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# Mnt2p and Mnt3p of *Saccharomyces cerevisiae* are members of the Mnn1p family of α-1,3-mannosyltransferases responsible for adding the terminal mannose residues of *O*-linked oligosaccharides

**Pedro A.Romero, Marc Lussier[1], Steeve Véronneau[1], Anne-Marie Sdicu[1], Annette Herscovics and Howard Bussey[1,2]**

McGill Cancer Centre, McGill University, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6 and the [1]Department of Biology, McGill University, 1205 Dr. Penfield Avenue, Montreal, Quebec H3A 1B1, Canada

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[2]To whom correspondence should be addressed

**The genome of *Saccharomyces cerevisiae* contains five genes that encode type II transmembrane proteins with significant amino acid similarity to the α-1,3-mannosyltransferase Mnn1p. The roles of the three genes most closely related to *MNN1* were examined in mutants carrying single and multiple combinations of the disrupted genes. Paper chromatographic analysis of [2-$^3$H]mannose-labeled *O*-linked oligosaccharides released by β-elimination showed that the *MNT2* (*YGL257c*) and *MNT3* (*YIL014w*) genes in combination with *MNN1* have overlapping roles in the addition of the fourth and fifth α-1,3-linked mannose residues to form $Man_4$ and $Man_5$ oligosaccharides whereas *MNT4* (*YNR059w*) does not appear to be required for *O*-glycan synthesis.**

*Key words:* O-glycosylation/α-1,3-mannosyltransferase/gene family/yeast

## Introduction

Glycosylation of secretory and membrane proteins in eukaryotic cells imparts a wide variety of biological functions and requires the participation of many different enzymes associated with the endoplasmic reticulum and the Golgi apparatus. The glycoproteins of *Saccharomyces cerevisiae* contain both *N*-linked and *O*-linked oligosaccharides. The *N*-glycans attached to asparagine residues have a core whose synthesis from $Glc_3Man_9GlcNAc_2$ in the endoplasmic reticulum is similar to that of other eukaryotes. However, modifications of this core in fungi differ from those of other eukaryotes with the addition by Golgi mannosyltransferases of a variable number of mannose residues. Fungal *O*-glycans are also distinct, and in *Saccharomyces cerevisiae* they consist of a linear oligosaccharide containing up to five mannose residues (Ballou, 1990; Herscovics and Orlean, 1993; Orlean, 1997; Herscovics, 1999).

The biosynthesis of *O*-linked oligosaccharides in *S.cerevisiae* is initiated in the ER with Dol-P-Man as the donor for the mannosyl residue transferred to the hydroxyl group of either serine or threonine. GDP-Man is utilized as the sugar donor in the subsequent elongation of the *O*-linked carbohydrate chains in the Golgi apparatus, resulting in a linear glycan in which the second and third mannose residues are α-1,2-linked while the fourth and fifth mannose residues are α-1,3-linked (Ballou, 1990; Häusler *et al*., 1992; Yamada *et al*., 1994).

Most of the enzymes involved in the addition of the first three mannose residues of the *O*-linked oligosaccharides have been identified and their structural genes isolated. At least four different genes of the seven-membered *PMT1-7* gene family encoding Dol-P-Man:protein O-D-mannosyltransferases are responsible for initiation of *O*-glycans in the ER (Gentzsch and Tanner, 1996, 1997). Protein *O*-glycosylation is essential for yeast cell function since mutants of *S.cerevisiae* lacking different combinations of three of the *PMT* genes are not viable (Gentzsch and Tanner, 1996). A family of enzymes with homology to the α-1,2-mannosyltransferase, Kre2p/Mnt1p has been identified. Of these Ktr1p, Ktr3p, and Kre2p/Mnt1p have overlapping roles in the addition of the α-1,2-linked mannose residues on *O*-linked oligosaccharides in the Golgi (Lussier *et al*., 1997a,b, 1999). *Mnn1* mutants lacking α-1,3-linked mannose residues on both *N*- and *O*-linked oligosaccharides were originally isolated by Antalis *et al*. (1973). The *MNN1* gene was cloned and shown to encode an α-1,3-mannosyltransferase (Graham *et al*., 1992, 1994; Yip *et al*., 1994). The enzyme responsible for the addition of the fifth α-1,3-linked mannose residue has not yet been identified.

