorescent banding from the DAPI/propidium iodide mixture localized the hybridization signal to mouse Chromosome 4D2 (data not shown).

Breeding of dyl mice; phenotypic and PCR analysis of N_2 affected mice. Male $dyl^{-/-}$ mice were crossed to normal C57BL/6J females. The resultant female F_1 mice ($dyl^{+/-}$) were then backcrossed to the original male $dyl^{-/-}$ parents (N_2 generation). All N_2 offspring were analyzed for the presence of cataracts at 3–5 weeks of age. The eyes of the mice were dilated with 1% atropine (Johnson & Johnson) and then examined using a slit lamp ophthalmoscope. Criteria for deciding whether a mouse was affected were based on previous phenotypic characterizations (Sanyal and Hawkins, 1979). Mice not exhibiting complete cataractous degeneration and/or extrusion of the lens nucleus were excluded from the study. DNA was extracted from the tails of the affected N_2 mice as described previously (Hogan *et al.*, 1986), and PCR was performed using primers flanking INPCA1 as described above. Additional PCR analysis was performed using primers (MapPairs) obtained from Research Genetics (Huntsville, AL) under the manufacturer's suggested conditions.

RESULTS

Screening of 2.4×10^5 colonies from the 129Sv mouse cosmid library yielded eight positive clones through tertiary screening. These clones were further analyzed by restriction mapping and Southern blotting and were determined to be identical (data not shown). One of these clones, cMI5P, was used for the remainder of the studies. This clone was biotin labeled and used as a probe in FISH, which localized the gene to mouse Chromosome 4 (Fig. 1). Further localization to band D2 of Chromosome 4 was performed by the fluorescent Q-banding revealed by the DAPI and propidium iodide counterstaining (data not shown).

To obtain a more accurate localization of *Inpp5b* on distal Chromosome 4, we performed analysis on a panel of DNA samples from a mouse interspecific backcross. This panel has been characterized for over 800 genetic markers throughout the genome. The genetic markers included in this map span between 50 and 80 cM on each mouse chromosome and the X chromosome (for example see Saunders and Seldin, 1990; Watson *et al.*, 1992). Initially, DNA from the two parental mice [C3H/HeJ-*gld* and (C3H/HeJ-*gld* x *M. spretus*)F_1] were digested with various restriction endonucleases and hybridized with the *Inpp5b* cDNA probe to determine restriction fragment length variants to allow haplotype analyses. Informative RFLVs were *Msp*I-digested parental DNAs: C3H/HeJ-*gld*, 4.0, 3.0, and 2.2 kb; *M. spretus*, 4.5, 3.5, and 1.6 kb.

All three of the *M. spretus* restriction endonuclease fragments detected with the *Inpp5b* probe cosegregated in the 114 interspecific backcross mice, indicating that the bands represented closely linked sequences in the mouse genome. Comparison of the haplotype distribution of *Inpp5b* with those determined for loci throughout the mouse genome indicated that in 111 of 114 meiotic events examined this locus cosegregated with the gene *Lmyc1* (Fig. 2), a locus previously mapped to mouse distal Chromosome 4 (Abbott *et al.*, 1992). The best gene order (Bishop, 1985) ± the standard deviation (Green, 1981) indicated the following relationships: (centromere)–*Lmyc1*–2.6 ± 1.4 cM–*Inpp5b*–4.4 ± 1.9 cM–*Grik5*.

The position of *Inpp5b* on Chromosome 4 is located near the mouse mutation *dysgenetic lens* (*dyl*). *dyl* was originally mapped distal to two markers, *b* (brown) and *Mup-1* (major urinary protein-1), and maps approximately 12 cM distal to *b* and 20 cM distal to *Mup-1* (Sanyal *et al.*, 1986). This mapping study places *dyl* near but proximal to *Lmyc1* on Chromosome 4. Since this mapping was performed with two widely spaced markers and since a 53% identical gene at the amino acid level (*OCRL1*) causes cataracts in humans, we wanted to test the possibility that *dyl* and *Inpp5b* are the same locus. Southern, Northern, and RT-PCR anal-

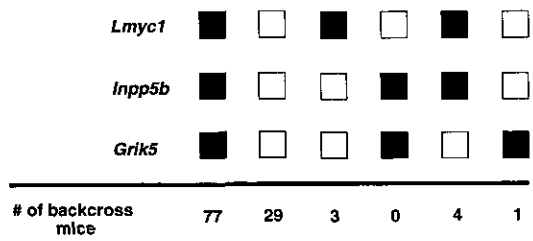

FIG. 2. Haplotype distribution of *Inpp5b*. The segregation of *Inpp5b* among distal mouse chromosome 4 loci in [(C3H/HeJ-*gld* × *Mus spretus*)F_1 × C3H/HeJ-*gld*] interspecific backcross mice is shown. The loci are listed from proximal to distal on the left side. Each column represents a possible haplotype, and the number of mice observed with each haplotype is indicated at the bottom of the column. The boxes indicate whether the mice were typed as C3H/HeJ-*gld* homozygotes (black) or F_1 heterozygotes (white) for each locus. Note that the larger number of mice typing as C3H homozygotes on mouse chromosome 4 represents a phenomenon of segregation distortion that has been described for another region of the mouse genome in this cross (Seldin *et al.*, 1989).

yses revealed no differences between *dyl* and normal mice (data not shown).

