**MNN6, a Member of the KRE2/MNT1 Family, Is the Gene for Mannosylphosphate Transfer in Saccharomyces cerevisiae**

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In yeast *Saccharomyces cerevisiae* the N-linked sugar chain is modified at different positions by the addition of mannosylphosphate. The *mnn6* mutant is deficient in the mannosylphosphate transferase activity toward mannolactose (Karson, E. M., and Ballou, C. E. (1978) *J. Biol. Chem.* 253, 6484–6492). We have cloned the *MNN6* gene by complementation. It has encoded a 446-amino acid polypeptide with the characteristics of type II membrane protein. The deduced Mnn6p showed a significant similarity to Kre2p/Mnt1p, a Golgi α-1,2-mannosyltransferase involved in O-glycosylation. The null mutant of *MNN6* showed a normal cell growth, less binding to Alcian blue, hypersensitivity to Calcofluor White and hygromycin B, and diminished mannosylphosphate transferase activity toward the endoplasmic reticulum core oligosaccharide acceptors (Man8GlcNAc2-PA and Man5GlcNAc2-PA) in vitro, suggesting the involvement of the *MNN6* gene in the endoplasmic reticulum core oligosaccharide phosphorylation. However, no differences were observed in N-linked mannoprotein oligosaccharides between ∆och1 Δmnn1 cells and ∆och1Δmnn1Δmnn6 cells, indicating the existence of redundant genes required for the core oligosaccharide phosphorylation. Based on a dramatic decrease in polylactosamine outer chain phosphorylation by *MNN6* gene disruption and a determination of the mannosylphosphorylation site in the acceptor, it is postulated that the *MNN6* gene may be a structural gene encoding a mannosylphosphate transferase, which recognizes any oligosaccharides with at least one α-1,2-linked mannohexose unit.

In yeast *Saccharomyces cerevisiae* the biosynthesis of N-linked oligosaccharides has been studied in detail. The core oligosaccharide (Man5GlcNAc2) synthesized in the endoplasmic reticulum (ER) is identical in yeast and mammals. The

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‡ The abbreviations used are: ER, endoplasmic reticulum; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; CPW, Calcofluor White; PA, pyridylamide; MS-PA, Man8GlcNAc2-PA; ManP-MS-PA, monomannosylphosphorylated M8-PA; M5-PA, Man5GlcNAc2-PA; ManP-M5-PA, monomannosylphosphorylated M5-PA; ManP-M6-PA, monomannosylphosphorylated Man6GlcNAc2-PA.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—Strain TO3-6D, used for the cloning of *MNN6*, was a meiotic segregant from a cross of LB1425-1B, kindly provided by C. E. Ballou (University of California, Berkeley) and a strain of LB1-10B, purchased from the American Type Culture Collection (ATCC). KK4 and its isogenic *mnn6* disruptant strain XW44 were used for the Calcofluor White (CFW; Sigma) and hygromycin B (Sigma) sensitivity test. Strains YS125-15B, XW27, YS131-30A, and YS131-30D were used for microsomal membrane preparation. YS126-47D and its isogenic disruptant strain XW43 were used for oligosaccharide analysis. Yeast strains used in this study are summarized in Table I.
Yeast strains with multigene disruptions were constructed by standard genetic methods (17, 18). Yeast strains were grown either in YPD (2% Bacto-peptone, 1% yeast extract, and 2% glucose) or in a complete minimal medium containing 4% glucose, 0.67% Bacto-yeast nitrogen base without amino acids (Difco), 0.3% sorbitol and supplemented with the appropriate auxotrophic requirements. Hydrophobic requirements were separately added to autoclaved YPD/agar to a final concentration of 50 μg/ml just prior to pouring plates.

Cloning of MNN6 by Modified Alcian Blue Staining—Yeast genomic DNA library in YCP50, “CEN BANK” A and B, were purchased from ATCC. Transformation of yeast cells was carried out by the lithium acetate method (19). Transformants were selected on an SD-ura plate and colonies were picked and stored on an SD-ura plate. The colonies from master plates were transferred to nitrocellulose filter and incubated for one more day at 30 °C. Those colonies on the filter were fixed by autoclaving at 120 °C for 1 h and stain by immersion into 0.1% Alcian blue solution until the blue color was developed on the wild type (MNN6) colonies at room temperature (20–30 min). Positive colonies were screened as those providing a blue stain (wild type phenotype). The putative clones were further reasserted and confirmed individually by conventional Alcian blue assay (11).

