Molecular and phenotypic analysis of the S. cerevisiae MNN10 gene identifies a family of related glycosyltransferases

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The Saccharomyces cerevisiae mnn10 mutant is defective in the synthesis of N-linked oligosaccharides (Ballou et al., 1989). This mutation has no effect on O-linked sugars, but results in the accumulation of glycoproteins that contain severely truncated N-linked outer-chain oligosaccharides. We have cloned the MNN10 gene by complementation of the hygromycin B sensitivity conferred by the mutant phenotype. Sequence analysis predicts that Mann10p is a 46.7 kDa type II membrane protein with structural features characteristic of a glycosyltransferase. Subcellular fractionation data indicate that most of the Mann10 protein cofractionates with Golgi markers and away from markers for the endoplasmic reticulum (ER), suggesting Mann10p is localized to the Golgi complex. A comparison of the Mann10 protein sequence to proteins in the two different databases identified five proteins that are homologous to Mann10p, including a well-characterized Schizosaccharomyces pombe α1,2 galactosyltransferase that resides in the Golgi complex. Taken together, these results suggest that MNN10 encodes a novel Golgi-localized mannosyltransferase contained in this previously unrecognized family of related sugar transferases.

Key words: glycosyltransferase/Golgi/yeast/glycosylation/MNN10

Introduction

Glycosylation is an essential modification that is highly conserved among eukaryotes. Most of the glycosyltransferases that catalyze the addition of sugars onto proteins are resident proteins of the endoplasmic reticulum and the Golgi complex. In the case of the Golgi enzymes, biochemical analyses have demonstrated that different glycosylation processing enzymes are compartmentalized within different Golgi cisternae (Kornfield and Kornfield, 1985). As a result of the spatial organization of these resident glycosyltransferases, nascent secretory proteins undergo progressive and sequential processing as they transit the secretory pathway.

In S. cerevisiae, following the initial glycosylation steps in the endoplasmic reticulum (ER), N-linked oligosaccharides are primarily elongated in the Golgi by mannosyltransferases to form glycoproteins with extended outer chains of 50 or more mannose units. The outer chain, consisting of an α1,6-linked mannose backbone, is normally highly branched with α1,2 and α1,3-linked mannoses (Herscovics and Orlean, 1993; Kukuruzinska et al., 1987). The structure and biosynthetic pathway of outer chain oligosaccharides in S. cerevisiae has been deduced largely from the analysis of the mnn mutants. These mutants were initially isolated based on changes in cell surface carbohydrate structure (Ballou et al., 1980) and have since been shown to be affected in the biosynthesis of the outer chain portion of N-linked oligosaccharides (Ballou, 1990). The mnn8, mnn9, and mnn10 mutants are similar to one another in that each mutant is severely defective in outer chain biosynthesis. Mnn9, which is epistatic to both mnn8 and mnn10, eliminates all but two mannoses of the outer chain (Ballou et al., 1989). The MNN9 gene has been cloned, but the precise mechanism by which Mnn9p regulates glycosylation is unknown (Yip et al., 1994). Mnn10 mutants accumulate oligosaccharides containing 10–14 mannoses. Similarly, mnn8 mutants accumulate heterogeneous oligosaccharides of a similar structure to mnn10, but of a somewhat wider size range (Ballou et al., 1989). These defects suggest that the mnn8 and mnn10 mutants may be defective in the activity or regulation of the α1,6 mannosyltransferases involved in the elongation of the outer chain.

Though the studies of the oligosaccharides that accumulate in these mutants have been instrumental in defining the structure of N-linked oligosaccharides in yeast, little is known about the regulation and subcellular organization of the glycosyltransferases that mediate these modifications in yeast. Molecular analyses of the MNN genes will be essential for a greater understanding of the mechanisms regulating glycosylation in yeast. In this report, we describe the cloning and analysis of the MNN10 gene, which encodes a novel, highly conserved glycosyltransferase that is localized to the Golgi compartment.

