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PMI40, an Intron-Containing Gene Required for Early Steps in Yeast Mannosylation

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We have previously described a temperature-sensitive *pmi40-1* mutant of *Saccharomyces cerevisiae* which is defective in glycosylation and secretion because of a thermolabile phosphomannose isomerase (PMI) activity. Inactivation of PMI at the restrictive temperature of 37°C prevents synthesis of the GDP-mannose and dolichol-phosphate-mannose required for a number of critical mannosyl transfer reactions and results in cell death. Here, we report the isolation of the *PMI40* gene by complementation of the corresponding mutation. The *PMI40* gene contains an efficiently spliced intron which differs from the majority of those so far identified in *S. cerevisiae* in that it is short and the branch-forming structure has an AACTAAC motif replacing the highly conserved consensus TACTAAC. The 48.2-kDa protein predicted to be encoded by *PMI40* contains amino acid sequences corresponding to those of internal peptides derived from purified *S. cerevisiae* PMI. Deletion of the *PMI40* coding sequence results in a strain requiring D-mannose for growth. The *PMI40* gene is located on chromosome V, and its transcription is increased 12-fold when cells are grown on D-mannose as sole carbon source instead of D-glucose. PMI enzyme activity, however, is not increased in D-mannose-grown cells, and PMI protein levels remain constant, suggesting that the *PMI40* gene is subject to additional levels of regulation.

Dolichol-phosphate-mannose (Dol-P-Man) and GDP-mannose (GDP-Man) serve as the mannose donors for a number of important mannosylation reactions found in eukaryotes. These include formation of the mannosyl residues found in glycoproteins, where the mannosyl residues can be attached to both serine/threonine (O-linked) and asparagine (N-linked) (9, 24, 33, 34) yeast mannosyl- and inositol-containing sphingolipids (1), and the glycosyl phosphatidylinositol moiety found linked to certain membrane proteins (5, 8, 18, 23). The pathway of GDP-Man and Dol-P-Man synthesis and many of the glycosylation reactions in which these molecules participate are similar in higher and lower eukaryotes, and *Saccharomyces cerevisiae* has provided a useful model system for the study of glycosylation (1a, 17). Indeed, it has been shown that the *S. cerevisiae* Dol-P-Man synthase *DPM1* gene (24) can complement a glycosylation-defective mammalian cell line (1a).

The first step in the synthesis of GDP-Man and Dol-P-Man from glycolytic intermediates is performed by phosphomannose isomerase (EC 5.3.1.8), which catalyzes the reversible isomerization of fructose-6-phosphate and mannose-6-phosphate (13) (Fig. 1). We have previously described a temperature-sensitive lethal mutant of *S. cerevisiae* which possesses a thermolabile phosphomannose isomerase (PMI) activity due to the *pmi40-1* mutation (26). At the restrictive temperature of 37°C, strains carrying *pmi40-1* display an abnormal clumped morphology, are unable to secrete extracellular glycoproteins, produce cell walls deficient in D-mannose, and undergo cell lysis as measured by the release of intracellular enzymes. All of these defects can be overcome by growth on medium containing 10 mM D-mannose in addition to D-glucose. Growth in the presence of D-mannose probably bypasses the requirement for PMI activity by allowing the direct production of mannose-6-phosphate through the uptake of D-mannose by the hexose sugar transport system and its subsequent phosphorylation, thereby permitting its entry into the GDP-Man–Dol-P-Man pathway (Fig. 1). This feature distinguishes strains carrying the *pmi40-1* mutation from those having the phenotypically similar *sec53* mutation, which produces a thermolabile phosphomannomutase (2, 10, 16). Phosphomannomutase performs the step following PMI in the GDP-Man–Dol-P-Man pathway whereby mannose-6-phosphate is converted to mannose-1-phosphate (Fig. 1). The *sec53* phenotype cannot be repaired by exogenous D-mannose, presumably because the cell is unable to phosphorylate D-mannose directly at the C-1 position and cannot use mannose-6-phosphate in GDP-Man synthesis.

Genes encoding PMI have been isolated from *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* (4, 6, 19), in which PMI is necessary for the synthesis of the capsular polysaccharide. The PMIs from *E. coli* and *S. typhimurium* have high similarity (4), whereas the *P. aeruginosa* PMI has no significant similarity with the *E. coli* and *S. typhimurium* proteins. It has been shown by Shinabarger et al. (31) that the *P. aeruginosa* PMI is a bifunctional protein with both PMI and GDP-Man pyrophosphorylase activity, which forms GDP-Man from mannose-1-phosphate and GTP (Fig. 1).

To assist in the analysis of the role of PMI in the pathway of GDP-Man and Dol-P-Man synthesis in *S. cerevisiae*, we have cloned the *S. cerevisiae PMI40* gene by complementation of the *pmi40-1* mutation, characterized the gene, and here present data showing that the gene contains an unusual intron and is transcriptionally regulated. The protein encoded by the *S. cerevisiae PMI40* gene is related to the PMIs from *E. coli* and *S. typhimurium* rather than that from *P. aeruginosa*.