DNA Sequencing—Bacterial strain JM109 was used for the preparation of plasmids. Restriction fragments containing a portion of the MNN6 gene were subcloned into plRSM16 vector (20). Sequencing was performed by the dye cycle terminator method with dye primers (21, 22). The SequiTherm II Sequencing Kit-LC by the LI-COR model 4000L Automated Sequencer. Sequences comparisons against the GenBank or GenPept sequence data bases were performed using the FASTA (22) and BLAST (23) programs. The comparisons against the GenBank or GenPept sequence data bases were performed by the dideoxy chain termination method with dye primers MNN6 and confirmed individually by conventional Alcian blue assay (11).

Gene Disruption—Disruption of MNN6 gene was made by the single-step gene replacement procedure (25). The 4.3-kb isola-ble pair HpaI-SacI fragment containing MNN6 gene from pSA9-7 was digested with BglII and BclI restriction endonucleases. The BclI site is located 333 base pairs upstream from the ATG, and the BclI site is found 558 base pairs upstream from the stop codon (see Fig. 4A). This digestion removed a 1116-base pair encompassing 261 amino acids of the MNN6 sequence and further replaced it with a BglII fragment containing the complete ADE2 gene from pAS111 (26). Haploid yeast strains were used to transform with the linearized mnn6::ADE2 DNA fragments (Fig. 4A). The disruption of MNN6 was confirmed by Southern hybridization (data not shown). Selection of mnn6 disruptants (Δmnn6) was done as follows: for strains carrying the ade2 mutation, Δmnn6 was selected on an SD-ade plate; for strains carrying no ade2 mutation (like K9700) Δmnn6 was detected by QAE-Sepharose adsorption according to the method described by Balou (11).

Construction of High Copy Plasmid Carrying MNN6—A complementing fragment (HpaI/NruI fragment) containing the entire MNN6 gene was excised by digestion with KpnI/SacI, whose sites are located in multicloning sites of plRSM16-based plasmid pRSMNN6 and then inserted into the multicopy vector of pET351 and named pETMNN6. The pETMNN6 high copy vector was constructed based on pYEp51 (27), in which a BamHI/HpaI fragment containing the LEU2 gene was replaced with a BamHI/PouII fragment carrying TRP1 gene from pJ4246 vector (28).

Mannosylphosphatase Transferase Assay—The microsomal membrane proteins containing mannosylphosphatase transferase activity were prepared according to the previous method (29), except that the cell pellets were frozen at –20 °C for 1 h before the cells were destroyed by glass beads using a B. Braun homogenizer. The enzyme assay was carried out by using 400 μg of protein of the high speed pellet (centrifugation at 100,000 × g for 60 min) in 50 μl of 50 mM Tris-HCl (pH 6.0), 10 mM MgCl2, 25 or 50 pmol of acceptor (depending on the acceptor used), 0.6% Triton X-100, 1 mM GDP-mannose, and 0.5 mM 1-deoxy-mannojirimycin as an inhibitor of α-mannosidase in yeast (30) at 30 °C for 60 min. The enzyme reaction was terminated by boiling for 5 min, and the reaction mixture was ultracentrifuged with ultrafree C3LG (Millipore). The filtrated solution was lyophilized and used for high performance liquid chromatography (HPLC) analysis. MassGlCNAC2-PA, purchased from Takara Shuzo Co. (Kyoto, Japan), was used as the acceptor (50 pmol) in the enzyme assay. Man5GlcNAc2-PA acceptor (25 pmol) prepared from strain YC10-1B containing the wild type MNN6 gene was digested by 1,2-α-mannosidase solution (Aspergillus saitoi, Oxford Glycosystems, Inc.) was added and then incubated for 15 h at 37 °C.