The yeast genome contains five genes, *YGL257c*, *YIL014w*, *YNR059w*, *YJL186w*, and *TTP1* (*YBR015c*) (Romero *et al*., 1994), encoding proteins with significant similarity to Mnn1p (Romero *et al*., 1997a; Lussier *et al*., 1999). Recently, it was established that *TTP1* and *YJL186w* correspond to the *MNN2* and *MNN5* genes, respectively (Rayner and Munro, 1998), previously shown to be required for the addition of α-1,2-linked mannose residues to the *N*-linked outer chains of mannoproteins (Raschke *et al*., 1973; Cohen *et al*., 1980). In the present work, functional characterization of the other members of the Mnn1p α-1,3-mannosyltransferase family is reported.

## Results and discussion

### *The MNN1 gene family*

The yeast genome contains five genes, *YGL257c*, *YIL014w*, *YNR059w*, *YJL186w*, and *TTP1* (Romero *et al*., 1994) (*YBR015c*), encoding proteins with significant amino acid similarity to Mnn1p (Romero *et al*., 1997a; Lussier *et al*., 1999). Each predicted sequence has a hydrophobic transmembrane region near the N-terminal and large lumenal domain, indicating that they all are type II transmembrane proteins, a topology characteristic of many Golgi glycosyltransferases (Kleene and Berger, 1993; Colley, 1997; Lussier *et al*., 1999). Initial characterization of this gene family was made by comparison of their deduced amino acid sequences (Figure 1) and construction of a relational homology tree (Figure 2). Two subfamilies are evident. The first consists of Ygl257p, Ynr059p, Yil014p, and Mnn1p. Among these, Ygl257p and Ynr059p are most homologous, sharing 44%