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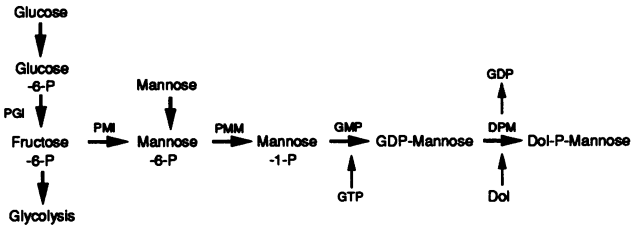

FIG. 1. Pathway of D-mannose utilization in *S. cerevisiae* leading to Dol-P-Man and fructose-6-phosphate. PGI, phosphoglucose isomerase; PMM, phosphomannomutase; GMP, GDP-Man pyrophosphorylase; DPM, Dol-P-Man synthase; Dol, dolichol.

MATERIALS AND METHODS

Chemicals and media. Media components were purchased from Difco. All other chemicals were purchased from Sigma or Fluka Biochemika and were of the highest grade obtainable.

Strains and culture conditions. *S. cerevisiae* S-150-2B (*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-1*) and X2180-1B (*MAT*α) were used as reference strains. The isolation and characterization of mutant S-40 from S-150-2B has been described previously (26). Mutant 17A (*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-1 pmi40-1*) was derived from a dissected tetrad resulting from a cross between S-40 and wild-type A6α (*MAT*α *ade6*). Strain SEY6210 (*MAT*α *leu2-3,112 ura3-52 trp1-Δ901 his3-Δ200 lys2-80 suc2-Δ9*) was used as a host for gene disruption experiments. Genetic manipulations were performed essentially as described by Sherman et al. (30). Strains were propagated on YPD (yeast extract [1%, wt/vol], peptone [2%, wt/vol], and D-glucose [2%, wt/vol] solidified with agar [2%, wt/vol] as required) at 30°C or, in the case of temperature-sensitive strains, at 25°C. Minimal medium contained yeast nitrogen base without amino acids (0.67%, wt/vol) and agar (2%, wt/vol) if required. Sugars as carbon source were supplied at 2% (wt/vol) or as indicated in Results. L-Amino acids were at 20 mg liter^{-1}, adenine was at 20 mg liter^{-1}, and uracil was at 40 mg liter^{-1} (final concentrations). D-Mannose was added at a final concentration of 10 mM to supplement *pmi40-1* or *pmi40::URA3* strains. *E. coli* DH1 (*hsdR17 supE44 recA1 endA1 gyrA96 thi-1 relA1*) or DH5α (*hsdR17 supE44 ΔlacU169* [Φ80*lacZ*ΔM15] *recA1 endA1 gyrA96 thi-1 relA1*) was used for propagation of plasmids.

Molecular biology techniques. Small- and large-scale plasmid isolation from *E. coli*, restriction enzyme digestion, *E. coli* transformation, Southern and Northern (RNA) blotting onto Hybond N (Amersham International, Zurich, Switzerland) membrane, plasmid construction, agarose gel electrophoresis, oligonucleotide 5′ end labeling, and primer extension transcript mapping were performed according to standard techniques (29). Isolation of DNA fragments from agarose gels was performed with a Geneclean II kit (Bio 101, La Jolla, Calif.) by following instructions supplied by the manufacturer. DNA probes were prepared by using an Amersham Multiprime DNA labeling kit and [α-^{32}P]dCTP (Amersham) at ~3,000 Ci/mmol. Southern and Northern blots were hybridized with labeled probes under conditions described in the text (below) in a Hybaid Standard Hybridization Oven (Hybaid Ltd., Teddington, United Kingdom). Autoradiography was performed using the Goos Special 200 intensifying screens on Amersham Hyperfilm MP. Isolation of DNA and RNA from *S. cerevisiae* was by established procedures (30), and transformation was by the lithium acetate technique (14). A nylon hybridization membrane containing separated *S. cerevisiae* chromosomes was obtained from Clontech Laboratories Inc., Palo Alto, Calif. Plasmid DNA was prepared for sequencing according to the method described by Smith et al. (32) and sequenced with a Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) according to the manufacturer's instructions. Oligonucleotide primers for sequencing and polymerase chain reaction (PCR) were synthesized on an Applied Biosystems 394 DNA/RNA Synthesizer or obtained commercially from Microsynth (Zurich, Switzerland). Perkin Elmer Cetus GeneAmp PCR reagent kits (RNA or Standard) were used under conditions recommended by the manufacturer to generate cDNA or genomic fragments which were then amplified in a Perkin Elmer Cetus DNA Thermal Cycler. For sequencing PCR-generated fragments directly, the fragments were first purified by agarose gel electrophoresis to remove the amplifying primers and then sequenced according to the protocol described above except for a primer annealing time of 2 min. An AMBIS Radioanalytic Imaging System (AMBIS Systems, Inc., San Diego, Calif.) was used for quantification of RNA samples on Northern blots. The resolution plate was 1.6 by 3.2 mm, movements were 8 by 9, and detection time was 1,440 min.