Results

Cloning the MNN10 gene

A number of spontaneous vanadate-resistant mutants with glycosylation defects have previously been isolated, including mnn10 (Ballou et al., 1991; Poster and Dean, unpublished results). All of these mutants are sensitive to the aminoglycoside, hygromycin B (Ballou et al., 1991; Dean, 1995). This drug sensitivity was exploited as a means of cloning the MNN10 gene. The mnn10 mutant strain, HTY10 was transformed with a yeast genomic library in a centromere-containing vector that carries the URA3 selectable marker. Uracil prototrophs were replate cloned onto media containing 50 μg/ml hygromycin B. Three hygromycin resistant colonies were isolated. Plasmid DNA obtained from each of these colonies was distinct, but contained overlapping restriction fragments. All three plasmids conferred hygromycin B resistance when retransformed into the mnn10 mutant. Further subcloning
Fig. 1. The cloned MNN10 gene rescues the hygromycin sensitivity of the mnn10 mutant. Strains were streaked for single colonies on YPAD with or without the addition of 50 μg/ml hygromycin B as indicated. Shown are wild type (SEY6210), mnn10-3 (HTY10), mnn10-3 (HTY10) harboring pMNN10U, which carries the 1.9kb SalI HindIII complementing fragment containing the MNN10 gene, and mnn10-3 (HTY10) carrying an integrated copy of the myc-tagged-MNN10 gene (pTIMNN10-myc).

indicated the activity that complements drug sensitivity was encoded by a 1.9 kb SalI/HindIII fragment in pMNN10 (Figure 1).

The mnn10 mutation has a severe effect on N-linked glycosylation (Ballou, 1990; Ballou et al., 1989). Complementation of this glycosylation defect was monitored by assaying the electrophoretic mobility of invertase. Invertase is a highly glycosylated protein whose rate of migration on acrylamide gels reflects the size and number of N-linked oligosaccharide chains it contains. A glycosylation defect can be detected by comparing the average rate of migration of invertase in extracts prepared from mutant and wild type cells (Ballou, 1990). Previous studies have demonstrated that N-linked oligosaccharides in mnn10 mutant cells are truncated, containing an average of 10–14 mannose residues as compared to 50 or more in wild type oligosaccharides (Ballou et al., 1989). Consequently, in mnn10 mutant cells, invertase migrates more rapidly, as a compressed species. We confirmed this result in our mutant mnn10-3 (Figure 2, lane 1). Mutant cells harboring a plasmid (pMNN10) containing the 1.9 kb SalI/HindIII fragment express invertase that migrates with a mobility indistinguishable from that of wild type cells (Figure 2, lane 3), demonstrating that this fragment confers complementation of the glycosylation defect.

We determined the sequence of the 1.9 kb fragment containing the mnn10 complementing activity. A comparison with the database revealed this sequence had been previously sequenced through the yeast genome sequencing project and was on chromosome IV. The nucleotide and predicted amino acid sequence of this region is shown in Figure 3A. Expression of a cloned fragment containing only this ORF rescued both the invertase glycosylation defect and the hygromycin B sensitivity in the mnn10 mutant (data not shown). A similar fragment, but containing a deletion of a unique 841 base pair XbaI fragment within this ORF failed to rescue either of these phenotypes (data not shown), demonstrating that the mnn10 complementing activity resides within this ORF.

The MNN10 gene is predicted to encode a 393 amino acid protein of 46.7 kDa. Hydrophobicity analysis (Figure 3B) suggests that the protein contains a single membrane spanning domain close to the amino terminus.

