Molecular cloning, gene organization, and expression of mouse Mpi encoding phosphomannose isomerase

Joseph A. Davis, Xiao-Hua Wu1, Ling Wang1, Charles DeRossi, Vibeke Westphal, Rongrong Wu, Gordon Alton, Geetha Srikrishna, and Hudson H. Freeze2

Glycobiology Program, The Burnham Institute, 10901 North Torrey Pines Rd., La Jolla, CA 92037, USA

Received on January 29, 2002; revised on April 3, 2002; accepted on April 11, 2002

Phosphomannose isomerase (PMI) interconverts fructose-6-P (Fru-6-P) and mannose-6-P (Man-6-P), linking energy metabolism to protein glycosylation. We have cloned the mouse Mpi cDNA, analyzed its genomic organization, and studied the expression in different tissues. The Mpi gene has eight exons covering 7.2 kb. The structure and intron–exon boundaries are essentially the same as its human ortholog with 85% amino acid identity. Mpi is alternatively spliced at the 3′ end, resulting in three messages with different 3′-untranslated regions. Mpi expression is regulated at both the transcription and translation levels, with the highest expression level in testis. Rabbit antibodies prepared against mouse PMI expressed in E. coli recognize a single 47-kDa band. Immunohistochemistry of mouse tissues shows general cytosolic staining in all cells. In testis, staining is intense in round spermatids and residual bodies, moderate in pachytene spermatocytes, and weak in spermatogonia and spermatozoa. In contrast, northern blot analysis shows comparable transcripts of 1.8 and 1.6 kb in pachytene spermatocytes and round spermatids, suggesting delayed translation of PMI. The stage-specific expression of PMI in testis may be important for KDN synthesis, which requires Man-6-P, or it may be needed to ensure sufficient glycosylation precursors in cells that do not utilize glucose and instead rely on lactate and pyruvate.

Key words: congenital disorder of glycosylation/KDN/Mpi/ phosphomannose isomerase/testis

Introduction

Biosynthesis of Man-6-P occurs by two routes: direct phosphorylation of mannose via hexokinase, or from Fru-6-P via phosphomannose isomerase (PMI) (E.C. 5.3.1.8). The latter enzyme interconverts Fru-6-P and Man-6-P, linking glucose catabolism to protein glycosylation. Man-6-P is converted to Man-1-P, and then to GDP-Man (Figure 1). Man-6-P is also a precursor for 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) synthesis in mammalian cells (Angata et al., 1999a), which can be enhanced by addition of exogenous mannose (Angata et al., 1999b). PMI is encoded by the mannose 6-phosphate isomerase gene (MPI), which has been cloned from several sources, including S. cerevisiae, C. albicans, and human, and shows highly conserved regions with 35–42% overall identity at the nucleotide level (Proudfoot et al., 1994). Loss of pmi40, the yeast homolog of MPI, is lethal unless mannose and glucose are provided in the growth medium (Payton et al., 1991). Mutations in human MPI cause congenital disorder of glycosylation type Ib (OMIM 602579) and result in inefficient glycosylation due to a limited amount of precursors (Niehues et al., 1998). The clinical symptoms can be treated with dietary mannose supplements (Niehues et al., 1998; Lonlay et al., 1999; Babovic-Vuksanovic et al., 1999; Westphal et al., 2001).

The mouse mannose 6-phosphate isomerase gene (Mpi) is located on chromosome 9, but it has not been cloned. Because it was assumed to be a housekeeping gene, little is known about its expression or regulation in different organs. To provide a foundation for PMI exploration in the mouse, we have cloned the Mpi cDNA and analyzed its genomic organization, expression pattern, and cellular distribution.

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1These authors contributed equally to this work.
2To whom correspondence should be addressed; E-mail: hudson@burnham.org

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Results

Cloning of the Mpi cDNA

To isolate mouse Mpi cDNA, the mouse expressed sequence tag (EST) database was searched using the human PMI protein sequence (accession CAA53657). We identified 58 mouse EST sequences that are highly homologous to human MPI. Two EST clones, AU080858 and AI875357, are 90% identical to the 5′ region of human MPI cDNA, including the translation start codon. Another EST clone (accession AK003289) is 87% identical to the human MPI cDNA including the stop codon and 3′-untranslated regions (3′-UTRs). The cDNA of Mpi was obtained by reverse transcription–polymerase chain reaction (RT-PCR) cloning with primers designed according to the two EST sequences and confirmed by sequencing the two independent PCR products. The 5′ and 3′-UTRs of Mpi were obtained by rapid amplification of cDNA ends (RACE)–PCR. The cDNA and predicted amino acid sequence are shown in Figure 2A and 2B, respectively.