PMI assay and immunoblot analysis. Cell extracts of *S. cerevisiae* were prepared and assayed for PMI activity as previously described (26). Immunoblotting was performed by electrophoretically transferring separated proteins onto nitrocellulose membrane after sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) on 12% gels. The membrane was blocked for 1 h in blocking buffer (1% [vol/vol] phosphate-buffered saline, 0.1% [vol/vol] Triton X-100, 1% [wt/vol] casein). The filter was then incubated in a 1:20 dilution in blocking buffer of a rabbit polyclonal antibody raised against purified, denatured *S. cerevisiae* PMI (28) for 1 h at 37°C. The filter was washed twice with blocking buffer (30 min per wash), incubated for 1 h with peroxidase-labeled goat anti-rabbit immunoglobulin G at 2 μg/ml (KPL, Gaithersburg, Md.), washed twice more in blocking buffer (30 min per wash), rinsed in 50 mM sodium acetate (pH 4.5) for 15 min, and developed with 0.5% (wt/vol) 3-amino-9-ethylcarbazole and 0.014% (vol/vol) hydrogen peroxide in the same buffer.

Computing. Computer programs supplied as part of the University of Wisconsin Genetics Computer Group Version 7.0 sequence analysis package (7) running on a VAX4000 were used for all sequence assemblies, analysis, and comparisons.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ data bases under accession number M85238.

RESULTS

Isolation of a gene complementing the *pmi40-1* mutation in strain 17A. *S. cerevisiae* 17A carrying the *pmi40-1* mutation was transformed with plasmid DNA prepared from an *S. cerevisiae* genomic bank constructed in the transformation vector YEp13 (22; obtained from J. Ernst). Approximately 10,000 17A Leu$^+$ transformants were obtained and screened for growth at the restrictive temperature of 37°C. One 17A transformant which grew at this temperature was identified, and total DNA prepared from it was used to transform *E. coli* DH1 to ampicillin resistance. Plasmid pSPMI2, which was isolated from the resulting ampicillin-resistant *E. coli*

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   L  G  K  D  I  I  D  K  F  H  A  T  N  E  L      PEP1
ATGTTGGGTAAGGACATTATTGACAAGTTCCACGCTACTAACGAATTG    DS1
          C  C                 C

   A  A  S  D  N  V  V  R  A  G      PEP2
ATGGCTGCTTCTGACAACGTTTTGAGAGCTGGT    DS3
      C     T              C
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FIG. 2. Sequences of cyanogen bromide-generated peptides PEP1 and PEP2 obtained from *S. cerevisiae* PMI purified to homogeneity (28) and the corresponding oligonucleotides, DS1 and DS3, used as hybridization probes.

colonies, was able to transform mutant 17A to growth at 37°C. This suggested that the DNA insert carried by pSPMI2 contained the structural gene *PMI40*.

The availability of sequence data from peptides of purified PMI protein obtained after cleavage with cyanogen bromide (28) allowed the design of two oligonucleotide hybridization probes, DS1 and DS3 (Fig. 2). These hybridized to unique DNA fragments on Southern blots of restriction digests of both pSPMI2 and *S. cerevisiae* genomic DNA and provided additional strong evidence that pSPMI2 contained DNA encoding PMI. A restriction map of a 5.2-kb *Sal*I fragment from the *S. cerevisiae* DNA insert contained in pSPMI2 was constructed, and the region to which both DS1 and DS3 hybridized was determined by further Southern hybridizations (Fig. 3).

Characterization of the *PMI40* gene. The region of DNA defined by using the oligonucleotides DS1 and DS3 as probably containing *PMI40* was sequenced by subcloning suitable DNA fragments into pUC19. The sequences at the ends of these fragments were determined by using M13 universal and reverse-sequencing primers, and the sequence thus generated allowed the synthesis of novel oligonucleotide primers capable of initiating second-strand synthesis on previously unsequenced DNA. In this way, the complete nucleotide sequence of the region corresponding to the *PMI40* gene and its flanking sequences were determined on both strands (Fig. 4).