Since *dyl* arose as a spontaneous mutant, it is possible that it will be a point mutation. The above-mentioned analyses might miss a point mutation, and therefore we decided to undertake a genetic approach. The *dyl* parental mice were bred to C57BL/6J mice, and the resulting N_1 were then backcrossed to the *dyl* parents. The N_2 offspring were scored for the presence of cataracts, and affected offspring were then used for genotype analysis. To differentiate between a C57BL/6J and a *dyl* chromosome at the *Inpp5b* locus, we searched for a polymorphism at the *Inpp5b* locus.

We isolated two $(CA)_n$ repeat regions (INPCA1 and INPCA2) from cMI5P. PCR analysis was performed on INPCA1 using two flanking primers on DNA isolated from mouse strains. The PCR analysis showed that INPCA1 is polymorphic between various mouse strains (Fig. 3a). The sizes of the amplified products in Fig. 3a are *dyl* (BALB/cHeA), 144 bp; C57BL/6J, 134 bp; SJL/J, 148 bp; DBA/2J, 146 bp; 129Sv/J, 144 bp; CAST/EI, 132 bp; and *M. spretus,* 136 bp.

These PCR primers were then used to amplify INPCA1 from the *dyl* backcross mice. Analyses of 98 affected N_2 mice showed four crossover events in which both a *dyl* and a C57BL/6J chromosome could be amplified from the *Inpp5b* locus (Fig. 3b). Further analyses of these four mice were then performed using MapPairs primers, *D4Mit124* and *D4Mit52,* known to map near *Inpp5b* and *dyl* on Chromosome 4. PCR analysis at *D4Mit124* showed that in three of the four N_2 mice there was also a crossover event at this locus. However, analysis with *D4Mit52,* which is located 2.1% recombination proximal on Chromosome 4, showed no recombination events between *dyl* and *D4Mit52* (data not shown). The sizes of the amplified products using *D4Mit124* were 157 bp (C57BL/6J) and 139 bp (*dyl*) and using *D4Mit52* 116 bp (C57BL/6J) and 108 bp (*dyl*). The sizes of the amplified products using *dyl* DNA (on BALB/cHeA) are the same as those reported using BALB/cJ DNA.

DISCUSSION

The family of inositol polyphosphate-5-phosphatases plays an important role in regulating signal transduction. They are capable of terminating the IP_3 and IP_4 signals that cause release of calcium (Berridge, 1993). There are at least three types of inositol polyphosphate-5-phosphatases that catalyze this reaction (Bansal and Majerus, 1990). Over the past few years, cDNAs encoding at least two of these, type I (Laxminarayan *et al.,* 1994) and type III (Ross *et al.,* 1991), have been isolated. The isolation of these cDNA clones permits the genetic and molecular characterization of these genes to be undertaken. The gene for the type III inositol 5-phosphatase is very similar (53% identical) to *OCRL1,* the gene responsible for Lowe syndrome in humans (Attree *et al.,* 1992). Thus it is possible that mutations in the type I or type III gene are responsible for other genetic disorders found in human or mouse. Mapping of these genes provides an initial approach to answer this hypothesis.

Two genes involved in phosphatidylinositol metabolism have been previously mapped. The gene for inositol polyphosphate-1-phosphatase has been mapped to human chromosome 2q32 (York *et al.,* 1993). The activity of this enzyme is inhibited by lithium, a common treatment for manic-depressive disorder. However this locus has not been linked to inherited depressive disorders (York *et al.,* 1993). The gene for the type III inositol polyphosphate-5-phosphatase (*INPP5B*) has been previously mapped to human chromosome 1p34 (Jänne *et al.,* 1994). This map position was not located near any obvious candidate genetic disorders.

In this paper we present evidence that the murine homologue of *INPP5B, Inpp5b,* maps to distal mouse Chromosome 4. This was achieved by two independent methods: fluorescence *in situ* hybridization and an interspecific backcross analysis. The interspecific backcross analysis provided us with a more detailed map position and showed that *Inpp5b* maps 2.6 ± 1.4 cM distal to *Lmyc1.* This map position is in the vicinity of the locus for *dysgenetic lens* (*dyl*), an autosomal recessive disorder causing cataracts in mice (Sanyal, 1986). Due to the high homology between *OCRL1* and *INPP5B* (Attree *et al.,* 1992) and since one of the phenotypic consequences of Lowe syndrome is cataracts, we wanted to investigate the possibility that *Inpp5b* and *dyl* are the same locus. Southern, Northern, and RT-PCR analyses showed no difference between *dyl* and normal mice.