Mass Spectrometry—Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed in the negative ion mode by using α-cyano-4-hydroxy cinnamic acid as a matrix. The mass spectrometer used in this work was a Finnigan Lasermat (Finnigan MAT Ltd., Hempstead, United Kingdom). Samples (100–1000 pmol) were desalted by HPLC using a Tosoh TSK-GER Carbon500 column (0.4 × 10 cm). Two solvents, A and B, were used. Solvent A was water containing 0.1% trifluoroacetic acid. The column was equilibrated with solvent B, and a linear gradient was run over 40 min, in which the percentage of solvent B increased from 20 to 100% with a flow rate of 1 ml/min. For analysis of mannosylphosphorylated Man5GlcNAc2-PA, buffer A contained a 10:90 (v/v) ratio of 200 mM acetic acid adjusted with triethylamine (pH 7.3). The initial solvent of 100% buffer A (0% buffer B) was run for 5 min, and then the percentage of buffer B was linearly increased from 0 to 50% within 30 min; finally, the percentage of buffer B was linearly increased from 50 to 100% for another 15 min with a flow rate of 1 ml/min.

α-1,2-Mannosidase Digestion—Samples (enzymatic reaction product or in vivo acidic oligosaccharide product prepared from Δoch/Δmann1 cells) were dissolved in 8 μl of 0.1 mM sodium acetate buffer (pH 5.0). One micromol/2 μl of α-1,2-mannosidase solution (from Aspergillus satoi, Oxford Glycosystems, Inc.) was added and then incubated for 15 h at 37 °C.

RESULTS

Cloning of the MNN6 Gene—The original mnn6 mutant exhibited a reduced amount of phosphomannan on the cell wall and showed less binding to Alcian blue, a dye that binds to a phospho moity of cell surface mannoproteins (11). The wild type cells exhibited a blue color after staining with Alcian blue, and showed less binding to Alcian blue, a dye that binds to a phospho- moity of cell surface mannoproteins. The chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but they were actually measured by reference to internal acetone (δ = 2.217 ppm).

Restriction maps of the insert DNA on the plasmids from two positive clones were identical. A ~10-kb DNA fragment that can complement the mnn6 mutation was isolated. To identify the smallest complementary region, further subcloning into

bulk yeast mannoproteins using glycopeptidase A (Seikagaku Kogyo Co., Tokyo, Japan), an enzyme specific to release N-linked oligosaccharides from glycoprotein or glycopeptide. Pyridylation of the oligosaccharides was performed using a commercial reagent kit (Takara Shuzo Co., Kyoto, Japan). The PA-oligosaccharides were obtained by gel filtration chromatography on Bio-Gel P-2 and purified by high voltage electrophoresis (1.0 × 40 cm) and used for detection by fluorescence (excitation ~ 310 nm; emission ~ 380 nm).

HPLC Analysis—The separation of PA-oligosaccharides was carried out by HPLC using a Tosoh C18-PM II, a Tosoh PX-8020 controller, and a Shimadzu spectrofluorometric detector, RF-550. Phosphorylated oligosaccharides were quantitated by their size and polarity with Asahipak NH-PF-50 (0.46 × 25 cm) (Asahi Chemical Co., Tokyo, Japan) at a flow rate of 1 ml/min. The retention time of oligosaccharide largely depends on the number of sugar residues in the amine-modified column chromatography. Samples were resuspended in buffer A and injected in up to 20-μl aliquots. For analysis of mannosylphosphorylated Man5GlcNAc2-PA, the ratio of 200 mM acetic acid adjusted with triethylamine (pH 7.3) to ammonium acetate, and buffer B was 100% 200 mM acetic acid adjusted with triethylamine (pH 7.3). The initial solvent of 100% buffer A (0% buffer B) was run for 5 min, and then the percentage of buffer B was linearly increased from 0 to 50% within 30 min; finally, the percentage of buffer B was linearly increased from 50 to 100% for another 15 min with a flow rate of 1 ml/min.
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**Fig. 1. Complementation analysis of isolated DNA.** The restriction map of the MNN6 clone is shown. Open areas and straight lines indicate cloned yeast DNA and vector DNA, respectively. The ability of DNA fragments (from pSA9-1 to pRS-MNN6) to complement the mnn6 mutant was assayed by Alcian blue dye binding (indicated by + or −). The DNA fragment of pRS-MNN6 was sequenced; the open reading frame (ORF) of MNN6 is indicated by the shaded box, and the direction of transcription is denoted by the arrow.