Mpi gene organization, expression, and alternative splicing

Using Southern blot assay, PCR, and sequencing, we found that the mouse Mpi gene consists of eight exons spanning...
approximately 7.2 kb (Figure 3A and Table I). Mouse genome analysis identified a TASS-1 element about 1.2 kb upstream of the Mpi translational start codon ATG (Figure 3B). This element is involved in regulated gene expression in pachytene spermatocytes and round spermatids (Charron et al., 1999). Interestingly, RT-PCR for the 3’ portion of Mpi and 3’ RACE of mouse Mpi produced multiple bands (data not shown). These are alternatively spliced forms that were confirmed by sequencing. They encode the same protein but differ only in the length of the 3’-UTR region (Figure 3C).

To determine the expression pattern of the alternate splice forms of Mpi, we performed RT-PCR to amplify the Mpi cDNAs from various mouse tissues (Figure 4A). The mRNA was present in all mouse tissues examined. It was most abundant in testis, followed by brain, heart, and muscle and expressed at lower levels in spleen, intestine, kidney, lung, and liver. We noticed that the full-length mRNA is detectable mainly in testis and brain. We found that mouse Mpi cDNA contains highly conserved sequences (Y and H elements), which have been shown to bind to testis-brain-RNA-binding protein (TB-RBP) that is involved in arresting the translation of mRNA in these organs (Wu et al., 1997). Human MPI contains such elements as well (data not shown).

**PMI activity and antigen**

PMI activities were measured in the 100,000 \( \times g \) supernatant of tissues. Pellets contained < 10% of the total activity. A native gel electrophoresis in situ assay showed a single band (data not shown). The soluble activity was very high in testis, followed by brain, heart, and kidney. Spleen, lung, intestine, and liver showed relatively low activity (Figure 4B, lower panel). PMI antigen was detected by semi-quantitative western blot analysis as a single band of 47 kDa using an affinity-purified anti-mouse PMI antibody (Figure 4B, middle panel). Preadsorption of the antiserum with yeast PMI abolished the band. Quantitation of band intensity showed that protein expression closely corresponds to PMI-specific activities (Figure 4B, upper panel). Antibody prepared against expressed human PMI also cross-reacted with mouse PMI (data not shown). In most cases, there is a close correlation between the intensity of the PCR products, antigen, and enzyme activity, suggesting primarily transcriptional regulation.

**Immunohistochemistry**

To further determine the PMI expression pattern, we immunohistochemically localized the PMI antigen using the purified anti-mouse PMI antibody. Most cell types showed diffuse cytosolic staining. The staining was specific, because sections treated with purified pre-immune IgG, yeast PMI preadsorbed antibody, or secondary antibody alone did not give positive staining. As expected, the strongest signal was observed in testis and the weakest in liver. Round spermatids and residual bodies showed very strong immunoreactivity. Pachytene spermatocytes showed moderate reactivity, whereas spermatogonia, primary spermatocytes, spermatozoa, and Sertoli cells, were faint even at high concentration of antibody (Figure 5).

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**Table I.** Exon–intron boundaries in the mouse Mpi gene

<table>
<thead>
<tr>
<th>Exon no.</th>
<th>Exon–intron boundary sequence</th>
<th>Exon size (bp)</th>
<th>Intron size</th>
</tr>
</thead>
<tbody>
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<td>AGTCCGCAGGtggaagcct...gttgcacctcAGTGTCCTGACT</td>
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<td>427 bp</td>
</tr>
<tr>
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<tr>
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<td>1.4 kb</td>
</tr>
<tr>
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<td>329 bp</td>
</tr>
<tr>
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<td>2 kb</td>
</tr>
<tr>
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<td>174</td>
<td>1 kb</td>
</tr>
<tr>
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<td>209</td>
<td>256 bp</td>
</tr>
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<td>8a</td>
<td></td>
<td>677</td>
<td>440 bp</td>
</tr>
<tr>
<td>8b</td>
<td></td>
<td>440 bp</td>
<td></td>
</tr>
<tr>
<td>8c</td>
<td></td>
<td>220 bp</td>
<td></td>
</tr>
</tbody>
</table>