The genetic studies using a backcross analysis provide evidence that *Inpp5b* and *dyl* are not the same locus. Our backcross analysis of *dyl* mice to C57BL/6J showed that in the N_2 generation we find 4/98 of the

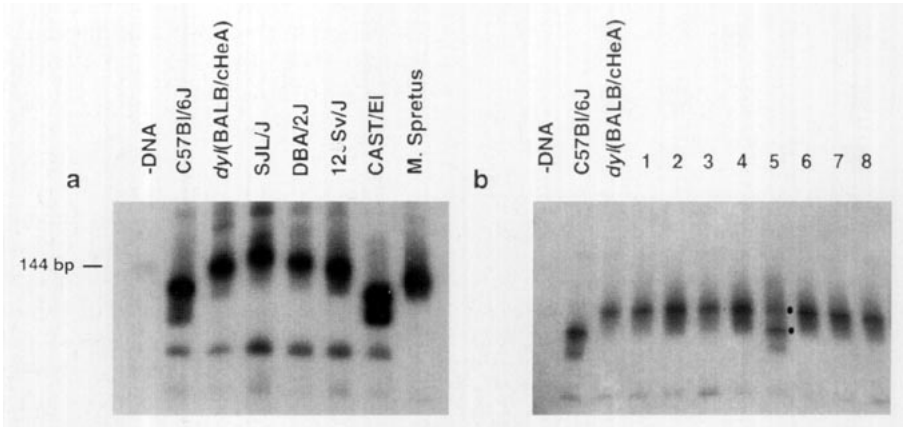

FIG. 3. (**a**) Analysis of INPCA1 from various mouse strains. PCR primers flanking INPCA1 were used to amplify DNA from various mouse strains. The forward primer was end labeled with [γ-^{32}P]ATP and the PCR carried out as described. The samples were then separated by electrophoresis in an 8% acrylamide/urea gel and exposed to X-ray film at −70°C. As can be seen, the amplified INPCA1 region is of variable size in these mouse strains. (**b**) Analysis of N_2 *dyl* mice. The same PCR primers flanking INPCA1 were used to analyze N_2 *dyl* mice. As can be seen, the parental alleles (C57BL/6J and *dyl*) are of different size. Analysis of DNA from eight N_2 *dyl* samples is shown. One of them (lane **5**) shows amplification from both a *dyl* and a C57BL/6 allele (marked by dots) and thus has undergone a recombination event that includes the *Inpp5b* locus.

affected mice to be recombinant at the *Inpp5b* locus. Therefore, *Inpp5b* cannot cause *dyl,* and it is formally excluded as a candidate gene. Our data suggest that *Inpp5b* is located ~4% recombination or approximately 4 cM distal to the *dyl* locus.

Further analysis of the four affected N_2 mice that had a crossover at the *Inpp5b* locus was performed using markers (SSRs from Map Pairs). This allowed us to map the crossover events further in these mice. The markers used, *D4Mit52* and *D4Mit124,* map in the vicinity of *Inpp5b,* but their exact position with respect to *Inpp5b* is unknown. However, three of these four mice also showed crossovers at *D4Mit124,* which places this locus proximal to *Inpp5b* on chromosome 4 since double crossovers are exceedingly rare in mice. Furthermore, at *D4Mit52,* all four mice did not exhibit a crossover. Thus, in three of the four mice, the recombination occurred between *D4Mit124* and *D4Mit52,* which are located 2.1% recombination (2.1 cM) apart from one another (Research Genetics). In the fourth mouse the recombination event occurred somewhere in between *Inpp5b* and *D4Mit124.*

Combined, these data suggest that *dyl* is located proximal to *Inpp5b* on mouse Chromosome 4 and that the *dyl* locus is proximal to *D4Mit124* but can be in the vicinity or including *D4Mit52.* In addition, we have described a new polymorphic SSR marker from the *Inpp5b* locus that can be used for further genetic studies of this region.

The map position of *Inpp5b* on Chromosome 4 is located within a region homologous to human chromosome 1p31–p34 (Abbott *et al.,* 1993). The mapping of the human homologue, *INPP5B,* to human chromosome 1p34 (Jänne *et al.,* 1994) demonstrates that this homologous relationship is maintained for these genes in the mouse.

ACKNOWLEDGMENTS

We thank Dr. Larry Charnas for the *Inpp5b* cDNA clone and Dr. Maja Bucan for use of her fluorescence microscope. This work was supported by the Howard Hughes Medical Institute (R.L.N.), National Institutes of Health Grants R01HD23245 (R.L.N.), T32-GM07170 (P.A.J.), and HG-00734 (J.M.R. and M.F.S.), National Science Foundation Grant MCB 9210351 (P.A.M.-D.), and the Division of Intramural Research of the National Center for Human Genome Research (R.L.N.).

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