**MNN10 defines a related family of proteins**

A sequence homology search identified six genes whose products are significantly homologous to Mnn10p (Figure 4). An alignment of these six proteins is diagrammed in Figure 4A and the similarity as compared to Mnn10p is summarized in Figure 4B. The first homologue is gma12, a well characterized α1,2 galactosyltransferase from Schizosaccharomyces pombe (Chappell et al., 1994; Chappell et al., 1994).
**MNN10 encodes a Golgi-localized glycosyltransferase**

Fig. 3. A. The nucleotide and predicted amino acid sequence of the MNN10 gene. The potential membrane spanning domain is underlined. B. Hydropathy plot of the predicted Mnn10p. Hydrophobicity was calculated according to the method of Kyte and Doolittle (32), using a window of 13 amino acids.
The *MNN10* gene identifies a family of related proteins. A. Proteins were identified in the Genbank and dbest data bases using the Blast algorithm (17) and aligned using the Clustal algorithm on DNA Star. The accession numbers for each are as follows: *MNN10* (L42540): \*pombe\* *gmal2*, labeled s.p.Gtase, (Z30917); \*pombe\* ORF1 (SPAc5H10_10-Acc.# Z49811); \*pombe\* ORF2 (SPAc5H10_13 Acc.#Z49811); *A. ihatmlia* cDNA 1, labeled Arab 1, (T76358); *A. ihatmlia* cDNA 2, labeled Arab 2, (T22705). B. A summary of the similarity and identity of each of the homologues to the MnnlO protein. The gap alignment program used the Needleman and Wunsch algorithm on the GCG program.
and Warren, 1989). gma12p is 26% identical and 54% similar to Mnn10p. Two other S. pombe genes show similar levels of homology to Mnn10p. These also display features characteristic of glycosyltransferases. Other members of the glycosyltransferase family are between 350–450 amino acids in length, predicted to be type II membrane proteins with a short N-terminal cytoplasmic domain, a single membrane spanning region near the N-terminus, followed by a large C-terminal lumenal domain containing the catalytic site. Most contain a disulfide bond and most, but not all are glycosylated (Kleene and Berger, 1993; Paulson and Colley, 1989).

In addition to these yeast genes, a search of the database of expressed sequences (dbest) identified two related plant cDNAs from Arabidopsis thaliana. Sequence alignments suggest that these cDNAs represent partial sequences, lacking the 5' ends of the genes. These cDNAs never the less clearly encode members of this family of proteins. MNN10, gma12, and the two uncharacterized S. pombe ORFs are all predicted to encode glycosyltransferases. Like S. pombe gma12p, Mnn10p is probably not modified by N-linked sugars. It contains one Asn/X/Ser sequence at position 195. However this sequence contains a disfavored proline in the second position. Immunoblot analysis of epitope-tagged Mnn10p (see below) indicates that the protein migrates with a molecular weight of about 47 kDa, as a single, discrete band, suggesting that Mnn10p is not glycosylated. Two conserved cysteines are invariant at positions 424 and 430 among the four yeast proteins and are candidates for involvement in disulfide bond formation. Although these proteins show some similarity throughout their length, the highest degree of homology is concentrated in the carboxyl portion where the predicted catalytic domain would lie (Kleene and Berger, 1993; Paulson and Colley, 1989).

The MNN10 gene is required for germination, but not for growth

We analyzed the effect of replacing the wild type version of the MNN10 gene with a null allele. A standard one-step gene disruption (Rothstein, 1983) was performed in order to replace the chromosomal copy of the MNN10 gene with an allele in which a Stu1 fragment, within the coding region was replaced with the LEU2 gene (See Figure 5A). A linear DNA fragment containing the disrupted mnn10 gene was transformed into a wild type diploid homozygous for the leu2–3,112 mutation. The disruption of one allele was confirmed by Southern blot analysis of genomic DNA (data not shown).

Diploids were sporulated and 10 dissected tetrads were analyzed for cell viability. The resulting tetrad analysis (Figure 5B) demonstrated that in each case, only two of the four spores survived and neither were Leu+. These results suggested that MNN10 encodes an essential gene. However, inviable spores failed to form microcolonies, suggesting the possibility that the MNN10 gene may be required for germination, rather than growth.