Capital letters represent exonic sequences; small letters represent splice donor and splice acceptor intronic sequences.
Mpi expression in spermatogenic cells

Northern blot analysis was done using total RNA derived from purified populations of cells from different stages of spermatogenesis. Pachytene spermatocytes showed one band of 1.8 kb and two faint bands of 1.95 and 1.55 kb. Round spermatids did not show the 1.8-kb band. However, there were bands of both 1.6 kb and 1.9 kb. No signal was detected in Sertoli cells (Figure 6).

Protein-bound KDN and Neu5Ac

Because Man-6-P is a precursor for KDN synthesis in mammalian cells (Angata et al., 1999a), increased PMI activity in testis may be needed to generate KDN. We compared the amount of protein-bound KDN and Neu5Ac in mouse testis and liver as shown in Figure 7. The total amount of KDN/mg protein in testis was 5.5-fold greater than that of liver (40.7 and 7.4, respectively). However, the total Neu5Ac/mg protein in liver was 1.6-fold higher than that of testis (462 and 284 respectively). These results suggest that though the sialic acid metabolism is more active in liver than testis, KDN metabolism seems to be more active in testis (Figure 7).

Discussion

In this study, we cloned the mouse Mpi cDNA encoding a protein of 423 amino acids that is 85% identical to human PMI protein (Figure 2B). Both human and mouse recombinant PMI proteins expressed in and purified from Escherichia coli showed compatible PMI activity, and the antibodies generated against them cross-reacted with each other. The expression level of PMI was regulated in different tissues with good correlation between enzymatic activity, antigen, and transcript, suggesting that control occurs primarily at the transcriptional level. Transcription was highest in testis and brain, where the
larger alternative transcripts were seen. All other tissues have only a single size transcript. The activity was also high in heart and intestine but low in kidney, spleen, lung, and ovary. Liver has the lowest activity, suggesting that a small amount of PMI is sufficient to produce the glycosylation precursors needed for the large daily output of secreted plasma glycoproteins. We noticed that there is a TASS-1 element 1189 nt upstream of Mpi translational start codon ATG (Figure 3A). TASS-1 was first identified in β4GalT-I gene (β1,4-galactosyltransferase-I), and shown to be essential for its transcription (Charron et al., 1999). Therefore, we surmise that Mpi gene expression could be regulated by this element.

Interestingly, we found that mouse Mpi mRNA is alternatively spliced in the 3′-UTR region. The 3′-UTR of mRNAs play important roles in regulating their translation, allowing them to be used at different times and in specific subcellular locations (Conne et al., 2000). Such uncoupling between transcription and translation is required, for example, in gametogenesis, embryogenesis, and for the targeting of specific mRNA into neuronal dendrites (Wu et al., 1997; Conne et al., 2000). Furthermore, mouse Mpi mRNA contains putative H and Y elements which were first identified in protamine genes (Wu et al., 1997). These elements bind to TB-RBP, resulting in mRNA transport to microtubules and arrested translation (Hecht, 2000). The level of mRNAs in pachytene spermatocytes and round spermatids is comparable. However, the level of protein in pachytene spermatocytes is much lower than it is in round spermatids, suggesting that PMI translation is delayed during spermatogenesis. PMI has a stage-specific expression pattern and may have a stage-specific role during spermatogenesis. We speculate that Mpi expression is regulated not only at the transcription level by the TASS-1 element but also at the translational level by H and Y elements and the 3′-UTR.