pRS316 vector was carried out. Plasmid pA9 (Fig. 1) contained a ∼10-kb Sau3AI fragment insert at the BamHI site on YCp50 vector. pSA series plasmids ranging from pSA9-1 to pSA9-7 and pRS-MNN6 (Fig. 1) were the subclones derived from plasmid pA9 and inserted into single-copy yeast vector pRS316. These plasmids were introduced into strain TO3-6D, and the complementation analysis was carried out by Alcian blue staining. The complementing region was assigned into a 2.7-kb HpaI-NruI fragment (pRS-MNN6) (Fig. 1). To exclude the possibility of the suppressor gene cloning of mnn6 mutation, strain XW13 (Δmnn6::ADE2) (Table I and Fig. 4A) was crossed with strain TO3-6D (mnn6), and after sporulation, tetradis were analyzed by Alcian blue staining. All of the segregants derived from 20 tetrads showed the mutant phenotype, which was not able to bind to Alcian blue, demonstrating the gene disruption at the original mnn6 locus and confirming the cloning of the MNN6 gene.

**MNN6 Is a Member of the KRE2/MNT1 Mannosyltransferase Gene Family**—Sequence analysis of a 2.7-kb HpaI-NruI fragment revealed one open reading frame of 1338 base pairs, which was translated to a protein of 446 amino acids (MNN6 accession number U43922). From the GenBank data base, the MNN6 gene was identical with the KTR6 gene, which was reported by the genome sequencing as a family of killer toxin related genes (accession number U39205). Two potential N-glycosylation sites were found in the Mnn6p sequence (Fig. 2). Kyte-Doolittle hydrophobicity analysis showed a potential membrane-spanning region near the N-terminus suggesting a type II membrane protein (Fig. 2B).

In addition, a homology search of the MNN6 sequence revealed that Mnn6p shares 38% identity and 79% similarity with Kre2p/Mnt1p, an α-1,2-mannosyltransferase responsible for O-linked glycosylation in yeast (32, 33). Sequence alignment of the Mnn6p and Kre2 protein families (Ktr1p, Ktr2p, Ktr3p, and Yur1p) (34–36) is shown in Fig. 3. Six cysteine residue positions in the latter half of Mnn6p were identical to those of the other proteins, suggesting a similarity of three-dimensional structures. Interestingly, Mnn6p has an additional cysteine at the 120th residue, which shares an identical position with Kre2p, but lacks one cysteine at the 229th residue, which is commonly located in the other five proteins.

**Disruption of the MNN6 Gene Results in Calcoflour White and Hygromycin B Sensitivities**—To study the function of Mnn6p, the MNN6 gene was disrupted by inserting the ADE2 gene (Fig. 4). MNN6 gene disruption did not affect the cell morphology and the rate of cell growth, indicating a nonessential gene for normal cell growth. The mnn6 null (Δmnn6) mutant displayed the same phenotype as the original mnn6 mutant, which provides a prominent loss of Alcian blue binding ability, while the isogenic wild type cells strongly bound the dye. A single copy plasmid containing the MNN6 gene restored the Alcian blue binding, which was lost in the Δmnn6 mutant (data not shown), suggesting the involvement of MNN6 in oligosaccharide phosphorylation. A sensitivity to a negatively charged fluorescent dye CFW and an aminoglycoside antibiotic hygromycin B was examined. As shown in Fig. 4B, the Δmnn6 mutant was sensitive to CFW and hygromycin B, while the isogenic wild type was not affected. A single copy of the MNN6 gene restored the growth defect of the Δmnn6 mutant by CFW and hygromycin B, respectively. Since CFW binds to nascent chains of chitin and prevents both microfibril formation and cell wall assembly (37), the result may suggest a lesser charge repulsion between CFW and the cell surface in Δmnn6 mutant.