To test the requirement of MNN10 for growth, we analyzed the effect of replacing the wild type version of the MNN10 gene with a null allele in a haploid strain. The same disruption plasmid described above (see Figure 5A) was introduced into a SEY6210 carrying the leu2–3,112 mutation. Leu+ transformants, carrying the disrupted mnn10 allele were recovered on plates lacking leucine. The disruption of the MNN10 allele was confirmed by PCR (data not shown), indicating that all contained a disrupted mnn10 allele. Analysis of the phenotype of the disrupted haploid strain indicated that it was defective in glycosylation (Figure 5C, compare lanes 1 and 2) and was hygromycin B sensitive (data not shown). These mutant phenotypes are complemented by the introduction of a plasmid-borne MNN10 gene, suggesting that the recovery of Leu+ transformants was not due to a second site suppressor of the mnn10Δ::LEU2 allele.

The haploid strain carrying the null allele (JPY12) was crossed to a wild type strain to produce a heterozygous diploid. This diploid was sporulated and dissected tetrads were analyzed for growth. The resulting segregation pattern was again 2+2 for growth, demonstrating that in our strain background, the MNN10 gene is only required for germination of spores and not for growth.

Table 1: Homology of plant genes to MNN10

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Fig. 4. Continued
Fig. 5. Disruption of the \textit{MNN10} gene. A. Schematic representation of the \textit{MNN10} gene and flanking sequences on chromosome IV, and the strategy used to construct the disruption plasmid, showing relevant restriction sites used to replace coding sequences with the \textit{LEU2} gene. B. Tetrad analysis of a diploid strain heterozygous for the \textit{MNN10} disrupted allele (JPY11). Tetrads obtained from the sporulation were dissected on YPAD plates, with four spore on one column. These were incubated for three days at 30° C. Each column is labeled numerically. C. Glycosylation state of invertase-myc in the \textit{mnn10\Delta::LEU2} disrupted haploid (JPY12) and isogenic wild type strain (SEY6210). Protein extracts from each strain were immunoblotted and invertase-myc detected using 9E10 monoclonal antibodies against the \textit{c-myc} epitope.

structure as a consequence of the glycosylation phenotype, and are common to a number of glycosylation mutants (Ballou et al., 1991).

\textit{MNN10} is not required for O-linked glycosylation

We also wished to determine whether or not the \textit{mnn10} mutant was defective in \textit{N}- but not \textit{O}-linked glycosylation, as expected for a glycosyltransferase involved in \textit{N}-linked glycosylation. To monitor \textit{O}-linked glycosylation, the electrophoretic mobility of chitinase was monitored. Chitinase is glycosylated exclusively by \textit{O}-linked sugars (Kuranda and Robbins, 1991), and therefore any effect on \textit{O}-linked glycosylation can be readily detected by a mobility shift on denaturing acrylamide gels. Whole cell extracts were prepared from wild type or mutant cells carrying the \textit{mnn10\Delta-3} allele and assayed by immunoblotting, using antichitinase antiserum. No differences in the mobility of chitinase were seen in extracts from wild type or mutant cells (Figure 6, compare lanes 1 and 2) demonstrating that the \textit{mnn10\Delta-3} mutation is specific for \textit{N}-linked glycosylation.

Expression and localization of Mnn10p

In order to determine the subcellular location of Mnn10p, a plasmid was constructed which encodes an epitope-tagged version of the protein (see Materials and Methods). The \textit{c-myc} epitope was added onto the C-terminus of the protein. As expected from the predicted protein sequence, the Mnn10p-myc protein ran as a single band (see below), with a molecular weight of about 47 kDa on SDS-acrylamide gels. Expression of the tagged protein rescued both the
Fig. 6. Immunoblot analysis of chitinase in wild type and mnnlO–3 mutant cells. Equivalent amounts of total protein extracts from the mnnlO–3 mutant (HTY10) (lane 1) and wild type (SEY6210) cells were prepared as described in Materials and methods, resolved by 8% SDS-PAGE and immunoblotted using anti-chitinase antibodies.

glycosylation defect (data not shown) and the hygromycin B sensitivity (Figure 1) when expressed in mnnlO–3 mutant cells, demonstrating that the addition of the epitope does not alter the normal function of the protein.