One long open reading frame (ORF) starting at position 240 and terminating at 1379 was contained within the sequence which, when translated, predicted amino acid sequences corresponding to those obtained from the sequence

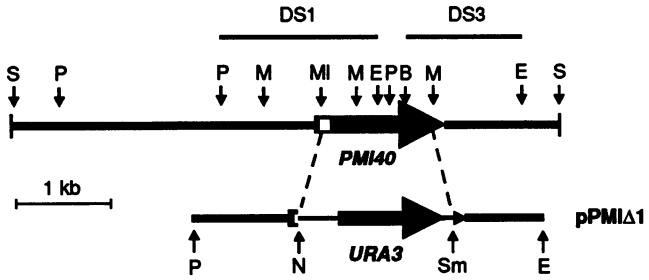

FIG. 3. Restriction map of a *Sal*I fragment from the *S. cerevisiae* DNA insert in pSPMI2 containing the *PMI40* gene. Closed box, protein-coding regions; open box, intron sequences. Arrows shows direction of transcription. Horizontal bars indicate the restriction enzyme fragments to which oligonucleotides DS1 and DS3 hybridized at a wash stringency of 4× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate)–0.1% (wt/vol) SDS at 60°C for 15 min. The strategy used to make the pPMIΔ1 construct used to delete most of the *PMI40* coding region is shown. S, *Sal*I; P, *Pst*I; M, *Msc*I; MI, *Mlu*I; E, *Eco*RI; B, *Bam*HI; N, *Nru*I; Sm, *Sma*I.

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-328 ACAAGCGCAAGGATGAGAATCATGAAAACCTTGCTACAACAAGATGCCAATAAAGTCGTT -269
-268 CCCTGATATAGCTAGTACTTTGCGGGAGTAATTTGTTATTCTGCTTTCTTTTGCACTTTG -209
-208 ATCATGCCAAGTCTCGTGGTGCACTATCCATTTCGTGCTGCTTGGTTATTGTAGCAATTT -149
-148 TTTGTCGCGTCCGTGTCCTTTTTCGCCGCGATTTACGTATTTAAATATACACGTCGCAAT -89
 -88 CTTAGTATATTTCTGTATAAAATGTTTGTTTCTTGTAATCATTCAACACTAAACACGTAA -29
 -28 AGAACATCAGACCAGAAAATTTTAAAACATGTCCAACAAGCTGTTCAGGTTAGATGCAGg 31
                                M S N K L F R L D A G
  32 tatgtgccatttttcgtcaaatattttgaatactttgagtatttttttttttttttttttg 91
  92 gtacactaactaacgcgtacgactctatgtagGCTACCAACAATACGACTGGGGTAAAAT 151
                                   Y Q Q Y D W G K I
 152 CGGCTCTTCTTCACGTGTCGCTCAATTTGCTGCCCATTCTGACCCCTCTGTTCAAATTGA 211
       G S S S R V A Q F A A H S D P S V Q I E
 212 ACAAGATAAACCATATGCAGAGTTATGGATGGGTACCCACAGCAAGATGCCTTCCTACAA 271
       Q D K P Y A E L W M G T H S K M P S Y N
 272 CCATGAGTCTAAGGAATCCCTGAGAGATATCATCTCCAAGAACCCCTCTGCCATGTTAGG 331
       H E S K E S L R D I I S K N P S A M L G
 332 TAAGGACATTATTGATAAGTTCCACGCCACAAATGAATTGCCCTTCCTTTTCAAAGTTTT 391
       K D I I D K F H A T N E L P F F L F K V L
 392 GTCCATTGAAAAAGTCTTGTCTATTCAAGCACATCCCGACAAAGCCTTGGGTAAAATATT 451
       S I E K V L S I Q A H P D K A L G K I L
 452 GCACGCTCAAGATCCTAAGAACTATCCTGATGATAATCACAAACCTGAAATGGCCATCGC 511
       H A Q D P K N Y P D D N H K P E M A I A
 512 TGTGACTGACTTTGAAGGTTTCTGCGGGTTCAAACCTTTGCAAGAGATTGCAGATGAATT 571
       V T D F E G F C G F K P L Q E I A D E L
 572 GAAACGTATTCCTGAATTACGCAACATTGTTGGTGAAGAAACTTCCAGGAATTTTATTGA 631
       K R I P E L R N I V G E E T S R N F I E
 632 GAACATTCAACCTTCTGCTCAGAAAGGTTCCCCAGAAGATGAGCAAAACAAAAAGCTATT 691
       N I Q P S A Q K G S P E D E Q N K K L L
 692 GCAAGCTGTTTTCAGCAGGGTCATGAACGCTTCGGATGACAAAATCAAGATTCAAGCTCG 751
       Q A V F S R V M N A S D D K I K I Q A R
 752 CTCCTTGGTCGAAAGATCAAAGAATTCTCCATCAGACTTTAACAAACCTGATTTACCAGA 811
       S L V E R S K N S P S D F N K P D L P E
 812 ATTAATTCAAAGACTGAATAAACAGTTCCCTGATGACGTGGGTTTGTTTTGTGGATGTTT 871
       L I Q R L N K Q F P D D V G L F C G C L
 872 ATTGTTGAATCACTGCAGATTGAATGCTGGTGAAGCCATCTTTTTAAGAGCTAAGGATCC 931
       L L N H C R L N A G E A I F L R A K D P
 932 TCACGCCTATATAAGCGGTGATATTATGGAATGTATGGCTGCTTCTGACAACGTAGTTAG 991
       H A Y I S G D I M E C M A A S D N V V R
 992 AGCAGGCTTCACTCCAAAATTCAAGGATGTTAAAAACTTGGTCTCCATGTTAACCTATAC 1051
       A G F T P K F K D V K N L V S M L T Y T
1052 ATATGATCCTGTGGAAAAGCAAAAAATGCAGCCTTTAAAGTTCGACAGGTCCTCTGGTAA 1111
       Y D P V E K Q K M Q P L K F D R S S G N
1112 CGGTAAGTCAGTTTTATATAACCCTCCAATCGAAGAATTTGCTGTATTGGAGACTACTTT 1171
       G K S V L Y N P P I E E F A V L E T T F
1172 TGATGAGAAACTTGGTCAAAGGCATTTTGAAGGTGTTGATGGTCCAAGTATCTTAATCAC 1231
       D E K L G Q R H F E G V D G P S I L I T
1232 TACAAAAGGTAATGGTTACATTAAAGCAGATGGCCAAAAATTGAAAGCTGAACCCGGTTT 1291
       T K G N G Y I K A D G Q K L K A E P G F
1292 TGTCTTTTTCATCGCTCCACATTTGCCTGTTGATTTGGAAGCTGAAGATGAGGCGTTTAC 1351
       V F F I A P H L P V D L E A E D E A F T
1352 TACCTATAGAGCCTTTGTGGAACCAAATTAGCTTTCTTTCTATTTCTTAATTTTCTTATA 1411
       T Y R A F V E P N *
1412 TCTATAATAGAGAAAATATTATACTTATTTATTTGAAGTCTACTTTATTTCACAGTTTTC 1471
1472 TATTTGCTTCTCACGTTCTATAATATAGTGGAATGGAAATATCAAACATAATTTGCTGTT 1531
1532 CTCTAAAACACTTGATATTGATGCATAAAAACATATATAGATACATATATAAATATTACG 1591
1592 TGGTAATCAATTGGTAGTTGCCATTGGTTTATTTTGATGCAATACCGTTTCTTTTTTCC 1651
1652 ACCTTAATAACC 1663
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FIG. 4. Sequence of the *S. cerevisiae PMI40* gene. Intron sequences are indicated in lowercase letters, with conserved elements underlined. The putative TATA box is double underlined. Transcription start sites determined by primer extension transcript mapping are indicated by arrows. Amino acid sequences corresponding to those found in sequenced peptides obtained from the purified protein are underlined.