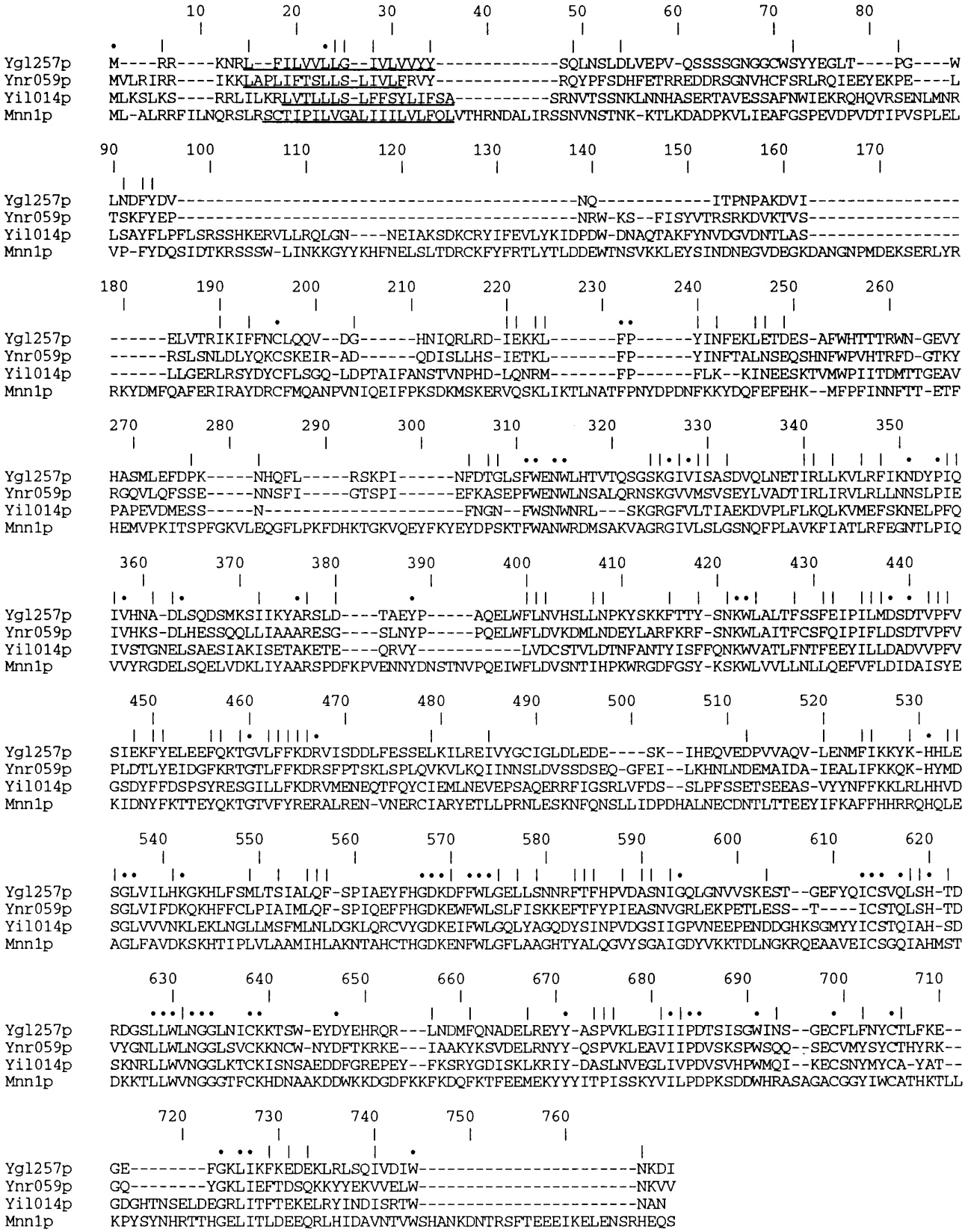



**Fig. 1.** Sequence similarities between Ygl257p (Mnt2p), Ynr059p (Mnt4p), Yil014p (Mnt3p), and Mnn1p. The amino acid residues are shown in single-letter amino acid code. Sequences were aligned with gaps to maximize homology. Dots represent identity between sequences, while a vertical slash indicates conservative substitutions. The hydrophobic putative transmembrane domains are underlined.

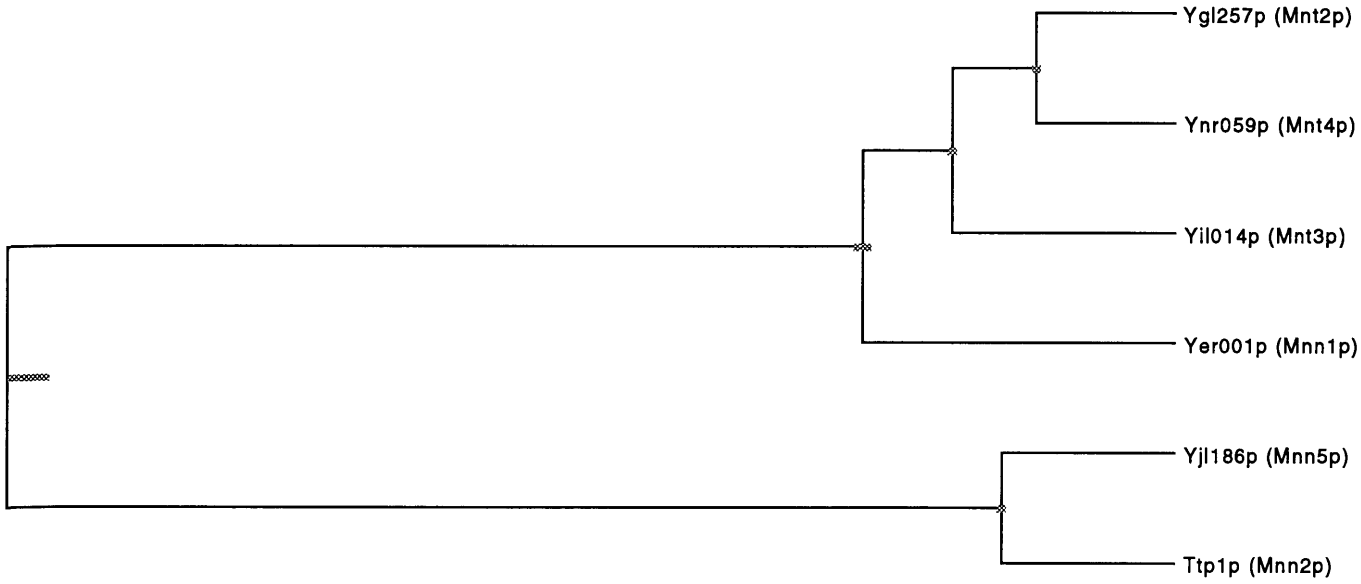



**Fig. 2.** Relational homology tree of the Mnn1p protein family. The tree was calculated with the unweighted pair group method with an arithmetic mean (Nei, 1987). The length of the horizontal lines linking the sequence of one family member to another is proportional to the estimated distance between the sequences.