To test for the localization of Mnnl0-myc protein, we fractionated membranes from cells expressing the myc-tagged protein by differential centrifugation to enrich for ER and Golgi-containing fractions. By this procedure (Horazdovsky and Emr, 1993) the majority of Mnnl0-myc (85%) pelleted at a 100,000 × g spin (P100 fraction) (Figure 7, top panel) fractionating with another Golgi marker, the α,2 mannose transferase, Mntlp. Under these same conditions, Mnnl0-myc fractionated away from an ER marker, Kar2p, found primarily in the 13,000 × g spin (P13 fraction) or in the 100,000 × g spin supernatant (S100, data not shown). These results suggest that Mnnl0p is normally localized in the Golgi complex.

Discussion

We have described a molecular and phenotypic analysis of the MNN10 gene. The mnn10 mutant has a severe defect in N-linked glycosylation, resulting in oligosaccharides containing only 10–14 mannose residues. Like other glycosylation mutants defective in the synthesis of the outer chain, this gene product is not essential for growth. The absence of MNN10 results in slow growth and a failure in spore to germinate. Unlike some other vanadate-resistant glycosylation mutants with pleiotropic defects that affect outer chain elongation (unpublished data), the mnn10 lesion is specifically defective in N-linked glycosylation. Mutations in MNN10 have no effect on O-linked glycosylation. Sequence analysis predicts a protein whose structure resembles that of a glycosyltransferase. Furthermore, Mnnl0p is highly homologous to one known glycosyltransferase, the S. pombe gna12 protein. While we have not yet demonstrated enzymatic activity of the purified Mnnl0 protein, taken together, these data strongly suggest that the MNN10 gene product is a sugar transferase.

Several S. cerevisiae genes that encode mannosyltransferases have been isolated, including OCH1 MNN1, and MNT1. OCH1 encodes an α,1,6 mannosyltransferase that is probably involved in outer chain initiation (Nakanishi-Shindo et al., 1993; Nakayama et al., 1992). MNN1 encodes an α,1,3 mannosyltransferase that is a terminal transferase (Graham et al., 1992; Yip et al., 1994). MNT1, also known as KRE2, encodes an α,1,2 mannosyltransferase involved in O-linked glycosylation (Hausler et al., 1992; Hausler and Robbins, 1992; Hill et al., 1992). Three yeast genes, including KTR1 (Hill et al., 1992), KTR2 (Lussier et al., 1993) and YUR1 (Foreman et al., 1991) encode proteins with significant homology to Mntlp, though the function of these related proteins is unknown. No homology between Mnnl0p and these other known yeast glycosyltransferases was observed.

Typically glycosyltransferases are grouped together by the type of sugar they transfer. In some cases sequence homology is displayed among those enzymes that transfer the same sugar. However, unlike S. pombe, S. cerevisiae oligosaccharides do not contain galactose (Ballou, 1990). Therefore the homology between Mnnl0p and the S. pombe galactosyltransferase, gna12p, cannot reflect a domain involved in the recognition of the donor nucleotide sugar, UDP-galactose. The identification of other MNN10-related genes suggests that the gene products serve a highly conserved function. One explanation is that the similarity between these proteins is based upon a common structure of the acceptor substrate recognized by all of these enzymes. The structure of the oligosaccharide that accumulates in the mnn10 mutant has been determined by a combi-