of peptides derived from the pure protein (Fig. 2 and 4). However, the predicted molecular mass of this protein (42.7 kDa) was not in good agreement with the estimated size of the purified protein of 47 kDa. In addition, the sequence surrounding the initiation codon of this ORF was not similar to the consensus sequence derived from the analysis of many *S. cerevisiae* genes (3). Analysis of codon usage in the

PMI40 sequence using the program Codon Preference and the University of Wisconsin Genetics Computer Group yeast codon usage table indicated that an ORF initiating translation a further ~250 bp upstream of the methionine codon at position 240 had *S. cerevisiae* codon bias (results not shown). This information suggested that the *PMI40* transcript may be spliced. Analysis of *S. cerevisiae* introns has shown three highly conserved elements, i.e., the 5′ splice site GTAPyGT, the 3′ splice site PyAG, and the TACTAAC branchpoint sequence, which is usually situated 20 to 40 bp upstream of the 3′ splice site. A consensus intron 5′ splice site starting at position 31 in the *PMI40* sequence and a potential 3′ splice site starting at position 121 and defining an intron of 93 bp were found (Fig. 4). The branch site of this putative intron was not the consensus but a closely related sequence (AACTAAC) which was located 15 bp upstream of the 3′ splice site.

Removal of the putative intron resulted in a single ORF of 1,286 bp encoding a protein of 48.3 kDa, which is in close agreement with the size estimate of the pure protein (47 kDa), that still contained amino acid sequences corresponding to those obtained from the pure protein (Fig. 2 and 4). The nucleotides surrounding the initiation codon of the first exon were those often found in *S. cerevisiae* genes (3). The primary sequence of the protein encoded by the spliced *PMI40* gene was compared with the *E. coli*, *S. typhimurium*, and *P. aeruginosa* PMIs. Significant similarity was found only with the proteins from *E. coli* (35.2% identity) and *S. typhimurium* (34.9% identity). In contrast, extensive similarity searching has shown that PMI40 has no significant identity with the *P. aeruginosa* PMI–GDP-Man pyrophosphorylase bifunctional protein or with the reported sequence of phosphoglucoisomerase from *S. cerevisiae* (35), which shares the same substrate (fructose-6-phosphate) as PMI (Fig. 1).