identity, while Mnn1p is most related to Yil014p, with 30% identity. In the second group Yjl186p and Ttp1p have 37% amino acid sequence identity, but only about 20% identity with the other members of the family. Because of their similarities with the *MNN1* gene product, the *YGL257c*, *YIL014*, and *YNR059w* genes were named *MNT2*, *MNT3*, and *MNT4* respectively (for Mannosyltransferase).

### *Roles of the MNT2, MNT3, and MNN1 genes in O-linked oligosaccharide formation*

To determine whether Mnt2p, Mnt3p, and Mnt4p share functional similarities with Mnn1p, we examined the extent of *O*-glycosylation in yeast strains bearing mutations in these genes. Deletional disruptions of *MNT2* (*YGL257c*), *MNT3* (*YIL014w*), *MNT4* (*YNR059w*), and *MNN1* were obtained by a PCR procedure (see *Materials and methods*). To test for possible functional redundancies between these related genes, multiple disruptants were also constructed using standard genetic techniques. Analysis was then made of total *O*-linked [$^3$H]mannose-labeled oligosaccharides released by β-elimination in mutants carrying different combinations of the disrupted genes (Figure 3, Table I).

In the wild type strain, $Man_1$ to $Man_5$ oligosaccharides were seen following paper chromatography (Figure 3A). The extent of synthesis of $Man_5$ is likely strain-specific since in earlier studies the largest *O*-glycan observed in *Saccharomyces cerevisiae* was usually $Man_4$ (Nakajima and Ballou, 1974; Ballou, 1990). The *mnn1* null mutant also had $Man_1$ to $Man_5$ oligosaccharides, but the relative amount of $Man_4$ was highly reduced but not totally eliminated (Figure 3B, Table I). These results are consistent with previous work showing that the *MNN1* gene is required for the addition of the fourth α-1,3-linked mannose residue (Nakajima and Ballou, 1974; Ballou, 1990). However, they also indicate that one or more additional genes participate in the addition of the fourth and fifth mannose residues. In both the *mnt2* (Figure 3C, Table I) and *mnt3* (Figure 3D, Table I) null mutants, there is a considerable decrease in the formation of $Man_5$ while there is in *mnt3* a decrease in $Man_4$, demonstrating that both the *MNT2* and *MNT3* gene products participate in the addition of the terminal mannose residue. In contrast, the profile of oligosaccharides in the *mnt4* null mutant is more similar to that of the wild type cells (Figure 3E, Table I).

**Table I.** Composition of products from β-elimination

| Strain | Radioactivity (%) | | | | |
|---|---|---|---|---|---|
| | $Man_1$ | $Man_2$ | $Man_3$ | $Man_4$ | $Man_5$ |
| Wild type | 10 | 48 | 22 | 14 | 6 |
| *mnn1*Δ | 14 | 49 | 28 | 4 | 5 |
| *mnt2*Δ | 13 | 50 | 20 | 16 | 1 |
| *mnt3*Δ | 22 | 48 | 20 | 8 | 2 |
| *mnt4*Δ | 16 | 47 | 20 | 12 | 5 |
| *mnn1*Δ *mnt2*Δ | 16 | 46 | 38 | 0 | 0 |
| *mnn1*Δ *mnt3*Δ | 23 | 50 | 27 | 0 | 0 |
| *mnn1*Δ *mnt4*Δ | 9 | 43 | 34 | 6 | 8 |
| *mnt2*Δ *mnt3*Δ | 10 | 45 | 27 | 17 | 1 |
| *mnt2*Δ *mnt4*Δ | 11 | 45 | 22 | 21 | 1 |
| *mnt3*Δ *mnt4*Δ | 18 | 41 | 25 | 12 | 4 |

The % of radioactivity recovered in each fraction was calculated from the chromatographic profiles shown in Figure 3.