Fig. 7. Subcellular fractionation of myc-tagged Mntlp and Mnn10p. Membranes were fractionated by differential centrifugation as described (20). Equivalent amounts of the P13 and P100 fractions were subjected to immunoblot analysis with the monoclonal antibody, 9E10 to detect myc-tagged Mntlp and Mnn10 proteins (top panel) or anti-HDEL antisera to detect Kar2p (bottom panel). In top panel 1/10 of an equivalent of starting material (−) was loaded, while in the bottom panel equivalent amounts of material were loaded in each lane. The position of the Mntlp-myc, Mnn10p-myc and Kar2 proteins are indicated by an arrow.
nation of NMR and size chromatography of purified oligosaccharides (Ballou et al., 1989). It is a Man\GlcNAc\ beta to which is added an average of four mannoses in an \alpha,6 linkage, terminated by a single mannose in an \alpha,2 linkage, that probably acts as a stop signal (Ballou et al., 1989). Based upon this structure, a strong prediction is that Mnn10p is an elongating \alpha,6 mannosyltransferase. Characterization of the acceptor substrate recognized by both Mnn10p and gma12p will be required in order to understand what the functional ties are between these two proteins.

A major question is how Mnn10 and other Golgi-localized glycosyltransferases are correctly compartmentalized to enable the stepwise series of reactions that lead to correct glycan synthesis. Data from fractionation experiments suggest that Mnn10p is localized to the Golgi complex. There is evidence from studies in both yeast (Whitters et al., 1995) and higher eukaryotes (Nilsson et al., 1993) that some resident Golgi proteins are not restricted to specific subcompartments but instead exhibit a substantial degree of compartment overlap. This has led to the suggestion that compartment identity may be dictated by a particular mixture of proteins that overlap rather than by a unique set of enzymes (Nilsson et al., 1993). The availability of MNN10 and other cloned glycosyltransferases from S. cerevisiae provide the best compartment markers and should rapidly advance knowledge of Golgi targeting and retention mechanisms in yeast.

Materials and methods

Yeast strains, media and genetic methods

Yeast strains were grown in either YPAD (1% yeast extract, 2% peptone, 2% dextrose, 50 \mu g/liter adenine sulfate), or synthetic medium which contained the appropriate auxotrophic requirements (Guthrie and Fink, 1991). YPAD liquid medium was supplemented with 0.5 M KCl for the growth of the mnn10 mutant strains which are osmotically sensitive. Hygromycin B (Boehringer Mannheim) was added to YPAD agar after autoclaving to a final concentration of 50 \mu g/ml. Yeast strains were transformed using the LCI procedure (Ito et al., 1983).

All yeast strains used in this study are listed in Table 1. The strain NDY4, which carries the mnn10-3 allele, was isolated as a spontaneous vanadate-resistant mutant on YPAD plates containing 7.5 mM sodium orthovanadate (Fisher). NDY4 was out crossed twice to SEY6210 to obtain the strain HTY10. HTY10 was used for all analyses of the mnn10-3 phenotype.

Plasmid constructions

All DNA manipulations were carried out according to standard protocols (Sambrook et al., 1989). The 1.9 kb SalI HindIII fragment containing the entire MNN10 gene and upstream regulatory sequences was subcloned into the vectors pRS316 and pRS315, containing either theURA3 or LEU2 selectable markers (Sikorski and Hieter, 1989) to generate the CEN based plasmids, pMNN10U and pMNN10L.

The disruption plasmid, pMNN10::LEU2 was constructed by replacing a 371 bp fragment in the MNN10 gene with a BamH1/BamH1 fragment (blunt-ended with Klenow) containing the LEU2 gene (see Figure 5). A c-myc tagged version of MNN10 was constructed using a N-terminal PCR primer to introduce a unique BamH1 site just upstream the translational start site (5' - CCGGGATCCACCATGTCTAGTGTACCTTAT- 3') and a C-terminal PCR primer to replace the stop codon with an Neo1 site (5' - CACCATGATGTTAGAAGAAAATCTCGTG- 3'). This PCR generated fragment was then cut with BamH1 and Neo1 and cloned into the plasmid pF16 (Dean and Pelham, 1990) to allow the in-frame fusion of the sequence encoding the c-myc epitope. The resulting plasmid (pTHMN10-myc) encodes an Mnn10p protein which contains the amino acid sequence TRFFFP—followed by the c-myc epitope, SMEKLISEEDLNSAEARL-stop at the C terminal junction. Expression of Mnn10p is under the regulation of the constitutive tritose phosphate isomerase (TPI) promoter. This plasmid is a derivative of the URA3-containing Ylp56 plasmid, but contains an Xho1 linker in the SalI site of the URA3 gene, causing its inactivation (Hardwick et al., 1990). Linearization of this plasmid with Xho1 allows integration at the ura3-1 or ura3-52 locus. URA3 activity requires recombination of the plasmid and the chromosomal copy, enabling selection on plates lacking uracil.