Isolation and sequence of a *PMI40* cDNA. A cDNA of a part of *PMI40* was produced by using 1 μg of total RNA from *S. cerevisiae* S-150-2B as starting material for a cDNA synthesis reaction. An oligonucleotide corresponding to a region on the 3′ side of the intron was used as primer for the synthesis. A PCR amplification was then performed to generate a fragment from this cDNA after addition of a second oligonucleotide primer situated on the 5′ side of the intron. The following amplification conditions were used: 35 cycles of 94°C for 2 min, 40°C for 1 min, and 72°C for 1.5 min. A portion of this fragment was sequenced directly (see Materials and Methods) by using a third primer directed to give sequence data across the expected intron-exon boundaries. The resulting sequence was identical to that predicted for the *PMI40* gene after excision of the intron (results not shown).

PMI N-terminal sequence analysis. A peptide containing the N terminus of purified *S. cerevisiae* PMI protein was isolated and subjected to electrospray ionization mass spectrometric analysis (28). The sequence obtained corresponded to that of the first eight amino acids of the protein predicted from the ORF of the first exon in the *PMI40* DNA sequence (Fig. 4) but indicated that the N-terminal initiation methionine had been removed and the following serine had been acetylated.

Chromosomal location and transcript analysis of *PMI40*. Southern blots of restriction enzyme digests of total *S. cerevisiae* DNA were probed with a labeled 1.2-kb *Pst*I fragment containing most of the *PMI40* gene. Only the predicted hybridizing bands were detected, indicating that *PMI40* is probably unique (results not shown). The *PMI40* gene was located on chromosome V by hybridization of a *PMI40* probe to a Southern blot of *S. cerevisiae* gel-separated chromosomes (Fig. 5).

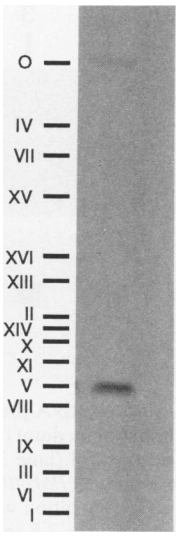

FIG. 5. Southern blot of *S. cerevisiae* gel-separated chromosomes hybridized to a 1.2-kb *Pst*I fragment containing *PMI40* DNA. Wash stringency used was 0.1% (wt/vol) SDS–0.2× SSC at 65°C for 2 h. The positions of all chromosomes visible on the ethidium bromide-stained gel are marked. O marks the origin of the gel.

The transcription start points of the *PMI40* gene were determined by primer extension transcript mapping. Five transcript initiation sites were found between positions −31 and −45 (Fig. 4), and the first ATG downstream of these corresponds to that of the initiation codon of the first exon of *PMI40*. A consensus TATA region (3) is found 22 bp upstream of the transcript initiation site at −45 (Fig. 4).

Disruption of the *PMI40* gene. A gene deletion construct (pPMIΔ1) was made by replacing the sequence between *PMI40 Mlu*I and *Msc*I restriction sites with an *Nru*I-*Sma*I fragment containing the *URA3* gene of *S. cerevisiae* (Fig. 3). The *Pst*I-*Eco*RI fragment from pPMIΔ1 containing the deletion construct was used to transform *S. cerevisiae* SEY6210. Ura$^+$ transformants were isolated on medium containing 10 mM D-mannose, and the integration of the construct at the chromosomal location of *PMI40* was confirmed by Southern analysis of DNA prepared from one of the transformants (results not shown). The resulting strain carrying a *pmi40*::*URA3* null allele and lacking 85% of the *PMI40* gene was able to grow on YPD or minimal medium containing D-glucose only when supplemented with 10 mM D-mannose. Transfer of the *pmi40*::*URA3* strain from medium with D-mannose to medium lacking D-mannose resulted, within 1.5 h, in cessation of growth, clumping, and arrest of many cells with unseparated buds. Continued incubation of the cells in medium without D-mannose resulted in a gradual decrease in viability.

***PMI40* gene expression.** Total RNA was prepared from exponentially growing *S. cerevisiae* S-150-2B cells grown on minimal medium containing D-glucose, D-mannose, or glycerol. Approximately equivalent amounts of RNA were gel separated under denaturing conditions and transferred to a nylon hybridization membrane. The filter was probed with the *PMI40* gene (Fig. 6A), and the number of bound counts was determined by quantitative radioanalytical image scanning of each RNA-containing track of the filter (Table 1). A DNA fragment containing the *ACT1* gene, which encodes