**Alcian Blue Staining of Various Strains**—The original mnn6 mutant was deficient in the mannosephosphate addition to the mannose outer chain (15). This is supported by the Alcian blue staining of the isogenic pairs with or without the MNN6 gene (Δmnn1 cells and Δmnn1Δmnn8 cells in Table II, G-1). To further examine whether the MNN6 gene may affect phosphorylation of the oligosaccharide lacking a mannose outer chain, other isogenic pairs of double and triple mutant cells (Δoch1Δmnn1 and Δoch1Δmnn1Δmnn6) were constructed. The Δoch1Δmnn1 cells showed a significant dye binding, while the Δoch1Δmnn1Δmnn6 cells failed to bind the dye (Table II, G-2). The introduction of a multicopy plasmid containing the MNN6 gene (pETMNN6) into Δoch1Δmnn1Δmnn6 cells recovered the...
dye binding ability, but the effect of multicopy gene dosage was not observed on Alcian blue staining (Table II, G-2). These results suggest that the MNN6 gene may be involved in the phosphorylation in vivo not only at the outer chain portion but also the N-linked core and/or O-linked oligosaccharides. In contrast, the Alcian blue staining was not changed by the introduction of the Dmnn6 mutation into Doch1Dmnn1Dmnn6 cells, which produces the N-linked core oligosaccharide (Man8GlcNAc2) (31) and truncated O-linked chains (Man2) (32), suggesting the possibility of no apparent effect of the MNN6 gene on N-linked core oligosaccharide phosphorylation in vivo.

Reduction of Mannosylphosphate Transferase Activity toward N-Linked Core Oligosaccharide Acceptors in a mnn6 Null Mutant—The assay conditions for the mannosylphosphate transferase were established by using 1 mM GDP-mannose as a donor and 50 pmol of pyridylaminated core oligosaccharide Man8GlcNAc2-PA (M8-PA; see structure shown in Fig. 8, A-1) as an acceptor in 50 μl of reaction mixture (see “Experimental Procedures”). Under these assay conditions, microsomal membranes from MNN6 wild type cells (Doch1Dmnn1, strain YS125–15B) showed two reaction products (peaks 1 and 2) (Fig. 5, A-1), which were already identified as a monomannosylphosphorylated Man8GlcNAc2-PA (ManP-M8-PA) (16). In contrast, microsomal membranes from isogenic Dmnn6 cells (Doch1Dmnn1Dmnn6, strain XW27) diminished corresponding peaks (Fig. 5, A-2), and the enzyme activity was restored after the introduction of the MNN6 gene into Dmnn6 cells (Fig. 5, A-3), indicating more directly the involvement of the MNN6 gene in the mannosylphosphate transferase activity toward Man8GlcNAc2-PA in vitro. However, introduction of MNN6 in a multicopy plasmid did not produce any higher enzymatic activities in wild type cells (Fig. 5, A-4), suggesting the presence of some limiting factors for this enzyme reaction.

Furthermore, the enzyme activity toward the N-linked core-like oligosaccharide derivative Man5GlcNAc2-PA (prepared from Doch1Dmnn1Dmnn4::lys2 alg3::His3 cells, 25 pmol) (see structure shown in Fig. 8, A-1) was also compared by using microsomal membranes prepared from isogenic pairs of Dmnn1 cells (strain YS131–30A) and Dmnn1Dmnn6 cells (strain YS131–30D). The MNN6 cell exhibited the enzyme activity toward the Man5GlcNAc2-PA (M5-PA) acceptor and gave the reaction

### Table I

| Strain          | Genotype                | Source            |
|-----------------|-------------------------|-------------------|
| LB1425–1B       | MATa mnn6               | C. E. Ballou      |
| LB1–10B         | MATa suc2 mal2gal2cup1mnn1 | ATCC              |
| TO3–6D          | MATa leu2 ura3 his1mirhis3 mnn1 mnn6 | This study       |
| XHW13           | MATa leu2 ura3 trp1 his3 ade2 lys2 mnn6:ADE2 | This study       |
| YS125–15B       | MATa leu2 ura3 trp1 his3 ade2 lys2 och1::LEU2 mnn1::URA3 | This study       |
| YS126–47D       | MATa leu2 ura3 trp1 his3 ade2 lys2 och1::LEU2 mnn1::URA3 mnn6::ADE2 | This study       |
| XW27            | MATa leu2 ura3 trp1 his3 ade2 lys2 och1::LEU2 mnn1::URA3 mnn6::ADE2 | This study       |
| YS131–30A       | MATa leu2 ura3 trp1 his3 ade2 lys2 mnn1::URA3 k2::TRP1 | This study       |
| YS131–30D       | MATa leu2 ura3 trp1 his3 ade2 lys2 mnn1::URA3 k2::TRP1 mnn6::ADE2 | This study       |
| KK4             | MATa ura3 his1 or his3 trp1 leu2 gal80 | Y. Nogi           |
| XW44            | MATa ura3 his1 or his3 trp1 leu2 gal80 mnn6::ADE2 | This study       |
| YS133–1D        | MATa leu2 ura3 trp1 his3 ade2 lys2 och1::LEU2 mnn1::URA3 k2::TRP1 mnn6::ADE2 | This study       |
| mnn4::lys2 alg3::His3 |                      |                   |