The synthesis of $Man_4$ and $Man_5$ oligosaccharides is eliminated in the double mutants *mnn1 mnt2* (Figure 3F, Table I) and *mnn1 mnt3* (Figure 3G, Table I), demonstrating that the *MNN1* gene in combination with either *MNT2* or *MNT3* are required for the addition of the fourth and fifth mannose residues. All the double mutants containing a *mnn1* disruption reveal an accumulation of $Man_3$ oligosaccharides as does the *mnn1* single mutant (see Table I and Nakajima and Ballou, 1974). In the *mnt2 mnt3* double mutant (Figure 3I, Table I), there is a very large inhibition

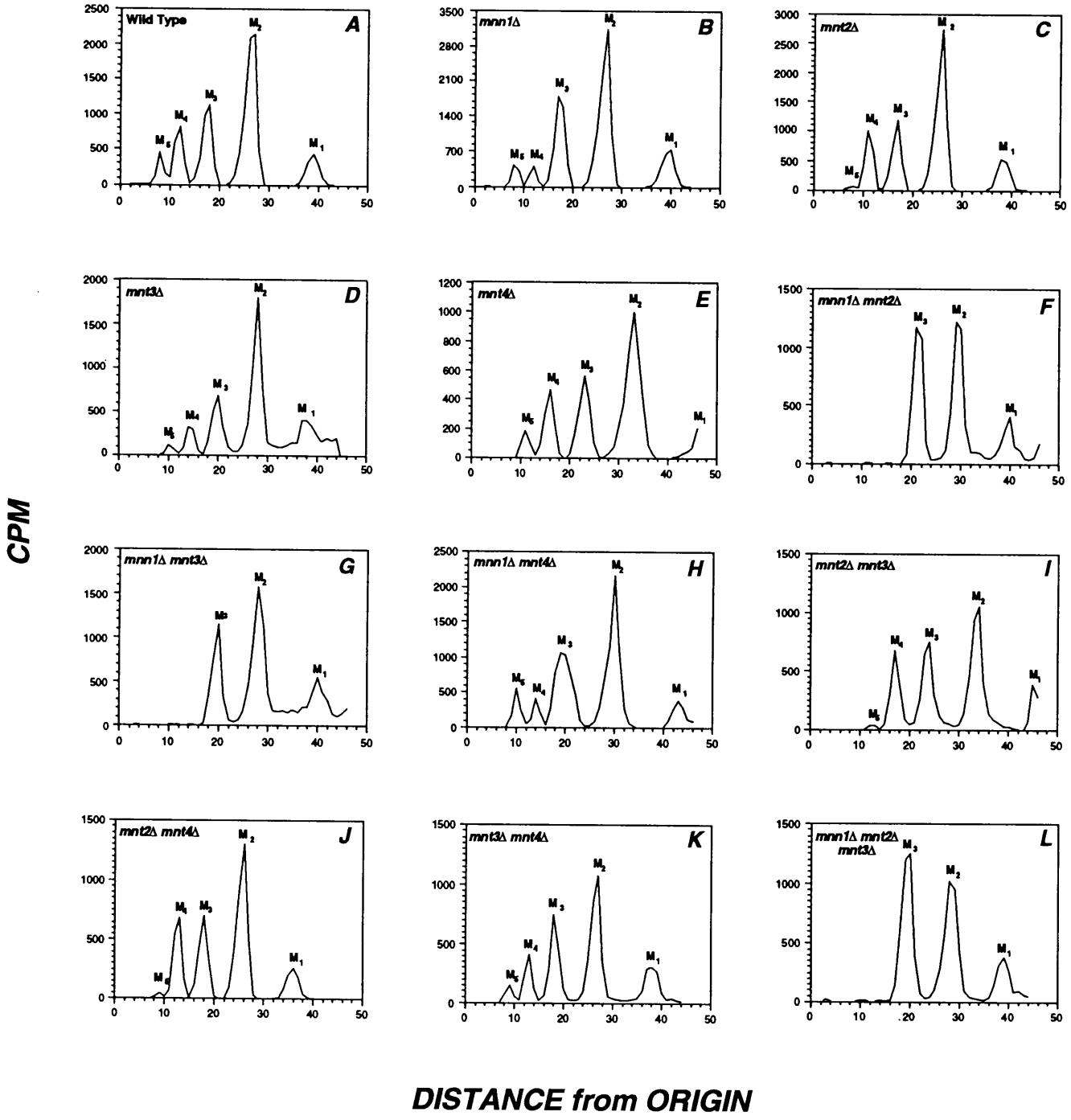
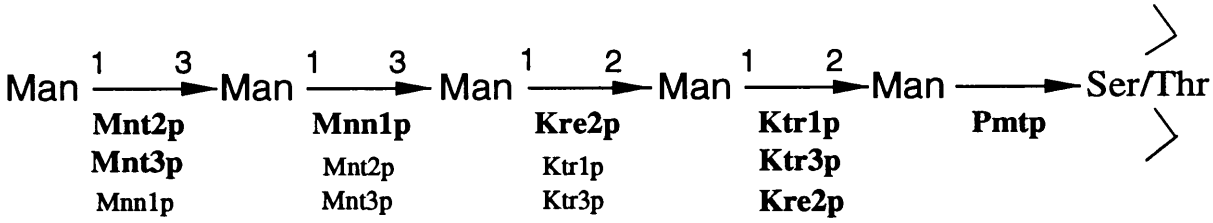