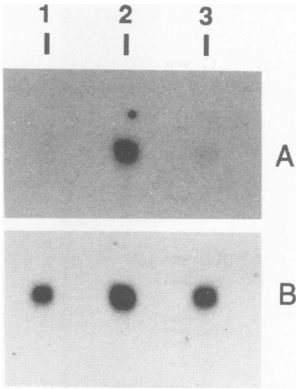

FIG. 6. Northern blot of total RNA prepared from *S. cerevisiae* S-150-2B cells grown on minimal medium containing D-glucose (lane 1), D-mannose (lane 2), or glycerol (lane 3) probed with the 0.8-kb *Mlu*I-*Bam*HI fragment containing *PMI40* DNA (A) or the *ACT1* gene of *S. cerevisiae* (B). Hybridization wash stringency was 0.1% (wt/vol) SDS–0.2× SSC at 65°C for 2 h in both cases.

the constitutively expressed actin (27), was used as a second probe (Fig. 6B), and the number of bound counts was determined as described above to establish the relative amounts of RNA loaded onto the gel (Table 1). RNA prepared from the D-mannose-grown cells contained 12 times as much *PMI40* mRNA as did RNA from cells grown on D-glucose (Table 1).

Because the *PMI40* intron does not contain a consensus branch site, we were interested in determining if there was any evidence for its inefficient splicing resulting in accumulation of unspliced transcript. A Northern blot of *S. cerevisiae* total RNA was hybridized with a labeled oligonucleotide corresponding to sequences 44 to 70 within the *PMI40* intron. No hybridizing message was detected in the expected position at ~1.6 kb (results not shown), indicating that the *PMI40* pre-mRNA is rapidly spliced under the conditions tested.

Assays of PMI activity in cell extracts prepared from *S. cerevisiae* S-150-2B cells grown under conditions identical to those used for the preparation of RNA were performed. Surprisingly, no increase in PMI activity was detected in cells grown on D-mannose as sole carbon source, despite the 12-fold-greater amount of *PMI40* mRNA found in these cells compared with D-glucose grown cells (Table 1). These results indicated either that there are further levels of control of expression of the *PMI40* gene, perhaps at the level of translation, or that the protein is posttranslationally modified. To determine which of these was most likely, an immunoblot of the cell extracts used for the PMI assays was probed with rabbit polyclonal antiserum raised against purified *S. cerevisiae* PMI protein (Fig. 7). The antibody was specific for PMI because it cross-reacted with purified PMI protein and was absent in a cell extract prepared from the strain carrying a *pmi40*::*URA3* deletion (Fig. 7). The intensities and mobilities of the bands were the same in the other two lanes containing material prepared from D-mannose- and D-glucose-grown cells. This suggested that equivalent amounts of PMI40 protein are made, that the protein is not posttranslationally modified, and that translation of the *PMI40* mRNA is regulated in D-mannose-grown cells.

TABLE 1. Effect of carbon source on *PMI40* mRNA levels and enzyme activity in *S. cerevisiae* S-150-2B

Carbon source	Total counts[a]		Relative level of *PMI40* mRNA[b]	PMI sp act[c]
	PMI probe	Actin probe		
D-Glucose	1,180	52,676	1.0	0.112
D-Mannose	33,228	120,218	12.4	0.088
Glycerol	2,182	50,257	1.9	ND

[a] Total bound counts of probe on the Northern blot shown in Fig. 6 as determined by radioanalytic imaging and quantitation.
[b] Relative levels of *PMI40* mRNA (glucose = 1) corrected for differences in gel loading by quantitation of an actin-probed Northern blot.
[c] Micromoles of NADPH per minute per milligram of protein at 25°C. ND, not determined.

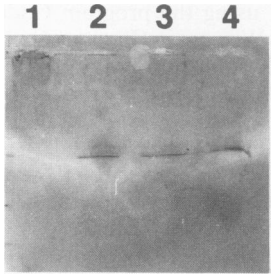

FIG. 7. Immunoblot of SDS-PAGE-separated cell extracts prepared from the *S. cerevisiae* strain carrying the *pmi40*::*URA3* allele (lane 1) or *S. cerevisiae* S-150-2B cells grown on minimal medium plus D-glucose (lane 2), D-mannose (lane 3), or purified PMI protein (lane 4). Equal amounts of protein were applied to each track (except for lane 4), and the blot was probed with polyclonal rabbit antiserum against purified *S. cerevisiae* PMI protein (see Materials and Methods).

DISCUSSION

We have isolated the *PMI40* gene by complementation of a strain carrying a temperature-sensitive *pmi40-1* mutation. Integration of a construct into the *S. cerevisiae* genome to delete 85% of the *PMI40* coding sequence results in a *pmi40*::*URA3* strain displaying a phenotype similar to that of strains containing the *pmi40-1* mutation at the restrictive temperature (26). Evidence that *PMI40* is the structural gene encoding PMI in *S. cerevisiae* is based on the following observations. First, strains possessing the temperature-sensitive *pmi40-1* mutation which was complemented by *PMI40* display thermolabile PMI activity, and therefore, *pmi40-1* is likely to define the structural gene for this enzyme (26). Second, the predicted sequence of the PMI40 protein contains sequences corresponding to those of peptides derived from PMI purified to homogeneity. Finally, the PMI40 protein has significant identity with the *E. coli* and *S. typhimurium* PMIs, indicating that they are homologous enzymes.