**Fig. 2.** The predicted Mnn6p sequence showed a feature of type II membrane protein. A, the deduced amino acid sequence of the MNN6 gene. The putative membrane spanning region is underlined. Two potential N-glycosylation sites are indicated by boldface type. B, hydropathy profile of Mnn6p according to Kyte and Doolittle (24) using a window of 16 amino acids. The hydrophilic region is indicated by positive values, and the hydrophobic portion is shown by negative values.
product of peak 3 (Fig. 5, B-1). This reaction product was identified as a monomannosylphosphorylated Man5GlcNAc2-PA (ManP-M5-PA) by alkaline phosphatase digestion and subsequent mild acid treatment (data not shown). Disruption of the MNN6 gene diminished mannosylphosphate transferase activity toward M5-PA (Fig. 5, B-2), indicating the involvement of MNN6 in mannosylphosphorylation of core-like oligosaccharide Man5GlcNAc2.

Characterization of Enzymatic Reaction Products—To determine the structure of reaction products, peaks 1 and 2 in Fig. 5 were analyzed by MALDI-TOF mass spectrometry. The molecular ion peaks were observed at m/z 2048.9 for peak 1 and 2042.5 for peak 2, respectively. These mass values were nearly identical to the molecular mass of Man5GlcNAc2-PA (calculated M_r 2041.8). The 1H NMR spectra of peak 2 show the mannosylphosphate signal at δ 5.44 (Fig. 6B). The intensity of this signal indicates the presence of one mannosylphosphate group in peak 2. Measurement of 1H NMR spectra of peak 1 was not successful due to the loss of material during the purification process.

The core-like oligosaccharide has two mannosylphosphorylation sites (38), two structures of Man5GlcNAc2-PA are possible. One is the structure in which mannosylphosphate attaches to the side of the α-1,6-branch of core Man8GlcNAc2 (structure I); the other is the structure in which mannosylphosphate attaches to the side of the α-1,3-branch of the same Man8GlcNAc2 (structure II). By α-1,2-mannosidase digestion, structure I releases three mannoses and yields ManP-M5-PA, but structure II releases two mannoses and yields ManP-M6-PA. The α-1,2-mannosidase digestion products of peaks 1 and 2 were analyzed by MALDI-TOF mass spectrometry. The molecular ion peak of each product was detected at m/z 1724.7 (ManP-M6-PA, calculated M_r 1717.5) for peak 1 and 1558.9 (ManP-M5-PA, calculated M_r 1555.4) for peak 2, respectively. These results revealed the site of monomannosylphosphorylation at Man8GlcNAc2. Peak 1 product was mannosylphosphorylated at the α-1,6-branch of core Man8GlcNAc2 (structure I) and peak 2 product was mannosylphosphorylated at the α-1,3-branch of core Man8GlcNAc2 (structure II).

Since the N-linked core oligosaccharide profiles were not changed by MNN6 gene disruption in vivo—Further to examine the effect of MNN6 gene disruption on the mannosylphosphate addition in the N-linked core portion, we analyzed the oligosaccharides of mannoproteins prepared from D. och1Dmnn1.
We have reported the cloning and analysis of the MNN6 gene. For the cloning, the original Alcian blue dye binding assay in a test tube was not appropriate for the colony screening due to the laborious work. To solve this problem, a modified procedure for Alcian blue staining was developed on plates. The method established in this work should be applicable to clone other yeast genes, especially genes related to the biosynthesis of cell wall components.