Differential expression of PMI activity, especially in the testis, suggests that it may be needed either to supply precursors for glycosylation or for catabolism. It seems unlikely that increased PMI activity converting Man-6-P to Fru-6-P would make a significant contribution to glycolysis. The very sharp rise in PMI antigen in round spermatids, however, suggests a particularly important function at that stage. One possibility for this increase is that more Man-6-P is needed for synthesis and accumulation of KDN, which has been found in large amounts in rainbow trout testis (Angata et al., 1999a) and in small amounts in mouse melanoma B16 and African green monkey kidney COS-7 cell lines (Angata et al., 1999b). In the latter, KDN synthesis is stimulated when mannose is provided in the culture medium (Angata et al., 1999b). We found that bound KDN in testis is nearly sixfold higher than in liver when normalized to protein. However, we do not know if increased KDN synthesis occurs in the round spermatids. Another possibility is that Man-6-P is needed for N-linked oligosaccharide synthesis by round spermatids engaged in acrosome formation and packaging of degradative enzymes into this specialized lysosome (Tulsiani et al., 1998). Lysosomal enzymes typically have glycan chains of the high-mannose type, rather than complex type. Recent results (Chayko and Orgebin-Christ, 2000) suggest that the targeting of several acrosomal proteins does not require either of the Man-6-P receptors.

Increased PMI activity in round spermatids may also be needed to ensure sufficient Man-6-P production from Fru-6-P generated through gluconeogenesis (Figure 1). This may be necessary because the glycolytic enzymes, specifically hexokinase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase, known to be rate-limiting enzymes in glycolysis, are very low in round spermatids (Nakamura et al., 1982). In fact, glucose is toxic in round spermatids because it depletes ATP supplies (Nakamura et al., 1986). Lactate or pyruvate are the preferred sources for energy (Jutte et al., 1981; Mita and Hall, 1982; Grooteboer et al., 1984) and can be catabolized through the tricarboxylic acid cycle. Alternatively, they can yield Fru-6-P through the gluconeogenesis pathway. Labeling experiments using isolated guinea pig spermatocytes and spermatids failed to show [3H]mannose incorporation into glycoconjugates, whereas other monosaccharides showed substantial incorporation (Joshi et al., 1990). The reason radio-labeled mannose was not incorporated is unknown, but high PMI activity would favor diverting [2-3H]Man-6-P into catabolism through the hexose monophosphate pathway (HMP pathway), leaving less for incorporation into glycoproteins.

The HMP pathway begins with glucose-6-P dehydrogenase (G6PDH) which generates NADPH that is used for reduction of highly reactive and potentially damaging lipid hydroperoxides (Sikka, 1996, 2001). There is evidence that human sperm may be limiting in G6PDH (Storey et al., 1998). Postmeiotic male germ cells contain NADPH-requiring lanosterol
Glucose-6-phosphate dehydrogenase (G6PDH) and 5′, 14α-demethylase (CYP51), which is required for sterol synthesis and thought to be involved in signaling (Stromstedt et al., 1998; Rozman and Waterman, 1998). On the other hand, reactive oxygen species are required for fertilization (Aitken et al., 1995). Therefore, it may be important to balance the amount of highly reactive species at different stages of spermatogenesis. High expression of PMI, G6PDH (Peter et al., 1997), and CYP51 (Rozman and Waterman, 1998) in the same population of cells may balance the needs of the HMP shunt and protein glycosylation. Additional studies including isolation of round spermatids will be needed to clarify the role of PMI in tests. These studies may be important for determining whether male CDG-Ib patients may have impaired fertility.

**Materials and methods**

Glucose-6-phosphate dehydrogenase (G6404), phosphoglucoisomerase (P5381), NADP (N 0505), Man-6-P, yeast PMI, Protein G Sepharose 4B, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, Affigel 10, all buffers and salts were obtained from Sigma (St. Louis, MO), pGEX-5X-2 vector, glutathione Sepharose 4B, and Factor Xa were from Pharmacia Biotech. D-amnose was obtained from Hoffman International (Calgary, Alberta, Canada). Hanks balanced salt solution was from Irvine Scientific (Santa Clara, CA). Age-matched female and male Sprague-Dawley mice (C57BL/6) were obtained from Harlan (San Diego, CA). RNeasy Mini Kit is from Qiagen, Germany. All of the reagents for PCR were obtained from Harlan (San Diego, CA). RNeasy Mini Kit was from Life Technologies (Gaithnburg, MD). The 129/SvJ λ phage library was provided by Dr. Jamey D. Marth, University of California, San Diego.