**Fig. 3.** Profile of the [$^3$H]mannose-labeled oligosaccharides released by β-elimination from total glycoproteins. Wild type cells (BY4742) and the different disruptants were labeled with [$^3$H]mannose, β-eliminated and submitted to paper chromatography as indicated in Materials and methods. $M_1$ to $M_5$ indicate oligosaccharides containing from one to five mannose residues. $M_1$, $M_2$, and $M_3$, comigrate with mannose, maltose, and maltotriose or raffinose used as internal standards. Similar profiles were obtained with 3 or 24 h of β-elimination.

of $Man_5$ synthesis similar to that observed in each of the *mnt2* and *mnt3* single mutants (Figure 3C,D) but little effect on $Man_4$. Therefore, the *MNT2* and *MNT3* genes are the major genes required for the addition of the fifth mannose residue. However, there is a residual amount of $Man_5$ formed in the *mnt2 mnt3* double mutant that disappears in a *mnn1* background, showing that Mnn1p is also involved in the addition of this fifth mannose. In contrast, the profiles of oligosaccharides in the *mnt4*-containing double mutants are similar to those observed in the *mnn1*, *mnt2*, and *mnt3* single mutants (compare Figure 3H,J,K with Figure 3B–D, respectively); thus, *MNT4* does not appear to play a major role in these steps of *O*-glycan synthesis.

The profile of oligosaccharides obtained in the *mnt2 mnt3 mnn1* triple null mutant (Figure 3L) is similar to that observed in either of the *mnt2 mnn1* or the *mnt3 mnn1* double mutants (Figure 3F,G) confirming that collectively Mnn1p, Mnt2p, and Mnt3p are




**Scheme 1.** Elaboration of the *S.cerevisiae O*–linked oligosaccharide. The sequence of *O*–glycans is depicted as well as the enzymes attaching the various mannose residues. Arrows represent α–1,2– and α–1,3–linkages between the mannose residues. The main enzymes of each step are represented in bold. Pmtp represents a family of 7 Dol–P–Man:protein–O–D–mannosyltransferases. Treatment of the *O*–linked oligosaccharides in wild type and in *mnn1* cells with different α–mannosidases, as described in *Materials and methods*, showed that the fourth and fifth mannose residues were sensitive to the *Xanthomonas* α–1,2,3–mannosidase, but not to the *Penicillium* α–1,2–mannosidase.

responsible for adding the fourth and fifth mannose residues on *O*-linked glycans. A *mnt2 mnt3 mnt4 mnn1* quadruple mutant had a similar profile of *O*-glycans as the triple null mutant (data not shown). These results indicate that Mnn1p is largely responsible for $M_4$ synthesis. Mnt2p and Mnt3p are also involved in adding this particular mannose (see Figure 3B) but they may act as a complex since the presence of both enzymes is required in a *mnn1* background (Figure 3F,G).