Sequence analysis of the MNN6 gene predicted a type II membrane protein with 446 amino acids, which is highly homologous to \( \alpha \)-1,2 mannosyltransferase, Kre2p/Mnt1p, involved in O-glycosylation in \( S. \) cerevisiae (32, 33). A significant homology between Mnn6p and \( KRE2/MNT1 \) gene family proteins suggests the presence of similar domain involved in the recognition of a common structure of the acceptor (\( \alpha \)-1,2-linked...
mannobiose) recognized by all of these enzymes. The functional relations between Mnn6p and Kre2p/Mnt1p will be investigated in future work.

Disruption of \textit{MNN6} caused a hypersensitivity to CFW and hygromycin B (Fig. 4). The former phenotype is caused by the loss of charge repulsion between the cell surface and drug, leading to the penetration of drug through the outermost mannoprotein portion in the cell wall. It is noteworthy that hygromycin B-sensitive mutants involve not only the defects in sugar chain length, as reported (39, 40), but also in oligosaccharide phosphorylation, although its mechanism is still unclear.

We have shown that \textit{MNN6} is involved in core oligosaccharide phosphorylation by demonstrating the loss of mannosylphosphate transferase activity \textit{in vitro} toward Man8GlcNAc2 and Man5GlcNAc2 in \textit{\Delta mnn6} cells. Two reaction products (peaks 1 and 2) corresponding to ManP-M8-PA were identified when M8-PA was used as an acceptor. The mannosylphosphorylation site was determined by time-of-flight mass spectrometry after the \(\alpha\)-1,2-mannosidase treatment. These sites were identical to the phosphorylation sites observed in dimannosylphosphorylated oligosaccharide \textit{in vivo} (peak \(x\) compound in Fig. 7) described in this paper and to those reported for the \(N\)-linked core-like Man10GlcNAc2 oligosaccharide from carboxypeptidase Y and \textit{mnn1 mnn9} strain mannoproteins (38, 41). When M5-PA was used for acceptor substrate, only one peak corresponding to monomannosylphosphorylated product was observed (Fig. 5, peak 3, B-1). Although the phosphorylation site could not be determined by \(\alpha\)-1,2-mannosidase treatment due to the limited amount of purified material, based on combined results on both the phosphorylation sites determined for ManP-M8-PA \textit{in vitro} and the structure of dimannosylphosphorylated oligosaccharide determined \textit{in vivo}, the most reasonable phosphorylation site in ManP-M5-PA is shown in Fig. 8 (A-2).

Apparently, \(\alpha\)-1,2-linked mannotriose (Man1,2Man1,2Man) (mannose residue for the phosphorylation is shown in boldface type) is a common structure for the phosphorylation of Man8GlcNAc2, Man5GlcNAc2, and mannose outer chain branch. Consistent with the previous result (15), we found that \textit{\Delta mnn6} mutant diminished the enzyme activity toward the \(\alpha\)-1,2-linked mannotriose (Man1,2Man1,2Man), which mimics...
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of mannose 1-phosphate and galactose from GDP-mannose and UDP-galactose, respectively (13). Since these reactions involve transfer of mannose 1-phosphate from GDP-mannose, this study may also contribute to the understanding of other mannosylphosphate transferases, such as those in Leishmania.