**Isolation of mouse Mpi cDNA**

To isolate mouse Mpi homolog, we searched EST database with human PMI protein sequence (accession: CAAS3657). With mouse Mpi gene-specific primers designed according to EST sequences, the forward primers 5′-ACGTTCTCGAGGCGGT (accession AU080858), the reverse primer, 5′-CTGTTCTCTCCAACTAG (accession AK003289), RT-PCR was performed to amplify mouse Mpi cDNA. Two independent PCR products were cloned and sequenced. Using mouse testis Marathon-Ready cDNA as a template, RACE–PCR was used to confirm the sequences of 5′ and 3′ flanking regions of the open reading frame (ORF).

**Expression and alternative splicing assay of mouse Mpi with RT-PCR**

Mouse (five male Sprague-Dawley mice C57BL/6) mRNAs from various tissues were isolated with RNeasy Mini Kit following the instructions of manufacturer. Three primers were designed according to mouse Mpi cDNA sequence: one forward 5′-TACGTTCCTCGAGGCGG, and two reverse primers, 5′-CTACAGCAGAGGACGCGG for the coding region; 5′-TACGTTCCTCGAGGCGG for the full-length cDNA. A set of primers against mouse β-actin cDNA was used as RT-PCR control (the forward primer 5′-TGTAGACCTGACCTAGACCCC, the reverse primer 5′-TGATCCACATCTGCGGAAG according to cDNA; accession X03765). RT-PCR was performed with Superscript one-step RT-PCR kit and the conditions were 50°C for 30 min, 94°C for 1 min, 30 (β-actin) or 35 (Mpi) cycles of 94°C for 20 s, 55°C for 20 s, 72°C for 2 min. The RT-PCR products were separated with agarose gel and visualized with ethidium bromide. The products from testis were eluted from agarose gels and confirmed by sequencing.

**Organization of mouse Mpi gene**

The mouse Mpi genomic clones were isolated from the 129/SvJ λ phage library, using mouse Mpi cDNA as a probe. A 12-kb genomic DNA fragment that covers exon 3 to exon 8 was isolated and subcloned into pBluescript KS vector at NotI site. Restriction endonuclease digestion and Southern blot analysis using 32P-labeled oligonucleotides corresponding to mouse Mpi cDNA determined exon–intron boundaries. Exon–intron boundaries of exon 1 to intron 3 were defined by sequencing of PCR products of genomic DNA, using 129/SvJ mouse genomic DNA library as template. Six primers were used: primer 1, 5′-TTTCTACGGCAACCTGGGATGGC-3′; primer 2, 5′-GCCACCTCTACTTTTGAGC-3′; primer 3, 5′-CTTCTCTGTGTGGGATAG-3′; primer 4, 5′-GTTAGGTGTGTCCCTGGTATAG-3′; primer 5, 5′-GCTCAAGGTCTAAACAC C-3′; and primer 6, 5′-ATTGACCACTGGAACTC-3′.

**Construction of prokaryotic expression plasmids**

The full length cDNA encoding the entire ORF of mouse Mpi was expressed as a glutathione S-transferase (GST) fusion protein with a Factor Xa recognition site using the pGEX-5X-2 system at EcoRI site (Pharmacia Biotech, Inc., Uppsala, Sweden). This generated the sequence encoding an N-terminal GST tag followed by a Factor Xa recognition site, followed by the Mpi gene under the control of the inducible lac promoter.

**Preparation and purification of recombinant enzyme**

The pGEX-5X-2 plasmid encoding PMI-GST was transformed into E. coli strain BL21 (DE3) as described (Sambrook et al., 1989). A single colony of transformed cells was picked and cultured at 37°C overnight in Luria Bertani (LB) medium supplemented with ampicillin. The colony was propagated by incubating 100 μl of the transformed cells in 5 ml LB medium. The expression was induced by adding isopropyl-1-thio-B-D-galactopyranoside to a final concentration of 0.1 mM. After 3 h at 37°C, the cells were harvested and frozen at −20°C or processed immediately.