Most of the gene products responsible for the assembly of the linear *O*-linked oligosaccharides in *S.cerevisiae* have now been identified as outlined in Scheme 1. *MNN1* together with the *MNT2* and *MNT3* genes are responsible for the addition of the fourth and fifth mannose residues to the linear mannose *O*-linked carbohydrate chain. All three genes have overlapping roles in the addition of the α-1,3-linked mannose residues. Additional work will be required to determine whether these genes have protein-specific functions.

The Mnn1p mannosyltransferase is also involved in the elaboration of *N*-linked carbohydrate chains (Antalis *et al*., 1973; Ballou, 1990; Yip *et al*., 1994). Mnt2p, Mnt3p, and Mnt4p may also have roles in these type of modifications. Analysis of the oligosaccharides attached on the specifically *N*-modified protein invertase produced in a *mnn1* background showed that *N*-linked glycans produced in this mutant still possessed α-1,3-linked mannose residues (Verostek and Trimble, 1995). Hence, Mnt2p, Mnt3p, and Mnt4p are candidates to participate in the elaboration of *N*-linked glycans with the *MNT4* gene possibly having a role that is exclusive to these processes.

*O*-Modified mannoproteins form an intrinsic part of the yeast extracellular matrix. Mutants defective in the addition of $Man_2$ and $Man_3$ on *O*-mannosyl chains show resistance to the cell wall binding of K1 killer toxin (Häusler *et al*., 1992; Hill *et al*., 1992; Lussier *et al*., 1997b, 1999). Mutants of the *MNN1* gene display wild type sensitivity in their response to this toxin as do mutants in the *MNN1* family, including the *mnn1 mnt2 mnt3* triple mutant (data not shown). Thus, the presence of the terminal α-1,3-mannose residues is not required for a functional killer toxin receptor, while the first three α-1,2-linked mannose residues are.

Roles have now been found for 5 members of the 6-membered *MNN1* family. Three genes, *MNN1*, *MNT2*, and *MNT3* have products and phenotypes that are consistent with their being α-1,3-mannosyltransferases. The related *MNT4* gene remains of unknown function. The *MNN2*/*MNN5* subfamily are mannosyltransferases which interestingly have a different specificity, acting as α-1,2-mannosyltransferases (Raschke *et al*., 1973; Cohen *et al*., 1980; Ballou, 1990; Rayner and Munro, 1998). Presumably, these subfamilies possess a common ancestral gene and have diverged to the point where they have acquired a different linkage specificity.

In *S.cerevisiae*, the entire *O*-mannosylation biosynthetic pathway of just five mannose additions requires enzymes in the *PMT*, *KTR*, and *MNN1* gene families that contain a total of 22 genes (Gentzsch and Tanner, 1996, 1997; Lussier *et al*., 1997a,b, 1999; Romero *et al*., 1997a). The abundance of genes required to accomplish complex mannosylation processes is presumably a useful evolutionary adaptation for the fungal cell. These mannosyltransferase families may allow, for example, a wider use of serine and threonine residues in various sequence contexts in glycoproteins changing their properties. This remains a speculation that needs to be tested, but such a high level of apparent redundancy for a biosynthetic pathway is not a general feature of the yeast genome.

## Materials and methods

### *Material*

D-[2-$^3$H]Mannose (specific radioactivity, 20 Ci/mmol) was purchased from ICN (Irvine, CA); recombinant α-1,2,3-mannosidase from *Xanthomonas manihotis* was from New England Biolabs (Mississauga, Ontario, Canada); recombinant α1,2-mannosidase from *Penicillium citrinum* was a gift of Dr. T.Yoshida, Tohoku University, Sendai, Japan.

### *Yeast strains and culture conditions*

All yeast manipulations were based on strains BY4741 (MATa, his3-Δ1, leu2-Δ0, met15-Δ0, ura3-Δ0) and BY4742 (MATα, his3-Δ1, leu2-Δ0, lys2-Δ0, ura3-Δ0) (Brachmann et al., 1998) which were grown under standard conditions (yeast extract, peptone, dextrose, yeast nitrogen base) as described previously (Brown *et al*., 1994).