We have demonstrated that the *PMI40* gene contains an intron and is thus, to our knowledge, the first *S. cerevisiae* glycolytic gene in which an intron has been identified. The presence of introns in nuclear pre-mRNAs from *S. cerevisiae* has been well documented (12, 37) and has provided a model system for the study of splicing mechanisms (25, 37). Most *S. cerevisiae* introns are in genes encoding ribosomal proteins and have a number of features in common. They are all situated toward the 5′ end of the gene, are between 300 and 500 bp in length, and have highly conserved elements important for splicing (12, 37). In yeast genes which do not encode ribosomal proteins, introns have been found in only

11 of the ~400 genes so far characterized (37). They appear to be always in genes encoding proteins with critical functions and often in those for structural and cytoskeletal proteins. The features of these introns are very similar to those of the introns found in genes encoding ribosomal proteins except that some are shorter.

The *PMI40* intron is located between the same two nucleotides of the same codon as the first intron of the *Aspergillus nidulans manA* gene encoding PMI (32a). This indicates that the intron may have been present in a common progenitor of the two now-diverged ascomycete species.

The *PMI40* intron does not contain the consensus TAC TAAC branch site found in the majority of introns so far identified in *S. cerevisiae* (37) but instead has the sequence AACTAAC. Three other *S. cerevisiae* introns which contain mutations in one of the first two nucleotides of the consensus branch site have now been identified (21, 37). Mutations at some points in the consensus branch site have been shown to be deleterious for splicing, although the T_1 or A_2 positions can be changed with little effect on splicing efficiency and such mutants accumulate only small amounts of unspliced transcripts (11, 15). It has been reported that a *Drosophila* alcohol dehydrogenase intron with the same branchpoint sequence as that of *PMI40* can be spliced correctly in *S. cerevisiae* (36), and we could detect no evidence for inefficient splicing of the *PMI40* gene under the conditions tested. If the first two branchpoint nucleotides can be mutated with little effect on splicing efficiency, then why are they conserved in all but 4 of the ~40 *S. cerevisiae* introns so far identified? Perhaps these conserved nucleotides are essential for maximal splicing efficiency in natural environments or in ways that cannot be detected by the assay systems currently used. Alternatively, the expression levels of genes containing variant intron branchpoint sequences may be such that they do not require being spliced with high efficiency. However, as we found no evidence for inefficient splicing of the *PMI40* intron, this indicates that a consensus branch site may not be essential for efficient splicing.

The steady-state level of *PMI40* mRNA varies according to the carbon source on which cells are grown, indicating that PMI activity is regulated, at least in part, at the level of transcription of the *PMI40* gene. When D-glucose or a gluconeogenic carbon source such as glycerol is used, the level of *PMI40* mRNA appears to be maintained at a basal and presumably constitutive level. However, when D-mannose is used, transcription of *PMI40* increases 12-fold over the basal level. This is perhaps not surprising, as one would anticipate a need for more PMI40 when cells are grown on D-mannose, so that fructose-6-phosphate could be generated to provide glycolytic intermediates for metabolism and growth. Alternatively, when grown on D-glucose, PMI40 would have to supply mannose-6-phosphate only for input into the GDP-Man–Dol-P-Man synthesis pathway. Transcription of the *PMI40* gene does not appear to be subject to glucose repression, because *PMI40* mRNA levels are still maintained at a low level when cells are grown on glycerol. In contrast, the increase in *PMI40* transcription when cells are grown on D-mannose indicates D-mannose induction of *PMI40* transcription mediated through unknown factors. The 5′ region of the *PMI40* gene does not appear to contain recognized motifs for transcription factors known to affect the expression of several glycolytic genes such as GCR1 or RAP1 (20).

Interestingly, PMI enzyme activity is not increased in D-mannose-grown cells compared with D-glucose-grown cells. The levels of PMI protein, as determined by immunoblotting, also appear to be constant, and therefore we propose that the *PMI40* gene is additionally regulated at the posttranscriptional level, probably at the level of translation, as has been observed for some other glycolytic genes (20). We have not been able to identify conditions under which increased levels of PMI activity can be detected.

The reaction performed by PMI40 represents a key branchpoint in the pathway from glycolytic intermediates through to Dol-P-Man. It has been shown that the expression of a number of glycolytic genes is regulated, but those for key steps, particularly phosphofructokinase and pyruvate kinase, are, like *PMI40*, regulated at both the transcriptional and posttranscriptional levels and are subject to high levels of induction (20).

We are currently extending our studies on PMI to other fungi and analyzing further the enzymology and role of this essential enzyme in the pathway of GDP-Man and Dol-P-Man synthesis.

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