For the purification of the recombinant enzyme, the bacterial cells were suspended in cold phosphate buffered saline (PBS), pH 7.2, containing 1 μg/ml each of aprotinin, leupeptin, and pepstatin A and 1% Triton X-100; sonicated; and spun at 15,000 × g for 20 min at 4°C. The supernatant was applied to glutathione Sepharose 4B column (bed volume 8 ml) equilibrated with PBS. After washing with PBS/1% Triton X-100 and PBS, the fusion protein was eluted with 5 mM glutathione followed by second elution with 3 M NaCl, 6 M urea in PBS. The A280nm peak was pooled; dialyzed against 10 mM NaCl, 100 mM NaCl in 50 mM Tris–HCl, pH 8.0; and digested with 40 U of Factor Xa for 60 h at room temperature. The digest was reapplied over glutathione Sepharose 4B to remove the GST tag, and the run-through was pooled as enzyme source. Purity of the enzyme was confirmed by SDS–PAGE and enzyme assays confirmed that the expressed protein had PMI activity.
Preparation of PMI antibodies

The purified recombinant mouse PMI was used as antigen in New Zealand rabbits. The animals were boosted repeatedly with 50 µg antigen. The rabbit antiserum was partially purified by the caprylic acid method, followed by Protein G-Sepharose column (Harlow and Lane, 1988). The GST-Affigel-10 column eluate was affinity purified on a yeast PMI-Affigel-10 column; washed with 20 volumes of 0.1 M Tris–HCl, pH 8.0; eluted with 0.1 M glycine-HCl, pH 3.0; and neutralized with 1 M Tris–HCl, pH 8.0. The A280nm peak was pooled, concentrated to 750 µg/ml, and stored frozen at −20°C and used for the experiments.

PMI assay

PMI activity was measured in 100,000 × g supernatant of freshly homogenized tissues using a coupled assay (Westphal et al., 2001). In brief, the assay was carried out in 50 mM HEPES, pH 7.1, containing 50 µg protein, 5 mM MgCl₂, 0.25 mM NADP, and 1 U/ml each of PGI and G6PDH, and the NADPH produced was measured at A340nm.

Western blot

Western blots were carried out as described (Towbin et al., 1979). In short, 10 µg of 100,000 × g supernatant of mouse tissue proteins were resolved on 8% SDS–PAGE and transferred to nitrocellulose, incubated with purified anti-mouse PMI (0.2 µg/ml) and anti-rabbit IgG–alkaline phosphatase and detected with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. The intensity of the immunoreactivity was integrated using the NIH Image program.

Native gel electrophoresis zymograms

The zymograms were performed as described with slight modifications (Hendriksen et al., 1997). Briefly, the frozen tissues were homogenized in PBS, pH 7.2, containing protease inhibitors (Boehringer Mannheim, Germany, cat. no. 1697498). The tissue homogenates were 5% goat serum, and treated with purified anti-mouse PMI (0.5 µg/ml) and stored frozen at −20°C and used for the experiments.

PMI activity was measured in 100,000 × g supernatant of freshly homogenized tissues using a coupled assay (Westphal et al., 2001). In brief, the assay was carried out in 50 mM HEPES, pH 7.1, containing 50 µg protein, 5 mM MgCl₂, 0.25 mM NADP, and 1 U/ml each of PGI and G6PDH, and the NADPH produced was measured at A340nm.

Acknowledgments

This work was supported by the National Institutes of Health, R01-GM55695, to H.L.F. We are indebted to Dr. Joel Shaper for northern blot of spermatogenic cells for Mpi and many helpful comments on the manuscript. We thank Robbin Newlin for the immunohistochemistry staining; Dr. Nissi Varki for suggestions on immunohistochemical studies; Drs. Jose Luis Millan, Sonoko Narisawa, and Clotilde Huet for useful suggestions; and Drs. Herman Higa and Darshini Mehta for the initial work on PMI antibody. The valuable suggestions from the members of the authors’ laboratory are gratefully acknowledged.
Abbreviations
DAB, diaminobenzidine; DMB, 1,2-diamino-4,5-methylene-dioxybenzene dihydrochloride; EST, expressed sequence tag; G6PDH, glucose-6-phosphate dehydrogenase; GST, glutathione-S-transferase; HMP, hexose monophosphate; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; LB, Luria-Bertani medium; MPI, mouse mannose 6-phosphate isomerase gene; ORF, open reading frame; PBS, phosphate-buffered saline; PBST, Tween 20/PBS; PGlu, phosphoglucomutase; PGI, phosphoglucose isomerase; PGI, phosphoglucose isomerase; PMI, phosphomannomutase isomerase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; SAGE, serial analysis of gene expression; TDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; UTR, untranslated region.

References

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