A plasmid containing the myc-tagged MNNT1 gene (pTiMNT-myc) was constructed by digesting a plasmid containing the MNNT1 gene (Haupler and Robbins, 1992) with SpeI, which allowed the in-frame fusion to a sequence encoding the c-myc epitope. The resulting plasmid encodes the Mnt1 protein which contains the amino acid sequence —QQGSCKGKE—followed by the C-terminal epitope, LEQKLISEEDLNSAEARL-stop at the C terminal junction. The MNT1-myc ORF was cloned into the same Ylp56 derivative described above and integrated at the ura3-52 locus in SEY6210.

The myc-tagged invertase plasmid (pTiINV-myc) was constructed by digesting Ylp56 by Ssp1 and ligating it into the Ylp56 derivative described above. After stable integration of the linearized plasmid at the ura3-52 locus in SEY6210 or HTY10, expression of secreted invertase was constitutive, under the control of the TPI promoter.

Cloning and sequencing the MNN10 gene

Strain HTY10 (MATa ura3-52 leu2-211 mnn10-3) was transformed with a yeast genomic CEN-based library in YCP50, carrying the URA3 selectable marker (Rose et al., 1983). Prototrophic transformants were selected on medium lacking uracil. These transformants were then replica plated onto medium containing 50 \mu g/ml hygromycin B. Plasmid DNA from hygromycin resistant colonies was isolated, amplified in a 371 bp fragment in the MNN10 gene with a BamH1/BamH1 fragment (blunt-ended with Klenow) containing the LEU2 gene (see Figure 5).

A 1.9 kb SalI/HindIII fragment, containing the complementing activity, was identified and the DNA sequence determined by the dideoxy method (Sanger et al., 1977). The SalI site in this fragment was derived from the library vector. A nested deletion series was constructed using the ExolI/EcoRVII method (Yannusch-Perron et al., 1985). DNA and predicted protein sequence comparisons against an updated data base were made using the BLAST algorithm (Altschul et al., 1990) and analyzed using the GCG software package.

### Table 1. Strains used in this study

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80
programs. Protein alignments were made with the DNASTAR program, using the Clustal algorithm.

Immunoblotting and subcellular fraction
Preparation of whole cell extracts and Western blotting were performed as described (Dean and Pelham, 1990). Culture supernatants containing the monoclonal anti-myc antibody, 9E10, were used at a 1:10 dilution for the detection of mvc-tagged invertase and mvc-tagged Mnn10p for immunoblotting. Anti-chitinase antibodies (kindly provided by Martina Gentzsch) were used at a 1:1000 dilution. Secondary anti-rabbit or antimouse antibodies were conjugated to horseradish peroxidase and the immune complex detected by chemiluminescence (ECL, Amersham), followed by autoradiography.

Subcellular fractionation of SEY6210, expressing either the mvc-tagged Mnn10p or the mvc-tagged o1.2 mannosyltransferase, Mnt1p. (Hausler et al., 1992, Hausler and Robbins, 1992) was performed as described (Horazdovsky and Emr, 1993), except that cells were dounced in lysis buffer containing 0.8 M sorbitol and a protease inhibitor cocktail resulting in a final concentration of 200 μM leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mM Chloro-(4-tosylamido)-7-amino-2- heptanone-HCl (TLCK). 1 μM pepstatin) (all protease inhibitors were from Sigma).

The nucleotide sequence data of MNN10 reported in this paper appears in the EMBL, GenBank and DDB1 Nucleotide Sequence Databases under the accession number L42540.

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References