### *Gene disruptions*

Deletional disruptions of the *YGL257w* (*MNT2*), *YIL014c* (*MNT3*), *YNR059w* (*MNT4*), and *MNN1* loci were made using a PCR-mediated approach (Baudin *et al*., 1993; Wach *et al*., 1994). The coding sequences of *YGL257w*, *YIL014c*, *YNR059w*, and *MNN1* were replaced by the *URA3*, *LEU2*, *HIS3*, and kan$^r$ (permitting geneticin resistance) genes, respectively. In all cases, the oligonucleotides used for the production of the disrupted DNA fragment contained two sections: (1) a 5′ region of about 50 nucleotides that corresponds either to the region immediately upstream of the start codon or to the region directly downstream of the stop codon; (2) a 3′ region (~20 nucleotides) identical to the

DNA flanking the disruption module. Haploid yeast cells were transformed with the PCR products. Integrants were selected on minimal medium lacking uracil, leucine, histidine, or on YEPD supplemented with G418 in the case of the *MNN1* disruption. Gene disruptions were confirmed by PCR analysis (data not shown).

### *[2-$^3$H]mannose-labeling and β-elimination*

The conditions for labeling with [2-$^3$H]mannose and for analysis of neutral *O*-glycans were as described previously (Byrd *et al*., 1982; Lussier *et al*., 1993; Romero *et al*., 1997b). For each mutant studied, profiles were obtained at least twice from independent spore progeny. Yeast cells were grown at 30°C in YPD (1% yeast extract, 2% peptone, and 2% glucose), harvested during exponential phase and incubated in YPG (1% yeast extract, 2% peptone, and 1% galactose) for 30 min. Cells ($A_{600}$ = 25) were incubated for 90 min with 100 μCi of [2-$^3$H]mannose in 2 ml of YPG. Cell pellets obtained by centrifugation at 4°C were washed twice with ice cold water containing 0.02% sodium azide and were stored at –80°C. The frozen cells were thawed and resuspended in 0.15 ml of 10 mM Tris–HCl pH 7.2. The cells were mechanically disrupted by vortex-mixing (10 pulses of 30 s; cooling in ice-water for 60 s between pulses) in the presence of 0.1 ml acid-washed glass beads. The supernatants were collected with washes of the glass beads and centrifuged in a microfuge at 4°C for 75 min. The resulting pellets were resuspended in 0.45 ml of water containing 0.02% sodium azide and 0.025 ml of 2 M NaOH was added for β-elimination (Sentandreu and Northcote, 1968; Byrd *et al*., 1982; Lussier *et al*., 1993). The samples were mixed for 3 h at room temperature and then neutralized with HCl and centrifuged for 15 min in a microfuge. The supernatants were subjected to gel filtration on Bio-Gel P6 as described previously (Romero *et al*., 1985). The labeled included fractions were pooled, freeze dried, resuspended in water, and analyzed by descending paper chromatography on Whatman No 1 paper in ethyl acetate:n-butanol:acetic acid:water (30:40:25:40 by volume) (Babczinski and Tanner, 1973) for about 50 h with internal standards of mannose, maltose and maltotriose or raffinose, that were detected with the alkaline silver nitrate reagent (Trevelyan *et al*., 1950). Radioactivity was determined by placing 1 cm strips in vials containing 0.75 ml of water and 4 ml of Universol (ICN, Irvine, CA) and counting in a Pharmacia LKB Biotechnology liquid scintillation spectrometer model 1218.

### α-*Mannosidase treatments*

Aliquots of the oligosaccharides obtained from Bio-Gel P6 were desalted using AG50W (X8, $H^+$ form, 200–400 mesh) and AG1 (X8, formate form, 200–400 mesh) resins and then incubated with the following enzymes: *Xanthomonas manihotis* α1,2,3-mannosidase: 8 units at 0, 2, and 4 days of incubation in an initial volume of 0.020 ml of 50 mM sodium citrate pH 6.0 containing 5 mM $CaCl_2$, 0.1 mg/ml of bovine serum albumin, and 0.02% sodium azide for a total of 7 days at 37°C. *Penicillium citrinum* α1,2-mannosidase: 0.285 μg of recombinant enzyme in a total volume of 0.050 ml of 50 mM sodium acetate pH 5.0 containing 5 mM $CaCl_2$ for 8h at 30°C. The samples were then submitted to paper chromatography, as described above.

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## Abbreviations

ER, endoplasmic reticulum; Dol-P-man, dolichyl mannosyl phosphate.

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