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**REFERENCES**

1. Nakajima, T., and Ballou, C. E. (1974) *J. Biol. Chem.* 249, 7665–7664
2. Kukuruzinska, M. A., Bergh, M. L. E., and Jackson, B. J. (1993) *Annu. Rev. Biochem.* 62, 915–944
3. Herscovics, A., and Orlean, P. (1993) *FASEB J.* 7, 540–550
4. Lehle, L., and Tanner, W. (1995) in *Protein Glycosylation in Yeast, in Glycoproteins* (Montreuil, J., Schachter, H., and Vliegenthart, J. F. G., eds) pp. 475–509, Elsevier, Amsterdam
5. Trimble, R. B., and Verostek, M. F. (1995) *Trends Glycobiol.* Glycotech. 7, 1–30
6. Mill, P. J. (1966) *J. Gen. Microbiol.* 44, 329–341
7. Cawly, T. N., and Ballou, C. E. (1972) *J. Bacteriol.* 111, 690–695
8. Thieme, T. R., and Ballou, C. E. (1971) *Biochemistry* 10, 4121–4129
9. Rasche, W. C., and Ballou, C. E. (1971) *Biochemistry* 10, 4130–4135
10. Ballou, C. E., Kern, K. A., and Raschke, W. C. (1973) *J. Biol. Chem.* 248, 4671–4673
11. Ballou, C. E. (1990) *Methods Enzymol.* 185, 440–470
12. Nakajima, T., and Ballou, C. E. (1975) *Proc. Natl. Acad. Sci. U. S. A.* 72, 3912–3916
13. Carver, M. A., and Turco, S. J. (1992) *Arch. Biochem. Biophys.* 293, 309–317
14. Rosenfeld, L., and Ballou, C. E. (1974) *J. Biol. Chem.* 249, 2319–2321
15. Karson, E. M. and Ballou, C. E. (1978) *J. Biol. Chem.* 253, 6484–6492
16. Okami, T., Shimma, Y., Tanaka, A., and Jigami, Y. (1986) *Glycobiology* 6, 805–810
17. Sherman, F., and Hicks, J. (1991) *Methods Enzymol.* 194, 21–37
18. Sherman, F., Fink, G. R., and Hicks, J. B. (1986) *Methods in Yeast Genetics,* pp. 163–167, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Ito, F., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Biol. Chem.* 105, 163–168
20. Sikorski, R. S., and Hieter, P. (1989) *Genetics* 122, 19–27
21. Prober, J. M., Trainer, G. J., Dam, R. J., Holb, F. H., Robertson, C. W., Zagursky, R. J., Cocuzza, A. J., Jensen, M. A., and Baumeister, K. (1987) *Science* 236, 336–341
22. Pearson, W. B., and Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 2444–2448
23. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410
24. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* 157, 103–132
25. Rothstein, R. (1991) *Methods Enzymol.* 194, 281–301
26. Stutz, A., and Linder, P. (1990) *Gene (Amst.)* 95, 91–98
27. Hill, J. E., Myers, A. M., Koerner, T. J., and Tsagolof, A. (1993) *Year* 2, 163–167
28. Jones, J. S., and Prakash, L. (1990) *Year* 6, 363–366
29. Nakazawa, K., Nagawa, T., Shimma, Y., Kurihara, J., and Jigami, Y. (1992) *EMBO J.* 11, 2511–2519
30. Jelinek-Slinko, Y., Nakayama, K., Tanaka, A., Toda, Y., and Jigami, Y. (1993) *J. Biol. Chem.* 268, 26338–26345
31. Hausler, A., Ballou, L., and Robbins, P. W. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 6846–6850
32. Hill, K., Boone, C., Geohi, M., Pueiras, R., Sdicu, A.-M., and Bussey, H. (1992) *Genetics* 130, 273–283
33. Lussier, M., Sdicu, A.-M., Camirand, A., and Bussey, H. (1996) *J. Biol. Chem.* 271, 11091–11098
34. Lussier, M., Camirand, A., Sdicu, A.-M., and Bussey, H. (1993) *Year* 9, 1057–1063
35. Mallet, L., Busseraveau, F., and Jacquet, M. (1994) *Year* 10, 819–831
36. Ram, F. J., Wohlers, A., Hoepen, R. K., and Kls, F. M. (1994) *Year* 10, 1019–1030
37. Hernandez, L. M., Ballou, L., Alvarado, E., Tsai, P.-K., and Ballou, C. E. (1989) *J. Biol. Chem.* 264, 13648–13659
38. Ballou, L., Hitzeman, R. A., Lewis, M. S., and Ballou, C. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 3209–3212
39. Dean, N. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 1257–1261
40. Hashimoto, C., Cohen, R. E., Zhang, W. J., and Ballou, C. E. (1987) *J. Biol. Chem.* 262, 4810–4817
41. Desceux, A., Luo, Y., Turco, S. J., and Beverley, S. M. (1995) *J. Biol. Chem.* 270, 24810–24817
42. Hou, W., and Turco, S. J. (1997) *Science* 269, 1869–1872